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The Effects of Reduced Insulin/IGF-like Signalling in the CNS on Lifespan, Brain Ageing and Stress Resistance in *Drosophila melanogaster*

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Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted in substantially the same form for any other degree or professional qualification elsewhere.

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Abbreviations

AKT – Protein Kinase B

AMP - Adenosine Monophosphate

ANOVA - Analysis Of Variance

CNS – Central Nervous System

CR – Calorie Restriction

Da – Daughterless

Dah - Dahomey

DILP – Drosophila Insulin-Like Peptide

DN – Dominant Negative

DNA – Deoxyribonucleic Acid

DR – Dietary Restriction

ELAV - Embryonic Lethal Abnormal Visual system

ER – Endoplasmic Reticulum

FOXO - Forkhead Box-O transcription factor

GFP – Green Fluorescent Protein

GS - GeneSwitch

HSD – Honest Significant Difference

IGF – Insulin-like Growth Factor

IIS - Insulin/IGF like Signalling

InR – Insulin Receptor

IPC – Insulin Producing Cell

IRS – Insulin Receptor Substrate

JNK - Jun amino-terminal Kinase

LTP – Long Term Potentiation

PBS – Phosphate-Buffered Saline

PDK - Phosphoinositide-Dependent Kinase

PI3K - Phosphoinositide 3-Kinase

PIP_2 - Phosphatidylinositol (4,5)-bisphosphate

PIP_3 - Phosphatidylinositol (3,4,5)-trisphosphate

PKB – Protein Kinase B

S6K – S6 Kinase

SYA – Sugar, Yeast, Agar

Tβh – Tyramine-β-hydroxylase

TF – Transcription Factor

TNT - Tris-NaCl-Tween buffer

TOR – Target Of Rapamycin

TSC - Tuberous Sclerosis Complex

UAS - Upstream Activation Sequence

UV – Ultraviolet

VNC – Ventral Nerve Cord

w^{Dah} – $white^{Dahomey}$

4E-BP – 4E-Binding Protein

1. Abstract

Single gene mutations have been discovered with the capability to improve lifespan in organisms such as *Drosophila melanogaster, Caenorhabditis elegans,* and *Mus musculis*. The first of these mutations was reduction of the Insulin/Insulin-like growth factor-1 (IGF-1) signalling (IIS) pathway. Tissue specific IIS reduction is sufficient to extend lifespan, in *Drosophila* pan-neural IIS reduction improves lifespan but negatively effects locomotor behavioural senescence. Further study has shown disconnection between the effects on lifespan and healthspan. It has been hypothesised that due to the differential IIS sensitivities of individual neuronal subtypes, the overall effects of pan-neural IIS reduction on lifespan and healthspan are the summation of positive, negative, and neutral effects on each subtype. To further investigate this hypothesis, previous work has been completed which studied the lifespan and behavioural effects of IIS reduction in specific neuronal subtypes. The four subtypes tested so far are Dopaminergic, Glutamatergic, Cholinergic, GABAergic, none of which have been found to positively affect longevity or behavioural senescence. The aim of this project is to investigate the lifespan and behavioural effects of IIS reduction specifically in serotonergic neurons in *D. melanogaster* and to further understand effects of pan-neural IIS reduction on stress resistance. We found that IIS reduction in serotonergic neurons is sufficient to extend lifespan in female flies but has no effect on male lifespan, similarly to the effects of pan-neural IIS reduction. Unlike pan-neural IIS reduction, serotonergic IIS knockdown is not detrimental to exploratory walking senescence, suggesting that this method is not damaging to the neural circuitry underlying those behaviours. The results of this study also show that pan-neural IIS reduction does not affect oxidative stress resistance, constitutive IIS reduction in neurons increases starvation resistance in females, but adult-specific pan-neural IIS reduction does not affect starvation resistance.

2. Literature Review

2.1 Introduction

Life expectancy has seen a sustained increase in developed countries over the past 150 years, which shows no signs of stopping (Partridge, 2010). The Office for National Statistics claims that the life expectancy at birth was 79.4 years for males and 83.1 for females between 2017 and 2019, an increase of approximately 2 years over the previous 10 year period (*Ons.gov.uk*, 2020). Though many predictions have been made as to when this steady linear increase in life expectancy will plateau, they have been continually proved wrong as an increase of roughly 3 months per year has continued over the past 16 decades (Oeppen and Vaupel, 2002). The increase we have seen in life expectancy is a direct result of advancements made in public health such as improvements in sanitation and access to clean water, as well as biomedical advancements such as vaccines and antibiotics (Oeppen and Vaupel, 2002). For instance, the first vaccine was created by Edward Jenner in the late 18th century when he used small amounts of the mild cowpox virus to give a high level of immunity to smallpox, this discovery not only led to the eradication of smallpox two centuries later but also gave way to the development of vaccines to protect against many other transmittable and often deadly diseases such as diphtheria, polio, typhoid, measles, mumps, and rubella (Plotkin, 2005). Vaccines are directly responsible for saving the lives of many millions of people and reducing the mortality rate of many infectious diseases (Worboys, 2007). In regard to antibiotics, despite there being evidence of moulds and plant extracts being used to treat infections as far back as the ancient Egyptians, it wasn't until the late 19th century that scientists could observe the mechanisms by which these antibacterial agents operated (Adedeji, 2016). Since those first observations many antibiotics have been developed, some with specific bacterial targets and others with a broader spectrum. Despite the problems faced in the form of antibiotic resistant bacterial strains, antibiotics have been, and remain, a key tool in the battle against communicable bacterial diseases (Clardy, Fischbach and Currie, 2009). This success in the battle against infectious diseases has resulted in more people surviving long enough to become at risk of developing age-related diseases such as cancer or cardiovascular disease (Partridge, 2010).

Due to these advancements people are healthier overall today at any given age than they were 150 years ago (Partridge, 2010). Whilst these improvements to health and life expectancy are often seen as great achievements, they have revealed new

problems that must now be solved by further research and development. The larger proportion of society living to older ages presents unprecedented socioeconomic problems, the cost of pension schemes has increased, there is an increased pressure on the social care system, and economic production is at risk of falling due to a smaller proportion of the population being of prime working age (Nagarajan, Teixeira and Silva, 2016). The World Health Organisation estimates that between 2015 and 2050 the proportion of the global population over 60 will double from 12% to 25% (*Who.int*, 2020).

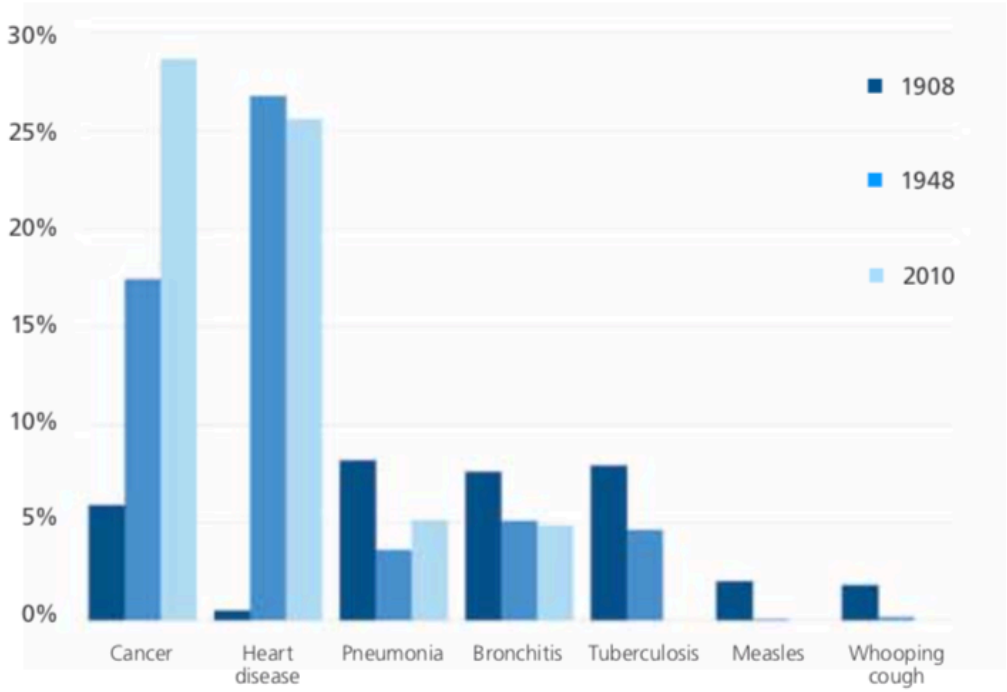


Figure 1. The most common causes of death from 1908 to 2010 in England and Wales. Between 1908 and 2010 there was a major shift in the most common causes of death, from communicable diseases such as infections and parasites to non-communicable diseases such as cancer and cardiovascular disease. This was due to the introduction of new methods for treatment and prevention such as: antibiotics, vaccines, and sanitation. Due to these interventions, life expectancy increased from under 50 to over 75 over this time period resulting in more people living to an age where they were at higher risk of developing age-related non-communicable diseases. (Thompson *et al.*, 2012)

With more people making it to older ages, a much higher proportion of the population is now at risk of developing age-related diseases. As shown in **Fig.1**, over the past 100 years there has been a seismic shift in the leading causes of death, from communicable to non-communicable diseases (Thompson *et al.*, 2012). Globally 7 of the 10 leading causes of death in 2019 were non-communicable (*Who.int*, 2020), whereas in 1908 infections such as pneumonia, tuberculosis and bronchitis were the leading causes of death (Thompson *et al.*, 2012). This is because in 1908 the life expectancy was below 50 and so people did not live long enough to be at high risk of non-communicable conditions such as cardiovascular disease, strokes, or Alzheimer's disease, all of which are now in the top 10 causes of death globally (*Who.int*, 2020). Research into the ageing process itself has been undertaken in order to further understand why and how we age, and more specifically whether any aspects of this process are modifiable to improve health at later ages and alleviate the effects of age-related diseases on the ageing population.

2.2 What is ageing?

Despite ageing being such an integral part of the human experience, we still know relatively little about how organisms age. Ageing can be defined in biological terms as the age-progressive decline in intrinsic physiological function, leading to an increase in age-specific mortality rate (Flatt, 2012). This broad definition owes its lack of specifics to the vast amounts of variability within the ageing process, not only between species and individuals, but also within even singular tissues (Kirkwood, 2002). Ageing is not something that can be easily and distinctly defined due to its broad spectrum of presentation, for this reason it is often described more simply as the accumulation of damage in tissues (Partridge, 2010).

For many years the decline in function seen during the ageing process was often thought to simply be due to the wearing out of tissues, but today ageing is more commonly understood as a genetically modifiable process as different species have wildly different lifespans (Kenyon, 2005). Evolutionary theory strongly disagrees with the notion that ageing is programmed as it argues that organisms are programmed to survive rather than die (Kirkwood, 2002). Though there is still not one universally accepted evolutionary theory of ageing, the three classical theories acknowledge that ageing and lifespan are at least partially determined by genetics. These three evolutionary theories of ageing are: Peter Medawar's mutation accumulation theory; George Williams' antagonistic pleiotropy theory; and Tom Kirkwood and Robin

Holliday's disposable soma theory (Gavrilov and Gavrilova, 2002). The mutation accumulation theory explains that harmful mutations which are only expressed later in life can accumulate in the genome. This is because the negative effects of their expression are only observed after reproduction and therefore are not selected against by natural selection, causing senescence to occur (Zwaan, 1999). The antagonistic pleiotropy theory is based on the idea that some genes which are beneficial in early life can also produce detrimental effects later in life (Kirkwood *et al.*, 1991). They are therefore selected for by natural selection due to the benefits that they give to fitness prior to and during reproduction. The disposable soma theory states that the resources of an organism are better invested in growth and reproduction (germline) rather than somatic tissue maintenance. This results in the accumulation of mutations and damage within somatic tissues leading to senescence (Shefferson, Jones and Salguero-Gómez, 2017). These theories are not mutually exclusive and it is likely that as more is uncovered about the ageing process these theories will come together to explain the full situation (Gavrilov and Gavrilova, 2002).

Because of the broad and complex nature of the ageing process, it was believed for many years that ageing and lifespan could themselves not be manipulated (Partridge, 2010). For this reason, biomedical research focused its efforts on developing treatments for these diseases individually of which ageing was a major risk factor, rather than targeting the ageing process itself (Butler *et al.*, 2008). This approach changed when single gene mutations were found with the ability to extend lifespan in the nematode worm, *C. elegans (Klass, 1983)*. The Insulin/IGF signalling pathway is the first in which single gene mutations have been found with the capability to effect the lifespan and often the healthspan of several organisms (Partridge, 2010). Research already completed studying the effects of alterations to this pathway have uncovered mechanisms of the ageing that were previously unknown (Bartke, 2011).

2.3 Ageing research in model organisms

Lifespan extension has been studied in model organisms since the 1930s (Partridge, 2010). Calorie restriction (CR: reduction of calorie intake without malnutrition) was the first intervention found to increase lifespan in laboratory rodents (McCay, Crowell and Maynard, 1935). Studies since have shown that CR is also capable of extending lifespan in other model organisms such as the nematode worm, *Caenorhabditis elegans* (Lakowski and Hekimi, 1998; Kennedy, Steffen and Kaeberlein, 2007). Later work has shown that dietary restriction (DR: reduction of dietary intake of specific

nutrients without malnutrition) of specific nutrients such as amino acids, is also capable of improving longevity in *Drosophila melanogaster* (Partridge, Piper and Mair, 2005), and several other model organisms (Partridge, 2010). Since the original discovery of these longevity altering interventions, they have been found to have similar beneficial effects on healthspan and lifespan in many different organisms, from simple yeasts to complex mammals (Mair and Dillin, 2008). Though there is a wide body of research into both DR and CR, the mechanism by which these methods affect longevity is still not entirely understood (Partridge, 2010). The mechanism by which CR results in lifespan extension is now thought to be mostly mediated by specific nutrient restriction such as essential amino acids, rather than the calorie reduction alone (Mirzaei, Suarez and Longo, 2014).

Studies of DR in rodents have shown that they benefit from a variety of health improvements such as a lesser impact of various ageing related diseases (Masoro, 2005). They also exhibit protection against cancer, motor function decline, and osteoporosis (Weindruch and Walford, 1988). DR has also been shown to ameliorate the age-related cognitive decline in function seen in many organisms via mechanisms such as the promotion of neurogenesis and increased synaptic plasticity (Hadem *et al.*, 2019). The beneficial effects on healthspan and longevity suggest that the evolutionary conserved pathways underlying DR and CR were protective mechanisms which protected against damage during times when sustenance was scarce (Mirzaei, Suarez and Longo, 2014). Studies in flies found that DR increased lifespan but simultaneously decreased fecundity (Partridge, Gems and Withers, 2005), this was thought to be due to a trade-off between the maintenance of somatic and germline cells when resources were low. However work completed since then has shown that both lifespan extension and reduced fecundity in DR flies are caused by amino acid imbalances, and it is possible to maintain lifespan extension whilst improving fecundity to levels of full feeding via the addition of methionine (Grandison, Piper and Partridge, 2009). Due to the obvious difficulty of reliably restricting the dietary intake of humans over an extended period of time, the aim of current research is to further elucidate the complex genetic pathways which mediate the DR response. This is done in order to develop therapeutic and pharmaceutical interventions safe for humans that could mimic the DR response thereby ameliorating a range of age-related pathologies (Mair and Dillin, 2008).

There are several organisms that are commonly used in ageing research, these are the budding yeast *Saccharomyces cerevisiae*, the nematode worm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster,* and the mouse *Mus musculus*

(Partridge, 2010). The highest aim of modern ageing research is to improve the health of the oldest section of the human population and decrease the effects of ageing related pathologies on the population, it is for these reasons that these model organisms are used for this research (Partridge, 2010). All of the previously mentioned organisms exhibit some form of growth before reproduction occurs, and most of them possess a fairly complex organ system, these features make them good models for ageing research, and somewhat similar to humans. The parameters of ageing in these organisms are also well understood and easily identifiable (W. Vaupel *et al.*, 2004). Due to the evolutionary conservation of many genes and their functions, studies of model organisms have improved our understanding of many different processes which also occur in humans (e.g. embryonic development) (Partridge, 2010).

Ageing research in model organisms led to the discovery that the alteration of a single gene in a metabolic pathway could affect the longevity of an organism. This was previously thought not possible due to the highly polygenic nature of the ageing process (Partridge, 2010). With the assumption that humans and the nematode worm share a common ancestor, and the knowledge that humans have a lifespan roughly 2,000 times longer than the nematode worm, it must be the case that the mutations that have occurred during the two species evolutionary diversion must have created this vast difference in longevity (Kenyon, 2010). The first single gene mutation found to have the capability of increasing longevity in model organisms was an alteration to the insulin/IGF-1 signalling (IIS) pathway in the nematode worm *Caenorhabditis elegans (C.elegans*) (Klass, 1983). This mutation decreased the activity of the gene *daf-2* which codes for an insulin-like hormone receptor (Kenyon, 2010). By reducing the activity of *daf-2*, or reducing the activity in other areas of the downstream IIS pathway, the lifespan of the worm could be doubled as well as the worms appearing much younger at older ages (Kenyon, 2005). This longevity altering mutation to the *daf-2* gene was found to be reliant on the *daf-16* gene which codes for a forkhead transcription factor (Kimura *et al.*, 1997). This research showed that in *C.elegans* ageing is at least partially regulated by hormonal activity (Kenyon, 2005). These findings in *C.elegans* showed that by altering the activity of a pathway that is responsible for the regulation of growth and metabolism in mammals, you could double lifespan in a nematode worm (Partridge, 2010). Despite it being thought that the effects of mutations of this sort were specific to worms, the pathway has since been studied in many other organisms and similar single gene mutations have been found which have the capability to affect longevity (Partridge, 2010).

The discovery of mutations to the IIS pathway with similar effects in other organisms, such as flies (Tatar *et al.*, 2001) and mice (Blüher, Kahn and Kahn, 2003) shows that the role that the IIS pathway plays in regulating lifespan is evolutionarily conserved. Other studies have revealed that Forkhead Box-O (FOXO) transcription factors are involved in the regulation of ageing in humans via insulin signalling (Martins, Lithgow and Link, 2016; Murtaza *et al.*, 2017). This means that the lifespan and healthspan improvements that have been achieved in model organisms could be transferable to humans (Partridge and Gems, 2002). The evolutionary conservation of these genes is what has allowed for the research done on nutrient signalling pathways to improve our understanding of how the ageing process is regulated in humans. It was not always thought to be the case that the results in model organisms would be transferrable to humans, but findings across several species have shown strong evidence for the evolutionary conservation of these nutrient signalling pathways, their roles in the regulation of longevity are also similar between species (Partridge, 2010).

2.4 The IIS Pathway and Ageing

The Insulin/insulin-like growth factor-1 (IGF-1) signalling (IIS) pathway shows strong evolutionary conservation from simple organisms such as *C.elegans* to more complex organisms such as the mouse (Giannakou and Partridge, 2007), though the pathway has been shown to be rather more complex in mammals than in more simple organisms (Broughton and Partridge, 2009). The IIS pathway is responsible for the regulation of several different systems in the body, an example of one such system is metabolic homeostasis (Saltiel and Kahn, 2001), the pathways role in regulating glucose homeostasis in mammals means that diabetes can occur as a detrimental effect of alterations to IIS activity (Broughton and Partridge, 2009). The IIS pathway also has regulatory roles in development, stress resistance (Giannakou and Partridge, 2007), and growth (Butler and Roith, 2001), as well as fecundity and lifespan (Flatt *et al.*, 2008).

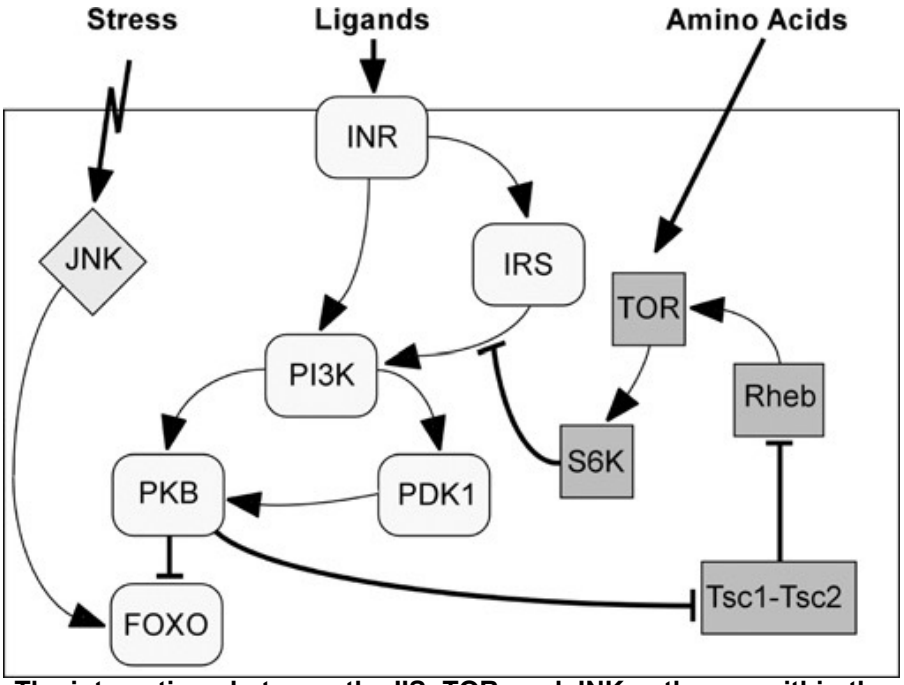


Figure 2. The interactions between the IIS, TOR, and JNK pathways within the complex signalling cascade. TOR is a nutrient sensing pathway regulated by the presence of amino acids and growth factors. TOR interacts with the IIS pathway at multiple points, when activated TOR activates S6 kinase (S6K) which inhibits activation of phosphoinositide 3 kinase (PI3K). When PKB is activated by PI3K, both TSC1 and TSC2 (tuberous sclerosis complexes 1 and 2) are inhibited which results in the further activation of TOR via a positive feedback loop. The JNK pathway responds to various environmental factors such as oxidative stress, heat, UV radiation, and DNA damage. The presence of such factors causes JNK to become activated, which in turn results in the nuclear localisation of FOXO. This is thought to occur via either the direct phosphorylation of FOXO or inhibition of IRS, though the exact mechanism is yet to be confirmed. (Broughton and Partridge, 2009)

The IIS pathway was the first pathway found to have a role in the regulation of ageing and lifespan (Klass, 1983). Since the initial discovery of its role in lifespan regulation the pathway has been studied in depth in many different organisms, this is possible because it is a highly evolutionarily conserved pathway (Barbieri *et al.*, 2003). Of all the pathways currently found to play a role in the regulation of lifespan the IIS pathway shows the strongest evidence of evolutionary conservation, although alterations to other pathways such as the TOR (target of rapamycin) and JNK (Jun amino-terminal kinase) pathways have been found to have similar beneficial effects on longevity (Wang, Bohmann and Jasper, 2003; Kapahi and Zid, 2004; Kapahi *et al.*, 2004). These effects have been shown to be reproducible across several different organisms, giving sufficient evidence that the regulatory roles both pathways play in ageing are conserved throughout evolution (Vellai *et al.*, 2003; Kapahi *et al.*, 2004; Partridge, 2010). Much like the IIS pathway, the TOR pathway is also involved in nutrient sensing, it is specifically responsible for the regulation of protein synthesis, metabolism, and growth in response to the presence of growth factors and amino acids (Wullschleger, Loewith and Hall, 2006). The JNK pathway is involved in stress response, it can be activated by the presence of cytokines or exposure to environmental stresses such as UV radiation or oxidative stress (Davis, 2000). As shown in **Fig.2**, the TOR, JNK, and IIS pathway all interact within a complex signalling network (Broughton and Partridge, 2009).

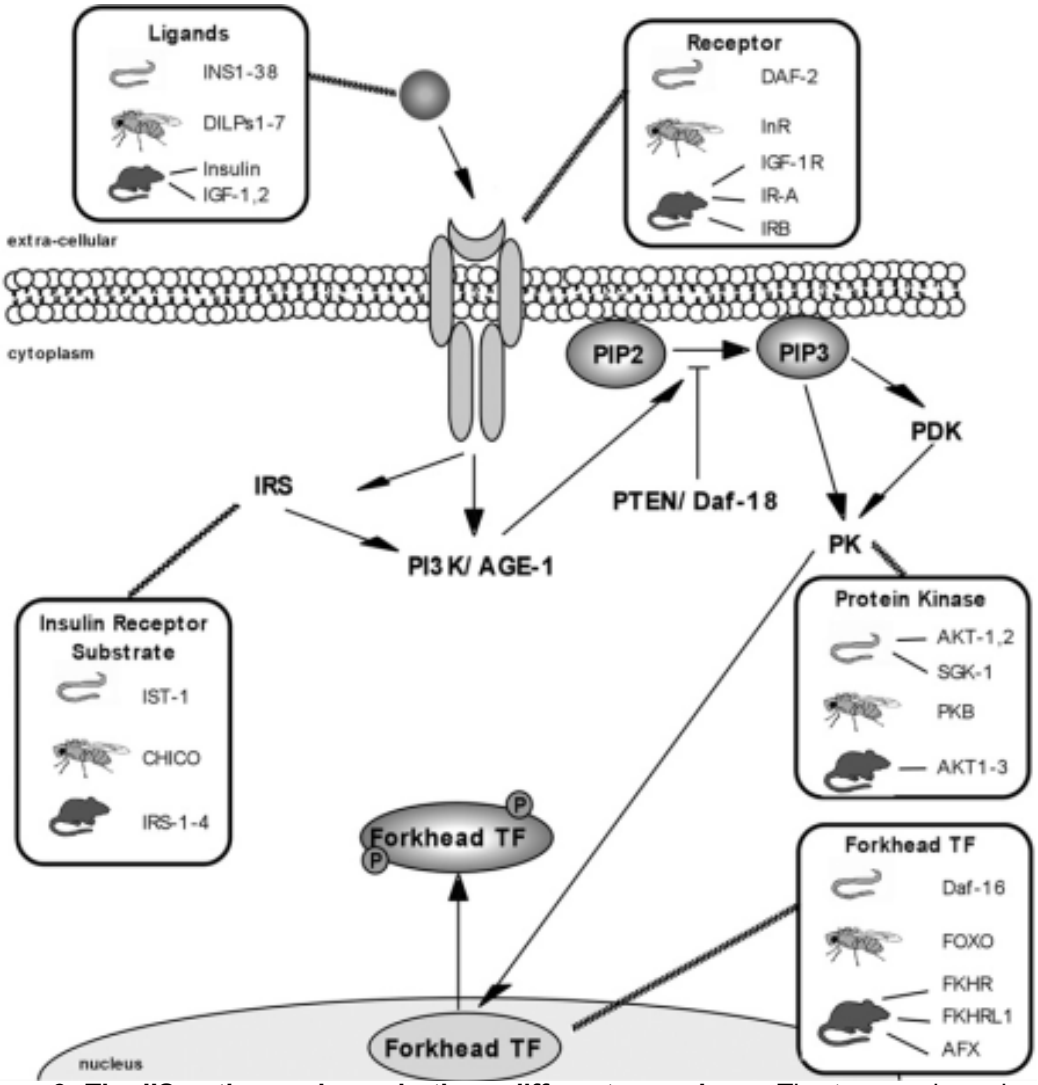


Figure 3. The IIS pathway shown in three different organisms. The type and number of ligands which bind with the receptor vary between organisms, there are 38 in worms (INS 1-38), but only 7 in flies (DILPs 1-7), and only 3 in mice (Insulin and IGF 1 and 2). There are also notable differences in receptors, worms and flies only have one (DAF-2 and InR respectively), whereas mice have 3 different receptors (IGF-1R, IR-A and IRB), which can dimerise to form a heterodimer. The receptor signal can then be transduced directly to PI3K or AGE-1 in flies and worms, or it can be transduced indirectly via an insulin receptor substrate in worms (IST-1), flies (CHICO), and mice (IRS1-4). PI3K then converts PIP_2 to PIP_3 thereby increasing levels of PIP_3 which activates PKB and PDK, PDK then further phosphorylates PKB to fully activate it. Flies have only one protein kinase (PKB) whereas worms have 3 (AKT-1 and 2, and SGK-1) and mice also have 3 (AKT1-3). Once fully activated the protein kinase then phosphorylates the forkhead transcription factor (Forkhead TF) which causes it to be excluded from the nucleus which results in its inactivation. Worms and flies have only one forkhead TF (Daf-16 and FOXO respectively), mice have 3 (FKHR, FKHRL1 and AFX). (Broughton and Partridge, 2009)

Since the discovery of the longevity influencing alterations to the IIS pathway in *C.elegans*, similar alterations have been found in the same pathway in the fruit fly (*D. melanogaster*) (Partridge *et al.*, 2011), and the mouse (*Mus musculus*) (Blüher, Kahn and Kahn, 2003). Though the individual components of the pathway differ between the organisms, the overall mechanism of the pathway as it is currently known remains relatively unchanged throughout (Broughton and Partridge, 2009), as shown in **Fig.3**. The extracellular ligand in the form of insulin or an insulin-like growth factor or peptide binds with its complementary receptor, this binding causes the receptor to become dimerised and autophosphorylated. Activation of the receptor causes the signal to be transduced directly (or indirectly via an insulin receptor substrate) to PI3K which in turn converts PIP_2 to PIP_3. The resulting increase in intracellular PIP_3 causes PKB and PDK to become activated, the activation PDK causes the phosphorylation and full activation of PKB. Fully activated PKB phosphorylates the FOXO transcription factor which means it becomes excluded from the nucleus (Broughton and Partridge, 2009).

When IIS activity is reduced, the result is that FOXO is not deactivated by phosphorylation and so does not exit the nucleus. FOXO in the nucleus is capable of upregulating the activity of pro-longevity genes resulting in an increase in lifespan (Mathew, Pal Bhadra and Bhadra, 2017). In *D. melanogaster* the forkhead transcription factor present is dFOXO (a representative of the forkhead box O family of transcription factors), it has been shown that activation of dFOXO causes an increase in longevity (Alic *et al.*, 2014). This connection between the nuclear localisation of FOXO and increased longevity is known to be evolutionarily conserved as the same connection can been seen between the human forkhead box O3A gene (FOXO3A) and longevity (Willcox *et al.*, 2008).

2.5 The tissue specific role of IIS in ageing

Although early studies altered IIS ubiquitously in order to extend lifespan, later studies have shown that modulation of IIS activity in individual tissues such as the brain, fat body or muscle is sufficient to increase longevity (Mathew, Pal Bhadra and Bhadra, 2017). To alter lifespan, individual tissues are capable of synthesising secreted factors such as insulin ligands. Once secreted from the cell these factors can either affect: the cells that the factors were secreted from (autocrine); locally to where they were released from (paracrine); or at a distance from where they were released (endocrine). When IIS activity is reduced, a tissue-autonomous response can occur. This can either

improve the overall operation of the tissue itself or lead to the production of secreted factors (Broughton and Partridge, 2009).

It is possible to extend lifespan by either downregulating positive IIS regulators such as CHICO, DILPs (*Drosophila* insulin-like peptides), or DInR, or upregulating negative IIS regulators such as dFOXO or dPTEN (Mathew, Pal Bhadra and Bhadra, 2017). Hwangbo et al. (2004) showed that activation of dFOXO specifically in the adult pericerebral fat body in *Drosophila* was sufficient to increase lifespan. This modification also resulted in reduced neuronal synthesis of DILP-2 and reduced IIS in the peripheral fat body which suggests that IIS alters longevity via a combination of cell-autonomous and non-autonomous effects (Hwangbo *et al.*, 2004). IIS reduction within adult adipose tissues via modification of FOXO activity or other elements of the IIS pathway has been shown to increase lifespan in worms, flies, and mice (Blüher, Kahn and Kahn, 2003; Libina, Berman and Kenyon, 2003; Giannakou *et al.*, 2004). This shows that the role of IIS within adult adipose tissue is evolutionarily conserved, however some studies have shown that the lifespan extension effect of dFOXO overexpression in the fat body extends female lifespan but also reduces fecundity and has no effect on male lifespan (Giannakou *et al.*, 2004). The work of Alic et al. (2014) showed that activation of dFOXO in the fat body does not require dFOXO to be present in other tissues in order to extend lifespan or ameliorate certain functional decline. This suggests that lifespan extension is not the result of non-autonomous dFOXO to dFOXO signalling across tissues, but instead is a result of dFOXO signalling to other factors in distal tissues in order to promote healthy ageing, the mechanism of which is still not fully understood (Alic *et al.*, 2014). In *Drosophila*, age-related muscle decline is associated with the accumulation of protein aggregates, dFOXO and its target 4E-BP (Binding Protein) are responsible for the removal of damaged proteins, maintenance of proteostasis, and preservation of muscle function (Demontis and Perrimon, 2010). In addition to this, increasing dFOXO expression in muscle tissue results in increased lifespan and ameliorates the age-related decline in muscle function. dFOXO activity within muscle tissue also reduces feeding and reduces DILP production, showing that the activity of dFOXO within muscle tissue is capable of regulating ageing of the whole organism as opposed to just muscle tissue (Demontis and Perrimon, 2010).

The capabilities of these tissue specific IIS modifications to improve lifespan and healthspan show that it is not a requirement to alter IIS in every tissue to achieve beneficial effects. By manipulating the correct signalling component, at the correct time, in the correct tissue, lifespan and healthspan can be improved whilst avoiding damage to the organism (Broughton and Partridge, 2009).

2.6 The IIS pathway and the CNS

The CNS is known to vary in structure and organisation in worms, flies, and mice, however the neuronal tissue plays a similar endocrine role across all of those model organisms in order to alter IIS in distant tissues (Tatar, Bartke and Antebi, 2003). IIS reduction in the CNS has been shown to be capable of extending lifespan in several animal models (Alcedo and Kenyon, 2004; Taguchi, Wartschow and White, 2007; Ismail *et al.*, 2015). It is currently thought that the method by which CNS activity affects longevity is mostly endocrine, with cell-autonomous mechanisms also being partially responsible (Broughton and Partridge, 2009; Mathew, Pal Bhadra and Bhadra, 2017; Satoh, Imai and Guarente, 2017). Although IIS reduction in the CNS is beneficial to lifespan, IIS is also known to be neuroprotective and is important for neuronal growth and survival, therefore IIS reduction could be detrimental to neuronal health (Bateman and Mcneill, 2006). In *C.elegans*, lifespan can be extended by inhibition of the function of sensory neurons, specifically some gustatory neurons are known to promote longevity, this is thought to occur via the IIS pathway (Alcedo and Kenyon, 2004). In mice, it has been shown that reduction of insulin receptor substrate-2 (IRS2) signalling specifically in the brain is sufficient to extend lifespan by up to 18%, these long-lived mice were more insulin sensitive and weighed more at later ages than their wild-type counterparts, but were also more active (Taguchi, Wartschow and White, 2007).

Ismail et al. (2015) studied the effects of pan-neural IIS reduction on lifespan and several measures of behavioural senescence in *Drosophila*, pan-neural IIS reduction was found to be sufficient to extend lifespan in female flies, but had no effect on male lifespan. Despite the beneficial lifespan effects both male and female flies with pan-neural IIS reduction exhibited an increased decline in locomotor and cognitive function with age, the detrimental effect was shown in several parameters of exploratory walking behaviour (shown in **Table.2**). Due to the detrimental effect on these behaviours, it is hypothesised that pan-neural IIS reduction is damaging to the neural circuitry underlying these behaviours (Ismail *et al.*, 2015).

The cause of age-related cognitive decline during normal ageing is not yet known, however it is thought that it is due to plasticity mechanism changes such as synapse formation and long term potentiation (LTP) (Barnes, 2011), rather than neuronal cell death such as that seen in individuals suffering from neurodegenerative diseases (Batlevi and La Spada, 2011). There is evidence from previous studies that IIS activity in the CNS is involved in the regulation of synaptogenesis and cognitive function throughout normal ageing (Martín-Peña *et al.*, 2006; Cuesto *et al.*, 2011, 2015). In *Drosophila*, the activity levels of PI3K and other components of the IIS pathway are

responsible for regulation of synaptogenesis within the CNS, this has been shown in both adult and larval neurons (Oldham and Hafen, 2003; Martín-Peña *et al.*, 2006). Overexpression of PI3K is sufficient to induce the formation of functional synapses in adult neurons which are capable of eliciting behavioural changes, continual PI3K activity is also required for synaptic maintenance (Martín-Peña *et al.*, 2006). Genome-wide gene expression studies across many different species have revealed several conserved functional changes throughout ageing such as reduced mitochondrial function and increased expression of stress resistance genes which are thought to be part of a neuroprotective mechanism (Miller, Oldham and Geschwind, 2008; Yankner, Lu and Loerch, 2008; Bishop, Lu and Yankner, 2010). Understanding how modifications to IIS activity exacerbate normal cognitive decline and what mechanisms are involved, will help us to understand what causes normal cognitive decline and therefore find ways to ameliorate its effects.

2.7 Drosophila based ageing research

The fruit fly *Drosophila melanogaster* has been a vital research tool for the past 100 years in a variety of different areas of scientific research (Piper and Partridge, 2018). Qualities such as being cheap and easy to maintain in large populations, as well as possessing a complex organ structure have made them the perfect model organism, especially in the fields of genetics and development (Piper and Partridge, 2018). In order to further elucidate the complex genetic functions underlying physical and neurological behaviours, a vast array of species specific genetic and molecular tools have been developed (Beckingham *et al.*, 2007; Piper and Partridge, 2018). The work already completed using flies has been vital in helping us develop a further understanding of the neural pathways and functions responsible for the control of many complex behaviours that they perform (Busto, Cervantes-Sandoval and Davis, 2010).

The fruit fly, along with several other model organisms, has been especially important in the field of ageing research. Drosophila have a relatively short lifespan compared to humans and unlike humans there are little to no ethical concerns regarding their use in scientific research. Despite these differences between flies and humans, the ageing related pathways that can be studied in flies show strong evidence of evolutionary conservation and are present with similar roles in humans (Martins, Lithgow and Link, 2016; Murtaza *et al.*, 2017). This means that the findings of fly-based research can be transferrable to humans, making the insight into the ageing process that we can achieve through the use of flies invaluable (Iliadi, Knight and Boulianne, 2012).

The progression of the ageing process often presents as a decline in the function of an organism over time, in *Drosophila* there are numerous observable and measurable markers of this age-related physiological decline (Piper and Partridge, 2018). Examples of these such markers include: metabolic changes such as reduced synthesis of fats and proteins; reduced fecundity; behavioural changes such as reductions in exploratory walking activity and courtship behaviours; decline in cognitive ability; and impaired locomotor functions (Piper and Partridge, 2018). These markers are important as they allow for the effects of lifespan altering modifications on health and the decline of health and function throughout the ageing process, they also allow us to understand whether any of these individual modifications could be utilised as a method by which to slow the ageing process.

Drosophila possess many homologues of mammalian tissues (Piper and Partridge, 2018), flies possess a heart which shares both functional and developmental homologies with vertebrate hearts (Piazza and Wessells, 2011). The *Drosophila* brain contains more than 100,000 neurons which form circuitry and neuropil in order to mediate many complex behaviours such as learning and memory, sleep, and feeding, not unlike more complex mammalian organisms (Pandey and Nichols, 2011). The study of learning and memory in flies has allowed for them to be used as a valuable model for human neurodegenerative diseases and the associated genetic pathways (Bonini and Fortini, 2003; Ali *et al.*, 2011). Several different assays are commonly used to quantify learning and memory in flies, these often make use of behavioural conditioning techniques (Ali *et al.*, 2011). This work has allowed for several parts of the fly CNS (e.g. mushroom bodies) and certain genes to be linked to learning and memory in *Drosophila* (Kahsai and Zars, 2011). Flies have also been suggested as a model for studying emotional behaviours such as anxiety (Iliadi, 2009). When flies are placed into an novel enclosed environment, they spend more time exploring the outer walls, this has been proposed as an anxiety related behaviour (Iliadi, 2009; Mohammad *et al.*, 2016). Similarly to rodent models, when flies are treated with the anxiety reducing drug Diazepam, wall following behaviour is reduced (Mohammad *et al.*, 2016).

The endocrine tissues present in flies are also comparable to their mammalian counterparts, which makes them a useful tool for the study of endocrine regulation of ageing (Broughton and Partridge, 2009). The IPCs (Insulin producing cells) present in the pars intercerebralis of the fly brain are responsible for the production of several DILPs, these cells can be considered homologous to the β-cells of the mammalian pancreas which also produce insulin-like peptides in order to regulate metabolic homeostasis (Broughton and Partridge, 2009). Genetic removal of IPCs has been

shown to increase lifespan (Broughton *et al.*, 2005), and several other studies have provided further evidence of the endocrine role that DILP production plays in the regulation of lifespan (Broughton *et al.*, 2010; Kannan and Fridell, 2013).

2.8 The GAL4-UAS system for spatial and temporal control of genetic alterations

In order to reduce IIS within specific tissues in *Drosophila* a method was needed with the capability to give spatial and possibly also temporal control over gene expression. The GAL4-UAS system is a method of genetic alteration which allows for spatial control of target gene expression to be achieved in *Drosophila* (Phelps and Brand, 1998).

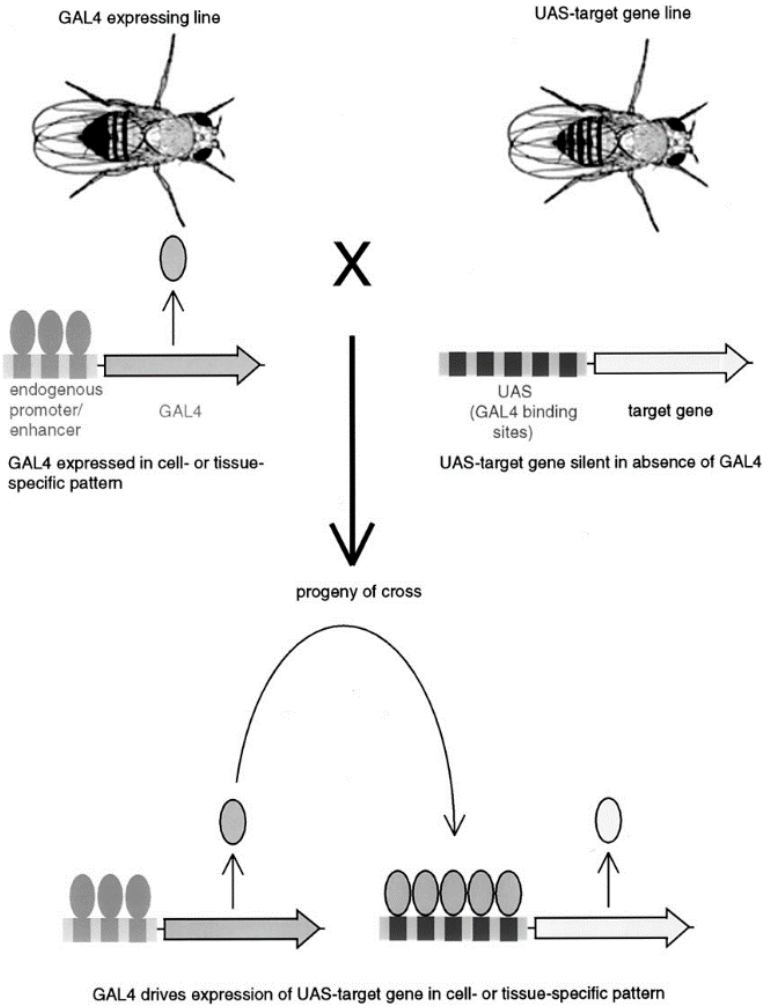


Figure 4. The GAL4-UAS system mechanism. The target gene is only expressed in an individual when GAL4 is produced and able to bind to the UAS binding site (upstream of the target gene sequence) leading to the expression of the target gene. In the individual lines of transgenic flies used for the cross, the target gene will not be expressed as one line only possesses the GAL4 transcription factor, and the other only possesses the target gene downstream of the UAS binding site which is silent without the presence of GAL4. (Phelps and Brand, 1998)

The GAL4 transcription factor was originally found in yeast and is not naturally present within the *Drosophila* genome, in order to direct gene expression GAL4 binds with the GAL4 binding site produced by the upstream activation sequence (UAS) (Fischer *et al.*, 1988; Brand and Perrimon, 1993; Nicholson *et al.*, 2008). The binding of GAL4 to the UAS results in the expression of the gene directly downstream of the UAS (Phelps and Brand, 1998). As shown in **Fig.4**, two independent transgenic lines are used, one of which possesses the GAL4 transcription factor, the other line contains the UAS and cloned target gene which is not expressed without GAL4 present to bind to the UAS binding site (Phelps and Brand, 1998). These two transgenic lines are crossed to produce a progeny which expresses the GAL4, UAS, and target gene, resulting in the GAL4 binding to the UAS and directing expression of the target gene immediately downstream of the UAS site. Spatial control of this system is achieved by using promotor regions in the GAL4 line, an example of this is the ELAV (embryonic lethal abnormal visual system) gene which is exclusively expressed in *Drosophila* neuronal cells and so can be used as a promotor region to direct GAL4 expression exclusively in neurons (Koushika, Lisbin and White, 1996).

To achieve temporal control over the expression of the target gene, an RU486 (mifepristone) dependent version of the GAL4 yeast transcription factor (GeneSwitch) is used (Osterwalder *et al.*, 2001). Without the presence of RU486, GAL4 will not be produced and so the target gene will not be expressed. Expression is dependent on the chimeric gene which encodes for the GAL4 DNA-binding domain; the human progesterone receptor-ligand-binding domain; and the activation domain of p65 (human protein) (Roman *et al.*, 2001). Due to the chimeric gene the GAL4 DNA-binding domain is only capable of binding to the UAS binding site in the presence of RU486, resulting in ligand-inducible transactivation of the target gene downstream of the UAS sequence (Roman *et al.*, 2001). Use of this system means that the addition of RU486 to the diet of the transgenic flies can induce the expression of the target gene at any point throughout their lifespan.

These methods can be used to achieve temporal and spatial control of IIS reduction within the *Drosophila* CNS. The target gene used by Ismail et al. (2015) and also used in this current study encodes for a dominant-negative insulin-like receptor, which when expressed in cells results in a reduction in IIS. GAL4 transgenes with different promotors can be used to enable expression of the mutant insulin-like receptor either in specific neuronal sub-types (e.g. serotonergic – TrhGAL4), or in all neuronal cells (ElavGAL4). The GeneSwitch method (RU486-dependent GAL4) can be used in order for the effects of both adult-specific and developmental IIS reduction to be studied.

2.9 Preliminary Data – work leading up to this project

In order to investigate the role of IIS in the regulation of lifespan and healthspan, Ismail et al. (2015) studied the effects of ubiquitous and neuron specific IIS reduction on lifespan, negative geotaxis, and exploratory walking in *Drosophila.* Two methods were used to achieve systemic IIS reduction: (1) d2GAL4/UAS-rpr, IPCs in the brain are ablated to decrease production of DILPs. (2) daGAL4/UAS-InR^{DN}, the daughterless GAL4 driver is used in order to direct ubiquitous expression of a dominant negative insulin receptor (InR^{DN}). Neuron specific IIS knockdown was achieved by using the pan-neural (elavGAL4) driver to direct neuron specific expression of a dominant negative insulin receptor (InR^{DN}). Dravecz (2020) continued this work by studying the effects of pan-neural and also neuronal subtype specific IIS reduction on lifespan and several measures of age-related behavioural decline. The effects of both constitutive and adult-specific IIS knockdown in neurons were studied via use of the pan-neural (elavGAL4) driver and the RU486 dependent Gene-Switch (elavGS) driver to allow for IIS to be reduced in adult neurons. The effects of neuronal subtype specific IIS reduction were studied using four subtype specific GAL4 drivers (ThGAL4-Dopaminergic, VglutGAL4-Glutamatergic, ChATGAL4-Cholinergic, Gad1GAL4-GABAergic) in order to direct expression of InR^{DN}.

The results of Ismail et al, (2015) show that ablation of IPCs in the brain is sufficient to extend lifespan in males and females, whereas ubiquitous expression of InR^{DN} only improved lifespan in females and did not affect male lifespan. Neuron specific expression of InR^{DN} also only increased female lifespan and did not alter male lifespan, this was repeated by Dravecz (2020) with the same conclusions. The results of Dravecz (2020) also show that reduction of IIS in adult neurons improved female lifespan but possibly reduced male lifespan. Of the four neuronal subtypes tested by Dravecz (2020) for subtype specific IIS reduction, all but GABAergergic neurons (which did not alter lifespan in males or females) reduced lifespan in both males and females.

Table 1: Summary of the effects of ubiquitous (Ismail *et al.*, 2015)**, pan-neural** (Ismail *et al.*, 2015; Dravecz, 2020)**, and neuronal subtype specific (Dravecz, 2020) IIS reduction on lifespan in *Drosophila*. (Table taken from Dravecz, 2020)**

	Genotype		Gender	Effect on lifespan
Full body	d2GAL4/UAS-rpr (Ismail, et. al. 2015)	Ablation of insulin producing cells in brain	Male	Extended
			Female	Extended
	daGAL4/UAS-InR^{DN} (Ismail, et. al. 2015)	Ubiquitous expression of InR^{DN}	Male	Normal
			Female	Extended
Pan-neural	elavGAL4/ UAS-InR^{DN} (Ismail, et. al. 2015)	Neuron specific expression of InR^{DN}	Male	Normal
			Female	Extended
	elavGAL4/ UAS-InR^{DN} (Dravecz, 2020)	Neuron specific expression of InR^{DN}	Male	Normal
			Female	Extended
	elavGS/ UAS-InR^{DN} (Dravecz, 2020)	Neuron specific expression of InR^{DN} in adult flies	Male	Slightly reduced?
			Female	Extended
Neuronal subtype specific	ThGAL4/ UAS-InR^{DN} (Dravecz, 2020)	Expression of InR^{DN} in dopaminergic neurons	Male	Reduced
			Female	Reduced
	VglutGAL4/ UAS-InR^{DN} (Dravecz, 2020)	Expression of InR^{DN} in glutamatergic neurons	Male	Reduced
			Female	Reduced
	ChATGAL4/ UAS-InR^{DN} (Dravecz, 2020)	Expression of InR^{DN} in cholinergic neurons	Male	Reduced
			Female	Reduced?
	Gad1GAL4/ UAS-InR^{DN} (Dravecz, 2020)	Expression of InR^{DN} in GABAergic neurons	Male	Normal
			Female	Normal

Ismail et al. (2015) showed that ablation of IPCs in the fly brain resulted in improved negative geotaxis at later ages in both males and females but had no effect on parameters of exploratory walking, suggesting that the improved negative geotaxis was due to peripheral changes rather than improvements in brain ageing (Ismail *et al.*, 2015). Ubiquitous expression of InR^{DN} improved negative geotaxis in females but had no effect on male negative geotaxis, this method also improved several parameters of exploratory walking in females but did not affect male exploratory walking behaviour. The results of Ismail et al. (2015) also show that pan-neural constitutive InR^{DN} expression does not affect negative geotaxis ability in males or females but detrimentally effected performance in several parameters of exploratory walking in both males and females. Overall, the results of Ismail et al. (2015) show that despite the beneficial lifespan effects of pan-neural IIS reduction, it is detrimental to exploratory walking behavioural senescence suggesting it is damaging to the neural circuitry underlying exploratory walking behaviour.

The results of Dravecz (2020) show that IIS knockdown in adult neurons does not affect negative geotaxis in males or females but was detrimental to performance in several parameters of exploratory walking behaviour in females. Adult IIS knockdown was not detrimental to exploratory walking behaviour in males, however this result may be due to masking effects of the inducing drug, RU486. Of the four neuronal subtypes tested by Dravecz (2020), IIS reduction in all but cholinergic neurons (which was detrimental to negative geotaxis behaviour in males and females) resulted in no change to negative geotaxis behaviour. IIS reduction in all but cholinergic neurons (which was detrimental as a young age in males but had no effect in females) had no effect on exploratory walking behaviour.

The results of these two studies show that whilst IIS reduction is beneficial to longevity, it can be detrimental to certain areas of healthspan. In the CNS, IIS reduction is capable of extending lifespan but is also thought to be detrimental to the neuronal circuitry underlying certain behaviours. These results also show further evidence of the possible disconnection between effects of IIS reduction on lifespan and healthspan, however the cause of this is still not known as the exact role of IIS in the CNS is yet to be fully elucidated.

Table 2: Summary of the effects of ubiquitous (Ismail *et al.*, 2015)**, pan-neural** (Ismail *et al.*, 2015; Dravecz, 2020)**, and neuronal subtype specific (Dravecz, 2020) IIS reduction on negative geotaxis and exploratory walking in *Drosophila*. (Table taken from Dravecz, 2020)**

	Genotype	Gender	Negative geotaxis	Exploratory walking parameters
Full body	d2GAL4/UAS-rpr (Ismail, et. al. 2015)	Male	Positive	No effect
		Female	Positive	No effect
	daGAL4/UAS-InR^{DN} (Ismail, et. al. 2015)	Male	No effect	No effect
		Female	Positive	Positive (total distance, velocity)
Pan-neural	elavGAL4/ UAS-InR^{DN} (Ismail, et. al. 2015)	Male	No effect	Detrimental (total distance, velocity, walking duration, rotation frequency)
		Female	No effect	Detrimental (total distance, velocity, rotation frequency)
	elavGS/ UAS-InR^{DN} (Dravecz, 2020)	Male	No effect	No effect (masked by RU486?)
		Female	No effect	Detrimental (total distance, velocity, walking duration, rotation frequency)
Neuronal subtype specific	ThGAL4/ UAS-InR^{DN} (Dravecz, 2020)	Male	No effect	No effect
		Female	No effect	No effect
	VglutGAL4/ UAS-InR^{DN} (Dravecz, 2020)	Male	No effect	No effect
		Female	No effect	No effect
	ChATGAL4/ UAS-InR^{DN} (Dravecz, 2020)	Male	Detrimental	Detrimental at young age
		Female	Detrimental	No effect
	Gad1GAL4/ UAS-InR^{DN} (Dravecz, 2020)	Male	No effect	No effect
		Female	No effect	No effect

2.10 Project Aims and Objectives

The aim of this project is to further our current understanding of the effects of neuronal IIS reduction on stress resistance, lifespan, and age-related decline in cognitive and locomotor function, in *D. melanogaster*. The results of this project will help to further elucidate the mechanisms by which IIS reduction in the CNS can impact lifespan and healthspan in the *Drosophila* model. The objectives are as follows:

I. To determine whether IIS reduction, specifically in serotonergic neurons has any effect on lifespan or the age-related decline in cognitive and locomotor function.
II. To backcross an octopaminergic GAL4 driver stock into the w^{Dah} background to enable future studies to investigate the role of IIS in octopaminergic neurons.
III. To show how adult-specific and constitutive pan-neural IIS reduction impacts resistance to starvation and oxidative stresses.

2.11 Research Design

Based on the results of previous studies it is believed that despite the beneficial effects on lifespan. Reduced IIS is detrimental to neuronal circuitry underlying certain locomotor behaviours (Ismail *et al.*, 2015). It has been hypothesised that due to the differential IIS sensitivities and roles of individual neuronal subtypes, the overall effects of pan-neural IIS reduction on lifespan and healthspan are the summation of positive, negative, and neutral effects on each neuronal subtype (Dravecz, 2020). To investigate the effects of IIS reduction in serotonergic neurons, the TrhGAL4 serotonergic driver (Alekseyenko, Lee and Kravitz, 2010) was used in conjunction with the UAS-InR^{DN} transgene (Ismail *et al.*, 2015). The effects of this IIS reduction on lifespan were measured by regularly recording the survival of the flies. Any effects on locomotor and cognitive senescence were studied by observing the exploratory walking behaviour of the flies at regular points throughout the lifespan. The observation of this behaviour was completed by placing the flies into a novel environment and analysing a variety of behavioural parameters in order to measure the decline in cognitive and locomotor function. All of these experiments included several control groups (TrhGAL4/+ and UAS-InR^{DN}/+), the results of which were used to determine any significant effect of the IIS reduction. To study the effects of constitutive IIS reduction in neurons, the elavGAL4 pan-neuronal promoter (Ismail *et al.*, 2015) was used in conjunction with the UAS-InR^{DN} transgene (Ismail *et al.*, 2015). To investigate the effects of IIS reduction in adult

neurons, the RU486 inducible GeneSwitch system (elavGS/UAS-InR^{DN}) was used to allow for temporal control of expression (Bauer *et al.*, 2005). To determine the effects of these methods of IIS reduction on starvation and oxidative stress resistance, flies were fed food containing either no sugar (starvation) or H_2O_2 (oxidative), and the survival of the flies was recorded at regular intervals throughout their lifespan.

3. Materials and Methods

3.1 Fly Husbandry and Stock Maintenance.

Fly stocks were stored at 25°C and 70% relative humidity in a 12hr light/dark cycle, experiments also took place in these conditions. Flies were moved to new media every 3-4 days to avoid bacterial growth or large amounts of eggs gathering. The white^{Dahomey} (w^{Dah}) stock were used as the wild-type controls for experiments, this stock was originally created in the Partridge Lab by backcrossing a partial deletion mutation in the white gene (w^{1118}) with the wild-type Dahomey strain to produce flies with white eyes (Ziehm, Piper and Thornton, 2013). The UAS-GAL4 method was used to allow for spatial and temporal control over the reduction of IIS. A transgenic line was used that possessed the upstream activator sequence (UAS) and a mutant insulin like receptor (InR^{DN}). The UAS-InR^{DN} transgene expresses a mutated *Drosophila* insulin receptor which includes an amino acid substitution in the R1409A kinase domain, as described in (Ikeya *et al.*, 2009). The GAL4 stock used for the constitutive IIS reduction experiments (starvation and oxidative stress resistance) possessed the ELAV (embryonic lethal abnormal visual system) promotor (elavGAL4) which specifies for all neuronal cells. The GAL4 stock used for adult-specific IIS reduction experiments possessed an RU486 dependent version of the GAL4 transcription factor, allowing for temporal control over IIS reduction in the CNS via the addition of RU486 to fly media, this is referred to as a GeneSwitch System (elavGS) (Osterwalder *et al.*, 2001). The GAL4 stock used for the serotonergic neuron specific IIS reduction experiments possessed a promotor which specifies for serotonergic neurons (TrhGAL4) (Alekseyenko, Lee and Kravitz, 2010), these were backcrossed with the w^{Dah} wild-type stock to ensure a similar genetic background. The GAL4 stock used for IIS reduction specifically in octopaminergic neurons contained a promotor which specifies for octopaminergic neurons (Tdc2GAL4). GAL4/GS stocks were crossed with the UAS-InR^{DN} stock in order to produce progeny with reduced IIS either pan-neurally or in specific neural sub-types (octopaminergic/serotonergic).

All fly stocks were kept in disposable plastic bottles with sponge bungs and fed using standard media (Bass *et al.*, 2007). They were stored in the lab at room temperature with natural light, as opposed to the experimental flies which were maintained at 25°C and 70% relative humidity in a 12hr light/dark cycle. The stock flies were swapped into new bottles with new media roughly every three weeks to allow enough time for mating to occur between generations but to also avoid mould growth. When swapped to new

bottles the wild-type w^{Dah} stocks were mixed between bottles to ensure no genetic bottlenecks occurred.

Table 3. The transgenic fly lines used during experiments. The stock numbers given refer to the Bloomington Stock Centre.

Stock	Abbreviation	Stock Number	Reference
Dominant negative insulin receptor	UAS-InR^{DN}	15635	(Ismail *et al.*, 2015)
Membrane bound GFP	UAS-GFPCD8	5130	(Kim *et al.*, 2012)
Cytoplasmic bound GFP	UAS-GFPS65T	1521	(Sun, Xu and Salvaterra, 1999)
Serotonergic	TrhGAL4	38389	(Alekseyenko, Lee and Kravitz, 2010)
Octopaminergic	Tdc2GAL4	9313	(Zhou, Rao and Rao, 2008)
Pan-neuronal promotor	elavGAL4	25750	(Ismail *et al.*, 2015)
Daughterless (ubiquitous)	daGAL4	13991	(Ismail *et al.*, 2015)
Pan-neuronal GeneSwitch	elavGS	N/A	(Bauer *et al.*, 2005)

3.2 Genetic Backcrossing Protocol (serotonergic and octopaminergic)

Male homozygotes of the serotonergic driver genotype (38389 – TrhGAL4) were crossed with w^{Dah} virgin females because genetic recombination only occurs in females in *Drosophila melanogaster*. The virgin female heterozygous offspring from this cross were then crossed with w^{Dah} males. This cross was then repeated a further 4 times. Orange eyed (heterozygous) virgin females and males were collected from the final cross and crossed to obtain red eyed (homozygous) offspring to be used to build a stock. All of the crosses were done in separate vials and mixed in between crosses to avoid inbreeding and genetic bottlenecks, and to maintain genetic variability. This backcrossing procedure was also performed for the octopaminergic driver genotype (9313 – Tdc2GAL4), although this driver was not used in further experiments due to time restraints resulting from the Covid-19 pandemic.

3.3 Genetic Validation

GFP Expression

Validation of the expression pattern of the serotonergic driver after backcrossing was completed by crossing the backcrossed TrhGAL4 stock with a UAS-GFPS65T stock (1521 – cytoplasmic bound Green Fluorescent Protein). Male and female brains of the resulting TrhGAL4/UAS-GFPS65T progeny were dissected (7-10 of each), they were knocked out using CO_2 and placed into ethanol to remove the waxy cuticle before being dissected in PBS whilst still attached to the bodies. The brains were kept in PBS on ice before being put through a glycerol series (10% - 20% - 40% - 60%), the brains were then moved into 80% glycerol in PBS with 2% n-propylgallate, covered, and stored in the fridge in order to maintain fluorescence. The brains were removed from the bodies and placed onto microscope slides in glycerol to be analysed by confocal microscopy. Analysis of the expression pattern was performed using Zeiss LSM 880 and Zen 2.3 SP1 Software. ImageJ was then used for comparison with the distribution of serotonergic neurons in the fly brain in current literature.

The backcrossed TrhGAL4 line was also crossed separately with a UAS-GFPCD8 stock (5130 – membrane bound Green Fluorescent Protein). Male and female brains of the resulting TrhGAL4/UAS-GFPCD8 progeny were also dissected in the same way (7-10 of each) but were fixed using a different protocol. The brains first spent 15 minutes in 4% PFA at room temperature with gentle shaking whilst being protected from light to avoid any degradation of the fluorescence. The brains were then moved through three separate ten minute washes in PBS, before going through three ten

minute washes in TNT (which contains a light detergent to slightly permeabilise the membrane). The brains then once again went through three ten minute washes in PBS, before being held in 80% glycerol in PBS with 2% n-propylgallate, covered, and stored in the fridge in order to maintain fluorescence. The brains were then analysed by confocal microscopy in the same way as the TrhGAL4/UAS-GFPS65T brains, and the expression pattern was compared with that already recorded in current literature to confirm that the serotonergic driver was functioning as expected.

Analysis of Growth

The UAS-InR^{DN} (Dominant Negative Insulin-like Receptor) stock was crossed with the daGAL4 (ubiquitously expressed driver - daughterless) stock, 20 of each of the male and female daGAL4/UAS-InR^{DN} progeny of the cross were weighed and compared with daGAL4/+ wildtype flies. This was done in order to validate the genetic functionality of the UAS-InR^{DN} stock, which in conjunction with the ubiquitously expressed daughterless GAL4 driver should result in developmental restrictions because of ubiquitously expressed constitutive reduced insulin signalling (Ikeya *et al.*, 2009).

3.4 Fly Media Preparation

In order to make 1L of the standard fly media, 15g of agar powder was mixed with 700ml of water and brought to the boil in order to ensure all of the agar was melted. Once boiled, 50g of sucrose and 100g of brewer's yeast powder were added to the mixture, which was then brought to the boil again whilst stirring before being taken off the heat to cool. To help the mixture cool, a further 170ml of cold water was added. Once the mixture reached the temperature of 60°C, 30ml of nipagin (a 10% solution in 100% ethanol) and 3ml of propionic acid were added to inhibit mould growth. The food mixture was then poured into the appropriate containers before being allowed to cool fully and set (Bass *et al.*, 2007).

In order to make 1L of the 200mM RU486 food the preparation was identical to the standard media however after the anti-mould agents were added, 85.9mg of mifepristone/RU486 (dissolved in 5ml of ethanol) were added to the media mixture. In experiments where the RU486 was used, 5ml of ethanol was also added to the standard media used.

To make 1L of the grape juice media, 1L of grape juice was brought to the boil and 16g of agar powder was added in order to melt the agar into the mixture. The mixture was then poured into the appropriate containers and allowed to cool fully to set.

The procedure to make the starvation food was identical to the standard food procedure without any yeast or sugar being added. When making the H_2O_2 food, no yeast or anti-mould agents were used, and the food mixture was allowed to cool to 60°C before 33.3ml of 30% H_2O_2 solution was added. The full recipes for the different fly media used are shown in **Table.4**.

Table 4. Fly media recipes.

Ingredients\Diet	Standard Food	RU486 Food	Grape Juice Plates	Starvation Food	H_2O_2 Food
Water (ml)	700	700	0	1000	160
Grape Juice (ml)	0	0	1000	0	0
Agar (g)	15	15	16	15	3
Sucrose (g)	50	50	0	0	10
Yeast (g)	100	100	0	0	0
Water at the end (ml)	170	170	0	0	6.7
Nipagin – 10% in 100% Ethanol (ml)	30	30	0	30	0
Propionic Acid (ml)	3	3	0	3	0
100% Ethanol (ml)	0	5	0	0	0
RU486 (mg)	0	85.9	0	0	0
30% H_2O_2 (ml)	0	0	0	0	33.3

3.5 Genetic Crosses

Roughly 50 male and 50 virgin female flies were collected of the appropriate genotypes for the cross. These flies were then placed together in a cage with the grape juice-based food in a petri-dish covered with a yeast paste (to stimulate egg laying). The flies then proceeded to mate and lay fertilised eggs on the grape juice food. After 24 hours of laying, the eggs were collected by squirting PBS (phosphate buffer solution) onto the food and using a small brush to agitate the eggs from the surface of the food. The PBS containing the eggs was then poured off the surface of the food and into a falcon tube, the eggs were left to settle to the bottom of the tube. A micropipette set to 120µl was used with an enlarged tip to collect the eggs in PBS from the bottom of the tube, which were then put into a food bottle in which the eggs then developed.

3.6 Lifespan Analysis

Flies aged 3-4 days were sorted by genotype and sex under CO2 (N=100). Flies were kept at 10 per vial and were swapped onto fresh standard food media every 3-4 days. The number of dead flies in each vial were counted and recorded each time the flies were moved onto fresh media. The data collected from the survival experiments were expressed as the proportion of flies still alive against time.

3.7 Exploratory Walking Behavioural Analysis

At various timepoints throughout their lifespans (every 10-14 days), flies of each genotype (N=16) were individually aspirated into 40mm diameter circular arenas which were 10mm high, and on a base of 2% agar gel. They were filmed in this novel environment for 15 minutes. These experiments were started at the same time of day for each timepoint to avoid any circadian effect, due to the nature of exploratory walking different flies were used at each timepoint. Due to the length of time required to film the behaviour, females were filmed on separate days to males. EthoVision XT video tracking software (Noldus Information Technology, Wageningen, The Netherlands) was then used to analyse parameters of the exploratory walking behaviour. The parameters were as follows: The total distance walked by a fly during the 15 minute observation period; Velocity; The frequency of rotation (changes in walking direction); The amount of time elapsed before the first rotation; The amount of time a fly spent moving during the 15 minute observation period; The duration of time a fly spent exploring the central zone of the arena during the 15 minute observation period; The

frequency of times a fly explored the central zone of the arena; The duration of individual visits to the central zone of the arena. Analysis data from EthoVision were exported to Microsoft Excel.

3.8 Starvation and Oxidative Stress Resistance.

Flies of each genotype were separated by sex and sorted into vials. For starvation stress resistance assays N=110 per genotype for each sex, these were kept at 10 per vial. For oxidative stress assays N=105 per genotype for each sex, these were kept at 15 per vial. The GeneSwitch (elavGS) flies were kept on standard food until they were 4 days old, they were then split onto standard or RU486 food. At 10 days old the flies were moved onto 5% H_2O_2 food or starvation food (agar media with no sugar, yeast, or RU486). RU486 was not included in the starvation media as the experiment was testing the effect of previous treatment with the drug. The number of flies that had died were counted twice a day for 6-10 days (until all were dead). The data collected from these survival experiments were expressed as the proportion of flies still alive against the time on the food type.

3.9 Statistical Analysis

JMP (version 14 student edition) statistical analysis software (SAS Institute Inc, Cary, NC, USA) was used for all data analysis completed during this project. The software was used to verify the distribution of the data collected, and also to complete several different statistical tests to determine any statistically significant effects of experimental genotypes. For comparison related tests, a p value of <0.05 was used to establish statistical significance. Analysis of lifespan data was completed by survival analysis using Log-Rank tests in JMP statistical analysis software. Exploratory walking data parameters were tested for normality using the Shapiro Wilk W test on studentised residuals (Sokal and Rohlf, 1995). Data were then analysed using a two-way ANOVA with genotype and age as the main effects. This was followed by a planned pairwise comparison (Tukey-Kramer HSD) for each age timepoint, and for each parameter measured of the exploratory walking using EthoVision XT video tracking software (Noldus Information Technology, Wageningen, The Netherlands). Weight data were tested for normality, a one-way ANOVA was performed with genotype as the main effect. Planned pairwise comparisons were performed using Tukey-Kramer HSD.

4. Results – Backcrossing and Transgenes Validation.

4.1 Introduction

Backcrossing of the serotonergic (TrhGAL4) and octopaminergic (Tdc2GAL4) stocks was completed to ensure that the genetic background of the experimental flies was as similar to that of the controls as possible to make sure that any differences seen could reasonably be assumed to be due to the reduced IIS and not due to any differences within their genetic backgrounds. Differences in genetic backgrounds can effect lifespan and functional senescence (Grotewiel *et al.*, 2005), so it is very important that all mutant stocks to be used in experiments were first backcrossed with the wild-type stock to be used as the controls. The elavGS, UAS-InR^{DN}, elavGAL4, and daGAL4 stocks had all been backcrossed with the w^{Dah} (wild-type) stock previously in our lab. In this study, the serotonergic (TrhGAL4) and octopaminergic (Tdc2GAL4) transgenic lines were obtained and backcrossed to the w^{Dah} genetic background.

When the new stocks entered the lab, they were first placed into a period of quarantine in order to ensure they did not carry mites which could be passed onto other stocks within the lab and damage them. Once the period of quarantine was complete the backcrossing began, the flies were crossed with the w^{Dah} stock (wild-type) which was developed in the Partridge lab. The w^{Dah} stock were obtained by backcrossing w^{1118} into an outbred stock collected in Dahomey in 1970, and have been maintained in large outbred populations since (Partridge and Farquhar, 1983; Broughton *et al.*, 2005). This stock was chosen due to their genetic background being extremely well documented and their white eye colour allows for the red eye colour of the serotonergic/octopaminergic driver stocks to be used as a genetic marker throughout the backcrossing, to show that the driver is still present in the offspring.

Backcrossing was completed by first crossing male homozygotes of the serotonergic/octopaminergic driver genotype with virgin w^{Dah} females. Virgin female heterozygous progeny of this cross were then crossed with w^{Dah} males, this cross was repeated with the progeny of the previous cross a further 4 times. From the final cross, heterozygous males and heterozygous virgin females were collected and crossed, the homozygous progeny from this cross were then collected to produce a stock. These stocks then needed to be validated to confirm the transgenes were correctly expressed before they could be used for experiments.

4.2 Validation of the TrhGAL4 line Expression Pattern in Serotonergic Neurons

Once backcrossing was completed, flies from the resulting stock (TrhGAL4) were then crossed with two different GFP (Green Fluorescent Protein) stocks. This was done in order to examine the expression pattern of the serotonergic driver in the fly brain and validate it against the known distribution of serotonergic neurons in the fly brain documented in the current literature using images from the work of Alekseyenko, Lee and Kravitz. (2010). The two different GFP stocks were UAS-GFPCD8 (membrane bound GFP) (Kim *et al.*, 2012) and UAS-GFPS65T (cytoplasmic bound GFP) (Sun, Xu and Salvaterra, 1999). The brains of the offspring of the separate crosses of these GFP stocks and the TrhGAL4 stock were dissected and fixed in glycerol and analysed by confocal microscopy to determine the expression pattern of the serotonergic driver and validate its functionality within the stock produced after backcrossing. As shown in **Fig.5**, the membrane bound GFP (GFPCD8) presented very weak fluorescence, this may have been affected by the processing of the fly brains. Also shown in **Fig.5**, the cytoplasmic bound GFP (GFPS65T) showed expression in the correct pattern of cell bodies when compared with the distribution of serotonergic neurons from current literature.

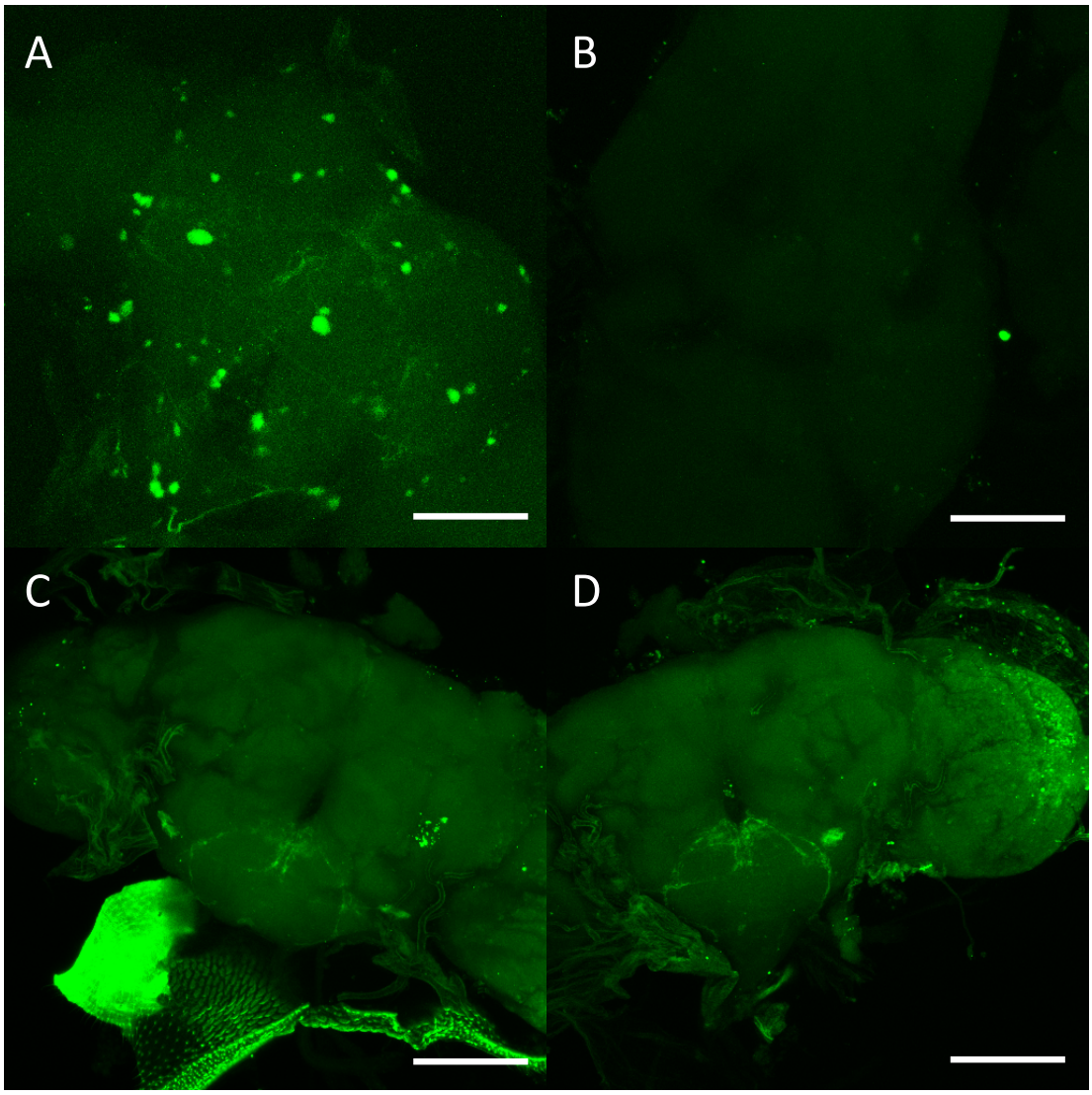


Figure 5: TrhGAL4 driven GFP expression in fly brains, with a control brain for comparison.

(A) The expression pattern of the TrhGAL4 stock backcrossed during this study, which was crossed with a cytoplasmic bound GFP transgenic line (UAS-GFPS65T), analysed by confocal microscopy. **(B)** A control (+/UAS-GFPS65T) fly brain which was fixed and analysed identically to the TrhGAL/UAS-GFP brains for comparison. **(C)** Image of a male fly brain, showing the expression pattern of the TrhGAL4 stock backcrossed during this study which was crossed with a membrane bound GFP transgenic stock (UAS-GFPCD8), analysed by confocal microscopy. **(D)** Image of a female fly brain, showing the expression pattern of the TrhGAL4 stock backcrossed during this study which was crossed with a membrane bound GFP transgenic stock (UAS-GFPCD8), analysed by confocal microscopy. (Scale bar, 100μm)

4.3 Validation of the UAS-InR^{DN} Transgene

The UAS-InR^{DN} stock was crossed with the daGAL4 stock which is a ubiquitously expressed GAL4 driver. This was performed to validate the functionality of the dominant negative insulin-like receptor mutation, IIS is required for normal growth throughout development and when this transgene is ubiquitously expressed it results in reduced growth due to reduced IIS in all tissues (Ikeya *et al.*, 2009). As an estimation of size, the progeny of this cross were weighed and compared with daGAL4/+ (wild-type) flies to determine if they suffered from reduced growth proving the functionality of the UAS-InR^{DN} transgene.

The data shown in **Fig.6** shows that both male and female daGAL4/InR^{DN} weighed significantly less than the daGAL4/+ wild-type control group flies ($p<0.0001$). This indicated that the UAS-InR^{DN} transgene was fully functional and its expression resulted in a reduction in IIS in the daGAL4/InR^{DN} genotype flies.

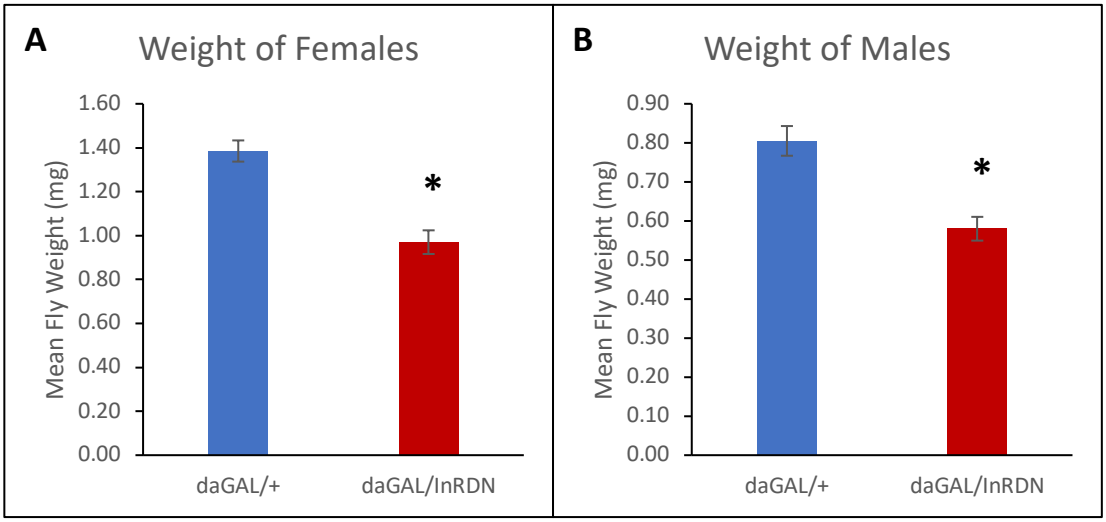


Figure 6: The weight of flies with ubiquitously reduced IIS compared with wild-type flies.

(A) Mean weight of daGAL4/UAS-InR^{DN} (ubiquitous IIS reduction) female flies compared with daGAL4/+ (wild-type) control female flies. The mean weights (+/- standard error) and sample sizes for each group were as follows: daGAL4/UAS-InR^{DN} = 1.39 +/- 0.05, N=20; daGAL4/+ = 0.97 +/- 0.05, N=20. The mean weight of the experimental group (daGAL4/UAS-InR^{DN}) was found to be statistically significantly lower than that of the control group (daGAL4/+) via t-test ($p<0.0001$), showing the dominant negative insulin-like receptor mutation to be functional in females. **(B)** Mean weight of daGAL4/UAS-InR^{DN} (ubiquitous IIS reduction) male flies compared with daGAL4/+ (wild-type) control male flies. The mean weights (+/- standard error) and sample sizes for each group were as follows: daGAL4/UAS-InR^{DN} = 0.58 +/- 0.03, N=20; daGAL4/+ = 0.81 +/- 0.04, N=20. The average weight of the experimental group (daGAL4/UAS-InR^{DN}) was found to be statistically significantly lower than that of the control group (daGAL4/+) via t-test ($p<0.0001$), showing the dominant negative insulin-like receptor mutation to also be functional in males. Data are presented as mean weight +/-SEM, and * indicates significant difference to control ($p<0.05$)

5. Results – The Effects of Constitutive IIS Reduction in Serotonergic Neurons on *Drosophila* Lifespan and Locomotive Behavioural Senescence.

5.1 Introduction

It has been shown that modulation of IIS in the CNS is sufficient to extend lifespan (Broughton and Partridge, 2009; Ismail *et al.*, 2015), but the mechanism is unknown. In addition, such lifespan extending IIS reductions are not always beneficial to CNS function and have sometimes been found to have detrimental effects (Taguchi, Wartschow and White, 2007; Ismail *et al.*, 2015). Ismail et al. (2015) showed that pan-neural reduction of IIS in Drosophila was sufficient to extend female lifespan at the same time as inducing detrimental effects on the decision making and locomotor abilities related to exploratory walking behaviour, indicating that pan-neural IIS reduction is not beneficial to the underlying neuronal circuitry. This study aimed to further investigate IIS in neurons, and its role in the regulation of lifespan and locomotor senescence.

As it has already been suggested that sensitivity to IIS varies throughout the CNS (Broughton and Partridge, 2009), one hypothesis as to why pan-neural IIS reduction positively effects lifespan but negatively affects neuronal circuitry and behavioural health is that different neuronal subtypes are differently effected by IIS reduction. The effects that we see as a result of pan-neural IIS reduction are therefore the sum of all the effects on the different neuronal subtypes present in the fly CNS (Dravecz, 2020). For this reason, further work was completed in our lab by Nikolett Dravecz which focused on studying the effects of IIS reduction in individual neuronal subtypes.

The cell bodies of descending neurons are based in the fly brain, the axons of these neurons carry signals from the brain to the thoracic ganglia allowing for control of peripheral behaviours (Hsu and Bhandawat, 2016). Hsu and Bhandawat (2016) estimated that there are approximately 1100 descending neurons in the fly brain which are split into 6 clusters, they also showed that each cluster contained several different neuronal subtypes. The localisation of the descending neurons which express each neurotransmitter is shown in **Fig.7**.

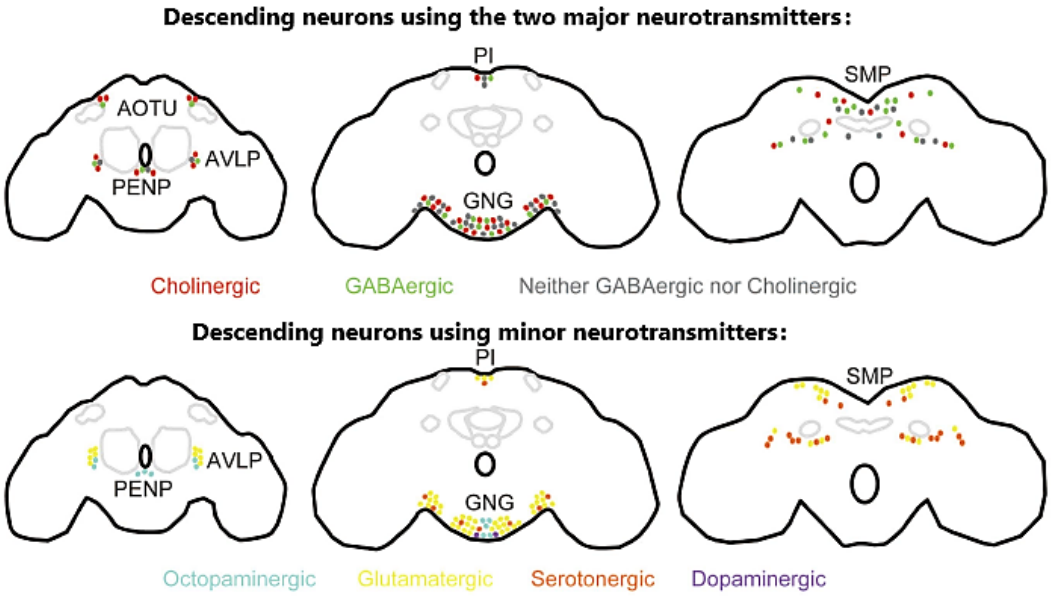


Figure 7: Distribution of descending neurons within the fly brain responsible for expressing specific neurotransmitters.

This image is taken from Dravecz (2020), this is an adaptation of a figure produced by Hsu and Bhandawat (2016). The colour coded dots represent the proportion of descending neurons expressing major neurotransmitters, and the individual descending neurons which express minor neurotransmitters.

The role of IIS in serotonergic and octopaminergic neurons has not yet been investigated and so it is possible that one or both of these neuronal subtypes plays a role in extending lifespan in response to IIS reduction. Both of these subtypes are known to be involved in the regulation of several different behaviours, for example octopamine is known to regulate aggression and motor behaviour in flies (Pflüger, Duch and Heidel, 2005; Zhou, Rao and Rao, 2008). Li et al. (2016) studied octopamine deficient flies (via defective expression of Tβh) and found that they have an increased resistance to starvation, decreased metabolic rate, and reduced physical activity when compared to control flies. The octopamine deficient flies also presented with a shorter lifespan and increased insulin release rates (Li *et al.*, 2016). Serotonin has also been shown to be involved in the regulation of aggression (Olivier, 2005; Alekseyenko *et al.*, 2014), sleep (Yuan, Joiner and Sehgal, 2006; Liu *et al.*, 2019), and memory formation (Sitaraman *et al.*, 2017; Scheunemann *et al.*, 2018). Serotonergic neurons are likely to be involved as previous studies have shown that they do modulate lifespan (Mattson, Maudsley and Martin, 2004; Ro *et al.*, 2016; Chakraborty *et al.*, 2019). De luca et al. (2003) mapped quantitative trait loci to determine genes responsible for the variation

in lifespan between two strains of *D. melanogaster*, dopa decarboxylase (an enzyme involved in serotonin synthesis) was identified as a candidate gene for longevity, suggesting that serotonin activity within the CNS is involved in the regulation of lifespan. Ro et al. (2016) showed that activity of the 5HT2a serotonin receptor is required for the lifespan effects of dietary choice in *Drosophila*, they achieved disruption in serotonergic signalling by treatment with the serotonin receptor antagonist, ketanserin (Colas *et al.*, 1995). There is also evidence from previous studies that DILP producing IPCs within the fly CNS are modulated by serotoninergic neurons, possibly via the 5-HT_{1A} serotonin receptor (Luo *et al.*, 2012, 2014; Nässel *et al.*, 2013). This suggests that altered activity of serotonergic neurons could result in changes in IPC DILP production which as mentioned previously is known to alter lifespan (Broughton *et al.*, 2005). In this study we reduced IIS specifically in serotonergic neurons using the TrhGAL4 driver (Alekseyenko, Lee and Kravitz, 2010) and the UAS-InR^{DN} transgene (Ismail *et al.*, 2015). We then studied these flies to determine whether IIS reduction in serotonergic neurons had beneficial or detrimental effects on locomotor function, decision making, and lifespan. As mentioned in chapter 3, backcrossing of the octopaminergic Tdc2GAL4 driver was also performed, although this driver was not used in further experiments due to time restraints resulting from the Covid-19 pandemic.

5.2 The constitutive reduction of IIS in serotonergic neurons increased the lifespan of female flies but showed no effect on males.

In this experiment, male and female flies with reduced IIS activity specifically in serotonergic neurons were compared with control flies to determine whether the reduction of IIS in serotonergic neurons had any effect on lifespan. The experimental genotype is the TrhGAL4/UAS-InR^{DN} group (serotonergic neuronal IIS reduction), the two control groups are the TrhGAL4/+ and the UAS-InR^{DN} groups. The experimental group was compared with both controls in order to determine any significant difference and therefore any effect of the serotonergic neuron IIS reduction on lifespan.

As shown in **Fig.8-9**, reduction of IIS in serotonergic neurons resulted in a significant increase in female lifespan in two independent experiments. However, there was no effect of reduced IIS in serotonergic neurons on male lifespan (**Fig.10**). In the first female group the TrhGAL4/UAS-InR^{DN} experimental group showed an increase in lifespan when compared to both control groups, this was confirmed as statistically significant by Log-Rank test, $p<0.0001$. In the second female group, the TrhGAL4/UAS-InR^{DN} experimental group also showed an increase in lifespan when compared to both control groups, this was confirmed as statistically significant by Log-Rank test, $p<0.0017$. The two female experiments were completed at separate times, they were therefore analysed separately to avoid effects of possible differences in experimental conditions. In the male group, the TrhGAL4/UAS-InR^{DN} experimental group was not statistically different from both control groups via Log-Rank test, $p=0.2589$.

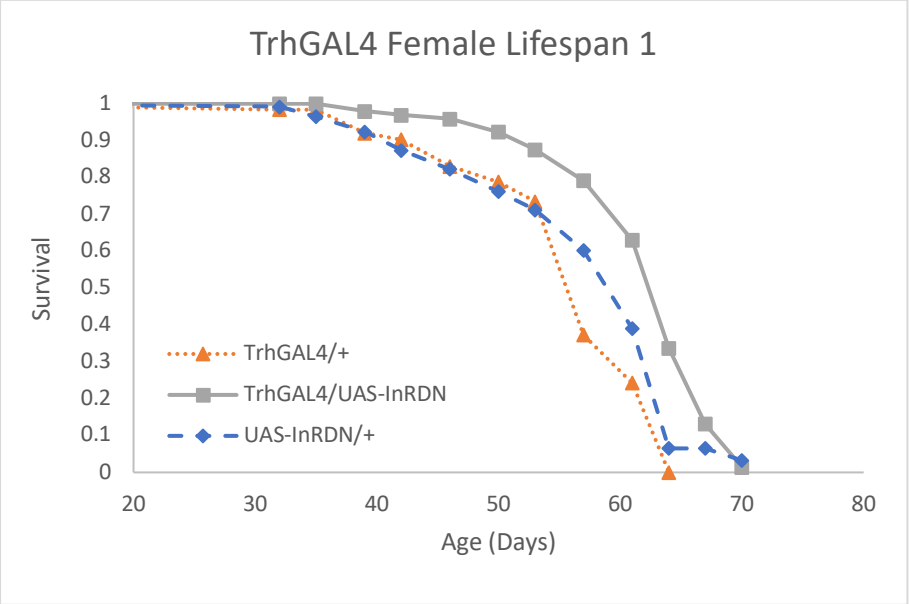


Figure 8: Survival of female flies with reduced IIS specifically in serotonergic neurons (TrhGAL4/UAS-InR^{DN}) compared with control (wildtype) female flies (TrhGAL4/+ and UAS-InR^{DN}/+).

Survival of TrhGAL4/UAS-InR^{DN} female flies compared with the TrhGAL4/+ and UAS-InR^{DN}/+ controls. The median lifespans and sample sizes for each group were as follows: TrhGAL4/UAS-InR^{DN} = 64 days, N = 112; TrhGAL4/+ = 57 days, N = 125; UAS-InR^{DN}/+ = 61 days, N = 113. The experimental group (TrhGAL4/UAS-InR^{DN}) had an increased lifespan compared to both controls, this was confirmed as significant by log rank tests ($p<0.0001$).

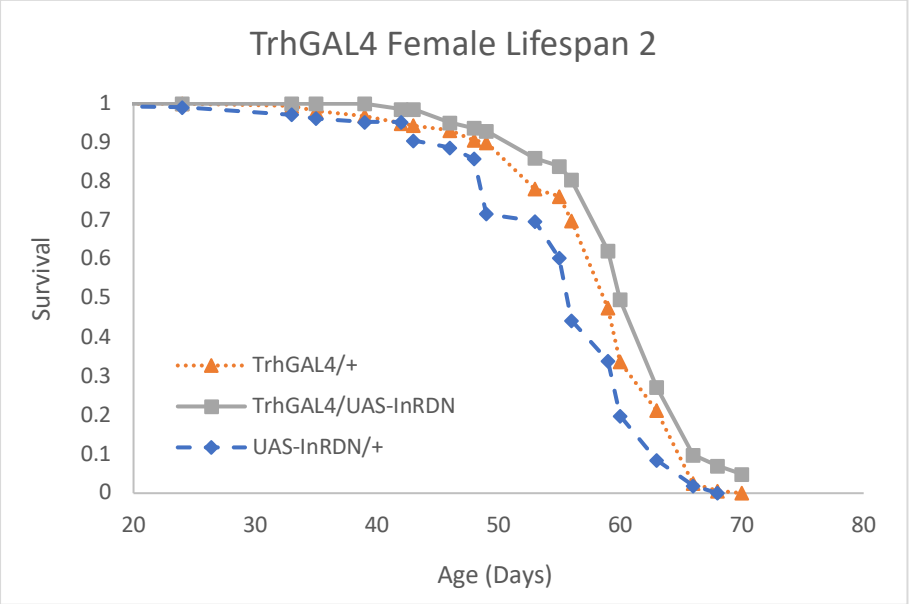


Figure 9: Repeated survival of female flies with reduced IIS specifically in serotonergic neurons (TrhGAL4/UAS-InR^{DN}) compared with control (wildtype) female flies (TrhGAL4/+ and UAS-InR^{DN}/+).

Survival of TrhGAL4/UAS-InR^{DN} female flies compared with the TrhGAL4/+ and UAS-InR^{DN}/+ controls. The median lifespans and sample sizes for each group were as follows: TrhGAL4/UAS-InR^{DN} = 60 days, N = 143; TrhGAL4/+ = 59 days, N = 160; UAS-InR^{DN}/+ = 59 days, N = 106. The experimental group (TrhGAL4/UAS-InR^{DN}) had an increased lifespan compared to both controls, similar to what was shown in the first TrhGAL4 survival experiment. The increased lifespan was confirmed as significant by log rank tests ($p<0.0017$).

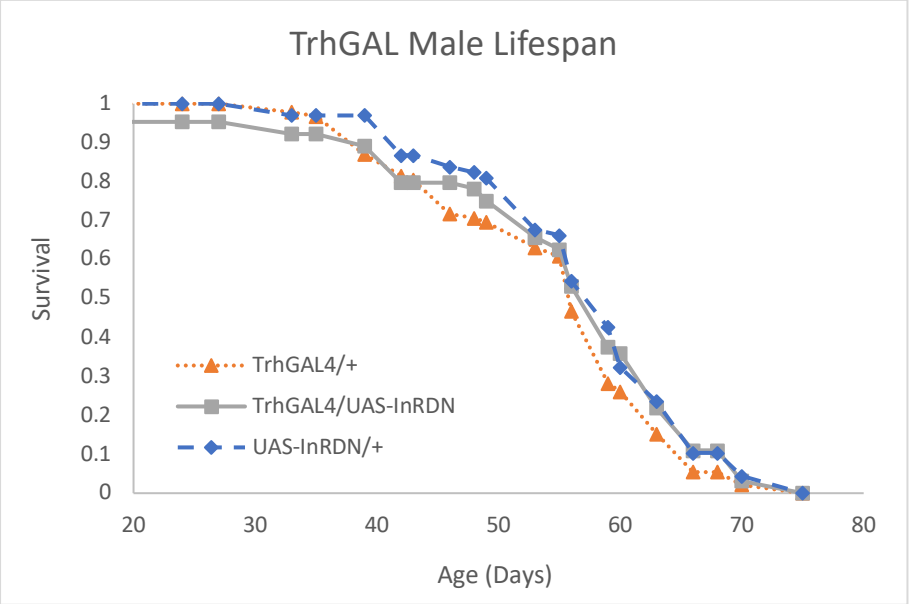


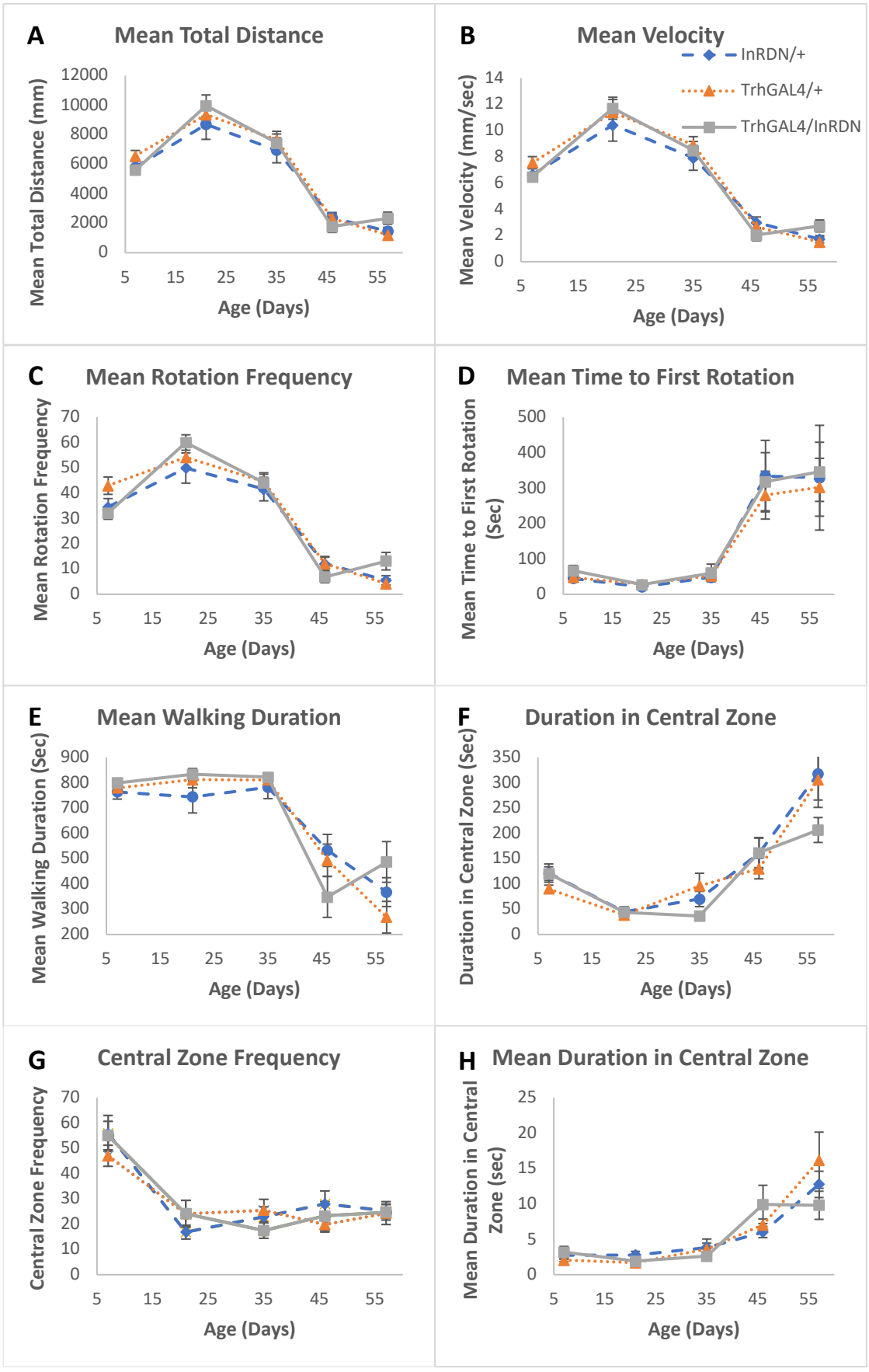
Figure 10: Survival of male flies with reduced IIS specifically in serotonergic neurons (TrhGAL4/UAS-InR^{DN}) compared with control (wildtype) male flies (TrhGAL4/+ and UAS-InR^{DN}/+).

Survival of TrhGAL4/UAS-InR^{DN} male flies compared with the TrhGAL4/+ and UAS-InR^{DN}/+ controls. The median lifespans and sample sizes for each group were as follows: TrhGAL4/UAS-InR^{DN} = 59 days, N = 65; TrhGAL4/+ = 56 days, N = 93; UAS-InR^{DN}/+ = 59 days, N = 69. Survival of the TrhGAL4/UAS-InR^{DN} flies was found to be the same as both controls by log-rank test (p=0.2589).

5.3 The serotonergic neuron specific reduction of IIS had no effect on the locomotive behavioural senescence seen during ageing in males or females.

In this experiment, male and female flies with reduced IIS activity specifically in serotonergic neurons were compared with two control groups of flies to determine whether the reduction of IIS in serotonergic neurons has any effect on their locomotor function or decision-making abilities. This was done by observing and analysing their exploratory walking behaviour. At several points throughout their lifespans (every 10-14 days) flies of each genotype were placed into a novel circular environment with a base of fresh agar media. They were then filmed whilst exploring this novel environment for 15 minutes. The videos were analysed using EthoVision XT video tracking software (Nodus), this was used to measure several different parameters of their exploratory walking behaviour. The parameters of exploratory walking allowed for a measurement of the age-related decline in cognitive and locomotor function, shown in **Fig.11-13**. The parameters of walking behaviour used to measure locomotor senescence were: Total Distance, Velocity, and Walking Duration. The decision-based parameters used to measure cognitive decline were: Rotation Frequency, Time to First Rotation, Duration in Central Zone, and Central Zone Frequency. The experimental genotype for this experiment was the TrhGAL4/UAS-InR^{DN} group (serotonergic neuronal IIS reduction), the two control groups were the TrhGAL4/+ and the UAS-InR^{DN} groups. The experimental group was compared with both controls in order to determine any significant differences and therefore any effect of the serotonergic neuron IIS reduction on the age-related decline in cognitive and locomotor function. The two female experiments were completed at separate times, they were therefore analysed separately to avoid effects of possible differences in experimental conditions.

As shown in **Fig.11-13**, the results of the exploratory walking experiments showed that all genotypes showed normal age-related changes in performance of walking parameters. However, no significant effects of IIS reduction in serotonergic neurons on any of the parameters measured, in both males and females, were seen. The TrhGAL4/UAS-InR^{DN} experimental group was compared with both the TrhGAL4/+ and the UAS-InR^{DN} control groups. Despite there being some differences at later ages in parameters such as the duration of time spent in the central zone, and mean rotation frequency, a two-way ANOVA followed by a planned pairwise comparison (Tukey-Kramer HSD) confirmed that the experimental group was not statistically significantly different from the control groups ($p>0.05$).


A
Mean Total Distance
Mean Total Distance (mm)
12000
10000
8000
6000
4000
2000
0
5
15
25
35
45
55
Age (Days)
B
Mean Velocity
InRDN/+
TrhGAL4/+
TrhGAL4/InRDN
Mean Velocity (mm/sec)
14
12
10
8
6
4
2
0
5
15
25
35
45
55
Age (Days)
C
Mean Rotation Frequency
Mean Rotation Frequency
70
60
50
40
30
20
10
0
5
15
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35
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55
Age (Days)
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Mean Time to First Rotation
Mean Time to First Rotation (Sec)
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300
200
100
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15
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35
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Mean Walking Duration
Mean Walking Duration (Sec)
900
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700
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500
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300
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5
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35
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Age (Days)
F
Duration in Central Zone
Duration in Central Zone (Sec)
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300
250
200
150
100
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Age (Days)
G
Central Zone Frequency
Central Zone Frequency
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60
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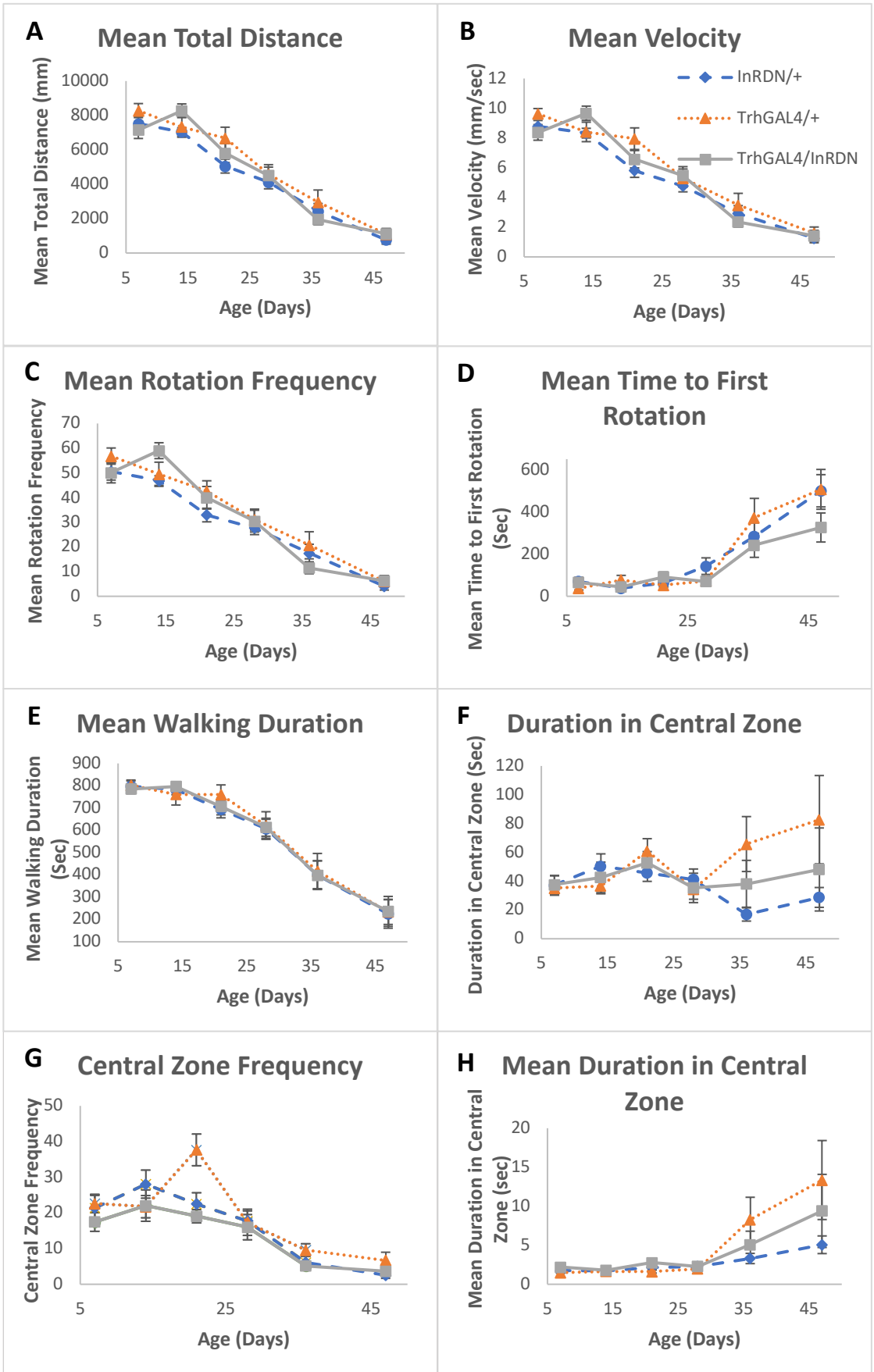
Age (Days)

Figure 11. Exploratory walking of TrhGAL4/InR^{DN} experimental females compared with control groups (TrhGAL4/+ and InR^{DN}/+) – experiment 1.

Graphs A-H show the effects of age and genotype on several measured parameters of exploratory walking within groups of female flies of given genotypic groups. The experimental group (TrhGAL4/InR^{DN}) is compared with the two control groups InR^{DN}/+ and TrhGAL4/+. N=12 for each genotypic group at each timepoint. **(A)** Mean average total distance walked by a fly during the 15-minute observation period, measured over the lifespan of female TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(B)** Mean average velocity (mm/sec) over the lifespan of female TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(C)** Mean average frequency of rotation (changes in direction of walking) over the lifespan of female TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(D)** Mean average time elapsed (sec) before the first change in walking direction, measured over the lifespan of female TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(E)** Mean average amount of time a fly spent walking (sec) during the 15-minute observation period, measured over the lifespan of female TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(F)** Duration of time a fly spent exploring the central zone of the enclosure (sec) during the 15-minute observation period, measured over the lifespan of female TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(G)** Frequency of times a fly explored the central zone of the walking enclosure, measured over the lifespan of female TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(H)** Mean average duration of a visit to the central zone of the walking enclosure (sec), measured over the lifespan of female TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+.


A
Mean Total Distance
Mean Total Distance (mm)
10000
8000
6000
4000
2000
0
5
15
25
35
45
Age (Days)
B
Mean Velocity
Mean Velocity (mm/sec)
12
10
8
6
4
2
0
InRDN/+
TrhGAL4/+
TrhGAL4/InRDN
Age (Days)
C
Mean Rotation Frequency
Mean Rotation Frequency
70
60
50
40
30
20
10
0
Age (Days)
D
Mean Time to First Rotation
Mean Time to First Rotation (Sec)
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Age (Days)
E
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Mean Walking Duration (Sec)
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500
400
300
200
100
Age (Days)
F
Duration in Central Zone
Duration in Central Zone (Sec)
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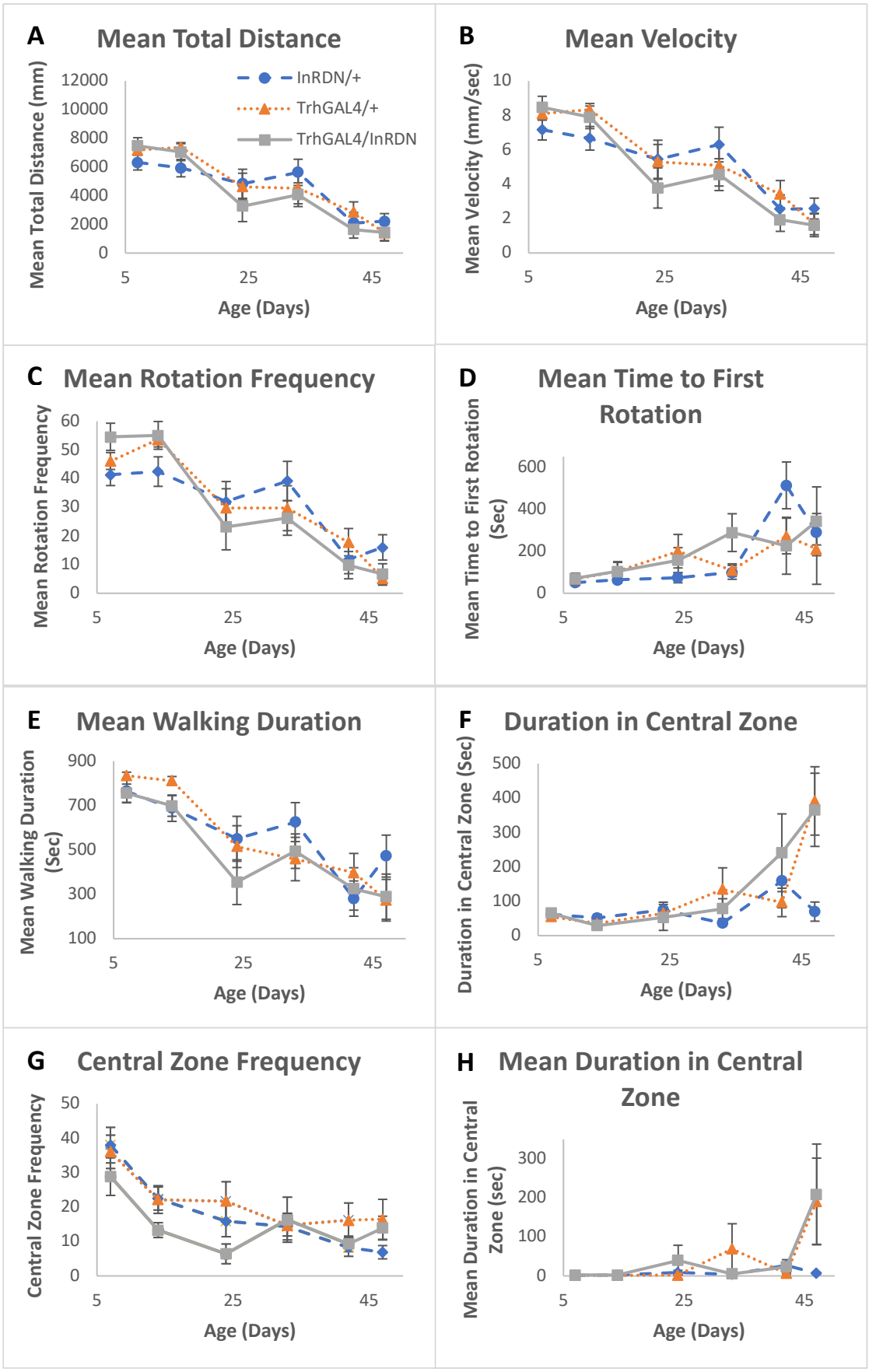
100
80
60
40
20
0
Age (Days)
G
Central Zone Frequency
Central Zone Frequency
50
40
30
20
10
0
Age (Days)
H
Mean Duration in Central Zone
Mean Duration in Central Zone (sec)
20
15
10
5
0
Age (Days)

Figure 12. Exploratory walking of TrhGAL4/InR^{DN} experimental females compared with control groups (TrhGAL4/+ and InR^{DN}/+) – experiment 2.

Graphs A-H show the effects of age and genotype on several measured parameters of exploratory walking within groups of female flies of given genotypic groups. The experimental group (TrhGAL4/InR^{DN}) is compared with the two control groups InR^{DN}/+ and TrhGAL4/+. N=12 for each genotypic group at each timepoint. **(A)** Mean average total distance walked by a fly during the 15-minute observation period, measured over the lifespan of female TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(B)** Mean average velocity (mm/sec) over the lifespan of female TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(C)** Mean average frequency of rotation (changes in direction of walking) over the lifespan of female TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(D)** Mean average time elapsed (sec) before the first change in walking direction, measured over the lifespan of female TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(E)** Mean average amount of time a fly spent walking (sec) during the 15-minute observation period, measured over the lifespan of female TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(F)** Duration of time a fly spent exploring the central zone of the enclosure (sec) during the 15-minute observation period, measured over the lifespan of female TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(G)** Frequency of times a fly explored the central zone of the walking enclosure, measured over the lifespan of female TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(H)** Mean average duration of a visit to the central zone of the walking enclosure (sec), measured over the lifespan of female TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+.


A
Mean Total Distance
Mean Total Distance (mm)
12000
10000
8000
6000
4000
2000
0
InRDN/+
TrhGAL4/+
TrhGAL4/InRDN
5
25
45
Age (Days)
B
Mean Velocity
Mean Velocity (mm/sec)
10
8
6
4
2
0
5
25
45
Age (Days)
C
Mean Rotation Frequency
Mean Rotation Frequency
60
50
40
30
20
10
0
5
25
45
Age (Days)
D
Mean Time to First Rotation
Mean Time to First Rotation (Sec)
600
400
200
0
5
25
45
Age (Days)
E
Mean Walking Duration
Mean Walking Duration (Sec)
900
700
500
300
100
5
25
45
Age (Days)
F
Duration in Central Zone
Duration in Central Zone (Sec)
500
400
300
200
100
0
5
25
45
Age (Days)
G
Central Zone Frequency
Central Zone Frequency
50
40
30
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45
Age (Days)
H
Mean Duration in Central Zone
Mean Duration in Central Zone (sec)
300
200
100
0
5
25
45
Age (Days)

Figure 13. Exploratory walking males. TrhGAL4/InR^{DN} experimental males compared with control groups (TrhGAL4/+ and InR^{DN}/+).

Graphs A-H show the effects of age and genotype on several measured parameters of exploratory walking within groups of male flies of given genotypic groups. The experimental group (TrhGAL4/InR^{DN}) is compared with the two control groups InR^{DN}/+ and TrhGAL4/+. N=12 for each genotypic group at each timepoint. **(A)** Mean average total distance walked by a fly during the 15-minute observation period, measured over the lifespan of male TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4. **(B)** Mean average velocity (mm/sec) over the lifespan of male TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(C)** Mean average frequency of rotation (changes in direction of walking) over the lifespan of male TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(D)** Mean average time elapsed (sec) before the first change in walking direction, measured over the lifespan of male TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(E)** Mean average amount of time a male fly spent walking (sec) during the 15-minute observation period, measured over the lifespan of male TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(F)** Duration of time a fly spent exploring the central zone of the enclosure (sec) during the 15-minute observation period, measured over the lifespan of male TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(G)** Frequency of times a fly explored the central zone of the walking enclosure, measured over the lifespan of male TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+. **(H)** Mean average duration of a visit to the central zone of the walking enclosure (sec), measured over the lifespan of male TrhGAL4/InR^{DN} flies compared with the two control groups InR^{DN}/+ and TrhGAL4/+.

5.4 Summary

In summary of the results of the lifespan experiment, the reduction of IIS in serotonergic neurons increased lifespan in females but had no effect on lifespan in males. The data are also very similar to the known effects of constitutive pan-neural IIS reduction on lifespan shown in previous studies, where lifespan in females was extended but there was no effect on male lifespan (Ismail *et al.*, 2015). These data support the hypothesis that different neuronal subtypes react differently to the reduction of IIS, as the subtypes previously tested for IIS reduction either had detrimental or no effect on lifespan (Dravecz, 2020). Despite extending female lifespan, reduced IIS in serotonergic neurons had no effect on the normal senescence of exploratory walking behaviour in females and males. This shows that modulation of IIS in serotonergic neurons is not sufficient to cause the detrimental effects to behavioural decline seen during pan-neural IIS knockdown. The lack of effect of IIS reduction in serotonergic neurons on exploratory walking behaviour shows that this method of IIS reduction is not detrimental to the neural circuitry underlying these behaviours. These findings give further evidence that the *Drosophila* insulin receptor independently modulates lifespan and areas of age-related locomotor behaviour (Ismail *et al.*, 2015).

6. Results – The Effects of Constitutive and Adult Specific Pan-Neural IIS Reduction on Oxidative and Starvation Stress Resistance.

6.1 Introduction

The role of IIS in modulating lifespan is well established and many studies have also shown a correlation with stress resistance (Longo and Fabrizio, 2002; Broughton *et al.*, 2005; Zhou, Pincus and Slack, 2011). In worms, long lived Daf-2 mutants have been shown to have increased resistance to oxidative and thermal stress (Lithgow *et al.*, 1995; Honda and Honda, 1999). In flies, it has been shown that the genetic ablation of the DILP producing IPCs in the fly brain is sufficient to extend lifespan (likely due to reducing levels of select DILPs in circulation), these long-lived flies also show resistance to starvation and oxidative stress (Broughton *et al.*, 2005). As pan-neural IIS knockdown has been shown to extend female lifespan (Ismail *et al.*, 2015), this study aimed to further investigate if stress resistance was altered by reduced IIS in neurons. Previous work completed by Dravecz (2020) has shown that reduced IIS in adult neurons has no effect on starvation resistance and results in sensitivity to oxidative stress. However, in these experiments, the inducing drug RU486 was found to have an effect on starvation resistance itself. This study repeated these preliminary experiments to determine whether constitutive or adult specific neuronal knockdown of IIS alters resistance to starvation or oxidative stress.

6.2 Reduced IIS in neurons had no effect on oxidative stress resistance.

This assay was used to determine whether the adult specific or constitutive reduction of IIS had any effect on resistance to oxidative stress. 105 flies of each genotype were placed into vials with media containing 5% H_2O_2 and 5% sugar (7 vials each containing 15 flies). The number of flies which had died were counted twice a day and recorded. Using these data, the survival of the flies was plotted over time and is shown in **Fig.14-15**. To constitutively reduce IIS in neurons, the experimental genotype was the elavGAL4/UAS-InR^{DN} group (constitutive pan-neural IIS reduction), the two control groups were the elavGAL4/+ and the UAS-InR^{DN}/+ groups. To knockdown IIS in adult neurons, the experimental genotype was the elavGS/UAS-InR^{DN} +RU486 (RU486-inducible adult-specific pan-neural IIS reduction), this was compared with the elavGS/UAS-InR^{DN} -RU486. The two elavGS/+ groups, with and without RU486 were compared and used to determine any effect of the inducing drug, RU486, itself on oxidative stress resistance. Any groups with RU486 were moved onto RU486 containing media at the age of 3 days. All elavGAL4 groups were moved to 5% H_2O_2 media at the age of 10 days. All elavGS groups were moved to 5% H_2O_2 media at the age of 12 days.

Constitutive reduction of IIS showed no effect on oxidative Stress Resistance.

The results of the constitutive reduction of IIS showed no significant effect on oxidative stress resistance in males or females. In the females, the elavGAL4/UAS-InR^{DN} experimental group was not statistically different from the UAS-InR^{DN}/+ control group via Log-Rank test, p=0.2516. In the males, the elavGAL4/UAS-InR^{DN} experimental group survival was in between both control groups.

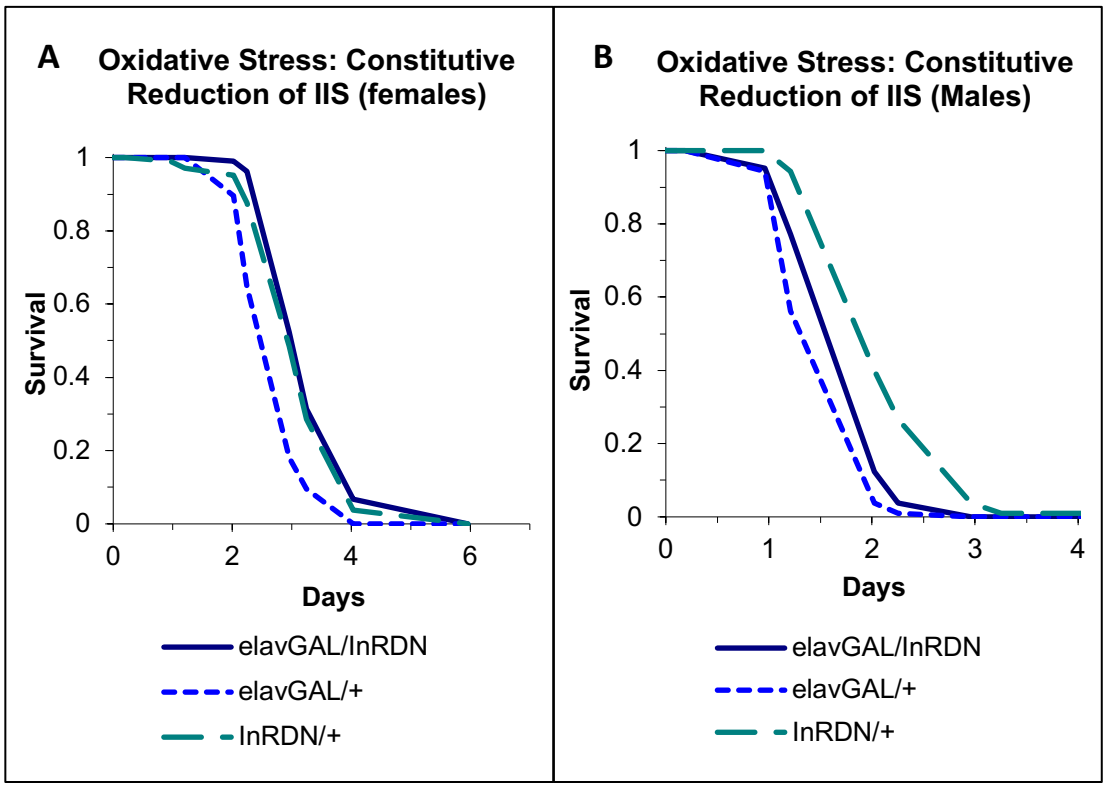

Figure 14: Survival of flies with constitutive pan-neural IIS reduction exposed to oxidative stress.

(A) Survival of elavGAL4/UAS-InR^{DN} (constitutive IIS reduction) female flies compared with the elavGAL4/+ and UAS-InR^{DN}/+ controls. Survival is expressed as the proportion of flies still alive against the time on the food type. The median lifespans and sample sizes for each group were as follows: elavGAL4/UAS-InR^{DN} = 3.25 days, N = 105; elavGAL4/+ = 2.96 days, N = 105; UAS-InR^{DN}/+ = 2.96 days, N = 105.The experimental group (elavGAL4/UAS-InR^{DN}) was found to be statistically the same as the UAS-InR^{DN}/+ control group via Log-Rank test (p=0.2516), showing no effect of constitutive IIS reduction on oxidative stress resistance in females. **(B)** Survival of elavGAL4/UAS-InR^{DN} (constitutive IIS reduction) male flies compared with the elavGAL4/+ and UAS-InR^{DN}/+ controls. Survival is expressed as the proportion of flies still alive against the time on the food type. The median lifespans and sample sizes for each group were as follows: elavGAL4/UAS-InR^{DN} = 2.02 days, N = 105; elavGAL4/+ = 2.02 days, N = 105; UAS-InR^{DN}/+ = 2.02 days, N = 104. The experimental group (elavGAL4/UAS-InR^{DN}) survival was in between both control groups, showing no effect of constitutive IIS reduction on oxidative stress resistance in males.

Adult Specific reduction of IIS showed no effect on oxidative stress resistance, nor did the addition of RU486.

The results of the adult-specific reduction of IIS showed no significant effect on oxidative stress resistance in either males or females (**Fig.15**). These results also showed no effect of RU486 on oxidative stress resistance in males or females. In the females, the elavGS/UAS-InR^{DN} +RU486 experimental group was not statistically different from the elavGS/UAS-InR^{DN} -RU486 control group via Log-Rank test, p=0.9821. The female elavGS/+ group with RU486 was not statistically different from the female elavGS/+ group without RU486 via Log-Rank test, p=0.1186. In the males, the elavGS/UAS-InR^{DN} +RU486 experimental group was not statistically different from the elavGS/UAS-InR^{DN} -RU486 control group via Log-Rank test, p=0.5492. The male elavGS/+ group with RU486 was not statistically different from the male elavGS/+ group without RU486 via Log-Rank test, p=0.9605.

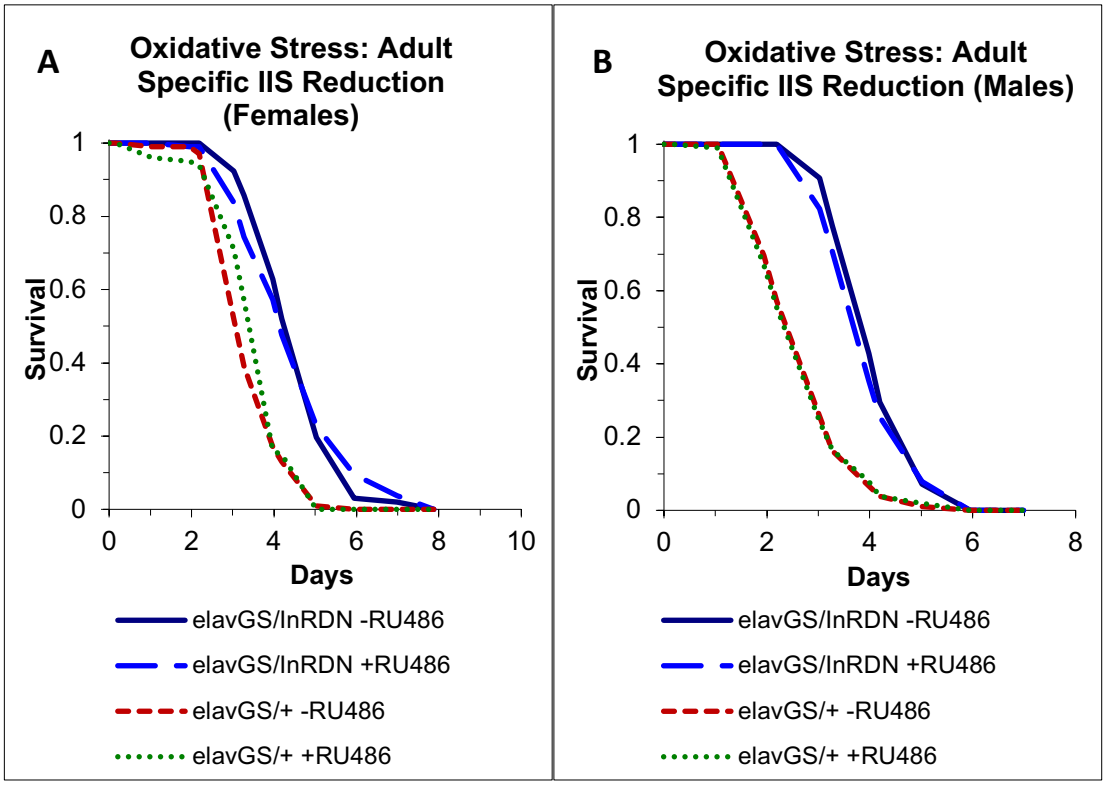

Figure 15: Survival of flies with adult specific pan-neural IIS reduction exposed to oxidative stress.

(A) Survival of elavGS/UAS-InR^{DN} +RU486 (adult specific IIS reduction) female flies compared with the elavGS/UAS-InR^{DN} -RU486; elavGS/+ +RU486; elavGS/+ -RU486 controls. Survival is expressed as the proportion of flies still alive against the time on the food type. The median lifespans and sample sizes for each group were as follows: elavGS/UAS-InR^{DN} +RU486 = 4.19 days, N = 105; elavGS/UAS-InR^{DN} -RU486 = 5.02 days, N = 105; elavGS/+ +RU486 = 3.98 days, N = 105; elavGS/+ -RU486 = 3.28 days, N = 105. The experimental group (elavGS/UAS-InR^{DN} +RU486) was found to be statistically the same as the elavGS/UAS-InR^{DN} -RU486 control group via Log-Rank test (p=0.9821), showing no effect of adult specific IIS reduction on oxidative stress resistance in females. The elavGS group with RU486 was found to be statistically the same as the elavGS group without RU486 via Log-Rank test (p=0.1186), showing no effect of RU486 on oxidative stress resistance in females. **(B)** Survival of elavGS/UAS-InR^{DN} +RU486 (adult specific IIS reduction) male flies compared with the elavGS/UAS-InR^{DN} -RU486; elavGS/+ +RU486; and elavGS/+ -RU486 controls. Survival is expressed as the proportion of flies still alive against the time on the food type. The median lifespans and sample sizes for each group were as follows: elavGS/UAS-InR^{DN} +RU486 = 3.98 days, N = 105; elavGS/UAS-InR^{DN} -RU486 = 3.98 days, N = 105; elavGS/+ +RU486 = 3.02 days, N = 105; elavGS/+ -RU486 = 3.02 days, N = 105. The experimental group (elavGS/UAS-InR^{DN} +RU486) was found to be statistically the same as the elavGS/UAS-InR^{DN} -RU486 control group via Log-Rank test (p=0.5492), showing no effect of adult specific IIS reduction on oxidative stress resistance in males. The elavGS group with RU486 was found to be statistically the same as the elavGS group without RU486 via Log-Rank test (p=0.9605), showing no effect of RU486 on oxidative stress resistance in males.

6.3 Starvation Stress Resistance

Flies with adult specific or constitutive reduction of IIS were used in this assay, to find out if either method of IIS reduction in neurons had any effect on resistance to starvation. 110 flies of each genotype were placed into separate vials (11 vials with 10 flies in each) with starvation media (containing no sugar or yeast). The survival of the flies was recorded twice daily. Using this data, the survival of the flies was plotted over time and is shown in **Fig.16-17**. To constitutively reduce IIS in neurons, the experimental genotype was the elavGAL4/UAS-InR^{DN} group (constitutive pan-neural IIS reduction), the two control groups were the elavGAL4/+ and the UAS-InR^{DN}/+ groups. To knockdown IIS in adult neurons, the experimental genotype was the elavGS/UAS-InR^{DN} +RU486 (RU486-inducible adult-specific pan-neural IIS reduction), this was compared with the elavGS/UAS-InR^{DN} -RU486. The two elavGS/+ groups, with and without RU486 were compared to determine any effect of the inducing drug, RU486, itself on starvation resistance. Any groups with RU486 were moved onto RU486 containing media at the age of 3 days. All elavGAL4 groups were moved onto starvation media at the age of 10 days. All elavGS groups were moved onto starvation media at the age of 12 days.

Constitutive IIS reduction increased the starvation stress resistance in females but showed no effect in males.

The results of the constitutive reduction of IIS in neurons showed an increase in starvation resistance in females but showed no effect in males. In females, the elavGAL4/UAS-InR^{DN} experimental group showed a statistically significant increase when compared with the UAS-InR^{DN}/+ control group (the closest control) by Log-Rank test, p=0.0393. In the males, the elavGAL4/UAS-InR^{DN} experimental group was not statistically different from the elavGAL4/+ control group, as shown by Log-Rank test, p=0.4461.

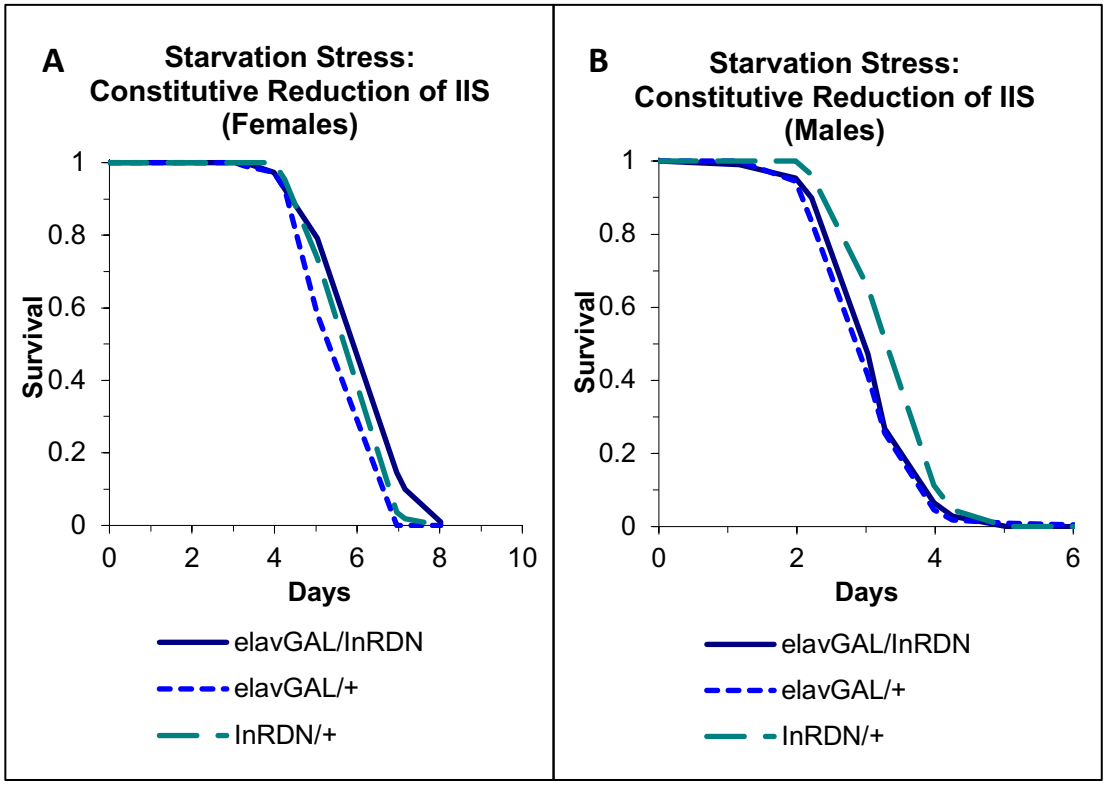

Figure 16: Survival of flies with constitutive pan-neural IIS reduction exposed to Starvation stress.

(A) Survival of elavGAL4/UAS-InR^{DN} (constitutive IIS reduction) female flies compared with the elavGAL4/+ and UAS-InR^{DN}/+ controls. Survival is expressed as the proportion of flies still alive against the time on the food type. The median lifespans and sample sizes for each group were as follows: elavGAL4/UAS-InR^{DN} = 6.96 days, N = 109; elavGAL4/+ = 6.96 days, N = 110; UAS-InR^{DN}/+ = 6.96 days, N = 110. The experimental group (elavGAL4/UAS-InR^{DN}) showed an increased resistance to starvation stress when compared to the control groups, this increase was shown to be significant via Log-Rank test (p=0.0393), showing that constitutive IIS reduction increases oxidative stress resistance in females. **(B)** Survival of elavGAL4/UAS-InR^{DN} (constitutive IIS reduction) male flies compared with the elavGAL4/+ and UAS-InR^{DN}/+ controls. Survival is expressed as the proportion of flies still alive against the time on the food type. The median lifespans and sample sizes for each group were as follows: elavGAL4/UAS-InR^{DN} = 3.03 days, N = 108; elavGAL4/+ = 3.03 days, N = 109; UAS-InR^{DN}/+ = 3.98 days, N = 107. The experimental group (elavGAL4/UAS-InR^{DN}) was not statistically different from the elavGAL4/+ control group via Log-Rank test (p=0.4461), showing no effect of constitutive IIS reduction on starvation stress resistance in males.

Adult specific IIS reduction did not affect starvation resistance, but RU486 increased starvation resistance.

As shown in **Fig.17**, the results of the adult-specific IIS reduction showed that RU486 increased starvation resistance in both males and females. The results also showed a significant difference between the two elavGS/UAS-InR^{DN} groups (with and without RU486), although due to the effect of RU486 on starvation resistance it is difficult to conclude that the reduction of neuronal IIS had any effect on starvation resistance. In females, the elavGS/UAS-InR^{DN} +RU486 experimental group showed a statistically significant increase in survival when compared with the elavGS/UAS-InR^{DN} -RU486 control group via Log-Rank test, $p<0.0001$. The female elavGS/+ group with RU486 also showed a statistically significant increase in survival compared to the elavGS/+ group without RU486 via Log-Rank test, $p<0.0001$. In males, the elavGS/UAS-InR^{DN} +RU486 experimental group showed a statistically significant increase in survival when compared with the elavGS/UAS-InR^{DN} -RU486 control group via Log-Rank test, $p<0.0001$. The male elavGS/+ group with RU486 also showed a statistically significant increase in survival when compared to the elavGS/+ group without RU486, this was shown by Log-Rank test, $p=0.0271$.

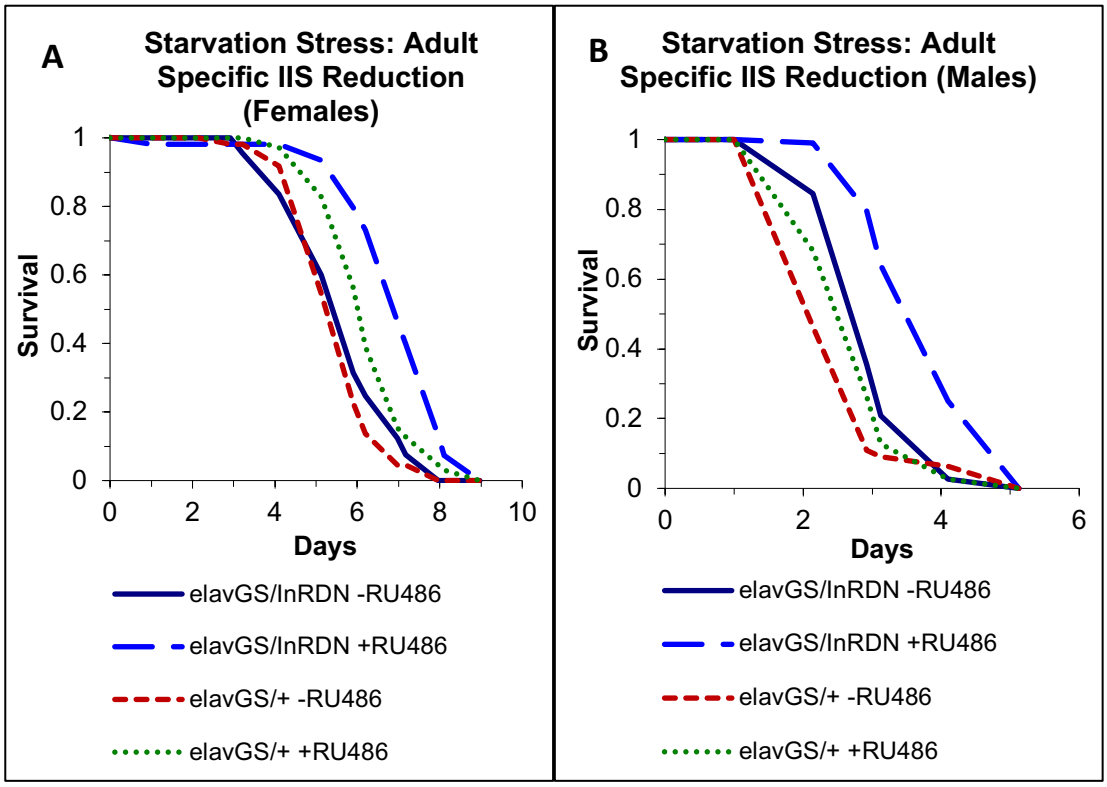

Figure 17: Survival of flies with adult specific pan-neural IIS reduction exposed to Starvation stress.

(A) Survival of elavGS/UAS-InR^{DN} +RU486 (adult specific IIS reduction) female flies compared with the elavGS/UAS-InR^{DN} -RU486; elavGS/+ +RU486; elavGS/+ -RU486 controls. Survival is expressed as the proportion of flies still alive against the time on the food type. The median lifespans and sample sizes for each group were as follows: elavGS/UAS-InR^{DN} +RU486 = 6.97 days, N = 110; elavGS/UAS-InR^{DN} -RU486 = 5.90 days, N = 110; elavGS/+ +RU486 = 6.19 days, N = 110; elavGS/+ -RU486 = 5.90 days, N = 110. The elavGS group with RU486 showed an increase in starvation stress resistance over the control groups without RU486, this was confirmed as significant via Log-Rank test ($p<0.0001$), showing that RU486 increases starvation stress resistance in females. The experimental group (elavGS/UAS-InR^{DN} +RU486) showed an increase in survival compared to the control groups, confirmed as significant by Log-Rank test ($p<0.0001$), due to the effect of RU486 on starvation resistance it is difficult to conclude that this difference was due to IIS reduction. **(B)** Survival of elavGS/UAS-InR^{DN} +RU486 (adult specific IIS reduction) male flies compared with the elavGS/UAS-InR^{DN} -RU486; elavGS/+ +RU486; and elavGS/+ -RU486 controls. Survival is expressed as the proportion of flies still alive against the time on the food type. The median lifespans and sample sizes for each group were as follows: elavGS/UAS-InR^{DN} +RU486 = 4.10 days, N = 110; elavGS/UAS-InR^{DN} -RU486 = 2.92 days, N = 110; elavGS/+ +RU486 = 2.92 days, N = 110; elavGS/+ -RU486 = 2.14 days, N = 110. The elavGS group with RU486 showed an increase in starvation stress resistance over the control groups without RU486, this was confirmed as significant via Log-Rank test ($p=0.0271$), showing that RU486 increases starvation stress resistance in males. The experimental group (elavGS/UAS-InR^{DN} +RU486) showed an increase in survival compared to the control groups,

confirmed as significant by Log-Rank test ($p<0.0001$), due to the effect of RU486 on starvation resistance it is difficult to conclude that this difference was due to IIS reduction.

6.4 Summary

Constitutive reduction of IIS showed no effect on oxidative stress resistance in males or females. Reduced IIS in adult neurons also showed no effect on oxidative stress resistance, nor did the addition of RU486. These results are not consistent with those of previous work completed, which showed that reduced IIS in adult neurons increased sensitivity to oxidative stress (Dravecz, 2020). The elavGS/InR^{DN} control group without RU486 appeared to show an increased resistance to oxidative stress, when compared to other control groups, this may have been due to non-neuronal effects of the dominant negative insulin receptor line. Constitutive reduction of IIS increased starvation stress resistance in females but there was no effect on male starvation resistance. This also is in contrast to previous findings, which showed no effect of constitutive or adult-specific IIS reduction on starvation resistance (Dravecz, 2020). However, similarly to the results of Dravecz (2020), there was no effect of adult specific IIS reduction on starvation resistance in this study. Due to the effect of RU486 on starvation resistance, it was concluded that reduced IIS in adult neurons could not be reliably determined as the cause of altered starvation resistance. The difference in magnitude of IIS reduction between the constitutive and inducible systems is not yet known, this could explain differences between the elavGS and elavGAL lines. More information is needed in order to determine any effect of reduced IIS in adult neurons on starvation resistance, it is likely that any difference seen was due to the effect of RU486 and that the IIS reduction has little to no effect on starvation resistance, this is consistent with previous findings (Dravecz, 2020).

7. Discussion

Despite the depth of work currently completed in the field of ageing research, specifically in model organisms, the mechanism by which single gene mutations or other alterations affect longevity is still not entirely understood, nor are the exact reasons as to why these organisms age and die at all (Piper and Partridge, 2018). However, it is becoming clear that ageing is influenced by nutrient sensing signalling pathways and that different organs and cell types play different roles in modulating lifespan and functional senescence. This project focused on the role of IIS in the CNS on lifespan and behavioural senescence. In order to further understand how pan-neural IIS reduction results in lifespan extension, it is important to determine how each neuronal subtype reacts to the IIS reduction and whether or not the function of neurons, rather than their ageing, is being compromised by reduced IIS resulting in the detrimental behavioural effects shown by Ismail et al. (2015). There are two current hypotheses to explain the disconnection between lifespan and healthspan seen as a result of reducing neuronal IIS. The first of which is that the detrimental effects on behavioural decline (seen in flies with pan-neural IIS reduction) are the result of the damaging effects of IIS reduction on neuronal function outweighing any beneficial effects on neuronal ageing. The second hypothesis is that due to the varying IIS sensitivities of the individual neuronal subtypes, the effects seen in flies with pan-neural IIS reduction are the sum of all of the negative, positive, and neutral effects on each of the subtypes. These two hypotheses are not mutually exclusive. Based on these hypotheses, Dravecz (2020) studied the effects of IIS reduction in specific neuronal subtypes on lifespan, behaviour, and function. The preliminary findings of this work have shown that IIS reduction in four neuronal subtypes independently (dopaminergic, cholinergic, glutamatergic, and GABAergic) results in detrimental or no effect on lifespan, exploratory walking, and negative geotaxis (Dravecz, 2020).

This study aimed to build on the previous work and further understand the effects of IIS reduction on the function of individual neuronal subtypes. During this study it was shown that constitutive reduction of IIS specifically in serotonergic neurons increases lifespan in female flies but had no effect on male lifespan. This serotonergic neuron specific IIS reduction also showed no effect on exploratory walking behavioural decline. This study also aimed to further understand how pan-neural IIS reduction (both constitutively and specifically in adult neurons) affected oxidative and starvation stress resistance. It was found that neither constitutive nor adult specific pan-neural IIS reduction had any effect on oxidative stress resistance in males or females, nor did the

addition of RU486. It was also found that constitutive IIS reduction in neurons was sufficient to increase starvation stress resistance in females but showed no effect on male starvation resistance. Flies with reduced IIS in adult neurons did show a significant increase in starvation resistance, however this was most likely due to the effects of the inducing drug RU486. RU486 alone was sufficient to significantly increase starvation resistance in both males and females.

7.1. Serotonergic IIS Reduction Lifespan and Exploratory Walking Data Analysis

The female lifespan extension and normal male lifespan in flies with reduced IIS in serotonergic neurons is similar to the effect of pan-neural IIS knockdown shown by Ismail et al. (2015). As mentioned previously, Dravecz (2020) studied the effects of IIS reduction in four separate neuronal subtypes: dopaminergic, GABAergic, cholinergic, and Glutamatergic. As shown in **Table.1**, IIS reduction in each of these subtypes resulted in reduced lifespan, with the exception of GABAergic neurons which showed no effect on lifespan. As shown in **Table.2**, IIS reduction in all but the cholinergic neurons had no effect on the decline in exploratory walking or negative geotaxis behaviours. IIS reduction in cholinergic neurons detrimentally effected negative geotaxis in both males and females, it was also detrimental to exploratory walking behaviour in males at a young age but showed no effect on exploratory walking behaviour in females. This study aimed to continue this work by studying the effects of IIS reduction in the serotonergic neuronal subtype on lifespan and exploratory walking behaviour. Serotonergic neurons are the only subtype tested so far in which IIS reduction results in increased lifespan, this suggests that the serotonergic neurons could be the subtype responsible for the lifespan extension seen during pan-neural IIS knockdown. The lifespan effects of IIS reduction in serotonergic neurons, in conjunction with the lack of effect on exploratory walking behaviour offers further evidence for the hypothesis that the healthspan and lifespan effects seen during pan-neural IIS knockdown are the result of the negative, positive, and neutral effects on each of the subtypes. These results also present the serotonergic neurons as a target by which the beneficial effects of IIS reduction on lifespan could be disconnected from the detrimental behavioural effects.

Serotonin activity within the CNS is responsible for the regulation of many different functions within the fly such as sleep, circadian rhythm, aggression, and certain forms of memory (Yuan *et al.*, 2005; Yuan, Joiner and Sehgal, 2006; Johnson, Becnel and Nichols, 2009; Lee *et al.*, 2011). The five serotonergic receptors present in *Drosophila*

are known to be expressed widely throughout the CNS and also within the thoracicoabdominal ganglion (Gnerer, Venken and Dierick, 2015). The serotonin 5-HT_{2A} receptor is expressed within the mushroom bodies where it maintains functional roles in sleep and anaesthesia resistant memory (Yuan, Joiner and Sehgal, 2006; Lee *et al.*, 2011). The serotonin d5-HT_{1B} receptor is expressed in clock neurons which function as part of the entrainment mechanism for circadian rhythm behaviour, serotonin utilises this receptor to inhibit the entrainment mechanism (Yuan *et al.*, 2005). The 5-HT_2Dro serotonin receptor is expressed by neurons within the ellipsoid body, the protocerebrum, and throughout the fly brain where it is known to play a modulatory role in aggression and circadian behaviours (Nichols, 2007; Johnson, Becnel and Nichols, 2009).

Several studies have provided evidence for the role which neuronal serotonergic activity plays in regulating feeding and hunger (Vargas *et al.*, 2010; Albin *et al.*, 2015; Lyu *et al.*, 2021). Activation of specific subsets of serotonergic neurons which project broadly throughout the fly brain has been shown to elicit a hunger response in recently fed flies, presenting these neurons as a key part of the neural circuitry responsible for the control of feeding behaviour (Albin *et al.*, 2015). Activation of S6 kinase (S6K) increases neuronal serotonin production in flies, this has been shown to alter dietary preference in flies towards yeast, flies fed a serotonin precursor exhibit the same alteration (Vargas *et al.*, 2010). The S6K is target of the TOR pathway which is an evolutionarily conserved pathway which is involved in growth and nutrient sensing in several species (Wullschleger, Loewith and Hall, 2006) and is also known to interact with the IIS pathway (Broughton and Partridge, 2009). Downregulating activity of the TOR pathway has also been shown to be sufficient to extend lifespan in worms and flies (Vellai *et al.*, 2003; Kapahi *et al.*, 2004). The results of Vargas et al. (2010) provide evidence that TOR signalling and serotonin activity are both involved in the maintenance of nutrient balance in *Drosophila*, and in doing so suggest a further possible link between a nutrient sensing pathway known to be involved in the regulation of lifespan and serotonin activity. The results obtained by Lyu et al. (2021) show that neuronal expression of the serotonin 5-HT_{2A} receptor is required for the lifespan effects of dietary choice in *Drosophila*. Allowing flies to choose their own diet composition by presenting the major dietary components separately reduces lifespan in males and females, these flies also exhibit altered activity, altered stress resistance, and changes to metabolic processes. When exposed to dietary choice, flies which lack the serotonin 5-HT_{2A} receptor express diet-dependent feeding changes similar to control flies, however their lifespan remains unchanged, this presents serotonergic activity within

the CNS as a key regulator of ageing and physiology in an environment where dietary choice is available (Lyu *et al.*, 2021).

The regulatory relationship between serotonergic activity in the CNS and lifespan extension has been established in previous studies (Ro *et al.*, 2016; Chakraborty *et al.*, 2019; Lyu *et al.*, 2021). The results of this study provide evidence that IIS reduction modulates the activity of serotonergic neurons to result in lifespan extension. Based on current knowledge of the role of serotonergic neurons in the CNS, there are several different elements which could be a part of the mechanism by which IIS reduction in serotonergic neurons results in lifespan extension. There is evidence from previous studies that insulin-producing cells (IPCs) in the fly brain, responsible for DILP production are regulated by the serotonin 5-HT_{1A} receptor (Luo *et al.*, 2012). This is an inhibitory pathway which could be the link between reduced insulin signalling in serotonergic neurons and lifespan extension. There is also a large amount of evidence from previous studies that the serotonergic pathway, much like the IIS pathway, is highly evolutionarily conserved and involved in the regulation of metabolism and the ageing process (Mattson, Maudsley and Martin, 2004). Serotonin is known to be related to cyclic AMP signalling in neurons, which has been shown in other insects to improve synaptic plasticity in the event of energy deprivation, giving a survival advantage (Friedrich, Thomas and Müller, 2004). This in conjunction with the body of evidence linking serotonergic activity with the regulation of diet and feeding mentioned previously, suggests that serotonergic activity in the CNS could be part of the mechanism by which DR affects longevity. Another study named the gene which encodes dopa decarboxylase (an enzyme involved in serotonin synthesis) as a candidate for longevity, as it was responsible for the variation in lifespan between two strains of *Drosophila* (De Luca *et al.*, 2003). This body of evidence suggests strongly that the serotonergic pathway within the CNS is highly involved in the regulation of lifespan and health throughout the ageing process. The results of this study regarding the effects of serotonergic neuron specific IIS reduction on lifespan and exploratory walking fit in well with the previous literature, these results also suggest strongly that the mechanism by which pan-neural IIS reduction effects longevity is reliant on the activity of serotonergic neurons.

The effects of serotonergic IIS reduction on age-related cognitive and behavioural decline were studied through the analysis of exploratory walking behaviour. The results of this analysis showed no significant effects of the IIS reduction on any of the measured parameters of exploratory walking. This means that IIS reduction in serotonergic neurons showed no effect on the age-related decline in decision making

and locomotor abilities, this is different to the detrimental effect of pan-neural IIS on these same parameters. The neural circuitry responsible for the control of most complex locomotor actions such as walking behaviours is located within the ventral nerve cord (VNC) (Court *et al.*, 2020). Descending commands are received by the VNC from the fly brain, output signals are then sent via motor neurons to the peripheral musculature (Baek and Mann, 2009). The serotonergic VNC subpopulation of neurons is responsible for innervating the leg neuropils, these neurons have been shown to play a multifaceted role in the modulation of walking behaviour in *Drosophila* via interactions with several specific receptors (Howard *et al.*, 2019). The results of this study therefore suggest that IIS is not required for the locomotor modulatory function of the serotonergic neurons.

Preliminary results of work previously completed in our lab has shown that pan-neural IIS reduction results in an increase in age-related neuronal apoptosis. This work was completed in flies with constitutive and adult-specific pan-neural IIS knockdown, both of which were found to have an increased amount of age-related neuronal apoptosis in the brain when compared to wild-type flies of the same age. This suggests that some neurons within the fly brain cannot survive the reduction in IIS, possibly due to the neuroprotective role that IIS is known to play within the CNS. This also provides an explanation for the detrimental behavioural and locomotor effects seen in these long-lived flies, as the IIS knockdown is damaging to the neural circuitry underlying these behaviours (Ismail *et al.*, 2015). The IIS pathway is known to be linked to apoptosis regulation via the phosphorylation of dFOXO (Fu and Tindall, 2008). Reduction of IIS signalling leads to a decrease in the downstream PI3K activity (shown in **Fig.3**), which means that dFOXO remains isolated within the nucleus which can cause apoptosis (Siegrist *et al.*, 2010). This offers an explanation for the increased neuronal apoptosis seen in flies with pan-neural IIS reduction. The lack of effect on behavioural decline in flies with serotonergic IIS reduction suggests that the serotonergic neurons are not responsible for the detrimental effects on behaviour seen in flies with pan-neural IIS reduction, and therefore not involved in the neural circuitry underlying those behaviours that is detrimentally affected by IIS reduction. Of the other four neuronal subtypes tested by Dravecz (2020), IIS reduction specifically in cholinergic neurons was the only subtype found to be detrimental to locomotor function. The exact detrimental effects on exploratory walking parameters which result from pan-neural IIS reduction are yet to be seen by IIS reduction in any individual subtype, this may be because these effects are the cumulative result of IIS reduction in several neuronal subtypes. It may therefore

be possible to achieve similar detrimental effects by modulating IIS activity in several specific neuronal subtypes simultaneously.

7.2. Oxidative Stress and Starvation Stress Resistance Data Analysis

It is well documented in current literature that the IIS pathway is related to the regulation of stress resistance in *Drosophila* (Broughton *et al.*, 2005; Wang *et al.*, 2008; Söderberg, Birse and Nässel, 2011). During this study, it was found that pan-neural IIS reduction had no effect on oxidative stress resistance in males or females, nor did the addition of RU486. Constitutive pan-neural IIS reduction was sufficient to increase starvation stress resistance in females but showed no effect on male starvation resistance. Although flies with reduced IIS specifically in adult neurons did show a significant increase in starvation resistance, this was likely due to RU486 (the addition of RU486 in both males and females was sufficient to increase starvation resistance). The experiments performed during this study were the repeats of those previously completed in our lab by Dravecz (2020). The results of Dravecz (2020) showed that IIS reduction in adult neurons reduced resistance to oxidative stress, no other methods of pan-neural IIS reduction were sufficient to alter resistance to oxidative or starvation stress. The results produced by this study are partially conflicting with those of the original experiments, the differences between the effects could be due to a number of factors, such as slightly altered genetic backgrounds of the flies, slight differences in food preparation, or the number of flies used for the experiments not being sufficient to reliably determine an effect. For this reason and the variable effects seen between the two repeats, it is important that these experiments are repeated again to determine whether constitutive or adult-specific pan-neural IIS reduction does affect resistance to oxidative or starvation stress. Further understanding of how IIS reduction in the CNS affects stress resistance is very important in determining how neurons are affected by IIS reduction and which mechanisms and pathways are being altered.

Effects on stress resistance in IIS mutants can be expected as the IIS pathway is known to be related to various pathways related to the stress response (Broughton and Partridge, 2009). Previous studies have shown that several methods of reducing IIS which extend lifespan also result in increased resistance to oxidative stress (Clancy *et al.*, 2001; Hwangbo *et al.*, 2004; Broughton *et al.*, 2005). In these models the pro-oxidant was included in the food media, it is possible that there is a genotype effect on feeding rates resulting in different doses being received by each group (Broughton *et al.*, 2005). Another possible explanation for the correlation seen between increased

longevity and oxidative stress resistance is altered activity of the JNK pathway which interacts with the IIS pathway (Broughton and Partridge, 2009). Increased JNK activity extends lifespan and improves tolerance to oxidative stress (Wang, Bohmann and Jasper, 2003). The results of this study showed no effect of IIS reduction in the CNS on oxidative stress resistance, this suggests that neuronal IIS reduction does not cause changes seen in the other long-lived models which result in alterations to the oxidative stress tolerance.

Although the effects shown by the results seem to be variable and further study is needed to confirm their validity, the effect on starvation resistance is similarly sexually dimorphic to the effect on lifespan seen previous work (Ismail *et al.*, 2015). When IIS is reduced systemically, the effect on lifespan is commonly sexually dimorphic, with males usually showing a lesser lifespan extension than females (Broughton *et al.*, 2005; Ikeya *et al.*, 2009). The reason for this commonly seen sexually dimorphic effect of reduced IIS is not currently known (Ikeya *et al.*, 2009; Ismail *et al.*, 2015; Dravecz, 2020). Previous studies have shown that IIS activity plays a regulatory role in female *Drosophila* remating, this offers a possible explanation for the sexually dimorphic lifespan effect as there is a documented link between lifespan and female reproductive rates (Barnes *et al.*, 2008; Wigby *et al.*, 2011; Duxbury, Rostant and Chapman, 2017). This also presents mating behaviour as a possible focus for future study of IIS knockdown flies to further understand the basis of the sexually dimorphic lifespan effects. The lifespan effects of reducing IIS in serotonergic neurons, as shown during this study are also similarly sexually dimorphic in nature, this suggests that the serotonergic neurons may be part of the neuronal circuitry underlying the effects seen in pan-neural IIS reduction models. Further research is needed to determine the oxidative/starvation stress resistance effects of IIS knockdown in serotonergic neurons in order to identify the mechanism which results in these effects.

It is possible that the effects of pan-neural IIS reduction on stress resistance may be due to altered DILP expression, several previous studies have linked the production of DILPs both in the CNS and in the periphery to altered levels of stress resistance (Broughton *et al.*, 2005; Enell *et al.*, 2010; Söderberg, Birse and Nässel, 2011; Gimenez *et al.*, 2013). IPCs within the CNS are responsible for the expression of DILPs 2,3, and 5 (Enell *et al.*, 2010), whilst these neurosecretory cells are not directly affected by the elavGAL4 driver, IPCs are regulated by neurons which are targeted by elavGAL4 (Nässel *et al.*, 2013). Previous studies have shown that genetic ablation of the IPCs in the fly brain is sufficient to increase lifespan. These long-lived flies also show increased resistance to starvation and oxidative stress (Broughton *et al.*, 2005).

DILP expression also presents a possible reason for the differences between the effects of constitutive vs adult-specific IIS reduction. The results of Ismail et al. (2015) show that constitutive IIS reduction has no effect on DILP expression levels at young ages. The results of Dravecz (2020) confirm the previous conclusions that constitutive pan-neural IIS reduction does not alter DILP expression, however the results of Dravecz (2020) also show that adult-specific pan-neural IIS reduction reduces expression of DILPs 6 and 2 in females and increases expression of DILPs 3 and 4 specifically in male heads. This is interesting as previous results have linked increased expression of DILP6 with increased lifespan (Bai, Kang and Tatar, 2012), and DILP6 is produced in the fat body showing that IIS in neurons is capable of altering DILP production in both the IPCs and the fat body via an unknown endocrine mechanism (Nässel *et al.*, 2013). Further work therefore needs to be completed to determine the effects of pan-neural IIS reduction on DILP expression throughout lifespan. This would help to understand whether alterations to DILP expression are responsible for any of the effects seen in flies with reduced neuronal IIS.

7.3. Summary of Findings, and Importance to Future Ageing Research

The overall results of this study have shown that it is possible to increase lifespan by reducing IIS in serotonergic neurons, without the detrimental effects seen during pan-neural IIS reduction. This gives a new target of study for the relationship between insulin signalling and lifespan, in the form of the serotonergic neuronal subtype and the pathways surrounding. These findings give further evidence to the previous hypothesis that different neuronal subtypes react differently to IIS reduction resulting in variable effects on longevity and behaviour.

The findings of this study are important for the future of ageing research, and more specifically the future of research involving lifespan extension and IIS reduction. The final aim of this body of research is to produce a therapeutic method which could improve lifespan and the health of humans at later ages by reducing the risk of developing ageing related diseases, which represents a major medical problem facing modern society. The results of this study show that it is possible to improve the lifespan of an organism without detrimentally effecting other behaviours or functions, serotonergic neurons are presented as regulatory body through which to achieve these longevity alterations. Obviously further study needs to be completed regarding the health of these flies, however the results currently obtained are promising and

represent a step forward in the journey towards understanding the exact mechanism by which IIS activity regulates lifespan.

7.4. Limitations of the Work

Though further behavioural analysis was planned for the flies with IIS reduction in serotonergic neurons, this could not be completed during this study due to the Covid-19 pandemic lockdown restrictions creating time restraints on lab work. It was originally planned for the sleeping behaviour and negative geotaxis of the flies to also be studied throughout their lifespan; this would have allowed for a broader picture of their overall health to be understood. This work is now planned to be completed at a later date, as well as analysis of synaptic plasticity by electron microscopy to further understand how brain health is affected by this modification.

Experiments to determine the lifespan and behavioural effects of reduction of IIS in octopaminergic neurons were also planned to be completed during this study. These were also unable to be completed due to the aforementioned lockdown restrictions. Previous studies have shown that octopaminergic receptors are responsible for the regulation of IPCs in the fly brain, alteration of the activity of octopaminergic neurons by IIS reduction could therefore result in alterations to DILP production (Luo *et al.*, 2014). This offers a mechanism by which octopaminergic neurons could be responsible for the lifespan effects seen during pan-neural IIS reduction. Octopaminergic activity is also known to be responsible for the regulation of other behaviours in flies, such as social behaviour, aggression, and locomotor function (Pflüger, Duch and Heidel, 2005; Hoyer *et al.*, 2008; Zhou, Rao and Rao, 2008; Luo *et al.*, 2014). It is therefore important that the lifespan and behavioural effects of IIS reduction in octopaminergic neurons are also studied in the future to determine whether that is the subtype (or part of a collective of subtypes) responsible for the variety of behavioural and lifespan effects seen during pan-neural IIS reduction. This further work is planned for future completion in order to give a full understanding of the effects of IIS reduction on each individual neuronal subtype. Flies containing the octopaminergic driver were backcrossed as part of this body of work, to prepare them for these future experiments.

As the oxidative and starvation stress resistance experiments produced results which show a variable effect when compared to the conclusions of previous work, it is required to conduct further repeats of these experiments. These repeats could benefit from further efforts to control the conditions and environments in order to fully determine any effects of pan-neural IIS reduction on oxidative and starvation stress

resistance. Resistance to other stresses could also be tested, for example tunicamycin-induced ER (endoplasmic reticulum) stress.

The male serotonergic IIS reduction lifespan and exploratory walking experiments were only able to be completed once. Repeats of these experiments should be completed in order to compare the results and determine reproducibility, similar to the way these experiments were repeated for the female groups.

7.5. Further Work Based on the Findings

For both the pan-neural and serotonergic neuron specific IIS reduction, more work needs to be done to understand the neuroendocrine effects of these alterations. One part of this work will be studying how levels of peripheral IIS activity are affected by IIS reduction in the CNS, throughout ageing. Peripheral tissues in which IIS activity should be tested are the musculature and the fat body, as these are both tissues in which IIS activity has been linked to the regulation of longevity (Mathew, Pal Bhadra and Bhadra, 2017). Western blots should be used to determine whether IIS is altered in the periphery, this can be done by measuring the proportion of phosphorylated AKT to non-phosphorylated AKT, as AKT is phosphorylated as part of the downstream pathway triggered by IIS activity. It also needs to be determined as to whether or not FOXO is required for the lifespan effect of these methods of IIS reduction, as it has been shown in previous studies that FOXO is not required in other tissues in order to achieve lifespan extension (Alic *et al.*, 2014). This could be done using a FOXO mutant background which lacks FOXO production in specific tissues. This would help to further our understanding of how pan-neural IIS reduction results in lifespan extension, and whether the mechanism is more endocrine, as previously thought, or cell autonomous within the CNS.

As it has previously been shown that pan-neural knockdown of IIS leads to alterations in DILP expression (Dravecz, 2020), and it is also known that ELAV is not expressed in the large neurosecretory cells. Therefore pan-neural IIS reduction must result in an endocrine effect on the IPCs, altering DILP expression. As mentioned previously, both serotoninergic neurons are involved in the regulation of IPCs which are responsible for DILP production (Luo *et al.*, 2012). DILP protein production and secretion should be analysed in flies with reduced IIS in serotonergic neurons in order to further understand whether reduced DILP expression is the mechanism by which IIS reduction in serotonergic neurons results in lifespan extension.

7.6. Conclusion

The main finding of this study is that constitutive reduction of IIS activity specifically in serotonergic neurons is capable of improving longevity in *Drosophila melanogaster*. This presents a new level of specificity within the CNS by which IIS can be reduced in order to extend lifespan. Further to this, the detrimental effects regarding exploratory walking behaviour seen in flies with pan-neural IIS reduction was not present. This shows that IIS reduction in serotonergic neurons is not damaging to the function of the neural circuitry underlying these behaviours. In addition to these findings, it was also shown during this study that pan-neural IIS reduction had no effect on oxidative stress resistance but did improve resistance to starvation stresses. This is important in further understanding how the pathways which regulate longevity and stress resistance are interlinked, however these results were contradictory to those obtained previously, therefore it is important that these experiments are repeated in the future.

Bibliography

Adedeji, W. A. (2016) 'The treasure called antibiotics', *Annals of Ibadan Postgraduate Medicine*, 14(2), pp. 56–57.

Albin, S. D. *et al.* (2015) 'A Subset of Serotonergic Neurons Evokes Hunger in Adult Drosophila', *Current Biology*, 25(18), pp. 2435–2440. doi: 10.1016/j.cub.2015.08.005.

Alcedo, J. and Kenyon, C. (2004) 'Regulation of C. elegans Longevity by Specific Gustatory and Olfactory Neurons', *Neuron*, 41(1), pp. 45–55. doi: 10.1016/S0896-6273(03)00816-X.

Alekseyenko, O. V. *et al.* (2014) 'Single Serotonergic Neurons that Modulate Aggression in Drosophila', *Current Biology*, 24(22), pp. 2700–2707. doi: 10.1016/j.cub.2014.09.051.

Alekseyenko, O. V., Lee, C. and Kravitz, E. A. (2010) 'Targeted Manipulation of Serotonergic Neurotransmission Affects the Escalation of Aggression in Adult Male Drosophila melanogaster', *PLOS ONE*, 5(5), p. e10806. doi: 10.1371/journal.pone.0010806.

Ali, Y. O. *et al.* (2011) 'Assaying Locomotor, Learning, and Memory Deficits in Drosophila Models of Neurodegeneration', *Journal of Visualized Experiments : JoVE*, (49). doi: 10.3791/2504.

Alic, N. *et al.* (2014) 'Cell-Nonautonomous Effects of dFOXO/DAF-16 in Aging', *Cell Reports*, 6(4), pp. 608–616. doi: 10.1016/j.celrep.2014.01.015.

Baek, M. and Mann, R. S. (2009) 'Lineage and Birth Date Specify Motor Neuron Targeting and Dendritic Architecture in Adult Drosophila', *Journal of Neuroscience*, 29(21), pp. 6904–6916. doi: 10.1523/JNEUROSCI.1585-09.2009.

Bai, H., Kang, P. and Tatar, M. (2012) 'Drosophila insulin-like peptide-6 (dilp6) expression from fat body extends lifespan and represses secretion of Drosophila insulin-like peptide-2 from the brain', *Aging Cell*, 11(6), pp. 978–985. doi: https://doi.org/10.1111/acel.12000.

Barbieri, M. *et al.* (2003) 'Insulin/IGF-I-signaling pathway: an evolutionarily conserved mechanism of longevity from yeast to humans', *American Journal of Physiology-*

Endocrinology and Metabolism, 285(5), pp. E1064–E1071. doi: 10.1152/ajpendo.00296.2003.

Barnes, A. I. *et al.* (2008) 'Feeding, fecundity and lifespan in female Drosophila melanogaster', *Proceedings of the Royal Society B: Biological Sciences*, 275(1643), pp. 1675–1683. doi: 10.1098/rspb.2008.0139.

Barnes, C. A. (2011) 'Secrets of aging: What does a normally aging brain look like?', *F1000 Biology Reports*, 3. doi: 10.3410/B3-22.

Bartke, A. (2011) 'Single-gene mutations and healthy ageing in mammals', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 366(1561), pp. 28–34. doi: 10.1098/rstb.2010.0281.

Bass, T. M. *et al.* (2007) 'Optimization of Dietary Restriction Protocols in Drosophila', *The Journals of Gerontology: Series A*, 62(10), pp. 1071–1081. doi: 10.1093/gerona/62.10.1071.

Bateman, J. and Mcneill, H. (2006) 'Insulin/IGF signalling in neurogenesis', *Cellular and molecular life sciences : CMLS*, 63, pp. 1701–5. doi: 10.1007/s00018-006-6036-4.

Batlevi, Y. and La Spada, A. R. (2011) 'Mitochondrial autophagy in neural function, neurodegenerative disease, neuron cell death, and aging', *Neurobiology of Disease*, 43(1), pp. 46–51. doi: 10.1016/j.nbd.2010.09.009.

Bauer, J. H. *et al.* (2005) 'Neuronal Expression of p53 Dominant-Negative Proteins in Adult Drosophila melanogaster Extends Life Span', *Current Biology*, 15(22), pp. 2063–2068. doi: 10.1016/j.cub.2005.10.051.

Beckingham, K. M. *et al.* (2007) 'Drosophila melanogaster - The model organism of choice for the complex biology of multi-cellular organisms', *Gravitational and Space Research*, 18(2). Available at: http://gravitationalandspaceresearch.org/index.php/journal/article/view/343 (Accessed: 21 December 2020).

Bishop, N. A., Lu, T. and Yankner, B. A. (2010) 'Neural mechanisms of ageing and cognitive decline', *Nature*, 464(7288), pp. 529–535. doi: 10.1038/nature08983.

Blüher, M., Kahn, B. B. and Kahn, C. R. (2003) 'Extended Longevity in Mice Lacking the Insulin Receptor in Adipose Tissue', *Science*, 299(5606), pp. 572–574. doi: 10.1126/science.1078223.

Bonini, N. M. and Fortini, M. E. (2003) 'Human neurodegenerative disease modeling using Drosophila', *Annual Review of Neuroscience*, 26, pp. 627–656. doi: 10.1146/annurev.neuro.26.041002.131425.

Brand, A. H. and Perrimon, N. (1993) 'Targeted gene expression as a means of altering cell fates and generating dominant phenotypes', *Development (Cambridge, England)*, 118(2), pp. 401–415.

Broughton, S. J. *et al.* (2005) 'Longer lifespan, altered metabolism, and stress resistance in Drosophila from ablation of cells making insulin-like ligands', *Proceedings of the National Academy of Sciences*, 102(8), pp. 3105–3110. doi: 10.1073/pnas.0405775102.

Broughton, S. J. *et al.* (2010) 'DILP-producing median neurosecretory cells in the Drosophila brain mediate the response of lifespan to nutrition', *Aging Cell*, 9(3), pp. 336–346. doi: https://doi.org/10.1111/j.1474-9726.2010.00558.x.

Broughton, S. and Partridge, L. (2009) 'Insulin/IGF-like signalling, the central nervous system and aging', *Biochemical Journal*, 418(1), pp. 1–12. doi: 10.1042/BJ20082102.

Busto, G. U., Cervantes-Sandoval, I. and Davis, R. L. (2010) 'Olfactory Learning in Drosophila', *Physiology*, 25(6), pp. 338–346. doi: 10.1152/physiol.00026.2010.

Butler, A. A. and Roith, D. L. (2001) 'Control of Growth by the Somatropic Axis: Growth Hormone and the Insulin-Like Growth Factors Have Related and Independent Roles', *Annual Review of Physiology*, 63(1), pp. 141–164. doi: 10.1146/annurev.physiol.63.1.141.

Butler, R. N. *et al.* (2008) 'New model of health promotion and disease prevention for the 21st century', *BMJ*, 337, p. a399. doi: 10.1136/bmj.a399.

Chakraborty, T. S. *et al.* (2019) 'Sensory perception of dead conspecifics induces aversive cues and modulates lifespan through serotonin in Drosophila', *Nature Communications*, 10(1), p. 2365. doi: 10.1038/s41467-019-10285-y.

Clancy, D. J. *et al.* (2001) 'Extension of Life-Span by Loss of CHICO, a Drosophila Insulin Receptor Substrate Protein', *Science*, 292(5514), pp. 104–106. doi: 10.1126/science.1057991.

Clardy, J., Fischbach, M. A. and Currie, C. R. (2009) 'The natural history of antibiotics', *Current Biology*, 19(11), pp. R437–R441. doi: 10.1016/j.cub.2009.04.001.

Colas, J. F. *et al.* (1995) 'Drosophila 5-HT2 serotonin receptor: coexpression with fushi-tarazu during segmentation', *Proceedings of the National Academy of Sciences*, 92(12), pp. 5441–5445. doi: 10.1073/pnas.92.12.5441.

Court, R. *et al.* (2020) 'A Systematic Nomenclature for the Drosophila Ventral Nerve Cord', *Neuron*, 107(6), pp. 1071-1079.e2. doi: 10.1016/j.neuron.2020.08.005.

Cuesto, G. *et al.* (2011) 'Phosphoinositide-3-Kinase Activation Controls Synaptogenesis and Spinogenesis in Hippocampal Neurons', *Journal of Neuroscience*, 31(8), pp. 2721–2733. doi: 10.1523/JNEUROSCI.4477-10.2011.

Cuesto, G. *et al.* (2015) 'GSK3β Inhibition Promotes Synaptogenesis in Drosophila and Mammalian Neurons', *PLOS ONE*, 10(3), p. e0118475. doi: 10.1371/journal.pone.0118475.

Davis, R. J. (2000) 'Signal transduction by the JNK group of MAP kinases', in Letts, L. G. and Morgan, D. W. (eds) *Inflammatory Processes:* Basel: Birkhäuser (Progress in Inflammation Research), pp. 13–21. doi: 10.1007/978-3-0348-8468-6_2.

De Luca, M. *et al.* (2003) 'Dopa decarboxylase (Ddc) affects variation in Drosophila longevity', *Nature Genetics*, 34(4), pp. 429–433. doi: 10.1038/ng1218.

Demontis, F. and Perrimon, N. (2010) 'FOXO/4E-BP Signaling in Drosophila Muscles Regulates Organism-wide Proteostasis during Aging', *Cell*, 143(5), pp. 813–825. doi: 10.1016/j.cell.2010.10.007.

Dravecz, N. (2020) *Insulin/IGF-like signalling and brain ageing in Drosophila melanogaster*. PhD. Lancaster University.

Duxbury, E. M. L., Rostant, W. G. and Chapman, T. (2017) 'Manipulation of feeding regime alters sexual dimorphism for lifespan and reduces sexual conflict in Drosophila melanogaster', *Proceedings of the Royal Society B: Biological Sciences*, 284(1854), p. 20170391. doi: 10.1098/rspb.2017.0391.

Enell, L. E. *et al.* (2010) 'Insulin Signaling, Lifespan and Stress Resistance Are Modulated by Metabotropic GABA Receptors on Insulin Producing Cells in the Brain of Drosophila', *PLOS ONE*, 5(12), p. e15780. doi: 10.1371/journal.pone.0015780.

Fischer, J. A. *et al.* (1988) 'GAL4 activates transcription in Drosophila', *Nature*, 332(6167), pp. 853–856. doi: 10.1038/332853a0.

Flatt, T. *et al.* (2008) 'Drosophila germ-line modulation of insulin signaling and lifespan', *Proceedings of the National Academy of Sciences*, 105(17), pp. 6368–6373. doi: 10.1073/pnas.0709128105.

Flatt, T. (2012) 'A New Definition of Aging?', *Frontiers in Genetics*, 3. doi: 10.3389/fgene.2012.00148.

Friedrich, A., Thomas, U. and Müller, U. (2004) 'Learning at Different Satiation Levels Reveals Parallel Functions for the cAMP–Protein Kinase A Cascade in Formation of Long-Term Memory', *Journal of Neuroscience*, 24(18), pp. 4460–4468. doi: 10.1523/JNEUROSCI.0669-04.2004.

Fu, Z. and Tindall, D. J. (2008) 'FOXOs, cancer and regulation of apoptosis', *Oncogene*, 27(16), pp. 2312–2319. doi: 10.1038/onc.2008.24.

Gavrilov, L. A. and Gavrilova, N. S. (2002) 'Evolutionary Theories of Aging and Longevity', *The Scientific World JOURNAL*, 2, pp. 339–356. doi: 10.1100/tsw.2002.96.

Giannakou, M. E. *et al.* (2004) 'Long-Lived Drosophila with Overexpressed dFOXO in Adult Fat Body', *Science*, 305(5682), pp. 361–361. doi: 10.1126/science.1098219.

Giannakou, M. E. and Partridge, L. (2007) 'Role of insulin-like signalling in Drosophila lifespan', *Trends in Biochemical Sciences*, 32(4), pp. 180–188. doi: 10.1016/j.tibs.2007.02.007.

Gimenez, L. E. D. *et al.* (2013) 'Modulation of methuselah expression targeted to Drosophila insulin-producing cells extends life and enhances oxidative stress resistance', *Aging Cell*, 12(1), pp. 121–129. doi: https://doi.org/10.1111/acel.12027.

Gnerer, J. P., Venken, K. J. T. and Dierick, H. A. (2015) 'Gene-specific cell labeling using MiMIC transposons', *Nucleic Acids Research*, 43(8), pp. e56–e56. doi: 10.1093/nar/gkv113.

Grandison, R. C., Piper, M. D. W. and Partridge, L. (2009) 'Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila', *Nature*, 462(7276), pp. 1061–1064. doi: 10.1038/nature08619.

Grotewiel, M. S. *et al.* (2005) 'Functional senescence in Drosophila melanogaster', *Ageing Research Reviews*, 4(3), pp. 372–397. doi: 10.1016/j.arr.2005.04.001.

Hadem, I. K. H. *et al.* (2019) 'Beneficial effects of dietary restriction in aging brain', *Journal of Chemical Neuroanatomy*, 95, pp. 123–133. doi: 10.1016/j.jchemneu.2017.10.001.

Honda, Y. and Honda, S. (1999) 'The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans', *The FASEB Journal*, 13(11), pp. 1385–1393. doi: https://doi.org/10.1096/fasebj.13.11.1385.

Howard, C. E. *et al.* (2019) 'Serotonergic Modulation of Walking in Drosophila', *Current Biology*, 29(24), pp. 4218-4230.e8. doi: 10.1016/j.cub.2019.10.042.

Hoyer, S. C. *et al.* (2008) 'Octopamine in Male Aggression of Drosophila', *Current Biology*, 18(3), pp. 159–167. doi: 10.1016/j.cub.2007.12.052.

Hsu, C. T. and Bhandawat, V. (2016) 'Organization of descending neurons in Drosophila melanogaster', *Scientific Reports*, 6(1), p. 20259. doi: 10.1038/srep20259.

Hwangbo, D. S. *et al.* (2004) 'Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body', *Nature*, 429(6991), pp. 562–566. doi: 10.1038/nature02549.

Ikeya, T. *et al.* (2009) 'The endosymbiont Wolbachia increases insulin/IGF-like signalling in Drosophila', *Proceedings of the Royal Society B: Biological Sciences*, 276(1674), pp. 3799–3807. doi: 10.1098/rspb.2009.0778.

Iliadi, K. G. (2009) 'The Genetic Basis of Emotional Behavior: Has the Time Come for a Drosophila Model?', *Journal of Neurogenetics*, 23(1–2), pp. 136–146. doi: 10.1080/01677060802471650.

Iliadi, K. G., Knight, D. and Boulianne, G. L. (2012) 'Healthy Aging – Insights from Drosophila', *Frontiers in Physiology*, 3. doi: 10.3389/fphys.2012.00106.

Ismail, M. Z. B. H. *et al.* (2015) 'The Drosophila Insulin Receptor Independently Modulates Lifespan and Locomotor Senescence', *PLOS ONE*, 10(5), p. e0125312. doi: 10.1371/journal.pone.0125312.

Johnson, O., Becnel, J. and Nichols, C. D. (2009) 'Serotonin 5-HT2 and 5-HT1A-like receptors differentially modulate aggressive behaviors in Drosophila melanogaster', *Neuroscience*, 158(4), pp. 1292–1300. doi: 10.1016/j.neuroscience.2008.10.055.

Kahsai, L. and Zars, T. (2011) 'Learning and Memory in Drosophila: Behavior, Genetics, and Neural Systems', in Atkinson, N. (ed.) *International Review of Neurobiology*. Academic Press (Recent advances in the use of Drosophila in neurobiology and neurodegeneration), pp. 139–167. doi: 10.1016/B978-0-12-387003-2.00006-9.

Kannan, K. and Fridell, Y.-W. (2013) 'Functional implications of Drosophila insulin-like peptides in metabolism, aging, and dietary restriction', *Frontiers in Physiology*, 4. doi: 10.3389/fphys.2013.00288.

Kapahi, P. *et al.* (2004) 'Regulation of Lifespan in Drosophila by Modulation of Genes in the TOR Signaling Pathway', *Current Biology*, 14(10), pp. 885–890. doi: 10.1016/j.cub.2004.03.059.

Kapahi, P. and Zid, B. (2004) 'TOR Pathway: Linking Nutrient Sensing to Life Span', *Science of aging knowledge environment : SAGE KE*, 2004(36), p. PE34. doi: 10.1126/sageke.2004.36.pe34.

Kennedy, B., Steffen, K. K. and Kaeberlein, M. (2007) 'Ruminations on dietary restriction and aging', *Cellular and molecular life sciences : CMLS*, 64, pp. 1323–8. doi: 10.1007/s00018-007-6470-y.

Kenyon, C. (2005) 'The Plasticity of Aging: Insights from Long-Lived Mutants', *Cell*, 120(4), pp. 449–460. doi: 10.1016/j.cell.2005.02.002.

Kenyon, C. J. (2010) 'The genetics of ageing', *Nature*, 464(7288), pp. 504–512. doi: 10.1038/nature08980.

Kim, S. E. *et al.* (2012) 'The role of Drosophila Piezo in mechanical nociception', *Nature*, 483(7388), pp. 209–212. doi: 10.1038/nature10801.

Kimura, K. D. *et al.* (1997) 'daf-2, an Insulin Receptor-Like Gene That Regulates Longevity and Diapause in Caenorhabditis elegans', *Science*, 277(5328), pp. 942–946. doi: 10.1126/science.277.5328.942.

Kirkwood, T. B. L. *et al.* (1991) 'Evolution of senescence: late survival sacrificed for reproduction', *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, 332(1262), pp. 15–24. doi: 10.1098/rstb.1991.0028.

Kirkwood, T. B. L. (2002) 'Evolution of ageing', *Mechanisms of Ageing and Development*, 123(7), pp. 737–745. doi: 10.1016/S0047-6374(01)00419-5.

Klass, M. R. (1983) 'A method for the isolation of longevity mutants in the nematode Caenorhabditis elegans and initial results', *Mechanisms of Ageing and Development*, 22(3), pp. 279–286. doi: 10.1016/0047-6374(83)90082-9.

Koushika, S. P., Lisbin, M. J. and White, K. (1996) 'ELAV, a Drosophila neuron-specific protein, mediates the generation of an alternatively spliced neural protein isoform', *Current Biology*, 6(12), pp. 1634–1641. doi: 10.1016/S0960-9822(02)70787-2.

Lakowski, B. and Hekimi, S. (1998) 'The genetics of caloric restriction in Caenorhabditis elegans', *Proceedings of the National Academy of Sciences*, 95(22), pp. 13091–13096. doi: 10.1073/pnas.95.22.13091.

Lee, P.-T. *et al.* (2011) 'Serotonin–mushroom body circuit modulating the formation of anesthesia-resistant memory in Drosophila', *Proceedings of the National Academy of Sciences*, 108(33), pp. 13794–13799. doi: 10.1073/pnas.1019483108.

Li, Yong *et al.* (2016) 'Octopamine controls starvation resistance, life span and metabolic traits in Drosophila', *Scientific Reports*, 6(1), p. 35359. doi: 10.1038/srep35359.

Libina, N., Berman, J. R. and Kenyon, C. (2003) 'Tissue-Specific Activities of C. elegans DAF-16 in the Regulation of Lifespan', *Cell*, 115(4), pp. 489–502. doi: 10.1016/S0092-8674(03)00889-4.

Lithgow, G. J. *et al.* (1995) 'Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress', *Proceedings of the National Academy of Sciences of the United States of America*, 92(16), pp. 7540–7544. doi: 10.1073/pnas.92.16.7540.

Liu, C. *et al.* (2019) 'A Serotonin-Modulated Circuit Controls Sleep Architecture to Regulate Cognitive Function Independent of Total Sleep in Drosophila', *Current Biology*, 29(21), pp. 3635-3646.e5. doi: 10.1016/j.cub.2019.08.079.

Longo, V. D. and Fabrizio, P. (2002) 'Visions & Reflections¶Regulation of longevity and stress resistance: a molecular strategy conserved from yeast to humans?', *Cellular and Molecular Life Sciences CMLS*, 59(6), pp. 903–908. doi: 10.1007/s00018-002-8477-8.

Luo, J. *et al.* (2012) 'Insulin-producing cells in the brain of adult Drosophila are regulated by the serotonin 5-HT1A receptor', *Cellular and Molecular Life Sciences*, 69(3), pp. 471–484. doi: 10.1007/s00018-011-0789-0.

Luo, J. *et al.* (2014) 'Drosophila Insulin-Producing Cells Are Differentially Modulated by Serotonin and Octopamine Receptors and Affect Social Behavior', *PLOS ONE*, 9(6), p. e99732. doi: 10.1371/journal.pone.0099732.

Lyu, Y. *et al.* (2021) 'Serotonin 2A receptor signaling coordinates central metabolic processes to modulate aging in response to nutrient choice', *eLife*. Edited by M. C. Wang, 10, p. e59399. doi: 10.7554/eLife.59399.

Mair, W. and Dillin, A. (2008) 'Aging and Survival: The Genetics of Life Span Extension by Dietary Restriction', *Annual Review of Biochemistry*, 77(1), pp. 727–754. doi: 10.1146/annurev.biochem.77.061206.171059.

Martín-Peña, A. *et al.* (2006) 'Age-Independent Synaptogenesis by Phosphoinositide 3 Kinase', *Journal of Neuroscience*, 26(40), pp. 10199–10208. doi: 10.1523/JNEUROSCI.1223-06.2006.

Martins, R., Lithgow, G. J. and Link, W. (2016) 'Long live FOXO: unraveling the role of FOXO proteins in aging and longevity', *Aging Cell*, 15(2), pp. 196–207. doi: https://doi.org/10.1111/acel.12427.

Masoro, E. J. (2005) 'Overview of caloric restriction and ageing', *Mechanisms of Ageing and Development*, 126(9), pp. 913–922. doi: 10.1016/j.mad.2005.03.012.

Mathew, R., Pal Bhadra, M. and Bhadra, U. (2017) 'Insulin/insulin-like growth factor-1 signalling (IIS) based regulation of lifespan across species', *Biogerontology*, 18(1), pp. 35–53. doi: 10.1007/s10522-016-9670-8.

Mattson, M. P., Maudsley, S. and Martin, B. (2004) ‘A neural signaling triumvirate that influences ageing and age-related disease: insulin/IGF-1, BDNF and serotonin’, *Ageing Research Reviews*, 3(4), pp. 445–464. doi: 10.1016/j.arr.2004.08.001.

McCay, C. M., Crowell, M. F. and Maynard, L. A. (1935) ‘The Effect of Retarded Growth Upon the Length of Life Span and Upon the Ultimate Body Size: One Figure’, *The Journal of Nutrition*, 10(1), pp. 63–79. doi: 10.1093/jn/10.1.63.

Miller, J. A., Oldham, M. C. and Geschwind, D. H. (2008) ‘A systems level analysis of transcriptional changes in Alzheimer’s disease and normal aging’, *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 28(6), pp. 1410–1420. doi: 10.1523/JNEUROSCI.4098-07.2008.

Mirzaei, H., Suarez, J. A. and Longo, V. D. (2014) ‘Protein and amino acid restriction, aging and disease: from yeast to humans’, *Trends in Endocrinology & Metabolism*, 25(11), pp. 558–566. doi: 10.1016/j.tem.2014.07.002.

Mohammad, F. *et al.* (2016) ‘Ancient Anxiety Pathways Influence Drosophila Defense Behaviors’, *Current Biology*, 26(7), pp. 981–986. doi: 10.1016/j.cub.2016.02.031.

Murtaza, G. *et al.* (2017) *FOXO Transcriptional Factors and Long-Term Living*, *Oxidative Medicine and Cellular Longevity*. Hindawi. doi: https://doi.org/10.1155/2017/3494289.

Nagarajan, N. R., Teixeira, A. A. C. and Silva, S. T. (2016) ‘The impact of an ageing population on economic growth: an exploratory review of the main mechanisms’, *Análise Social*, 51(218), pp. 4–35.

Nässel, D. R. *et al.* (2013) ‘Factors that regulate insulin producing cells and their output in Drosophila’, *Frontiers in Physiology*, 4. doi: 10.3389/fphys.2013.00252.

Nichols, C. D. (2007) ‘5-HT2 receptors in Drosophila are expressed in the brain and modulate aspects of circadian behaviors’, *Developmental Neurobiology*, 67(6), pp. 752–763. doi: https://doi.org/10.1002/dneu.20370.

Nicholson, L. *et al.* (2008) ‘Spatial and Temporal Control of Gene Expression in Drosophila Using the Inducible GeneSwitch GAL4 System. I. Screen for Larval Nervous System Drivers’, *Genetics*, 178(1), pp. 215–234. doi: 10.1534/genetics.107.081968.

Oeppen, J. and Vaupel, J. W. (2002) 'Broken Limits to Life Expectancy', *Science*, 296(5570), pp. 1029–1031. doi: 10.1126/science.1069675.

Oldham, S. and Hafen, E. (2003) 'Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control', *Trends in Cell Biology*, 13(2), pp. 79–85. doi: 10.1016/s0962-8924(02)00042-9.

Olivier, B. (2005) 'Serotonin and Aggression', *Annals of the New York Academy of Sciences*, 1036, pp. 382–92. doi: 10.1196/annals.1330.022.

Ons.gov.uk (2020). Available at: https://www.ons.gov.uk/peoplepopulationandcommunity/birthsdeathsandmarriages/lifeexpectancies/bulletins/nationallifetablesunitedkingdom/2017to2019#life-expectancy-at-birth-in-the-uk (Accessed: 10 October 2020).

Osterwalder, T. *et al.* (2001) 'A conditional tissue-specific transgene expression system using inducible GAL4', *Proceedings of the National Academy of Sciences*, 98(22), pp. 12596–12601. doi: 10.1073/pnas.221303298.

Pandey, U. B. and Nichols, C. D. (2011) 'Human Disease Models in Drosophila melanogaster and the Role of the Fly in Therapeutic Drug Discovery', *Pharmacological Reviews*, 63(2), pp. 411–436. doi: 10.1124/pr.110.003293.

Partridge, L. (2010) 'The new biology of ageing', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 365(1537), pp. 147–154. doi: 10.1098/rstb.2009.0222.

Partridge, L. *et al.* (2011) 'Ageing in Drosophila: The role of the insulin/Igf and TOR signalling network', *Experimental Gerontology*, 46(5), pp. 376–381. doi: 10.1016/j.exger.2010.09.003.

Partridge, L. and Farquhar, M. (1983) 'Lifetime mating success of male fruitflies (Drosophila melanogaster) is related to their size', *Animal Behaviour*, 31(3), pp. 871–877. doi: 10.1016/S0003-3472(83)80242-5.

Partridge, L. and Gems, D. (2002) 'Mechanisms of aging: public or private?', *Nature Reviews Genetics*, 3(3), pp. 165–175. doi: 10.1038/nrg753.

Partridge, L., Gems, D. and Withers, D. J. (2005) 'Sex and Death: What Is the Connection?', *Cell*, 120(4), pp. 461–472. doi: 10.1016/j.cell.2005.01.026.

Partridge, L., Piper, M. D. W. and Mair, W. (2005) 'Dietary restriction in Drosophila', *Mechanisms of Ageing and Development*, 126(9), pp. 938–950. doi: 10.1016/j.mad.2005.03.023.

Pflüger, H.-J., Duch, C. and Heidel, E. (2005) 'Neuromodulatory octopaminergic neurones and their functions during insect motor behaviour', *Acta Biologica Hungarica*, 55(1–4), pp. 3–12. doi: 10.1556/abiol.55.2004.1-4.2.

Phelps, C. B. and Brand, A. H. (1998) 'Ectopic Gene Expression in Drosophila Using GAL4 System', *Methods: A Companion to Methods in Enzymology*, 14(4), pp. 367–379.

Piazza, N. and Wessells, R. J. (2011) 'Drosophila Models of Cardiac Disease', *Progress in molecular biology and translational science*, 100, pp. 155–210. doi: 10.1016/B978-0-12-384878-9.00005-4.

Piper, M. D. W. and Partridge, L. (2018) 'Drosophila as a model for ageing', *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1864(9, Part A), pp. 2707–2717. doi: 10.1016/j.bbadis.2017.09.016.

Plotkin, S. A. (2005) 'Vaccines: past, present and future', *Nature Medicine*, 11(4), pp. S5–S11. doi: 10.1038/nm1209.

Ro, J. *et al.* (2016) 'Serotonin signaling mediates protein valuation and aging', *eLife*. Edited by A. Dillin, 5, p. e16843. doi: 10.7554/eLife.16843.

Roman, G. *et al.* (2001) 'P{Switch}, A System for Spatial and Temporal Control of Gene Expression in Drosophila melanogaster', *Proceedings of the National Academy of Sciences of the United States of America*, 98(22), pp. 12602–12607.

Saltiel, A. R. and Kahn, C. R. (2001) 'Insulin signalling and the regulation of glucose and lipid metabolism', *Nature*, 414(6865), pp. 799–806. doi: 10.1038/414799a.

Satoh, A., Imai, S. and Guarente, L. (2017) 'The brain, sirtuins, and ageing', *Nature Reviews Neuroscience*, 18(6), pp. 362–374. doi: 10.1038/nrn.2017.42.

Scheunemann, L. *et al.* (2018) 'Dunce Phosphodiesterase Acts as a Checkpoint for Drosophila Long-Term Memory in a Pair of Serotonergic Neurons', *Neuron*, 98(2), pp. 350-365.e5. doi: 10.1016/j.neuron.2018.03.032.

Shefferson, R. P., Jones, O. R. and Salguero-Gómez, R. (2017) *The Evolution of Senescence in the Tree of Life*. Cambridge University Press.

Siegrist, S. E. *et al.* (2010) 'Inactivation of Both foxo and reaper Promotes Long-Term Adult Neurogenesis in Drosophila', *Current Biology*, 20(7), pp. 643–648. doi: 10.1016/j.cub.2010.01.060.

Sitaraman, D. *et al.* (2017) 'Discrete Serotonin Systems Mediate Memory Enhancement and Escape Latencies after Unpredicted Aversive Experience in Drosophila Place Memory', *Frontiers in Systems Neuroscience*, 11. doi: 10.3389/fnsys.2017.00092.

Söderberg, J. A. E., Birse, R. T. and Nässel, D. R. (2011) 'Insulin Production and Signaling in Renal Tubules of Drosophila Is under Control of Tachykinin-Related Peptide and Regulates Stress Resistance', *PLOS ONE*, 6(5), p. e19866. doi: 10.1371/journal.pone.0019866.

Sokal, R. R. and Rohlf, F. J. (1995) *Statistical tables*. W.H. Freeman.

Sun, B., Xu, P. and Salvaterra, P. M. (1999) 'Dynamic visualization of nervous system in live Drosophila', *Proceedings of the National Academy of Sciences*, 96(18), pp. 10438–10443. doi: 10.1073/pnas.96.18.10438.

Taguchi, A., Wartschow, L. M. and White, M. F. (2007) 'Brain IRS2 Signaling Coordinates Life Span and Nutrient Homeostasis', *Science*, 317(5836), pp. 369–372. doi: 10.1126/science.1142179.

Tatar, M. *et al.* (2001) 'A Mutant Drosophila Insulin Receptor Homolog That Extends Life-Span and Impairs Neuroendocrine Function', *Science*, 292(5514), pp. 107–110. doi: 10.1126/science.1057987.

Tatar, M., Bartke, A. and Antebi, A. (2003) 'The Endocrine Regulation of Aging by Insulin-like Signals', *Science*, 299(5611), pp. 1346–1351. doi: 10.1126/science.1081447.

Thompson, G. *et al.* (2012) 'Olympic Britain'. Available at: https://commonslibrary.parliament.uk/research-briefings/rp12-38/ (Accessed: 10 December 2020).

Vargas, M. A. *et al.* (2010) 'A Role for S6 Kinase and Serotonin in Postmating Dietary Switch and Balance of Nutrients in D. melanogaster', *Current Biology*, 20(11), pp. 1006–1011. doi: 10.1016/j.cub.2010.04.009.

Vellai, T. *et al.* (2003) 'Influence of TOR kinase on lifespan in C. elegans', *Nature*, 426(6967), pp. 620–620. doi: 10.1038/426620a.

W. Vaupel, J. *et al.* (2004) 'The case for negative senescence', *Theoretical Population Biology*, 65(4), pp. 339–351. doi: 10.1016/j.tpb.2003.12.003.

Wang, B. *et al.* (2008) 'The Insulin-Regulated CREB Coactivator TORC Promotes Stress Resistance in Drosophila', *Cell Metabolism*, 7(5), pp. 434–444. doi: 10.1016/j.cmet.2008.02.010.

Wang, M. C., Bohmann, D. and Jasper, H. (2003) 'JNK Signaling Confers Tolerance to Oxidative Stress and Extends Lifespan in Drosophila', *Developmental Cell*, 5(5), pp. 811–816. doi: 10.1016/S1534-5807(03)00323-X.

Weindruch, R. and Walford, R. L. (1988) *retardation of aging and disease by dietary restriction*. C.C. Thomas. Available at: https://agris.fao.org/agris-search/search.do?recordID=US201300652687 (Accessed: 14 December 2020).

Who.int (2020). Available at: https://www.who.int/news-room/fact-sheets/detail/ageing-and-health (Accessed: 9 October 2020).

Wigby, S. *et al.* (2011) 'Insulin signalling regulates remating in female Drosophila', *Proceedings of the Royal Society B: Biological Sciences*, 278(1704), pp. 424–431. doi: 10.1098/rspb.2010.1390.

Willcox, B. J. *et al.* (2008) 'FOXO3A genotype is strongly associated with human longevity', *Proceedings of the National Academy of Sciences*, 105(37), pp. 13987–13992. doi: 10.1073/pnas.0801030105.

Worboys, M. (2007) 'Vaccines: conquering untreatable diseases', *BMJ*, 334(suppl 1), pp. s19–s19. doi: 10.1136/bmj.39045.558889.94.

Wullschleger, S., Loewith, R. and Hall, M. N. (2006) 'TOR Signaling in Growth and Metabolism', *Cell*, 124(3), pp. 471–484. doi: 10.1016/j.cell.2006.01.016.

Yankner, B. A., Lu, T. and Loerch, P. (2008) 'The aging brain', *Annual Review of Pathology*, 3, pp. 41–66. doi: 10.1146/annurev.pathmechdis.2.010506.092044.

Yuan, Q. *et al.* (2005) 'Serotonin Modulates Circadian Entrainment in Drosophila', *Neuron*, 47(1), pp. 115–127. doi: 10.1016/j.neuron.2005.05.027.

Yuan, Q., Joiner, W. J. and Sehgal, A. (2006) 'A Sleep-Promoting Role for the Drosophila Serotonin Receptor 1A', *Current Biology*, 16(11), pp. 1051–1062. doi: 10.1016/j.cub.2006.04.032.

Zhou, C., Rao, Yong and Rao, Yi (2008) 'A subset of octopaminergic neurons are important for Drosophila aggression', *Nature Neuroscience*, 11(9), pp. 1059–1067. doi: 10.1038/nn.2164.

Zhou, K. I., Pincus, Z. and Slack, F. J. (2011) 'Longevity and stress in Caenorhabditis elegans', *Aging (Albany NY)*, 3(8), pp. 733–753.

Ziehm, M., Piper, M. D. and Thornton, J. M. (2013) 'Analysing variation in Drosophila aging across independent experimental studies: a meta-analysis of survival data', *Aging Cell*, 12(5), pp. 917–922. doi: https://doi.org/10.1111/acel.12123.

Zwaan, B. J. (1999) 'The evolutionary genetics of ageing and longevity', *Heredity*, 82(6), pp. 589–597. doi: 10.1046/j.1365-2540.1999.00544.x.