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Elucidating the role of Insulin/IGF-like signalling in serotonergic neurons in the response of lifespan to dietary restriction in *Drosophila melanogaster*

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Declaration

This thesis is solely my own work and has not been submitted for the award of a higher degree or professional qualification elsewhere.

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1. Abstract

Mutations within the insulin/IGF-like signalling (IIS) pathway have been shown to influence lifespan in model organisms such as *Caenorhabditis elegans, Drosophila melanogaster,* and *Mus musculus*. Specifically, reductions in IIS, both systemic and tissue-specific, have been associated with increased lifespan, however some modulations have negatively impacted healthspan, including reproductive and behavioural health. In particular, the pan-neural reduction of IIS negatively affects the senescence of exploratory walking and negative geotaxis in *Drosophila* despite extending lifespan. The pan-neural effects of IIS reduction were hypothesised to be the sum of the positive, negative, and neutral effects of reduced IIS within individual neuronal subtypes and recent research supports this hypothesis. One recent study found that reducing IIS in specific neuronal subtypes in *Drosophila* resulted in varying effects on lifespan and locomotor senescence. Reducing IIS in serotonergic neurons was the only modulation investigated capable of extending lifespan, and it did so without negatively impacting locomotor senescence. This project addresses two hypotheses: 1) the response to dietary restriction involves insulin/IGF-like signalling in serotonergic neurons and therefore the response of flies with reduced IIS in serotonergic neurons will be reduced; and 2) reducing IIS in serotonergic neurons will alter serotonergic signalling to the IPCs resulting in altered DILP expression and/or secretion. To address these hypotheses, we used survival assays with standard, dietary restriction, fully fed, and low nutrient (0.1 x yeast) food conditions, immunohistochemical analysis of proteins in fly brains, and qPCR of fly heads. We also investigated other behaviours and phenotypes which could have been influenced by reduced serotonergic IIS including oxidative stress resistance, starvation resistance, negative geotaxis, feeding behaviour, and fecundity. The results showed that reduced serotonergic IIS increased lifespan in females on DR and SY diets and increased the lifespan of males on 0.1% yeast starvation and FF diets. No negative effects of reduced serotonergic IIS on stress resistance, negative geotaxis, or female fecundity were observed. Additionally, DILP2 and DILP5 expression was not altered in females with reduced serotonergic IIS, however, serotonin was. Together, the data suggest that the lifespan extension observed in females is likely not due to a modulation of the insulin producing cells (IPCs) and could be the result of altered serotonin signalling, however, further research is needed to determine the exact mechanism.

2. Introduction

2.1. Defining Ageing

Ageing is an inevitable part of the human experience and in the quest to understand, it must first be defined. The simplest definition of ageing is “the process of growing old”. However, the biological definition is “the time-dependent loss of physiological function at all levels, from molecular to organismal” (Kyriazis, 2020). The biological definition of ageing implies that ageing is not just how many years an organism has lived, but the accumulation of the effects of those years on organismal function and ability to adapt to the environment. It also allows for a distinction to be made between healthy and unhealthy ageing, where healthy ageing refers to developing and maintaining function at older ages, not just an absence of disease (Kyriazis, 2020).

The mechanisms underpinning ageing have still not been fully elucidated. However, twelve hallmarks of ageing which are conserved between numerous organisms including humans, have been identified, which are genomic instability, telomere attrition, epigenetic modifications, loss of proteostasis, disabled macroautophagy, mitochondrial dysfunction, stem cell exhaustion, cellular senescence, changes to intercellular communication, chronic inflammation, dysbiosis of the gut microbiome, and deregulated nutrient-sensing (López-Otín *et al.*, 2023). A tenth hallmark, compromised autophagy, has since been identified and is heavily associated with loss of proteostasis and mitochondrial dysfunction (Aman *et al.*, 2021). These hallmarks provide interesting potential focal points for ageing research and determining whether they are the result of ageing, or a causative factor is important as it could be the key to pharmaceutical interventions for healthy ageing (López-Otín *et al.*, 2023).

2.2. Ageing Research

2.2.1. Why study ageing?

In humans, life expectancy (LE), healthy life expectancy (HLE), and common causes of death have changed throughout history (WHO, 2022a, 2022b). Since the mid-1800s, lifespan has been steadily increasing. Initially, the increase was due to improvements in public hygiene and sanitation. This was then followed by the introduction of antibiotics and vaccines which reduced the number of deaths attributed to communicable diseases, such as polio,

diphtheria, and measles, which further increased life expectancy by reducing mortality rates among younger populations (Wilmoth, 2000; Partridge, 2010). This pattern of increasing life expectancy has continued into the modern day. Globally, LE increased by 6.6 years between 2000 and 2019. Despite this global HLE only increased by 5.4 years within the same period. This is because the increase was the result of declining mortality, not reduced years lived with disability (WHO, 2022a).

According to the ONS, (2019) the increase in LE also leads to changes within population structure, resulting in an “ageing population”. The phenomenon of the “ageing population” refers to the 65 and over age group growing faster than the working age group. By 2050, it is predicted that 1 in 4 people in the UK will be aged 65 or over and this will likely be accompanied by an increased old-age dependency ratio and an increased retirement age which could have major implications for the economy and healthcare providers (ONS, 2019).

Leading causes of death have also changed in more recent years. Between 2000 and 2019, more non-communicable diseases, such as Alzheimer’s and diabetes appeared within the global top 10 causes of death and communicable diseases such as tuberculosis and HIV/AIDS fell outside of it (Table 1) (WHO, 2022b). This move towards chronic illnesses such as cancer, diabetes, Alzheimer’s, and cardiovascular diseases, being leading causes of death is rooted in the fact that ageing is the major risk factor for their development.

Table 1: Global leading causes of death in 2000 and 2019. (WHO, 2022b).

2000		**2019**	
Cause	**% of total deaths**	**Cause**	**% of total deaths**
Ischaemic heart disease	13.2	Ischaemic heart disease	16.0
Stroke	10.7	Stroke	11.2
Neonatal conditions	6.2	Chronic obstructive pulmonary disease	5.8
Lower respiratory infections	6.0	Lower respiratory infections	4.7
Chronic obstructive pulmonary disease	5.8	Neonatal conditions	3.7
Diarrhoeal diseases	5.2	Trachea, bronchus, lung cancers	3.2
Tuberculosis	3.4	Alzheimer disease and other dementias	3.0
HIV/AIDS	2.7	Diarrhoeal diseases	2.7
Trachea, bronchus, lung cancers	2.4	Diabetes mellitus	2.7
Road injury	2.3	Kidney diseases	2.4

In conclusion, human lifespan and healthspan are not equal despite both increasing throughout history and in more recent years. The fact that HLE is not increasing at the same rate as LE indicates that the rate of human ageing has not decreased and explains the increasing prevalence of age-related diseases (Partridge, 2010). As a result, studying ageing, specifically the mechanisms involved in lifespan, healthspan, and the coupling and uncoupling of these two features is important as it could pave the way for broad-spectrum interventions capable of improving human health and function at old age.

2.2.2. Findings in model organisms

Research into ageing has been occurring for decades with caloric restriction (CR: reduction of caloric intake without inducing malnutrition) being the first lifespan extending intervention to be studied, in depth, during the 1930s (McCay *et al.*, 1935). McCay, Crowell and Maynard (1935) found that laboratory rats had extended lifespans when their caloric intake was reduced without causing malnutrition. Since these findings, further research into the effects of CR and dietary restriction (DR: the reduction of specific nutrient intake without causing malnutrition) has been carried out on a wide range of model organisms, from *Caenorhabditis elegans* to rhesus monkeys (Lakowski and Hekimi, 1998; Magwere *et al.*, 2004; Mattson, 2005; Mattison *et al.*, 2017).

In *C. elegans*, mutations in *eat* genes, which result in partial starvation due to disrupted pharyngeal function, were shown to extend lifespan (Lakowski and Hekimi, 1998). In *Drosophila*, DR has been shown to extend lifespan in both males and females, however, differences were observed between sexes (Magwere *et al.*, 2004). Furthermore, in rodents, CR and DR have been shown to both extend lifespan and ameliorate the onset and progression of many age-related diseases, including neurodegenerative diseases, diabetes, and cancer (Mattson, 2005). Rhesus monkeys also respond positively to DR, showing reduced incidences of some age-related diseases, including cardiovascular disease, cancer, and diabetes (Mattison *et al.*, 2017). Further research into DR has since found that diets containing reduced amounts of protein and certain amino acids, such as branched-chain amino acids (BCAAs) and methionine can promote longevity and health, suggesting that the effects of DR are at least in part in response to dietary protein content, more specifically BCAAs and methionine (Kitada *et al.*, 2019).

Nutrient-sensing pathways have been another focal point of ageing research, mainly due to the earlier work on DR indicating that nutrient intake can impact ageing. Four pathways have been identified to have an impact on ageing and lifespan and they are the Sirtuin (SIRT) pathway, AMP-dependent protein kinase (AMPK) pathway, target of rapamycin (TOR) pathway, and the insulin/IGF-like signalling (IIS) pathway (López-Otín *et al.*, 2023). All four have been linked to the control of lifespan in worms and flies, and the IIS and TOR pathways have been shown to impact lifespan and health in mice and associated between the IIS pathway and longevity have been observed in humans (López-Otín *et al.*, 2023).

The IIS pathway has also been shown to have a role in lifespan and healthspan determination. The initial association between the IIS pathway and lifespan was observed using mutant screening methods in *C. elegans* (Klass, 1983; Friedman and Johnson, 1988). Furthermore, the experiments carried out by Klass (1983), and Friedman and Johnson (1988) were the first experiments to show that lifespan altered through genetic manipulations alone. Since this discovery further research has been completed finding and evolutionarily conserved role for the pathway and tissue-specific effects (Broughton and Partridge, 2009). It is the most studied of the above pathways and has the strongest evidence for evolutionary conservation to humans (discussed further in 2.3. The Insulin/IGF-like pathway).

The TOR pathway is an evolutionarily conserved pathway first identified in set of *Saccharomyces cerevisiae* mutants which were resistant to growth inhibitory effects of rapamycin (Heitman *et al.*, 1991). It is evolutionarily conserved, and TOR exists in two complexes (TORC1 and TORC2) which have differing functions (Kapahi *et al.*, 2010). As shown in Figure 1, the pathway combines signals from growth factors and nutrients to control numerous processes including cell growth, protein synthesis, stress resistance, metabolism, cytoskeletal arrangements, and autophagy among others (Beauchamp and Platanias, 2013).

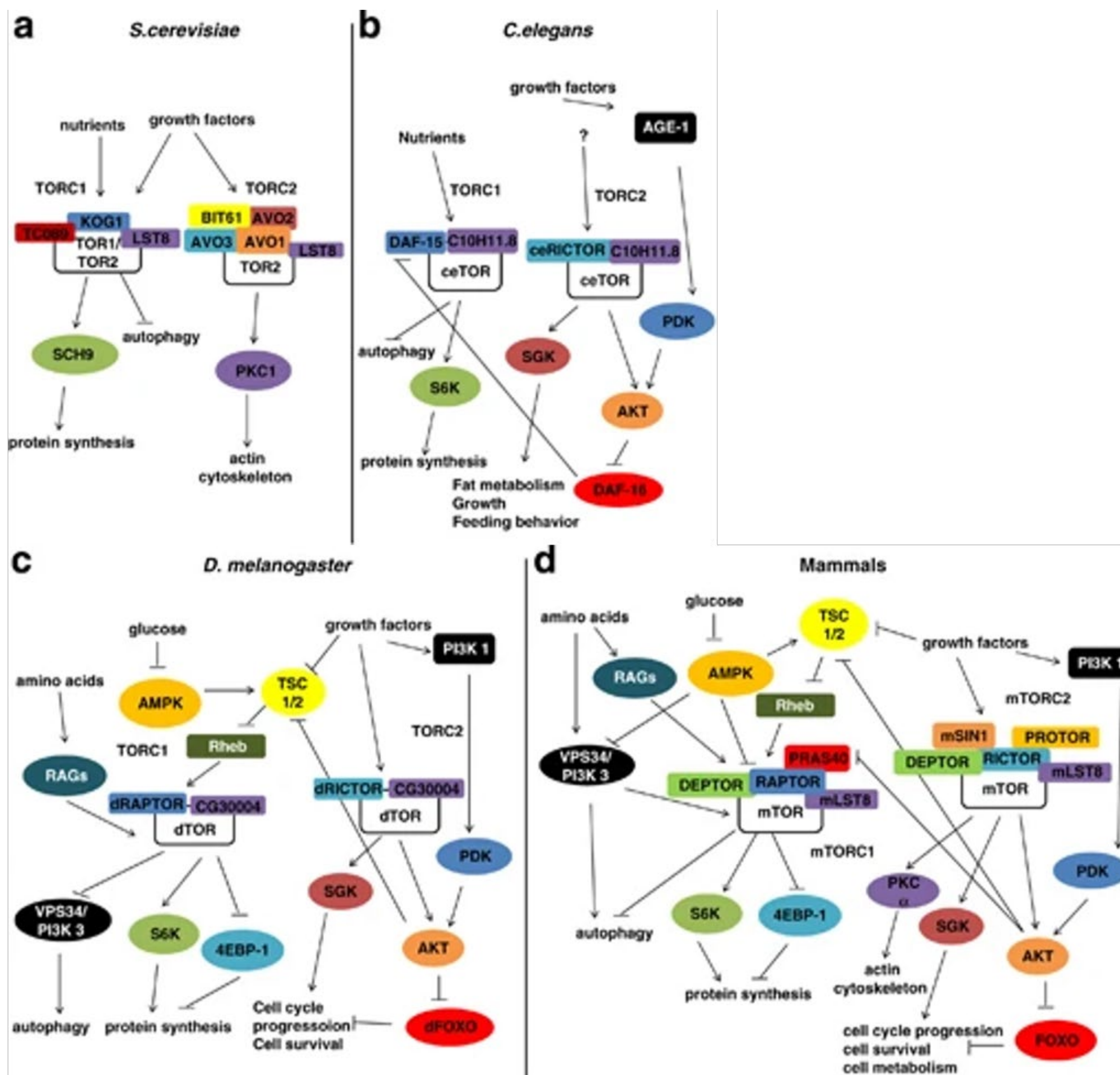


Figure 1: The TOR pathway. The TOR pathway in budding yeast, Saccharomyces cerevisiae (a); nematode worms, Caenorhabditis elegans (b); fruit flies, Drosophila melanogaster (c); Mammals, including mice and humans (d). The main components of the pathway are largely conserved (same shape and colour indicates homology between organisms) and the processes controlled are also consistent across organisms, however the pathway has evolved to become more complex with the mammalian pathway being much more complex than the yeast pathway. Image sourced from Beauchamp and Platanias, 2013.

Reducing TOR signalling via genetic manipulations has been shown to increase lifespan in *C. elegans* and *Drosophila* (Vellai *et al.*, 2003; Kapahi *et al.*, 2004). Pharmacological interventions using rapamycin have also been shown to extend lifespan in flies and mice of both sexes, and slow the development of age-related pathologies in mice (Harrison *et al.*, 2009; Bjedov *et al.*, 2010; Wilkinson *et al.*, 2012). Furthermore, genetic manipulation to reduce activity of S6 kinase (S6K), a downstream target of TORC1, extends lifespan in both

flies (specifically male flies) and mice and again, increases resistance to age-related pathologies in mice (Kapahi *et al.*, 2004; Selman *et al.*, 2009).

AMPK senses cellular energy levels and is activated in response to metabolic stress, more specifically, low levels of ATP and higher levels of ADP and AMP (Hardie *et al.*, 2012). This occurs when ATP production is inhibited, or ATP consumption is increased. When activated, AMPK modulates numerous processes to restore energy balance, including enhancing glucose uptake, inducing mitochondrial biogenesis, inducing autophagy, and inhibiting the biosynthesis of numerous biological molecules, such as proteins, lipids, ribosomal RNA, and carbohydrates (Hardie *et al.*, 2012). Studies into AMPK and lifespan have found that overexpression of *aak-2*, a catalytic subunit of AMPK, extend lifespan in *C. elegans*, and *aak2* mutants have a shorter lifespan than wild-types (Apfeld *et al.*, 2004). Likewise, increasing levels of AMP and ADP in flies of both sexes can extend lifespan by activating AMPK, and overexpression of the AMPK-α subunit in the fat body and muscles also extends lifespan (Stenesen *et al.*, 2013).

Sirtuins, which are NAD^+-dependent deacetylases, have also been implicated in the control of lifespan, and because of their reliance upon NAD^+ as a cofactor, they act as sensors of cellular metabolic state, indicating a link between metabolism and lifespan (Grabowska *et al.*, 2017). Their role in lifespan was first identified in *Saccharomyces cerevisiae*, a budding yeast, when SIR2 was shown to increase lifespan when overexpressed (Kaeberlein *et al.*, 1999). However, the role for sirtuins in longevity, specifically in *D. melanogaster* and *C. elegans*, is controversial. For example, increased expression of SIR2 homologues in *C. elegans* was initially shown to extend lifespan, and in both male and female *D. melanogaster* it was found to extend lifespan and be required for the response to DR (Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004). However, outcrossing of the *C. elegans* line used by Tissenbaum and Guarente (2001) abrogated the increase in longevity reported in the initial experiments (Burnett *et al.*, 2011). Furthermore, the lifespan extension seen in *D. melanogaster*, whilst repeated, was not significantly increased when compared to the necessary transgenic controls and was not required for lifespan extension via DR (Burnett *et al.*, 2011). Further research has found that overexpression of the *Drosophila* SIR2 homologue, Sirt1, in the adult fat body of male and female flies can extend lifespan and that

knockdown of Sirt1 in the fat body of female flies blocks lifespan extension caused by DR (Banerjee *et al.*, 2012; Hoffmann *et al.*, 2013). These conflicting results suggest that sirtuins may not be able to extend lifespan under all conditions.

The pathways discussed above interact as shown in Figure 2 (Pan and Finkel, 2017). Both AKT and AMPK are vital to the interconnectedness of the four pathways. AKT is activated by PI3K, TORC2, and SIRT1 and is inhibited by AMPK (Sarbassov *et al.*, 2005; Sundaresan *et al.*, 2011). AKT activates TORC1 by inhibiting TSC1/2, an inhibitor of TORC1, and inhibits FOXO activity via phosphorylation (Inoki *et al.*, 2002; Matsuzaki *et al.*, 2003). AMPK conversely inhibits TORC1 via phosphorylation of TSC1/2 and activates FOXO, both directly, via phosphorylation, and indirectly by inhibiting AKT (Inoki *et al.*, 2003; Greer *et al.*, 2007). AMPK can be activated indirectly by SIRT1 via LKB1 and can also activate SIRT1 by increasing intracellular levels of NAD^+ (Cantó *et al.*, 2009; Price *et al.*, 2012). SIRT1 also activates FOXO via deacetylation (Mouchiroud *et al.*, 2013). The complex interplay between these pathways means it can be difficult to untangle their individual effects on lifespan, however, this project focuses solely on the IIS pathway due to the numerous studied implicating it in the control of lifespan (Friedman and Johnson, 1988; Kenyon *et al.*, 1993; Clancy *et al.*, 2001; Holzenberger *et al.*, 2003; Broughton *et al.*, 2005; Selman *et al.*, 2008).

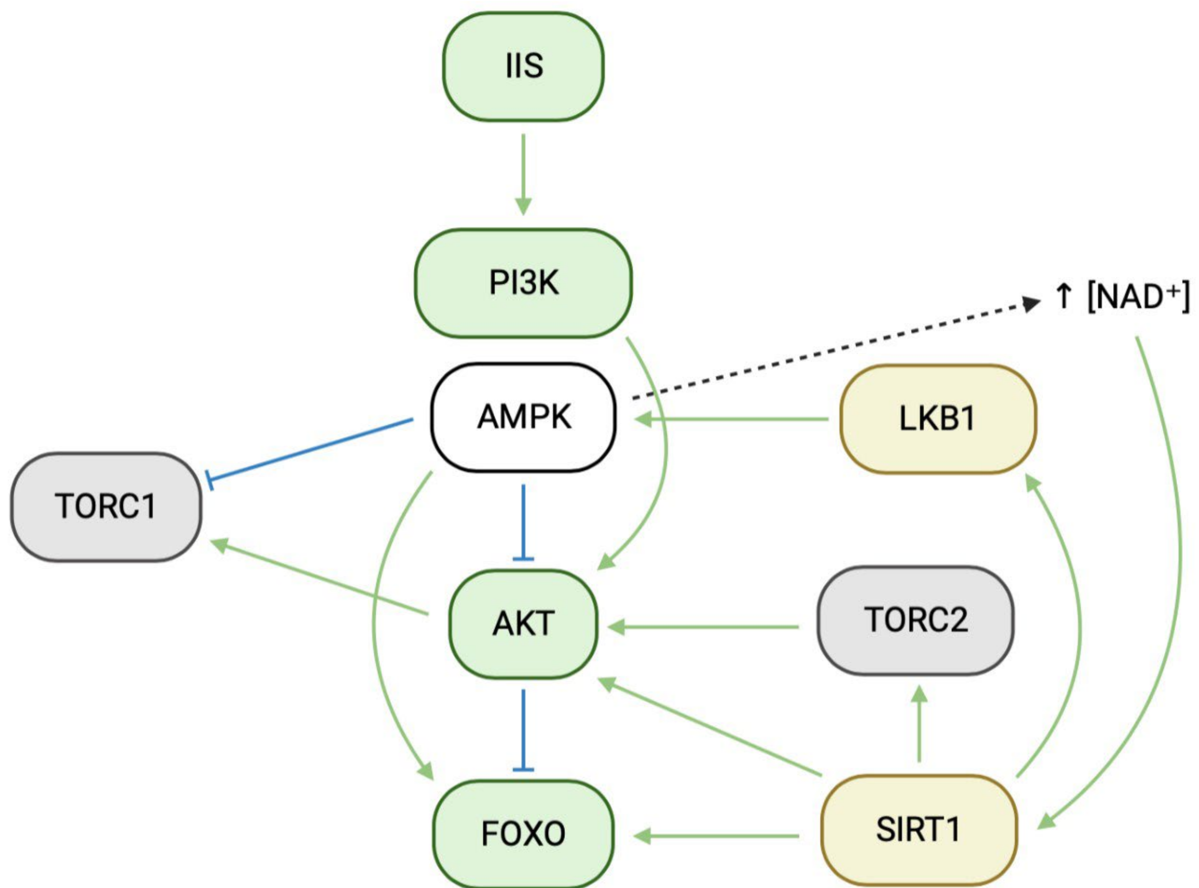


Figure 2: Interactions between IIS, TOR, sirtuins and AMPK. Some of the points of interaction between these signalling pathways are depicted here. Green arrows indicate activation and blue blunt-ended lines indicate inhibition. Image adapted from Pan and Finkel, 2017. Image created with BioRender.com.

2.3. The Insulin/IGF-like pathway

2.3.1. The pathway

The IIS pathway (Figure 3) can impact numerous processes including metabolic homeostasis, reproduction, growth, stress resistance, and longevity and ageing (Templeman and Murphy, 2018). Stimulation of the pathway initiates a signalling cascade mediated by phosphoinositide 3-kinase/protein kinase B (PI3K/AKT). The initial binding of insulin or insulin-like peptides leads to the dimerization and autophosphorylation of the receptor (InR) which then phosphorylates PI3K directly, or indirectly via the insulin receptor substrate (IRS). Phosphorylated PI3K catalyses the phosphorylation of PIP_2 (phosphatidylinositol 4,5biphosphate) to form PIP_3 (phosphatidylinositol 1,4,5-triphosphate) which can recruit AKT to the plasma membrane allowing phosphorylation and activation by phosphoinositide-dependent kinase (PDK) and TORC2. AKT phosphorylates the forkhead transcription factor

(FOXO/Daf-16) causing a conformational change that exposes a nuclear export signal and allows binding to 14-3-3, resulting in localisation of FOXO/Daf-16 to the cytosol. This inhibits expression of genes transcribed by FOXO meaning that IIS is a negative regulator of FOXO/Daf-16 transcription.

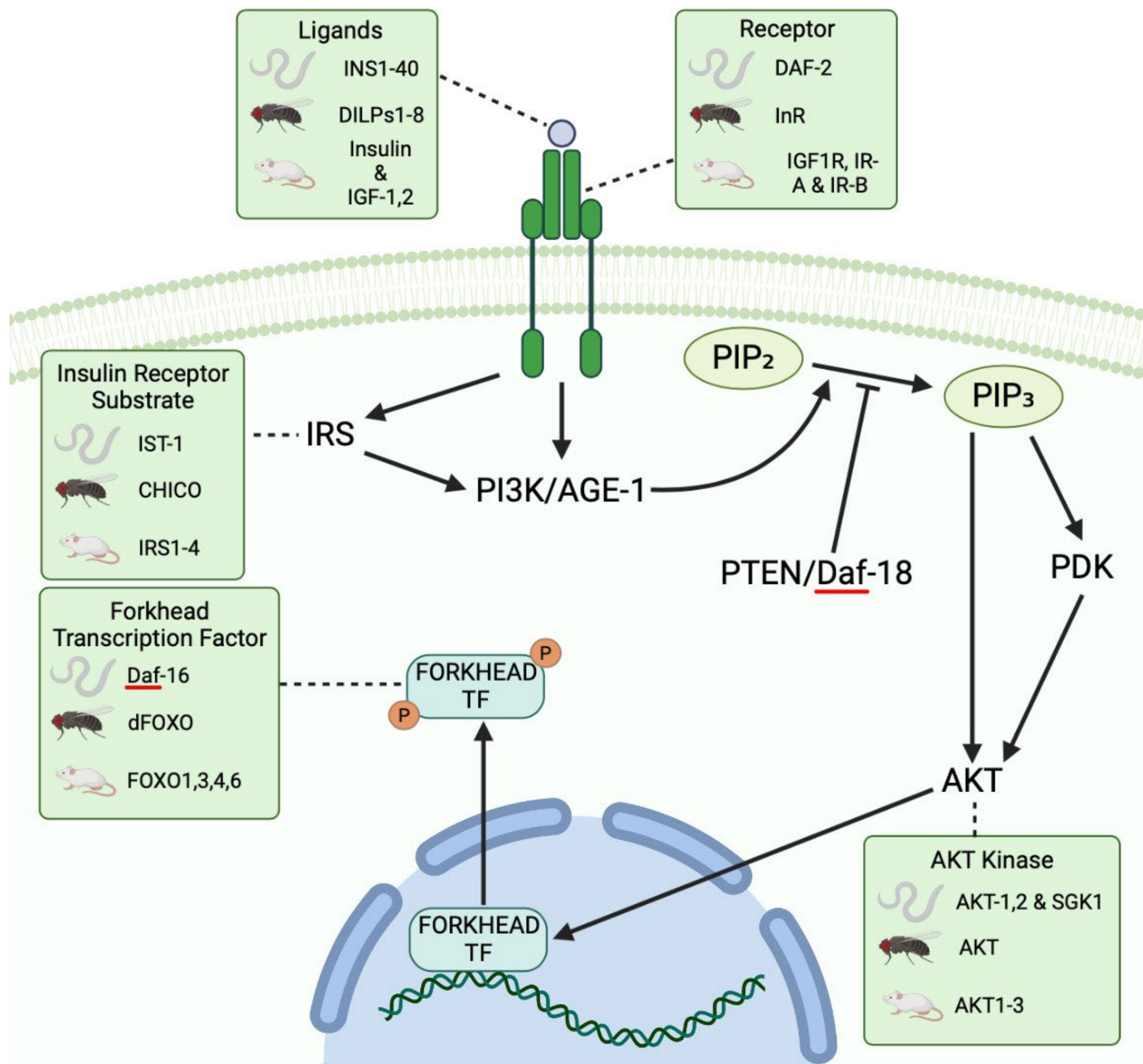


Figure 3: The insulin/IGF-like signalling pathway in Caenorhabditis elegans, Drosophila melanogaster, and mice (adapted from Broughton and Partridge, 2009). Image created with BioRender.com.

The IIS pathway is evolutionarily conserved, however, there are differences between organisms (Barbieri *et al.*, 2003). For example, *C. elegans* have 40 insulin-like peptides (Zheng *et al.*, 2018), *D. melanogaster* have eight (Colombani *et al.*, 2012; Garelli *et al.*, 2012), and mammals (including mice and humans) have three. Conversely, mammals have three

receptors which can dimerise in various combinations, whereas flies and worms only have one. Invertebrates also have only one forkhead transcription factor, Daf-16 in worms and dFOXO in flies, compared to the four seen in mammals (FOXO1,3,4,6) (Martins *et al.*, 2016).

2.3.2. Systemic modulation of IIS

One of the first genes involved in lifespan determination, later identified as *age-1*, was found using mutant screening methods in *C. elegans* (Klass, 1983; Friedman and Johnson, 1988). Following the discovery of long-lived *age-1* mutants, *daf-2* another gene with the ability to extend lifespan was identified. *daf-2* mutants were shown to have a lifespan over double that of wild-type *C. elegans* (Kenyon *et al.*, 1993). Furthermore, both *age-1* and *daf-2* mutants require the activity of *daf-16* to achieve their extended lifespans allowing researchers to conclude that *age-1*, *daf-2* and *daf-16* were working within a single pathway (Kenyon *et al.*, 1993; Dorman *et al.*, 1995). The subsequent determination of DNA sequences and cloning of all three genes revealed that they produced proteins involved in insulin/IGF-like signalling (IIS) with DAF-2 and AGE-1 antagonising the action of DAF-16, a FOXO transcription factor (Kimura *et al.*, 1997; Ogg *et al.*, 1997).

Since the discovery that modulation of some components of the IIS pathway could extend lifespan in *C. elegans* (see 2.3.1), further research has confirmed that the same can occur in both flies and mice (Tatar *et al.*, 2001; Holzenberger *et al.*, 2003). Specifically, systemic reductions in IIS achieved via genetic manipulation of the InR have been shown to extend the lifespan of female flies, and manipulation of the IRS has been shown to extend lifespan in both male and female flies (Clancy *et al.*, 2001; Tatar *et al.*, 2001). Similarly, in mice, homozygous knockout (KO) of IRS1 and heterozygous KO of IRS2 has been seen to extend lifespan, with the IRS1 KO effect present only in female mice (Taguchi *et al.*, 2007; Selman *et al.*, 2008). However, Selman *et al.* (2008) found that heterozygous IRS2 KO mice had a similar lifespan to that of wild type (WT) mice meaning IRS2 KO may not extend lifespan under all conditions.

Associations between the IIS pathway and longevity in humans also exist, however, it is difficult to study longevity in humans. Single nucleotide polymorphisms in *AKT*, *FOXO1*, and *FOXO3* occur more frequently in populations of long-lived humans (Flachsbart *et al.*, 2009; Li

et al., 2009; Pawlikowska *et al.*, 2009). Furthermore, analysis of IGF-1 levels and IGF-1 bioactivity has been investigated in centenarians and centenarians' offspring. Some studies concluded that lower IGF-1 levels were associated with longevity (Vitale *et al.*, 2012; Milman *et al.*, 2014). However, other studies have found no significant difference in IGF-1 levels or an increase (Paolisso *et al.*, 1997; Suh *et al.*, 2008). These opposing findings could be the result of using separate populations of centenarians and their offspring. Additionally, whilst centenarians are the best examples of long-lived humans, they are rare and there is no age-matched control group (Bucci *et al.*, 2016). The alternative is the study of centenarian offspring. This allows for age-matched controls and is justified by epidemiological data from numerous cohorts that shows relatives of centenarians have a higher chance of becoming long-lived and reduced risk of developing age-related diseases (Bucci *et al.*, 2016). However, using humans makes it difficult to carry out longitudinal studies due to longer lifespans and the unavoidable impact of environment.

Alongside effects on lifespan, systemic reduction of IIS also has impacts on certain measures of healthspan including locomotor function, age-related diseases, fecundity, and cognitive function. For example, *Drosophila* with systemically reduced IIS show attenuated age-related declines in locomotor function and female IRS1 KO mice show resistance to age-related locomotor dysfunction (Martin and Grotewiel, 2006; Selman *et al.*, 2008; Ismail *et al.*, 2015). Reduced IIS can also minimise or prevent the age-related changes in cardiac function that occur in *Drosophila* (Wessells *et al.*, 2004). IIS also protects worms and mice from Aβ accumulation, related proteotoxicity, and some of the symptoms associated with Aβ proteotoxicity, including paralysis in worms (Cohen *et al.*, 2006), and behavioural impairment, neuroinflammation and neuronal loss in mice (Cohen *et al.*, 2009; Freude *et al.*, 2009). Despite the positive effects of systemically reduced IIS on lifespan, locomotor function and the suggested positive impacts on cardiovascular disease and Alzheimer's disease, lifespan and healthspan are not always connected. For example, systemic reduction of IIS via expression of a dominant-negative insulin receptor, or by ablation of the insulin-like peptide producing median neurosecretory cells (IPCs), has little effect on the age-related senescence of exploratory walking (Ismail *et al.*, 2015). This occurred despite both interventions extending lifespan and improving negative geotaxis at old age (Ismail *et al.*, 2015). Fecundity is one of the best examples of a negative side effect seen to frequently

occur when reducing IIS and it has been observed in worms, flies, and mice (Partridge *et al.*, 2005b). However, reduced fecundity can be avoided based on tissue- and time-specific interventions. For example, adult-specific reduction of IIS via DAF-2 knockdown in worms and adult fat body specific *dFOXO* overexpression in flies do not reduce fecundity (Dillin *et al.*, 2002; Hwangbo *et al.*, 2004; Giannakou *et al.*, 2007). Furthermore, fat-specific insulin receptor knockout (FIRKO) mice have been reported to have normal fertility whereas neuronal insulin receptor knockout mice exhibit impaired spermatogenesis and ovarian follicle maturation (Brüning *et al.*, 2000; Partridge *et al.*, 2005b).

Numerous studies have been carried out investigating the effects of dietary restriction (DR) and reduced insulin/IGF-like signalling (IIS) on lifespan with the aim of determining the mechanisms responsible for the lifespan extension. One avenue that has been explored is the role of FOXO, a transcription factor that is downstream of the IIS pathway. We know that FOXO is required for lifespan extension via reduced IIS in *C. elegans* and *D. melanogaster* (Kenyon *et al.*, 1993; Slack *et al.*, 2011). Furthermore, FOXO is a nexus for many of the pathways involved in the response to DR, including the IIS, mTOR, AMPK, SIRT pathways (Greer and Brunet, 2008). Therefore, the genes under the control of FOXO and the processes they influence could identify potential mechanisms by which dietary restriction and reduced IIS can extend lifespan. For example, FOXO has been shown to influence autophagy, apoptosis, resistance to oxidative stress, cell cycle control, stem cell biology and tissue homeostasis, and inflammation (Martins *et al.*, 2016; Mathew *et al.*, 2017). However, some of the effects mediated by reduced IIS, such as the changes observed in growth, fecundity, and oxidative stress resistance, do not require FOXO in *D. melanogaster* (Slack *et al.*, 2011). As a result, there are still many outstanding questions about how reduced IIS and DR extend lifespan and modulate health and function during ageing. In particular, the relationship between lifespan extension and healthspan is complex and requires further study.

2.3.3. Tissue-specific modulation of IIS

Tissue-specific reductions in IIS have also been shown to extend lifespan. The IIS in adipose tissue has repeatedly been shown to impact longevity. In *C. elegans*, expression of *daf-16* in the intestine extends the lifespan of *daf-16(-);daf-2(-)* animals by 50-60% and rescues the lifespan of *daf-16(-)* mutants (Libina *et al.*, 2003). In flies, similar observations have been

made. Overexpression of dFOXO in the adult fat-body of flies extends the lifespan of females, however, constitutive overexpression of *dFOXO* causes developmental lethality, again showing the importance of both time- and tissue-specific modulation (Hwangbo *et al.*, 2004). Additionally, flies overexpressing *dFOXO* in the fat-body exhibited improved oxidative stress resistance (Giannakou *et al.*, 2004; Hwangbo *et al.*, 2004). Furthermore, FOXO is not required in every tissue for lifespan extension, for example activation of dFOXO in the fat body does not require dFOXO in other tissues to extend lifespan (Alic *et al.,* 2014). FIRKO mice also have extended lifespans and are protected against age-related, obesity, deterioration of glucose tolerance, and other metabolic abnormalities, making them comparatively healthier than their control counterparts despite similar food intake (Blüher *et al.*, 2003).

Overexpression of both *dPTEN* and *dFOXO* in the muscles of male flies has also been shown to extend lifespan and preserve muscle function at old age via FOXO/4E-BP signalling (Demontis and Perrimon, 2010). The overexpression is also associated with decreased feeding resulting in a fasting-like response which reduced insulin release from insulin-producing cells (IPCs) leading to systemic protection from age-related impairments in proteostasis (Demontis and Perrimon, 2010). Overexpression of *dPTEN* and *dFOXO* in cardiac muscle prevents the age-related decline in cardiac function, again via 4E-BP (Wessells *et al.*, 2009).

In *C. elegans*, animals with reduced *daf-2* expression in AB-derived cells, which includes almost all of the nervous system, have longer lifespans than controls (Apfeld and Kenyon, 1998). Additionally, restoring *daf-2* or *age-1* expression in neurons restored wild-type lifespan to that of *daf-2* or *age-1* mutants (Wolkow *et al.*, 2000). Brain-specific heterozygous and homozygous knockout of *IRS2* in mice has been shown to extend lifespan despite the mice being overweight, glucose intolerant, and hyperinsulinaemic (Taguchi *et al.*, 2007). Furthermore, male neuron-specific IRS1KO mice, whilst not long-lived, had improved insulin sensitivity, locomotor activity, and energy expenditure (Baghdadi *et al.*, 2023). Flies also show increased lifespan in response to neuron and brain specific modulations of IIS. For example, ablation of the IPCs has been shown to extend lifespan, however, the DILPs produced may be released into the circulatory system to exert systemic effects (Ikeya *et al.*,

2002; Broughton *et al.*, 2005). When IIS is reduced pan-neuronally using the GAL4-UAS system (UAS-InR^{DN} driven by elavGAL4), females, but not males, have increased lifespans (Ismail *et al.*, 2015). Lifespan extension was also seen in females when reduction of IIS was specific to adult neurons using the GeneSwitch variation of the GAL4-UAS system (Dravecz, 2020). Additionally, the pan-neural reduction of IIS had no effect on negative geotaxis or female fecundity, but constitutive and adult-specific reduction had detrimental effects on measures of exploratory walking in both sexes (Ismail *et al.*, 2015; Dravecz, 2020). The GAL4-UAS system is a gene expression system adapted from yeast which allows for both systemic and tissue-specific modulation of genes, and therefore proteins, of interest (Brand and Perrimon, 1993). In the case of tissue-specific expression, GAL4 is inserted near an enhancer or promoter within the fly genome, specific to a cell type, resulting in the expression of GAL4 being restricted to those cells. The upstream activating sequence (UAS), which is linked to the gene of interest, is present in all cells within the fly, however, the gene of interest will only be expressed when GAL4 binds to the UAS. Therefore, expression of the gene of interest is restricted to the cells where GAL4 is expressed (Roman *et al*., 2001). Temporal control using the GAL4-UAS system is difficult to achieve, however, a modified ligand-inducible GAL4-UAS system (GeneSwitch) which uses RU486 to induce expression allows for temporal and spatial control (Roman *et al*., 2001).

The effects of reduced insulin signalling on feeding behaviour, specifically food intake has also been investigated, however, the results are conflicting, with some findings showing that reduced insulin signalling promotes food intake and others showing no effect of reduced insulin signalling on food intake (Sudhakar *et al*., 2020). This link between the central nervous system (CNS) and IIS is not surprising given that worms, flies, and mice secrete factors from neuronal tissue capable of directly or indirectly modulating IIS locally and in distant tissues (Broughton and Partridge, 2009).

2.4. IIS in the Central Nervous System

2.4.1. Roles of IIS in the CNS

Reduced IIS in the CNS can extend lifespan in worms, flies, and mice (Wolkow *et al.*, 2000; Taguchi *et al.*, 2007; Ismail *et al.*, 2015). Alongside the effects of neuron-specific modulation of IIS on lifespan, IIS has numerous roles in the development and maintenance of the CNS.

For example, in the developing brain IIS is involved in axon guidance, differentiation of neuroblasts, synaptogenesis and neuronal outgrowth (Fernandez and Torres-Alemán, 2012). IIS also directly inhibits apoptosis by inhibiting GSK3 and FOXO, stimulating neuronal survival, and has been shown to be involved in synaptic plasticity, learning, and memory (Broughton and Partridge, 2009). However, reduced systemic IIS and overexpression of FOXO in the adult fat-body, and therefore potential increases in apoptosis, are associated with lifespan extension (Clancy *et al.*, 2001; Hwangbo *et al.*, 2004; Broughton *et al.*, 2005). These findings suggest that reducing IIS could negatively impact the integrity of the CNS despite extending lifespan, however, the effects of reduced IIS could be dependent upon the time at which it is reduced or the tissues in which it is reduced.

As previously stated, pan-neural reduction of IIS in *Drosophila* has no effect on locomotor function and extends lifespan in females (Ismail *et al*., 2015). Females with reduced pan-neural IIS had a median lifespan of 60 days compared to 52 and 47.5 days for the controls (Ismail *et al*., 2015), however no direct comparisons to systemic reductions in IIS have been made. Furthermore, it had detrimental effects on certain measures of exploratory walking, including the decision-making parameters, and the detrimental effects were still observed after adult-specific reduction suggesting the effects are not developmental (Ismail *et al.*, 2015; Dravecz, 2020). Furthermore, recovery from the effects of reduced pan-neural IIS was also observed suggesting that reduced IIS negatively affected neuronal function but did not accelerate neuronal ageing (Dravecz, 2020). With respect to learning and memory, impaired olfactory associative learning is observed in *chico*-null and insulin receptor mutants (Naganos *et al.*, 2012; Chambers *et al.*, 2015). Additionally, insulin signalling, specifically in the mushroom body and ellipsoid body, are required for normal learning and long-term memory (Naganos *et al.*, 2012; Chambers *et al.*, 2015). These findings suggest there is a fine balance between the positive, negative, and neutral effects of IIS within the CNS and further research has since identified that, in *Drosophila*, modulating IIS within individual neuronal subtypes produces different functional effects (Dravecz *et al.*, 2022).

2.4.2. Impact of IIS in specific neuronal subtypes

The CNS is made up of numerous components and contains multiple neuronal subtypes which produce and respond to various neurotransmitters. Studies in *C. elegans* have found

that IIS activity can have opposing effects on learning depending upon the neuronal subtype involved (Murakami *et al.*, 2005; Tomioka *et al.*, 2006). Additionally, in mice the four FOXO isoforms are expressed within the hippocampus in varying patterns and while the function of each isoform has not been completely elucidated it suggests that IIS may differentially modulate behaviour depending upon the region of the hippocampus being stimulated and the FOXO factors and cofactors present (Kim and Webb, 2017).

In *Drosophila* the effects of reduced IIS in different neuronal subtypes have been investigated (Dravecz *et al.*, 2022). Dravecz *et al.* (2022) reduced IIS in serotonergic, cholinergic, dopaminergic, octopaminergic, GABAergic, and glutamatergic neurons and recorded the effects on lifespan. Reduced IIS in dopaminergic and glutamatergic decreased female lifespan, however, the glutamatergic driver had a negative effect on lifespan in comparison to the UAS-InR^{DN} control. Additionally, the cholinergic driver also seemed to negatively impact lifespan in females. In males, reduced IIS in dopaminergic, glutamatergic, and cholinergic neurons reduced lifespan, however, the cholinergic driver also reduced lifespan. The reduction of IIS in octopaminergic and GABAergic neurons did not affect lifespan in either sex. Reduced IIS in serotonergic neuron was the only neuron-specific modulation capable of extending lifespan, however, it only extended lifespan in females.

Dravecz *et al.* (2022) also investigated the effects of reducing IIS in specific neuronal subtypes on negative geotaxis and exploratory walking. Reduced IIS in cholinergic neurons had detrimental impacts on negative geotaxis in both sexes, with females showing a faster decline than controls after 10 days old, and males showing reduced performance at all ages. Exploratory walking was also negatively impacted, but only in males, and the parameters were only affected significantly at 10 days old before they largely recovered and declined similarly to controls. However, none of the other neuronal subtypes, including serotonergic neurons, impacted either behaviour.

In summary, reducing IIS in different neuronal subtypes has different effects on lifespan and healthspan, with only serotonergic specific modulation capable of extending lifespan. However, reduced IIS in serotonergic neurons had no effect on negative geotaxis or exploratory walking. The differing effects suggest that, in *Drosophila*, individual neuronal

subtypes have specific roles in the modulation of lifespan and locomotor function. It is therefore likely that interplay between IIS and neuronal subtypes is responsible for the lifespan extension and behavioural effects seen in response to pan-neural reduction in IIS. This is because a pan-neural reduction in insulin signalling would decrease insulin in all neuronal subtypes, and therefore, be capable of influencing all downstream pathways and behaviours. However, as shown by Dravecz *et al*. (2022), when reducing insulin signalling in specific neuronal subtypes, some modulations reduced lifespan while others had no effect on lifespan or increased lifespan. Similarly, this varied response between different neuronal subtypes was also seen for negative geotaxis and exploratory walking. Therefore, to produce the phenotypes observed in response to reduced pan-neuronal IIS (Ismail *et al*., 2015; Dravecz, 2020), the effect of reduced IIS in each specific neuronal subtype could be interacting and either exacerbating or nullifying the effects of reduced IIS in the other neuronal subtypes. This summation of the effects of reduced IIS within each neuronal subtype could therefore produce the observed response to reduced pan-neural IIS, which would then contribute to the overall effect seen in response to systemic reductions in IIS.

2.5. Serotonin in behaviour and lifespan

2.5.2. Serotonin

Serotonin, also known as 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter produced within serotonergic neurons from L-tryptophan in a two-step process (Figure 4). The first step is the rate-limiting conversion of tryptophan to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (TRH) in the brain of tryptophan phenylalanine hydroxylase (TPH) in peripheral neurons (Kasture *et al.*, 2018). The second step is conversion of 5-HTP to 5-HT by aromatic amino acid decarboxylase which is also involved in the production of dopamine (Kasture *et al.*, 2018). Within the adult *Drosophila* brain there is approximately 90 to 100 serotonin-releasing neurons with their somas organised into numerous clusters (Figure 4B) and *Drosophila* have five separate G-protein coupled receptors which respond to serotonin: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{7} (Johnson *et al.*, 2009; Pooryasin and Fiala, 2015).

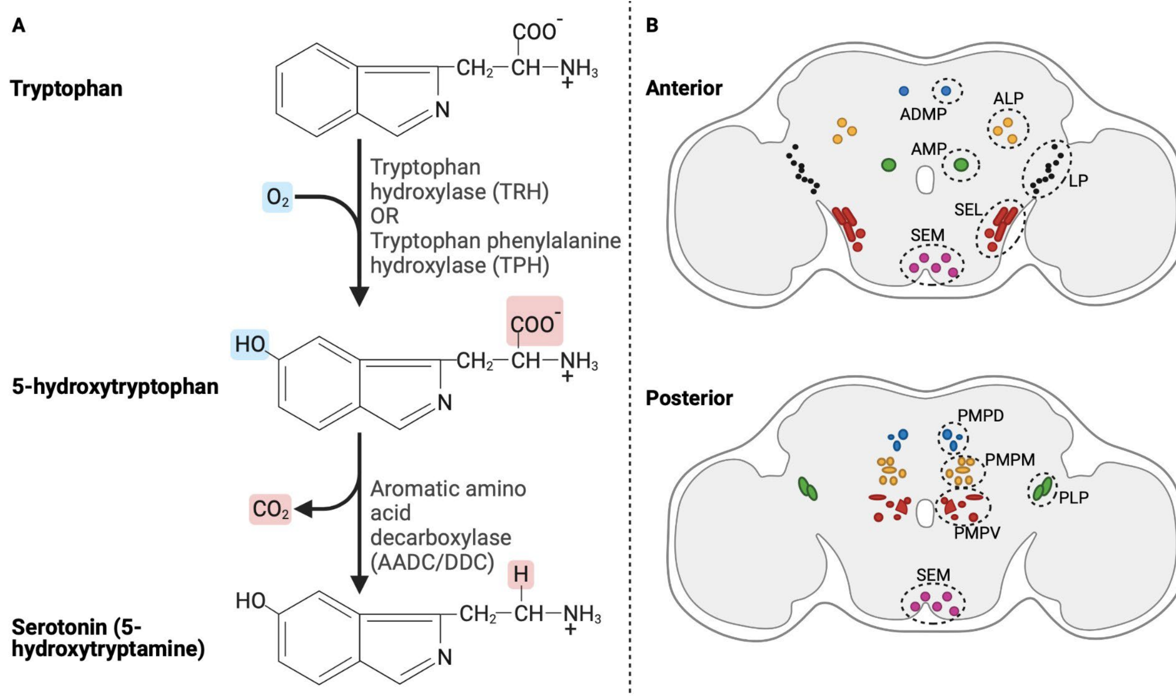


Figure 4: Serotonin synthesis and the localisation of the serotonergic neurons within the brain of Drosophila melanogaster. A: Synthesis of serotonin is a two-step process consisting of the conversion of tryptophan to 5-hydroxytryptophan and then to serotonin (5hydroxytryptamine; 5-HT). The neuronal production of serotonin is carried out by tryptophan hydroxylase (TRH) whereas the peripheral production of serotonin and tyrosine is carried out by tryptophan phenylalanine hydroxylase (TPH) (Kasture et al., 2018). B: Distribution of serotonin neurons in the adult Drosophila brain. ALP: Anterior lateral protocerebrum; AMP: Anterior medial protocerebrum; ADMP: Anterior dorsomedial protocerebrum; LP: Lateral protocerebrum; SEL: Lateral suboesophageal ganglion; SEM: Medial suboesophageal ganglion; PLP: Posterior lateral protocerebrum; PMPD: Posterior medial protocerebrum, dorsal; PMPM: Posterior medial protocerebrum, medial: PMPV: Posterior medial protocerebrum, ventral. Image created with BioRender.com.

2.5.2. Serotonin in behaviour

Activation of individual subsets or circuits of serotonergic neurons, or specific receptors can differentially modulate numerous behaviours in *Drosophila*, including feeding behaviour, motor activities, courtship, aggression, memory, circadian rhythms and sleep, and sensory perception (Bacqué-Cazenave *et al.*, 2020). Many of these behaviours are also modulated by serotonin in other organisms such as *C. elegans*, crayfish, and mammals suggesting that the function of serotonin is well-conserved between organisms, much like the function of IIS (Bacqué-Cazenave *et al.*, 2020).

For example, overall aggression in male *Drosophila* increases upon activation of 5-HT_{1A} receptors and in response to drug-induced increases in 5-HT and decreases upon activation of 5-HT_{2A} receptors (Dierick and Greenspan, 2007; Johnson *et al.*, 2009). Further research also found that a specific pair of neurons, the 5-HT PLP neurons, promote aggression but also interact with 5-HT_{1A} expressing cholinergic and GABAergic neurons to either increase or decrease aggression, respectively, indicating a complex role for serotonin in aggression (Alekseyenko *et al.*, 2014, 2019).

Circadian rhythms are also impacted by serotonin. Increased levels of serotonin have been shown to decrease circadian photosensitivity in *Drosophila* via the 5-HT_{1B} receptor (Yuan *et al.*, 2005). This is achieved by increasing the phosphorylation of SHAGGY which prevents its kinase activity and ultimately reduces the light-induced degradation of timeless, a *Drosophila* clock protein that is essential for circadian entrainment to light (Yuan *et al.*, 2005). Furthermore, the 5-HT_{1A} receptor and serotonin have been shown to play a role in sleep. *5-HT_{1A}* mutant flies have short, fragmented sleep and elevated levels of serotonin improved sleep in wild-type flies (Yuan *et al.*, 2006). Additionally, loss of *Trh*, *5-HT_{1A}*, or *5HT_{2B}* has been shown to decrease sleep, loss of *Trh* or *5-HT_{2B}* diminished sleep rebound after sleep deprivation, and *5-HT_{2B}* expression in a subset of dorsal fan-shaped body neurons has been shown to be essential for sleep homeostasis (Qian *et al.*, 2017).

Serotonin has a role in place learning and memory and short- and long-term olfactory learning and memory in *Drosophila* (Sitaraman *et al.*, 2008; Johnson *et al.*, 2011). In olfactory learning and memory, the 5-HT_{1A} receptor is required for consolidation and is important for recall, the 5-HT_{2A} receptor is important for both consolidation and recall, and the 5-HT_7 receptor is involved in acquisition, consolidation, and recall (Johnson *et al.*, 2011).

Sensory perception and the resulting behaviours and physiological changes are also modulated by serotonin. One example is *Drosophila* exposed to dead individuals develop aversive characteristics, modulated physiology and decreased longevity in females (Chakraborty *et al.*, 2019). The effects of exposure to dead flies rely upon both visual and olfactory cues and are mediated by serotonin signalling via the 5-HT_{2A} receptor (Chakraborty *et al.*, 2019). Sensory perception also has links to nutrient perception and feeding behaviour.

Serotonin signalling via the $5\text{-}HT_{2A}$ receptor is required for the development of protein preference in mildly starved flies (Ro *et al.*, 2016). Furthermore, the $5\text{-}HT_{2A}$ receptor modulates the effects of the metabolic reprogramming that occurs in *Drosophila* when they are fed on a choice diet where sugar and yeast are available separately (Lyu *et al.*, 2021). Serotonergic neurons also have a role in the translation of gustatory signals to physiological changes associated with nutrient regulation (Yao and Scott, 2022). Yao and Scott (2022) found two classes of 5-HT neurons capable of responding to sugars and bitter compounds. The neurons that responded to sugar activated insulin-producing cells and reduced food consumption, whereas the neurons that responded to bitter compounds activated enteric neurons capable of promoting gastric motility (Yao and Scott, 2022).

Numerous examples of the role of serotonin simple feeding behaviours, such as general levels of feeding, also exist (Albin *et al.*, 2015; Pooryasin and Fiala, 2015; Banu *et al.*, 2023). However, some of the results are conflicting or dependent on the specific serotonergic neurons being modulated (Albin *et al.*, 2015). This suggests that serotonin has a complex role in the control of feeding behaviour. For example, activation of a subset of neurons known as the R50H05 neurons, most of which are serotonergic, has been shown to induce feeding in sated flies and serotonin is required for their action (Albin *et al.*, 2015). However, the same study found that systemic activation of 5-HT neurons reduced feeding in starved and sated flies (Albin *et al.*, 2015). Another study also found that activation of the majority of 5-HT neurons inhibited feeding (Pooryasin and Fiala, 2015). Optogenetic assays have also shown similar results, with the activation of serotonergic cells in actively feeding flies being shown to suppress feeding in both sated and starved flies (Banu *et al.*, 2023). The microstructure of feeding behaviour also changes in food-deprived flies; an activated serotonergic system decreases sip duration and increases inter-sip intervals, and a suppressed system increases sip duration and decreases inter-sip intervals (Banu *et al.*, 2023). Additionally, the $5\text{-}HT_{1B}$ and $5\text{-}HT_7$ receptors have a role in suppressing and enhancing feeding, respectively (Banu *et al.*, 2023).

2.5.3. Serotonin in lifespan

Serotonin has also been linked to the control of lifespan. In *C. elegans* a deletion mutation in *ser-1* extends both mean and maximum lifespan by over 40% and this required the action of

daf-16 (Murakami and Murakami, 2007). Additionally, *ser-1* mutation did not further extend the lifespan of *daf-2*(RNAi) animals, however it further improved the lifespan of *eat-2* mutants (Murakami and Murakami, 2007). The same study found that a deletion in *ser-4* decreased survival of flies between 0-15 days old. This suggests serotonin signalling could have differing effects on lifespan based on the receptors being activated. Also, *ser-1* signalling is linked to the IIS pathway which could explain the influence of serotonin on lifespan, but no changes in *daf-28* expression were observed so the exact mechanism is still unknown (Murakami and Murakami, 2007). Pharmacological interventions using human serotonin receptor antagonists, specifically 5-HT_2 receptor antagonists, also extended lifespan in *C. elegans* (Petrascheck *et al.*, 2007). One compound tested, mianserin, required SER-4 action to extend lifespan and the lifespan extension observed involved mechanisms associated with DR, however, food intake was not affected (Petrascheck *et al.*, 2007).

In *Drosophila*, the 5-HT^{2A} receptor has been linked to lifespan via the modulation of protein intake. 5-HT^{2A} null *Drosophila* females have reduced protein consumption, extended lifespan, and a resistance to increases in dietary protein that usually reduce lifespan (Munneke *et al.*, 2022). Additionally, decreased longevity caused by chronic exposure to dead conspecifics is mediated via the 5-HT^{2A} branch of serotonin signalling and constitutive activation of 5-HT^{2A} signalling was sufficient to decrease lifespan (Chakraborty *et al.*, 2019).

2.6. IIS and Serotonin

2.6.1. Interplay between IIS and serotonin signalling

Alterations in serotonin signalling and IIS have been shown to modulate lifespan independently, however the interplay between the two has not been fully elucidated. The IPCs in *Drosophila* express the 5-HT_{1A} receptor and processes of serotonergic neurons impinge on branches of the IPCs suggesting signalling occurs between the two (Luo *et al.*, 2012). Additionally, reduced expression of the 5-HT_{1A} receptor in the IPCs resulted in increased IIS and reduced heat knockdown and starvation resistance and delayed cold coma recovery, as would be expected in response to increased IIS (Luo *et al.*, 2012). Further research, using qPCR to quantify *dilp* expression found that RNAi-induced knockdown of $5HT_{1A}$ increased levels of *dilp2* and *dilp5*, but not *dilp3* (Luo *et al.*, 2014). However, 5-HT_{1A}

RNAi does not completely inhibit 5-HT_{1A} signalling to the IPCs but likely results in the IPCs being less responsive to serotonin (Luo *et al.*, 2014).

NS3, a *Drosophila* nucleostemin family GTPase has been identified to have a role in the control of IIS via serotonergic neurons. Mutants exhibit reduced growth and various rescue experiments which targeted NS3-YFP to specific tissues found that NS3 acts specifically in serotonergic neurons to control growth. The *ns3* mutants also exhibited elevated levels of serotonin and DILP2, however, expression of *dilp2* did not change and the mutants had reduced peripheral insulin signalling (Kaplan *et al.*, 2008). This suggests *ns3* mutants have a defect in insulin release, however at the time of the study there were no assays capable of detecting circulating DILP levels. The findings of the study by Kaplan *et al.* (2008) suggest that elevated levels of serotonin observed in *ns3* mutants could inhibit DILP secretion, causing its build-up in the IPCs and the reduced peripheral insulin signalling. However, the results are also consistent with serotonin being a positive regulator of DILP secretion because *ns3* mutants could have a defect in serotonin release which would also explain the reduced DILP secretion (Kaplan *et al.*, 2008).

Serotonin levels have also been shown to decrease with age across multiple organisms, including flies and humans (Peters, 2006; Liao *et al.*, 2017; El Husseiny *et al.*, 2022). A study in *C. elegans* found that serotonin levels decrease with age and *eat-2* mutants but not *daf-2* or *age-1* mutants, did not experience the decline (Yin *et al.*, 2014). This identified a role for DR, but not IIS, in modulating 5-HT levels during ageing in *C. elegans*, and this may extend to *Drosophila* because of the evolutionary conservation of the IIS pathway and DR response. However, without experimental confirmation the exact influence of IIS on serotonin levels in *Drosophila* is still unknown.

The exact interactions between serotonin and IIS are difficult to untangle, however, there is interplay between the two signalling pathways. Combined with the influence serotonin and IIS have on lifespan and behaviour when investigated independently, and the findings of Dravecz *et al.* (2022), further research into the interactions between them and the impact of reducing IIS in serotonergic neurons is necessary to fully understand the mechanisms involved in ageing.

2.7. Aims and Objectives

The aim of this project is to confirm the lifespan extending effects of reduced IIS in serotonergic neurons and further investigate its effects on lifespan and behaviour, including stress resistance, in relation to diet, specifically protein intake. We chose to focus on oxidative stress and starvation with respect to the stress resistance assays. This is because oxidative stress and starvation resistance have previously been shown to be affected by reduced insulin signalling and have been used as measures of stress resistance in other experiments investigating the effects of reduced insulin signalling. Furthermore, this project aims to identify a potential pathway by which the reduction of serotonin-specific neuronal IIS extends lifespan.

Dravecz *et al.* (2022) showed that a reduction of IIS specific to serotonin neurons is sufficient to extend lifespan in females with no impact on locomotor senescence in either sex. However, the impact of diet was not investigated. Therefore, we wanted to investigate the effect of diet on lifespan and healthspan in flies with reduced IIS in serotonergic neurons because of known links between serotonin, feeding behaviour, metabolic programming, and lifespan, and DR, IIS, and lifespan. We hypothesise that the response to DR involves IIS in serotonergic neurons. Therefore, the response of flies with reduced IIS in serotonergic neurons to dietary restriction will be reduced, and they will not show an increase in lifespan due to DR.

Additionally, the reason for the lifespan extension is unknown. Previous research has identified a role for DILPs 2, 3 and 5 in lifespan extension and serotonergic neurons are known to signal to the IPCs which produce these proteins and alter their expression (Broughton *et al.*, 2005; Luo *et al.*, 2014). We hypothesise that reduced IIS in serotonergic neurons will alter serotonergic signalling to the IPCs resulting in altered DILP expression and/or secretion.

Research questions

Based on these hypotheses, this project addresses 2 questions:

1. Does diet influence lifespan or healthspan in flies with reduced IIS in serotonergic neurons?
2. How does reduced IIS in serotonergic neurons extend lifespan and is the mechanism influenced by diet?

Objective 1

To investigate the effects of different nutritional conditions on lifespan and healthspan in flies with reduced IIS in serotonergic neurons.

Research Design

IIS was reduced in serotonergic neurons using the UAS-InR^{DN} transgene targeted by the TrhGAL4 driver (Alekseyenko *et al*., 2010). The TrhGAL4 line drives expression of the UAS responder line, in this case UAS-InR^{DN}, in cells expressing the nervous system specific *tryptophan hydroxylase* enzyme (Alekseyenko *et al*., 2010). Flies were then maintained on foods containing varying quantities of yeast from 3-5 days old. Lifespan, negative geotaxis, feeding behaviour, oxidative stress, and starvation assay measurements were then compared between genotypes and food types.

Objective 2

To identify whether reducing IIS in serotonergic neurons alters the expression of DILPs 2, 3, and 5, and serotonin in the heads and brains of female flies under different dietary conditions.

Research Design

Real time qPCR was used to measure the expression of *dilp2, 3, 5* transcripts in fly heads in response to reduced IIS in serotonergic neurons under different dietary conditions. Immunostaining of serotonin and DILP5 in the brain and subsequent imaging using a confocal microscope was used to measure expression at the protein level under different dietary conditions.

3. Materials and Methods

3.1. Genetic background and maintenance of stocks

The wild-type strain used for this experiment was the *white* Dahomey (w^{Dah}) strain. The w^{Dah} strain was developed in the Partridge lab by repeatedly backcrossing the mutant *white* gene from the w^{1118} strain into the wild-type Dahomey strain (Ziehm *et al*., 2013). The wild-type Dahomey strain was initially obtained from Benin which has since been maintained in large cages with overlapping generations to ensure genetic variation (Ziehm *et al.*, 2013). To modulate insulin signalling in the serotonergic neurons, the UAS-GAL4 system was used. The specific *Drosophila* strains used were as follows: UAS-InR^{DN} (dominant-negative insulin receptor; Stock number: 8252, Bloomington Drosophila Stock Centre), TrhGAL4 (serotonergic GAL4 driver; Stock number: 38389, Bloomington Drosophila Stock Centre), and w^{Dah}.

All stocks were kept in disposable bottles (from FL Plastics), plugged with sponge bungs, containing standard sugar-yeast (SY) food. Yeast was from MP Biomedicals. Stocks were maintained at 25°C and 60-70% relative humidity on a 12:12 light-dark cycle.

3.2. Collecting virgin females

To ensure the experimental flies had the desired genotype, virgin females were collected and used for genetic crosses. This is necessary because female flies can store sperm after copulation meaning the genotype of their offspring is unknown unless further genetic analysis is completed (Pitnick *et al.*, 1999). After eclosion, females are unreceptive to male courtship for six to twelve hours depending on the temperature at which they are kept (Ashburner and Roote, 2007). They can be identified by a lack of pigment and different morphology. To maximise the number of virgin females collected, bottles with eclosing flies were kept at 25°C during the daytime and virgins were collected 2-3 times per day. Virgins were stored at 25°C at a density of 20 flies per vial and vials were checked for larvae after 2 days to ensure all flies collected were unmated at the time of collection.

3.3. Generation of experimental flies

Virgin females and young male flies were collected from the relevant genetic background and kept in vials until they were 3-4 days old. Approximately 100 virgin females and 50 males from the correct background were transferred into *Drosophila* cages with apple juice agar plates (Table 1) and live yeast as a food source. Cages were then left overnight at 25°C and 70% humidity, covered from light, to promote egg laying. Eggs were collected after 24 and 48 hours with the agar plate and live yeast being replaced after the first collection. Eggs were washed off the agar plates using Phosphate Saline Buffer (PBS) into a 50mL Falcon tube and allowed to settle to the bottom. Eggs were then pipetted, using a widened 200µL micropipette tip, into bottles containing SY food in 30µL aliquots. The bottles were then kept at 25°C until the flies eclosed. The eclosed flies were left to mate until the age of 3-4 days before being anaesthetised under CO_2 and sorted into vials by sex and genotype at a density of 10 flies per vial.

Table 1: UAS-GAL4 and w^{Dah} crosses.

Virgin females (~100)		**Males (~50)**	
UAS-InR^{DN}	×	TrhGAL4	**Experimental**
w^{Dah}	×	UAS-InR^{DN}	**Controls**
w^{Dah}	×	TrhGAL4	

3.4. Drosophila maintenance and media during experiments

Experimental flies were kept in disposable vials (from Regina Industries Ltd.) plugged with clean cotton balls at a density of 10 flies per vial with the flies being either male or female. The vials were maintained at 25°C, 70% humidity on a 12:12 light-dark cycle and were laid flat on their side to prevent flies from sticking to the media. The flies were transferred into fresh vials every two to three days.

Unless otherwise stated, flies were kept on a standard food containing 50g/L sugar and 100g/L yeast. The types of media and their recipes are summarised in Table 2.

Table 2: Recipes for fly media. The measurements of "Yeast" and "Water at the end" for the blue food are the same as Standard, DR, FF, or 0.1 depending on which food type the flies were previously fed with.

Ingredient \ Diet	Standard	DR	FF	0.1	Blue Food	H_2O_2	Starvation	Apple juice plates
Water (ml)	700	700	700	700	700	300	1000	500 (apple juice)
Agar (g)	15	15	15	15	15	4.5	15	7.5
Sugar (g)	50	50	50	50	50	15	0	0
Yeast (g)	100	50	200	10	varied	0	0	0
Water at the end (ml)	170	196	118	217	varied	12.6	0	0
Nipagin (ml)	30	30	30	30	30	0	30	0
Propionic acid (ml)	3	3	3	3	3	0	3	0
H_2O_2 (ml)	0	0	0	0	0	50	0	0
Brilliant Blue FCF (g)	0	0	0	0	25	0	0	0

To make the media, agar powder is added to hot water (or apple juice) and mixed thoroughly before being brought to the boil to ensure the agar dissolved completely. The sugar and yeast are added to the solution whilst constantly stirring and brought to the boil again. Once boiling, the mixture is removed from the heat and cold water is added to aid the cooling. The mixture is then left to cool below 60°C, being stirred occasionally to prevent the agar setting. After the mixture is cooled, nipagin and propionic acid are added as antimicrobials. The media is then poured into the appropriate container and left to set overnight at room temperature covered by breathable fabric.

3.5. Survival Analysis

Flies were sorted by CO_2 anaesthesia into vials containing the appropriate food at a density of 10 flies/vial (N=150). The flies were sorted when they were between 3 to 5 days old and separated by sex and genotype. They were transferred into fresh vials every Monday, Wednesday, and Friday and dead flies were counted during the transfers. Survival data are presented as a portion of surviving flies over time.

3.6. Fecundity

Female fecundity was measured by counting the number of eggs laid over a 48-hour period. The females were left to mate for 2-3 days after eclosion and then separated from males and sorted into vials. Ten vials from mated females on each treatment were sampled after lifespan transfers for egg counts. The same flies were monitored over their lifespan. The eggs counts were completed using vials collected on a weekly basis until the flies reached 39 or 40 days old. The data are presented as the mean number of eggs laid per female per day.

3.7. Negative Geotaxis

Flies kept at 10 flies/vial were transferred under light CO_2 anaesthesia into serological pipettes (25cm long, 1.5cm diameter) using a funnel (N=3 for each treatment) and left to recover for 30 minutes. After recovery pipettes were banged down one at a time hard enough so that the flies fell to the bottom. The pipettes were then stored upright for 45 seconds so the flies could be observed by eye. The flies staying at the bottom (not climbing more than 1cm) and the ones reaching the top (climbing more than 10cm) were recorded. Any flies that reached the top and fell were counted as reaching the top.

Each measurement was repeated three times, and the performance index was calculated using the formula 0.5 x (Ntotal+Ntop-Nbottom)/Ntotal. The experiment was carried out on a weekly basis throughout the lifespan, starting at the same time each day to avoid differences in activity levels caused by circadian rhythms.

3.8. Feeding Behaviour

At the age of 10 days, groups of 10 flies were transferred into vials (N=5 vials) containing DR, SY or FF food mixed with Brilliant Blue dye at a concentration of 2.5% w/v (Instant Sunshine FCF E133). They were left to feed on the food for 30 minutes before being frozen. Control flies were transferred into vials containing DR, SY or FF food with no dye.

The frozen flies were then transferred into 1.7mm Zirconium Bead ribolyser tubes (OPS Diagnostics) containing 250µL of PBS and homogenised using a ribolyser at 6.5m/s for 20 seconds. The samples were then centrifuged at 13,000rpm for 15 minutes (Eppendorf Centrifuge 5415 R), after which the supernatant was transferred out into 1.5mL Eppendorf tubes before being spun again at 13,000 rpm for 10 minutes. Following this, 100µL of each sample was pipetted into a CoStar 96-well flat transparent plate which was then read at 625nm using a Tecan Infinite 200 PRO plate reader.

A standard curve was produced using a 0.1mg/mL solution of Brilliant Blue with the start concentration being 0mg/mL and the final concentration being 0.025mg/mL. Data is presented as food ingested per fly (µg/mL).

3.9. Oxidative Stress Resistance – H_2O_2

At the age of 17 days, flies (N=100 flies) were transferred into vials containing H_2O_2 food and maintained at standard conditions. H_2O_2 (30% w/w in H_2O; Sigma Aldrich) food was at a concentration of 5% v/v. Deaths were recorded at least two times per day and the survival data are shown as a proportion of surviving flies over time.

3.10. Starvation Resistance

At the age of 17 days, flies (N=100 flies) were transferred into vials containing starvation food and maintained at standard conditions. Deaths were recorded at least twice a day, and the survival data are shown as a proportion of surviving flies over time.

3.11. RNA extraction from fly heads

At age 10 days, flies from each treatment were sorted into Eppendorf tubes under light CO_2 anaesthesia at a density of 20 flies per tube and immediately dropped into liquid nitrogen to snap freeze the flies. Once frozen tubes were removed with forceps and stored at -80°C. Separation of the fly heads and bodies was achieved by vigorously banging and shaking the tubes immediately after removal from the freezer. The heads and bodies were separated initially using a sieve, and then by eye, and 20 heads were counted and placed into 1.7mm Zirconium Bead ribolyser tubes (OPS Diagnostics) containing 1mL Tri Reagent (Sigma). The heads were homogenised using a ribolyser at 6.5m/s for 20 seconds before being left at room temperature for 5 minutes. 200µL of chloroform (Sigma-Aldrich) was added and the tubes were shaken vigorously for 15 seconds before being left to incubate at room temperature for 3 minutes. Samples were centrifuged at 12,000 rpm for 15 minutes at 4°C (Eppendorf Centrifuge 5415 R), following which 500µL (500µL = 1V) of the upper aqueous layer was removed to a clean Eppendorf tube. To the new Eppendorf 1V of isopropyl alcohol (Sigma) and 1/10V of 3M sodium acetate (NaOAc; EMD Millipore Corp.) were added and the samples were incubated at -80°C overnight for RNA precipitation.

Following the incubation, the samples were centrifuged at 12,000rpm for 15mins at 4°C. The supernatant was removed, and the remaining pellet was washed with 700µL of ice-cold 70% ethanol (EtOH; Fisher Scientific) in DEPC-water (Invitrogen). Samples were then spun at 10,000rpm for 10mins at 4°C. The 70% DEPC-EtOH wash was repeated twice more and after the third wash the ethanol was removed and the pellets were left to air-dry briefly before being resuspended in 10µL of DEPC-H_2O for heads. The RNA content and purity of the samples were measured at 260nm using Nanodrop and the samples were stored at -80°C.

3.12. cDNA generation

RNA samples were defrosted on ice and cDNA was generated from about 100ng of RNA in heads using the SuperScript III SuperMix kit (Invitrogen).

The following ingredients were added into 0.2mL PCR tubes on ice: 10 µL 2X RT Reaction Mix, 2µL RT Enzyme Mix, XµL RNA, and 8-XµL of DEPC-H_2O. The contents were then mixed and incubated at 25°C for 10 minutes, followed by incubation at 50°C for 30 minutes and then 85°C for 5 minutes. Following the 85°C incubation the samples were chilled on ice and 1 µL of RNase H was added. Samples were then incubated at 37°C for 20 minutes before being kept on ice or stored at -20°C.

3.13. Quantitative Polymerase Chain Reaction (qPCR)

The cDNA samples were defrosted, pulse centrifuged to collect their contents at the bottom and diluted with 10µL of ice-cold milliQ (MQ) water before being kept on ice until the PCR plates were ready to be loaded. Primer master mixes were made for each primer pair (all primers are from Invitrogen). *β-actin* and *Rpl32* were used as reference genes, and *dilp2, 3, 5* were tested for female fly heads.

β-actin primers:

Forward: CACACCAAATCTTACAAAA

Reverse: AATCCGGCCTTGCACATG

Rpl32 primers:

Forward: ATGCTAAGCTGTCGCACAAATG

Reverse: GTTCGATCCGTAACCGATGT

dilp2 primers:

Forward: GGCCAGCTCCACAGTGAAGT

Reverse: CGTTGGCGAGTTCGCCTC

dilp3 primers:

Forward: GGTGTCCAGGCCACCATGAA

Reverse: CGTCGAAGACTCCATCCCGA

dilp5 primers:

Forward: GCCTGTCCCAATGGATTCA

Reverse: CCTCAACGTGGAAAAGGAACA

The total reaction volume was 20µL and contained 10µL 2x SYBR Green Master Mix (Sigma-Aldrich), 6µL of MQ water, 1µL of 10µM forward primer, 1µL of 10µM reverse primer, and 2µL of cDNA. The samples were loaded into a BioRad Hard-Shell 96-well PCR plates and run on a BioRad CFX96 Real Time System C1000 Thermal Cycler machine using the following protocol (Broughton *et al.*, 2005).

10 minutes at 94°C
30 seconds at 94°C ←
30 seconds at 55°C — **Repeat 39 times**
60 seconds at 72°C. Plate read.
Melt curve 65°C to 95°C in increments of 0.5°C for 5 seconds
Read plate
End.

The readings were analysed using the Bio-Rad CFX Maestro software version 3.1.

3.14. Immunohistochemistry

CO_2 anaesthetised flies collected at 18 days old were dipped in 70% ethanol for 2 minutes before brains were dissected out in PBS and kept on ice. 4% paraformaldehyde (PFA) was made from frozen 20% PFA stock and PBS. The brains were incubated at room temperature with gentle shaking for 40 minutes in the 4% PFA to fix the brains. The brains were washed 3 times with PBS for 10 minutes with gentle shaking. This was repeated with TNT. The brains were blocked in blocking solution 1 for 2 hours at room temperature with gentle shaking before being incubated in the primary antibody solution, containing anti-serotonin (Sigma Aldrich) and anti-DILP5 antibodies (Broughton *et al.*, 2010), overnight at 4°C. After incubation the brains were washed 6 times with TNT for 10 minutes before being incubated with the secondary antibody solution for 2 hours in the dark at room temperature with

gentle shaking. The brains were washed 3 times with TNT for 10 minutes with gentle shaking and this was then repeated with PBS before the brains were soaked and held in 2% n-propylgallate and stored at 4°C in the dark. Ingredients of the reagents are shown in Table 3.

Table 3: Ingredients of the reagents used for the fixing, blocking, and staining of fly brains for DILP5 and serotonin. The anti-serotonin antibody is produced in rabbit and the antiDILP5 antibody is produced in rat.

TNT	Block solution 1	1° antibody solution	Block solution 2	2° antibody solution	2% n-propylgallate
0.1M Tris HCl 0.3M NaCl 0.5% Triton X-100	TNT 4% Normal Goat Serum	Blocking Solution 1 1:50 Anti-DILP5 antibody 1:500 Anti-serotonin antibody	TNT 2% Normal Goat Serum	Blocking Solution 2 1:20 Anti-rat antibody 1:20 Anti-rabbit antibody	80% Glycerol 20% PBS 2% n-propylgallate powder

Stained brains were mounted onto microscope slides and were imaged, and Z-stacks were generated using a Zeiss LSM880 confocal microscope. Images were then processed using FIJI Software (Schindelin *et al.*, 2012). Measures of integrated density were taken to quantify the levels of DILP5 and serotonin. DILP5 was measured in within the area of the IPCs (Fig.5a) and serotonin was measured within the central brain (Fig.5b). The areas taken for the measurements were designated manually in the FIJI Software.

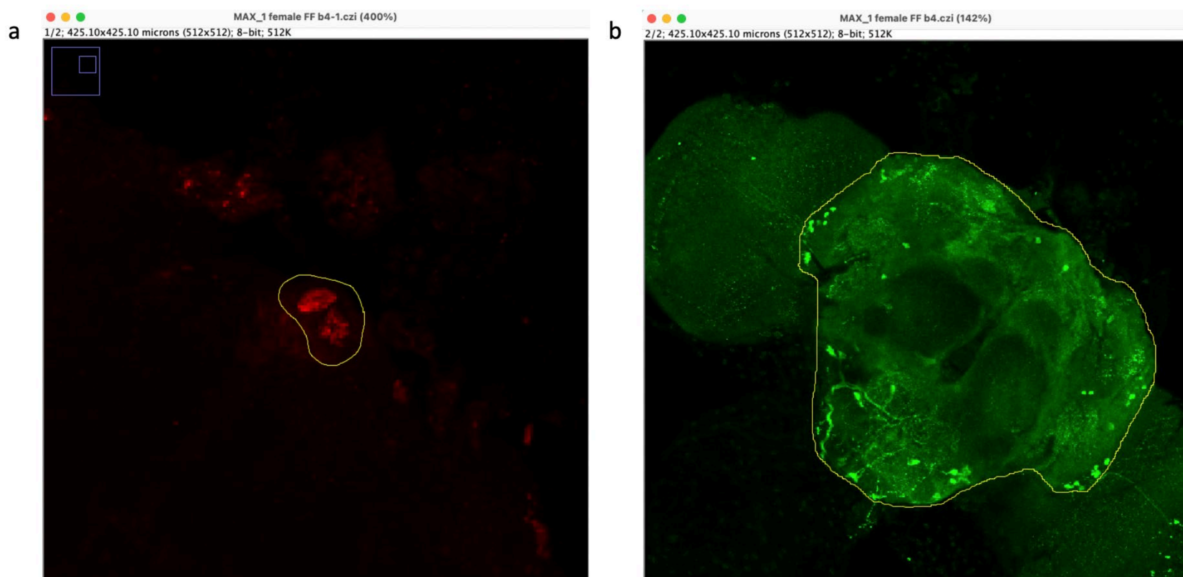


Figure 5. Representative ImageJ selections used to extract measures of Integrated Density. a) Selection for DILP5. b) Selection for serotonin within the central brain.

3.15. Statistical Analysis

Statistical analysis of the raw data was carried out using JMP Software 17.2.0 (SAS Institute, Cary, NC, USA, 1989-2023) and all graphs were generated using Excel. Lifespan and survival data were analysed using Kaplan-Meier estimates and Log Rank Chi-square statistics in JMP. Proportional hazards analysis was also carried out on the SY, DR, and FF lifespans to investigate the effects of genotype, food type and genotype*food type on survival. Where significant differences were observed, a data filter was used to carry out pairwise comparisons between genotypes. Proportional hazards analysis was also carried out using JMP.

Fecundity, negative geotaxis, feeding behaviour, qPCR and immunohistochemistry data were also analysed in JMP. Effect tests were carried out using Generalised Linear Model Fit looking for age, genotype, and food type effects (in all combinations) and where a significant effect was seen, post-hoc pairwise tests were carried out. Student's t-tests were used to compare two groups and Tukey-Kramer HSD tests were used to compare three or more groups at each age point. Repeated measures were carried out on the negative geotaxis and fecundity data with a focus on effects of food type, genotype, and food type*genotype effects across time. The repeated measures analysis used time points up to a maximum of 40 days to reduce the effects of reducing sample sizes due to deaths. When significant differences were observed in the repeated measures analysis, a data filter was used to carry out pairwise comparisons between genotypes.

Significant difference was defined as $p<0.05$. Due to lifespan experiments being carried out at separate times, data were analysed separately to avoid effects of potential changes in experimental conditions between the experiments.

4. Results – The effects of reduced IIS in serotonergic neurons on lifespan, behaviour, and stress resistance.

Previous research has shown that reduced serotonergic IIS can extend lifespan in female flies and has no impact on locomotor senescence in either sex (Dravecz *et al.*, 2022). Furthermore, IIS is known to impact fecundity and stress resistance (Partridge *et al.*, 2005b; Broughton *et al.*, 2005). However, the effect of reduced serotonergic IIS on stress resistance, fecundity, and on the resulting phenotypes in response to changes in diet has not been investigated. The aim of the experiments detailed below was to determine the effect of dietary restriction on lifespan, negative geotaxis, fecundity, and resistance to both starvation and oxidative stress in male and female flies with reduced IIS in serotonergic neurons.

To do this we carried out lifespan assays using flies with reduced serotonergic IIS (TrhGAL4/UAS-InR^{DN}) and the relevant controls (TrhGAL4/+ and UAS-InR^{DN}/+). The flies were maintained on standard sugar-yeast (SY), dietary restriction (DR), and fully fed (FF) food from the age of 3-5 days old under the conditions described in section 3.4. Alongside the lifespan, negative geotaxis assays were carried out every 7 days (section 3.7) and female fecundity was also recorded (section 3.6).

Resistance to starvation was investigated by maintaining flies on media containing yeast at a concentration of 10g/L (0.1) or media containing solely agar (0xSY). The flies on 0.1 media were maintained as a lifespan (section 3.5) (N=150). The flies on the 0xSY media were transferred from DR, SY or FF media at 10 or 17 days old and the number of deaths was scored one or more times a day (N=100). Oxidative stress resistance was investigated by transferring flies from DR or FF food onto media containing hydrogen peroxide (H_2O_2) and scoring the number of deaths 2 or more times a day (N=100).

4.1. Reducing IIS in the serotonergic neurons of flies on standard food produced the expected effect on lifespan.

4.1.1. Results

To confirm the effect of reduced serotonergic IIS on lifespan, the survival of TrhGAL4/UAS-InR^{DN} male and female flies on standard sugar yeast food (SY) was compared to controls (TrhGAL4/+ and UAS-InR^{DN}). As shown in Fig.5, reducing IIS in the serotonergic neurons of females (Fig.5a) extended lifespan significantly (to TrhGAL4/+ $p=0.0258$; to UAS-InR^{DN}/+ $p<0.0001$). However, in males (Fig.5b), reduced serotonergic IIS had no effect on lifespan.

Proportional hazard analysis of the female lifespan found a significant effect of genotype on survival ($p<0.0001$), and when analysed further it was shown that TrhGAL4/UAS-InR^{DN} flies were significantly different to UAS-InR^{DN}/+ flies ($p<0.0001$), but not TrhGAL4/+ flies ($p=0.0579$). Proportional hazards analysis of male lifespan found a significant effect of genotype ($p<0.0001$), however, further analysis showed that the effect was the result of UAS-InR^{DN}/+ flies having shorter lifespans than TrhGAL4/+ and TrhGAL4/UAS-InR^{DN} flies.

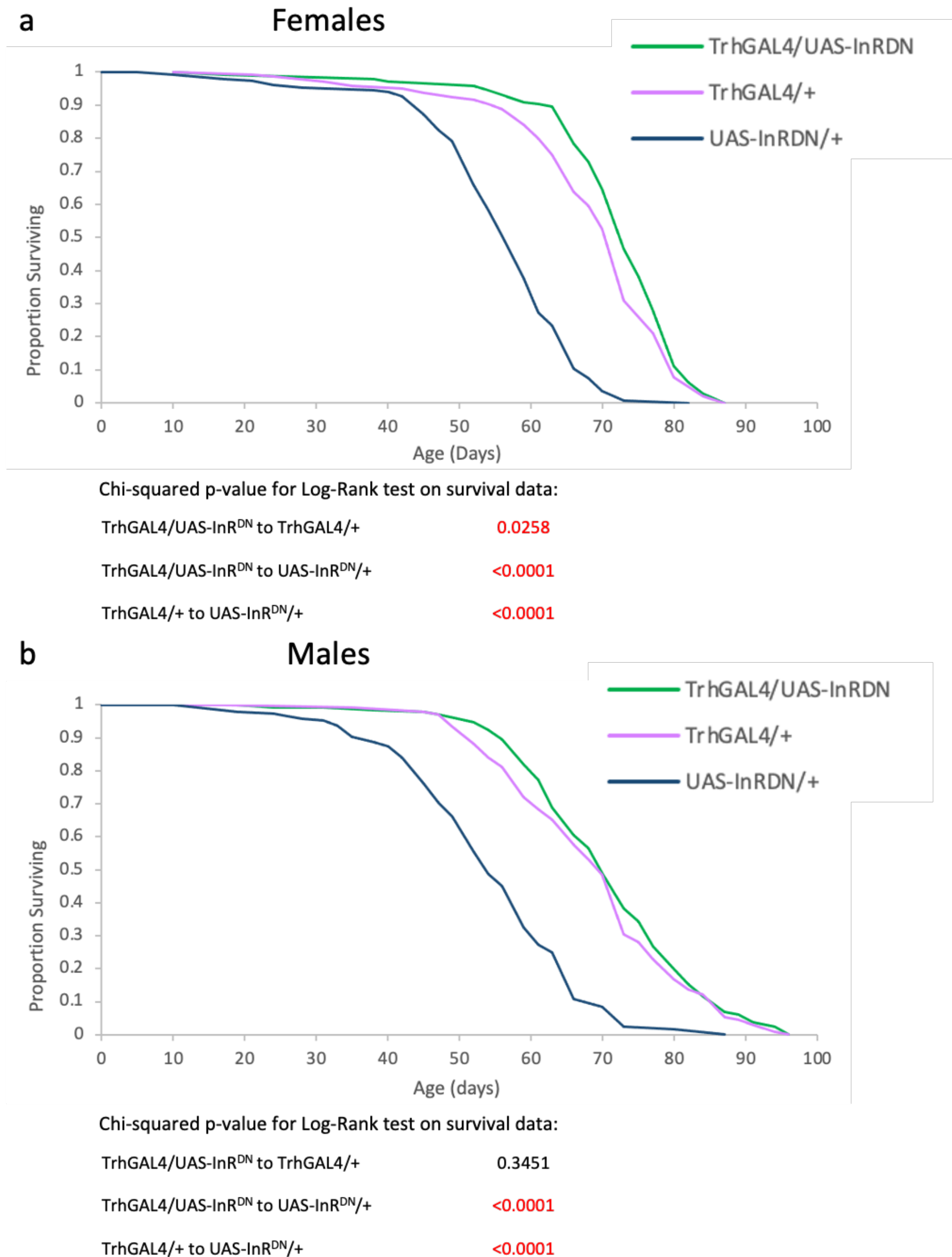


Figure 5. Survival of flies with reduced serotonergic IIS on SY. *Experimental flies with reduced serotonergic IIS are the TrhGAL4/UAS-InR^{DN} group, and the TrhGAL4/+ and UAS-InR^{DN}/+ groups are the controls for the driver and transgene respectively (N=150). a) Survival of female flies. Median lifespans: TrhGAL4/UAS-InR^{DN} = 73 days; TrhGAL4/+ = 73 days; UAS-InR^{DN}/+ = 59 days. b) Survival of male flies. Median lifespans: TrhGAL4/UAS-InR^{DN} = 70 days; TrhGAL4/+ = 70 days; UAS-InR^{DN}/+ = 54 days.*

4.1.2. Summary

Reducing serotonergic IIS in female flies on SY significantly extends lifespan in comparison to controls. However, proportional hazards analysis found no significant difference in survival between TrhGAL4/UAS-InR^{DN} flies and TrhGAL4/+ flies. Reduced serotonergic IIS in males on SY has no effect on lifespan.

4.2. Reduced IIS in serotonergic neurons does not alter response to dietary restriction.

4.2.1. Results

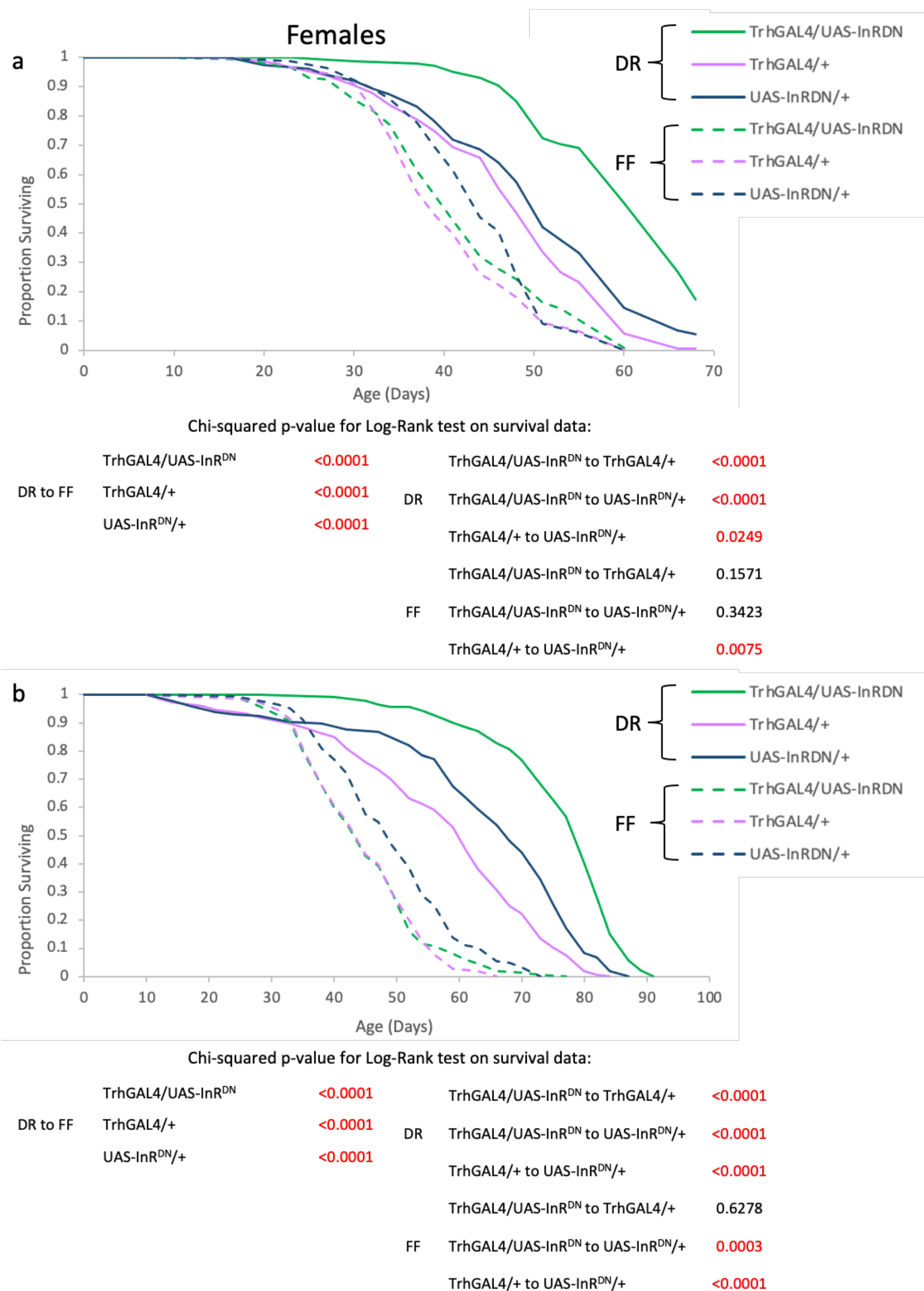
Two lifespan experiments were performed for males and females. In these experiments, flies with reduced IIS in serotonergic neurons were maintained on dietary restriction food (DR) or fully fed food (FF) and compared to controls to determine how reducing IIS in serotonergic neurons modulates lifespan under dietary restriction.

As shown in Fig.6 and Fig.7, control flies on DR showed the expected extended lifespans in comparison to controls on FF. There was some variation in the male controls between the two experiments (Fig.7), however, this is not abnormal. Furthermore, reduced serotonergic IIS did not affect this response in the experimental flies because male and female TrhGAL4/UAS-InR^{DN} flies were also long-lived on DR in comparison to FF, like the controls.

Alongside the expected effect of DR in females, genotype also had an effect (Fig.6). In two independent experiments, reduced IIS in serotonergic neurons resulted in lifespan extension in experimental flies on DR compared to control flies on DR, which did not occur in TrhGAL4/UAS-InR^{DN} flies under FF conditions. This extension of lifespan was additive to the lifespan extension seen in response to DR, when compared to FF conditions. In contrast, there was no effect of reduced IIS in serotonergic neurons on lifespan in females on FF.

In both experiments, proportional hazards analysis showed an effect of genotype ($p<0.0001$), food type ($p<0.0001$) and genotype*food type ($p<0.0001$) on survival. When the first experiment was analysed further, the TrhGAL4/UAS-InR^{DN} genotype did not impact survival when compared to controls of FF food (TrhGAL4/+ $p=0.2169$; UAS-InR^{DN}/+ $p=0.4095$), however it did in flies on DR food (TrhGAL4/+ $p<0.0001$; UAS-InR^{DN}/+ $p<0.0001$). Further analysis of the second experiment found an effect of genotype on survival in flies on both DR

and FF food, however, the effect of genotype on survival on FF food was a result of the UAS-InR^{DN}/+ females living significantly longer.



Chi-squared p-value for Log-Rank test on survival data:

DR to FF	TrhGAL4/UAS-InR^{DN}	<0.0001	DR	TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	<0.0001
	TrhGAL4/+	<0.0001		TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	<0.0001
	UAS-InR^{DN}/+	<0.0001		TrhGAL4/+ to UAS-InR^{DN}/+	0.0249
			FF	TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	0.1571
				TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	0.3423
				TrhGAL4/+ to UAS-InR^{DN}/+	0.0075

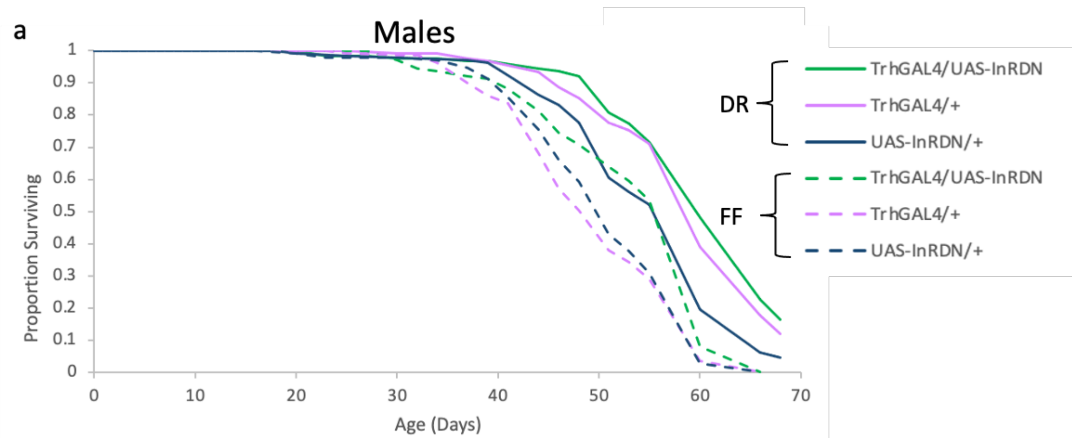
Chi-squared p-value for Log-Rank test on survival data:

DR to FF	TrhGAL4/UAS-InR^{DN}	<0.0001	DR	TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	<0.0001
	TrhGAL4/+	<0.0001		TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	<0.0001
	UAS-InR^{DN}/+	<0.0001		TrhGAL4/+ to UAS-InR^{DN}/+	<0.0001
			FF	TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	0.6278
				TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	0.0003
				TrhGAL4/+ to UAS-InR^{DN}/+	<0.0001

Figure 6. Survival of female flies with reduced serotonergic IIS on DR and FF. *Experimental flies with reduced serotonergic IIS are the TrhGAL4/UAS group, and the TrhGAL4/+ and UAS-InRDN/+ groups are the controls for the driver and transgene respectively (N=150). a) Median lifespans: DR TrhGAL4/UAS-InR^{DN} = 66 days; DR TrhGAL4/+ = 48 days; DR UAS-InR^{DN}/+ = 51 days; FF TrhGAL4/UAS-InR^{DN} = 41 days; FF TrhGAL4/+ = 39 days; FF UAS-InR^{DN}/+ = 44 days. b) Median lifespans: DR TrhGAL4/UAS-InR^{DN} = 80 days; DR TrhGAL4/+ = 61 days; DR UAS-InR^{DN}/+ = 68 days; FF TrhGAL4/UAS-InR^{DN} = 45 days; FF TrhGAL4/+ = 45 days; FF UAS-InR^{DN}/+ = 49 days.*

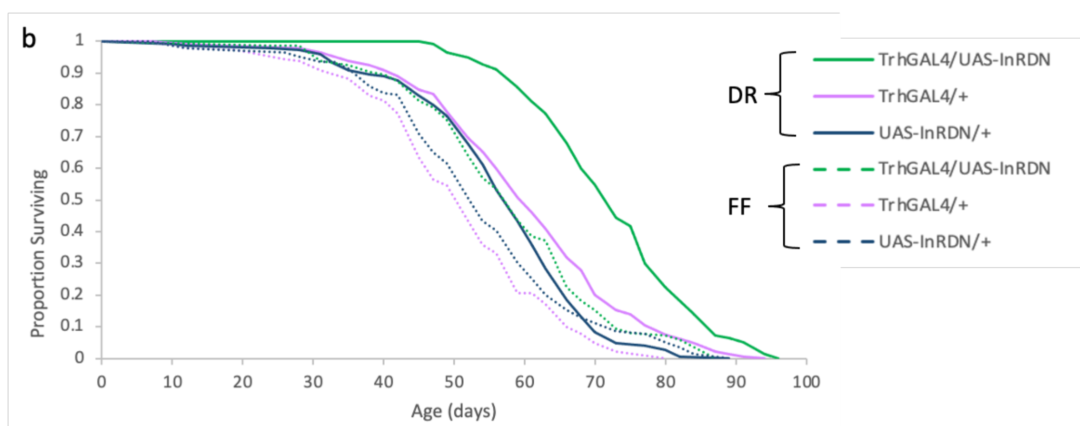
Genotype also influenced lifespan in males (Fig.7). Reducing IIS in serotonergic neurons extended lifespan in males on FF across two independent experiments. However, it did not consistently extend lifespan in males on DR.

Proportional hazards analysis of the first experiment (Fig.7a) found significant effects of genotype ($p<0.0001$), food type ($p<0.0001$), and genotype*food type ($p=0.0383$) on survival. When analysed further, the genotype effect observed in flies on DR food was shown to be the result of the UAS-InR^{DN}/+ flies having significantly shorter lifespans. However, reducing serotonergic IIS (TrhGAL4/UAS-InR^{DN}) had significant effects on survival when compared to both controls (TrhGAL4/+ $p=0.0039$; UAS-InR^{DN}/+ $p=0.0103$) in flies on FF food. The same analysis was carried out for the second experiment (Fig.7b) and it showed significant effects of genotype ($p<0.0001$), food type ($p<0.0001$), and genotype*food type ($p<0.0001$) on survival. Further analysis found that reducing IIS in serotonergic neurons (TrhGAL4/UAS-InR^{DN}) had significant effects on survival in flies on both DR (TrhGAL4/+ $p<0.0001$; UAS-InR^{DN} $p<0.0001$) and FF (TrhGAL4/+ $p<0.0001$; UAS-InR^{DN}/+ $p<0.0292$).



Chi-squared p-value for Log-Rank test on survival data:

DR to FF	TrhGAL4/UAS-InR^{DN}	<0.0001	DR	TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	0.2169
	TrhGAL4/+	<0.0001		TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	<0.0001
	UAS-InR^{DN}/+	<0.0001		TrhGAL4/+ to UAS-InR^{DN}/+	0.0002
			FF	TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	0.0002
				TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	0.0008
				TrhGAL4/+ to UAS-InR^{DN}/+	0.4905



Chi-squared p-value for Log-Rank test on survival data:

DR to FF	TrhGAL4/UAS-InR^{DN}	<0.0001	DR	TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	<0.0001
	TrhGAL4/+	<0.0001		TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	<0.0001
	UAS-InR^{DN}/+	0.2598		TrhGAL4/+ to UAS-InR^{DN}/+	0.0042
			FF	TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	<0.0001
				TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	0.0171
				TrhGAL4/+ to UAS-InR^{DN}/+	0.0259

Figure 7. Survival of male flies with reduced serotonergic IIS on DR and FF. *Experimental flies with reduced serotonergic IIS are the TrhGAL4/UAS group, and the TrhGAL4/+ and UAS-InRDN/+ groups are the controls for the driver and transgene respectively (N=150). a) Median lifespans: DR TrhGAL4/UAS-InR^{DN} = 60 days; DR TrhGAL4/+ = 60 days; DR UAS-InR^{DN}/+ = 60 days; FF TrhGAL4/UAS-InR^{DN} = 60 days; FF TrhGAL4/+ = 51 days; FF UAS-InR^{DN}/+ = 51 days. b) Median lifespans: DR TrhGAL4/UAS-InR^{DN} = 73 days; DR TrhGAL4/+ = 61 days; DR UAS-InR^{DN}/+ = 59 days; FF TrhGAL4/UAS-InR^{DN} = 59 days; FF TrhGAL4/+ = 52 days; FF UAS-InR^{DN}/+ = 54 days.*

4.2.2. Summary

Both male and female flies, including flies with reduced serotonergic IIS, showed the expected extension of lifespan in response to dietary restriction (DR). However, in males there was some variation in the response to DR of the control flies. In females under DR, reducing IIS in serotonergic neurons extended lifespan in comparison to controls across two independent experiments. However, TrhGAL4/UAS-InR^{DN} females were not long-lived under fully fed (FF) conditions when compared to controls. Conversely, males with reduced serotonergic IIS were long-lived under FF conditions in comparison to their controls across both experiments but were only long-lived under DR in one experiment.

These results also show a sexual dimorphism where reduced serotonergic IIS extends lifespan under FF conditions in males but not in females. Furthermore, an additive response to DR and reduced serotonergic IIS was observed in females across two experiments, where reduced serotonergic IIS extended lifespan in addition to DR when compared to FF controls. However, this was not observed across both experiments for males.

4.3. Fecundity in female flies with reduced serotonergic IIS responds normally to dietary restriction.

4.3.1. Results

Female fecundity was measured throughout lifespan on SY food to determine if reducing IIS in serotonergic neurons affects fecundity when compared to controls. Generalised linear modelling found a significant effect of genotype ($p<0.0001$), however, further analysis found that the number of eggs laid by TrhGAL4/UAS-InR^{DN} flies was not significantly different to both controls. At day 25, TrhGAL4/UAS-InR^{DN} flies on SY media produced significantly more eggs than the two controls (to TrhGAL4/+, $p=0.0133$; to UAS-InR^{DN}/+, $p=0.0002$), however no other significant differences were observed between TrhGAL4/UAS-InR^{DN} flies and both controls (Fig.8).

When analysed using repeated measures, significant effects of genotype ($p<0.0001$) and time ($p<0.0001$) were observed. When investigated further using a data filter to do pairwise comparisons between the genotypes, the significant difference could be attributed to UAS-

InR^{DN}/+ flies laying significantly less eggs that both TrhGAL4/UAS-InR^{DN} ($p<0.0001$) and TrhGAL4/+ flies ($p<0.0001$). TrhGAL4/UAS-InR^{DN} fly fecundity was not significantly different to that of the TrhGAL4/+ flies ($p=0.2785$).

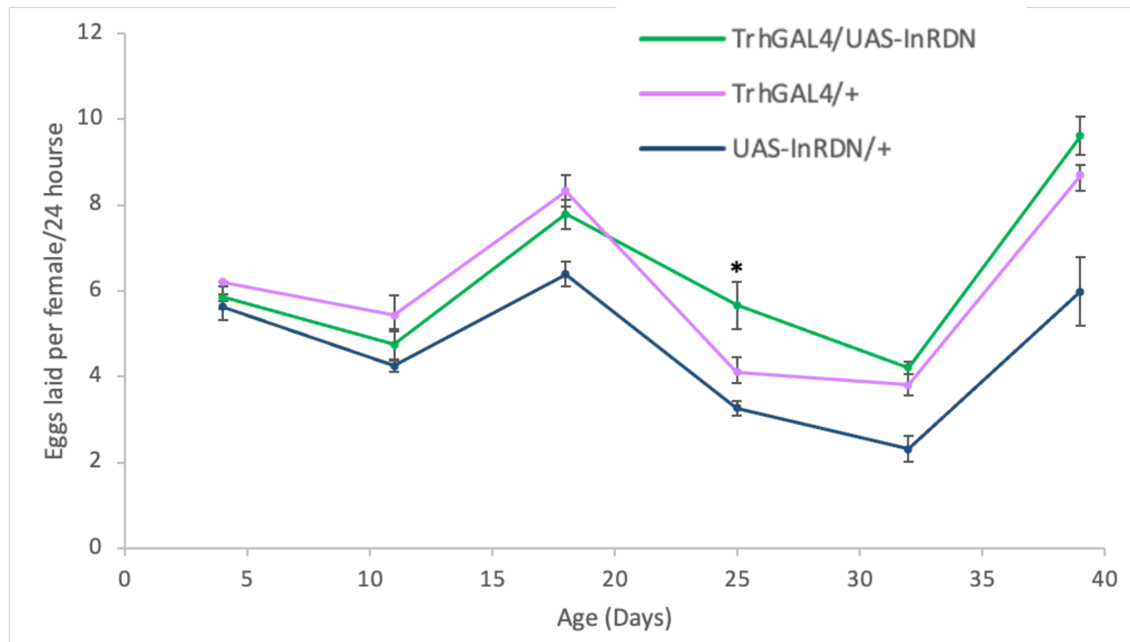


Figure 8. Fecundity of female flies with reduced IIS in serotonergic neurons on SY food. *Once-mated females were kept at 10 flies per vial (N=10 vials) and eggs laid over a 48-hour period were counted. Asterisks indicate significant difference ($p<0.05$ by log-rank test) between TrhGAL4/UAS-InR^{DN} and both controls (TrhGAL4/+ and UAS-InR^{DN}/+) on the same food type. Data are presented as mean no. of eggs laid per female per 24 hours ± SEM.*

Female fecundity was also measured in females on DR and FF food to determine the effects of dietary restriction on fecundity in flies with reduced serotonergic IIS. As shown in Fig.9, TrhGAL4/UAS-InR^{DN} flies responded similarly to controls, laying significantly more eggs under FF conditions compared to DR across all ages ($p<0.01$). At some age points, reduced IIS in serotonergic neurons did impact the number of eggs laid, however, the effects were not consistent between experiments.

When analysed using repeated measures, there was significant effects of genotype ($p=0.0005$), food type ($p<0.0001$), genotype*food type ($p=0.0060$) and time ($p<0.0001$) in the first experiment. When analysed within food type, TrhGAL4/UAS-InR^{DN} flies on laid significantly more eggs than TrhGAL4/+ ($p=0.0015$) or UAS-InR^{DN}/+ flies ($p=0.0022$) on DR

food. On FF food, TrhGAL4/UAS-InR^{DN} fly fecundity was not significantly different to TrhGAL4/+ fly fecundity (p=0.8988), however, it was significantly higher than UAS-InR^{DN}/+ fly fecundity (p=0.0014). In the second experiment, there was also significant effects of genotype (p<0.0001), food type (p<0.0001), genotype*food type (p<0.0001), and time (p<0.0001). When analysed further, TrhGAL4/UAS-InR^{DN} flies laid less eggs than TrhGAL4/+ flies (p=0.0063) on DR food, however there was no difference when compared to UAS-InR^{DN}/+ flies. On FF food, TrhGAL4/UAS-InR^{DN} flies laid less eggs than TrhGAL4/+ (p<0.0001) and UAS-InR^{DN}/+ flies (p=0.0004).

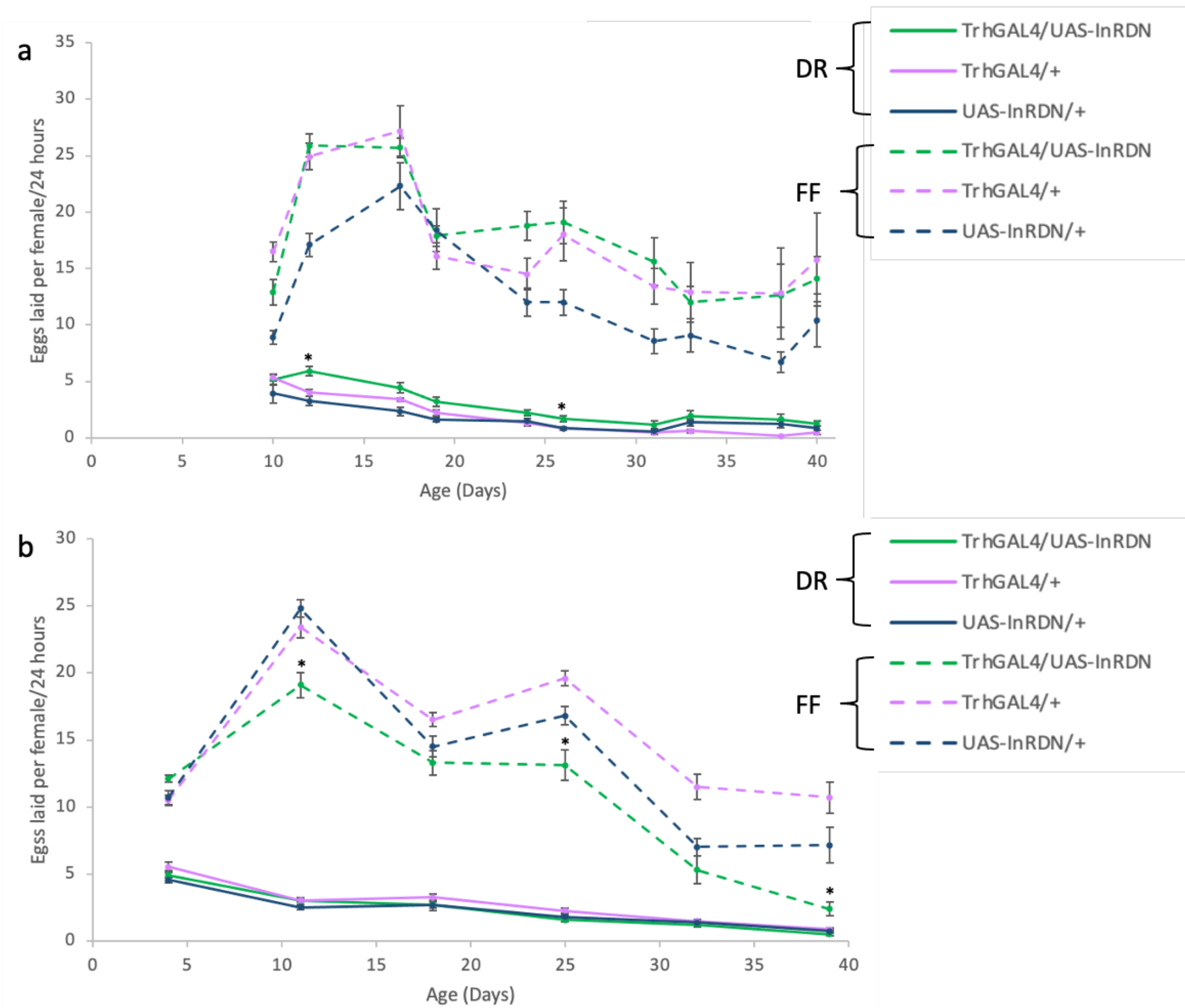


Figure 9. Fecundity of female flies with reduced serotonergic IIS on DR and FF. *Once-mated females were kept at 10 flies per vial (N=10 vials) and eggs laid over a 48-hour period were counted. a) Fecundity of females on FF and DR food throughout lifespan until age 40 days. b) Fecundity of females on FF and DR food throughout lifespan until age 39 days. Asterisks indicate significant difference (p<0.05 by log-rank test) between TrhGAL4/UAS-InR^{DN} and both controls (TrhGAL4/+ and UAS-InR^{DN}/+) on the same food type. Data are presented as mean no. of eggs laid per female per 24 hours ± SEM. Solid lines = DR food; dashed lines = FF food.*

4.3.2. Summary

Reducing IIS in serotonergic neurons did not influence female fecundity under SY or DR conditions. While there were some significant differences between TrhGAL4/UAS-InR^{DN} females and their controls at specific timepoints (Fig.8 and 9), these were not repeated between experiments, suggesting the differences seen could be attributed to natural variation within the fecundity of the sampled flies. Repeated measures analysis also differed between the two experiments with no consistent effect of reduced serotonergic IIS being

observed on DR or FF food. However, diet was shown to influence fecundity, with females on FF producing significantly more eggs than females on DR.

4.4. Reduced IIS specific to serotonergic neurons has no effect on negative geotaxis senescence in either sex.

4.4.1. Results

To confirm that reduced serotonergic IIS has no effect on negative geotaxis senescence, the performance index of TrhGAL4/UAS-InR^{DN} male and female flies on standard sugar yeast food (SY) was measured at multiple age points and compared to controls (TrhGAL4/+ and UAS-InR^{DN}). As shown in Fig.10, reducing IIS in serotonergic neurons did not alter negative geotaxis senescence in comparison to controls in either sex when flies were maintained on SY food. When analysed using repeated measures up to day 37, there was no significant effect of genotype in female flies (p=0.6223) or male flies (p=0.1074). In females, a significant time-dependent decline was observed (p=0.0081), however, this was not observed in males (p=0.0527).

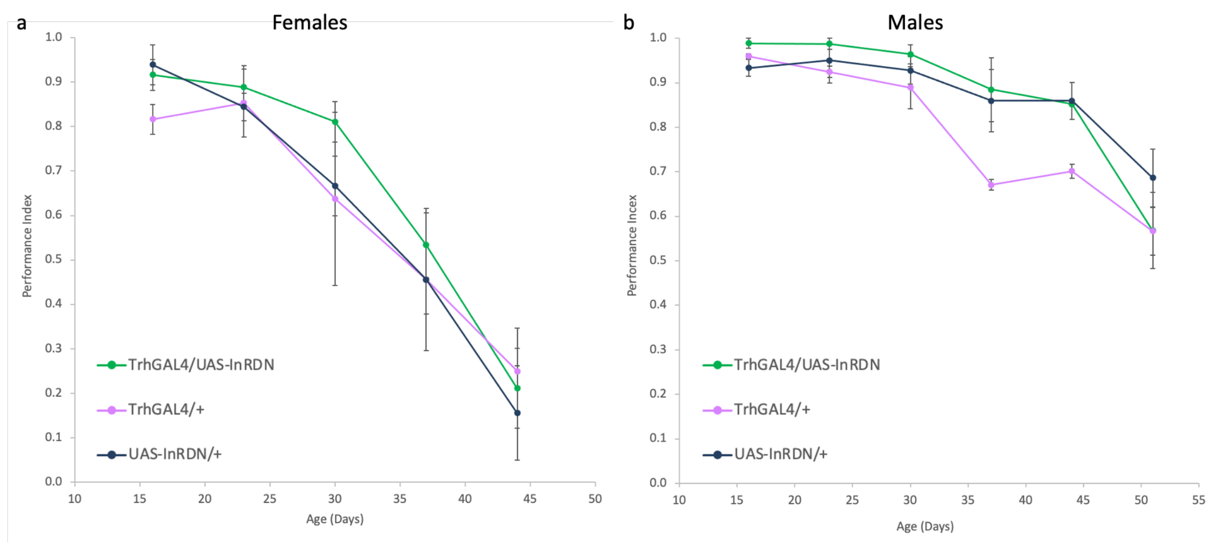


Figure 10. Negative geotaxis senescence of males and females with reduced serotonergic IIS on SY. *a) TrhGAL4/UAS-InR^{DN} females on SY food compared to TrhGAL4/+ and UAS-InR^{DN}/+ controls (N=3, groups of 10 flies). b) TrhGAL4/UAS-InR^{DN} males on SY food compared to TrhGAL4/+ and UAS-InR^{DN}/+ controls (N=3, groups of 10 flies). Data are presented as means ± SEM.*

In addition to the experiment confirming that reduced serotonergic IIS has no impact on negative geotaxis senescence, experiments investigating the effect of dietary restriction were carried out. As shown in Fig.10, negative geotaxis declined with age in females across all treatments. Generalised linear modelling found significant genotype (p=0.008), age*genotype (p<0.0001), and genotype*food type (p=0.0010) effects in the first experiment (Fig.10a). Further analysis of the effect of genotype via post-hoc Tukey-Kramer HSD tests found a significant difference between TrhGAL4/UAS-InR^{DN} females and their controls at age 37 (to TrhGAL4/+ p=0.0408; to UAS-InR^{DN}/+ p=0.0346) and 44 days (to TrhGAL4/+ p=0.0002; UAS-InR^{DN}/+ p=0.0002) on DR food, but not FF food. Generalised linear modelling using the results from the second experiment found significant genotype (p=0.0079), food type (<0.0001), and age*food type effects (p=0.0003) (Fig.11b). Post-hoc analysis using Tukey-Kramer HSD tests found flies fed FF food had a significantly higher performance index than those on DR (p=0.0198). However, there was no significant difference between TrhGAL4/UAS-InR^{DN} flies on DR or FF, despite the initial genotype effect observed. Furthermore, the results observed in the individual experiments did not mirror each other.

When analysed using repeated measures up to day 37, there was no significant effect of food (p=0.3374), genotype (p=0.3766) or genotype*food type (p=0.1232) observed in the first experiment, however, there was a significant effect of time (p<0.0001) (Fig.11a). Contrastingly, in the second experiment (Fig.11b), there was a significant effect of genotype (p=0.0311), food type (p=0.0226), and genotype*food type (p=0.0198). The effect of time remained significant (P<0.0001). When analysed further, TrhGAL4/UAS-InR^{DN} flies outperformed their controls across all age points (p=0.0082) on FF food, however, there was no effect of genotype on DR food (p=0.7333). Furthermore, there was no significant effect of genotype between UAS-InR^{DN}/+ and TrhGAL4/+ flies when compared within food type, and no significant effects of food type on either UAS-InR^{DN}/+ or TrhGAL4/+ flies.

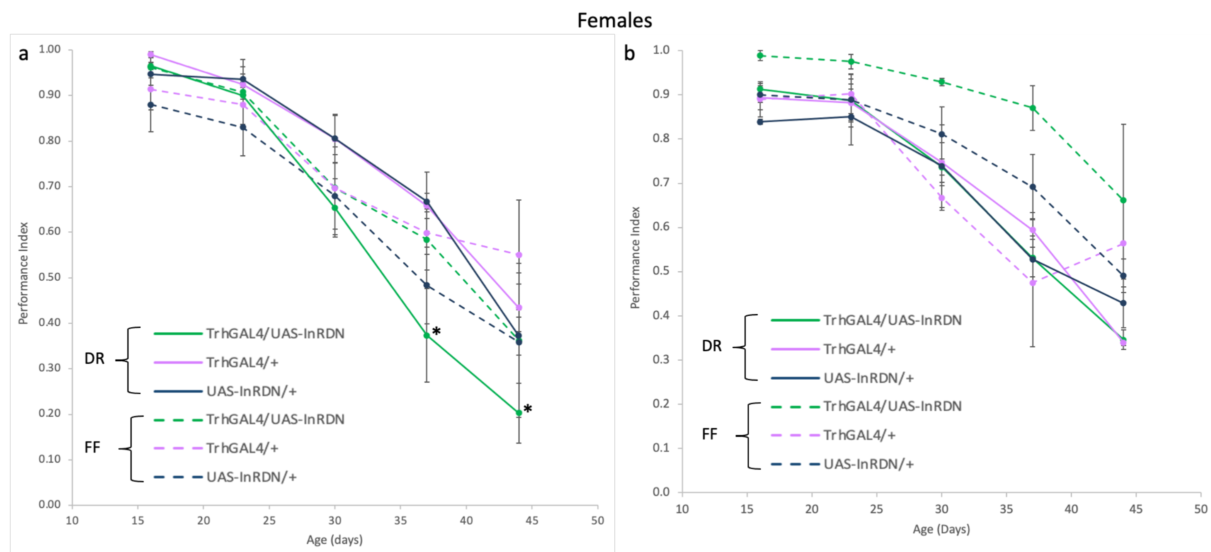


Figure 11. Negative geotaxis senescence of females with reduced serotonergic IIS on DR and FF.
Negative geotaxis performance index of female flies over lifespan. a) TrhGAL4/UAS-InR^{DN} females on DR and FF food compared to TrhGAL4/+ and UAS-InR^{DN}/+ controls (N=5, groups of 10 flies). b) TrhGAL4/UAS-InR^{DN} females on DR and FF food compared to TrhGAL4/+ and UAS-InR^{DN}/+ controls (N=3, groups of 10 flies). Data are presented as means ± SEM and asterisks indicate a significant difference (p<0.05 by Tukey-Kramer HSD) between TrhGAL4/UAS-InR^{DN} and both controls (TrhGAL4/+ and UAS-InR^{DN}/+) on the same food type.

Negative geotaxis declined with age in male flies across all treatments (Fig.12). Generalised linear modelling of the first experiment using male flies found a significant effect of food type (p=0.0026) and age*food type (p=0.0003) (Fig.12a). Post-hoc analysis found that flies fed DR food had a significantly higher performance index at age 37 (p=0.0398) and age 44 (p=0.0060). However, these findings were not repeated in the second experiment (Fig.12b). The second experiment showed an effect of genotype*food type (p=0.0015), age*food type (p=0.0146), and age*genotype*food type (p=0.0298). However, further analysis found no significant effect of dietary restriction for any of the genotypes at any age point.

When analysed using repeated measures up to day 37, there was no significant effect of food (p=0.1434), genotype (p=0.2757) or genotype*food type (p=0.6758) observed in the first experiment, however, there was a significant effect of time (p<0.0001) (Fig.12a). In the second experiment (Fig.12b), there was no significant effect of genotype (p=0.2803), food type (p=0.8857), however, there was a significant effect of genotype*food type (p=0.0091). When analysed within food type, there was a significant effect of genotype between

TrhGAL4/UAS-InR^{DN} and UAS-InR^{DN}/+ flies (p=0.0064), however, this was not seen with TrhGAL4/+ flies (p=0.0956). Furthermore, the significant effect of food type was only seen in UAS-InR^{DN}/+ flies (p=0.0292), where FF flies performed worse than DR flies. The effect of time was still observed (p=0.0043).

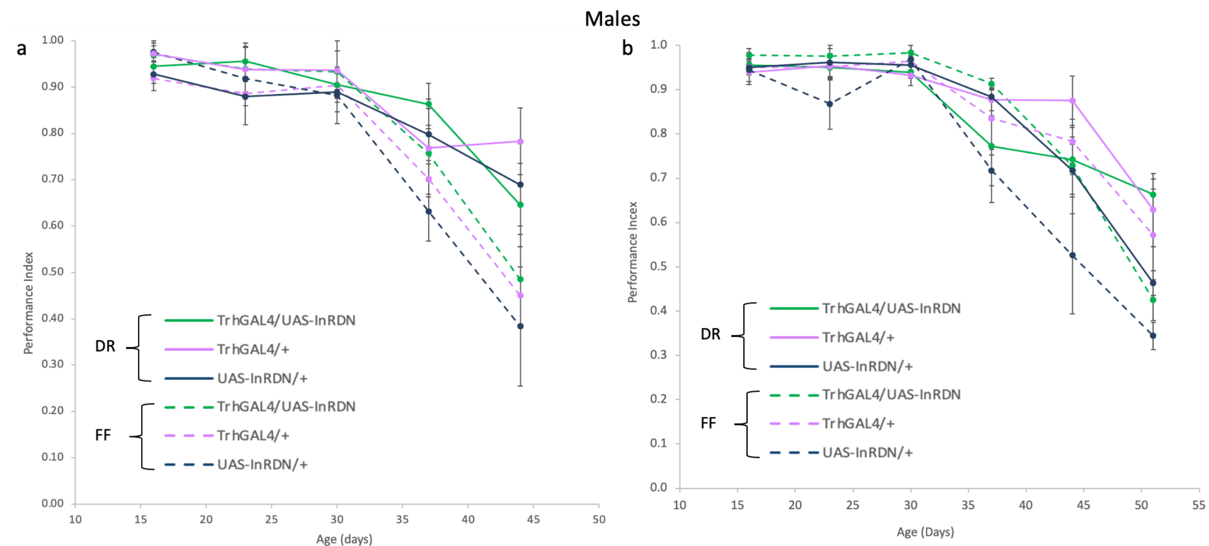


Figure 12. Negative geotaxis senescence of males with reduced serotonergic IIS on DR and FF. *Negative geotaxis performance index of male flies over lifespan. a) TrhGAL4/UAS-InR^{DN} males on DR and FF food compared to TrhGAL4/+ and UAS-InR^{DN}/+ controls (N=5, groups of 10 flies). b) TrhGAL4/UAS-InR^{DN} males on DR, SY, and FF food compared to TrhGAL4/+ and UAS-InR^{DN}/+ controls (N=3, groups of 10 flies). Data are presented as means ± SEM.*

4.4.2. Summary

In both sexes, negative geotaxis performance decreased with age and reduced serotonergic IIS had no effect on this trend. Furthermore, in females, no consistent effect of food type or genotype was observed across all experiments suggesting negative geotaxis senescence is not affected by reducing IIS in serotonergic neurons or dietary restriction. This finding was repeated in the male experiments, again suggesting no effect of reduced serotonergic IIS or dietary restriction on negative geotaxis senescence.

4.5. Feeding behaviour is not affected by diet or reduced IIS in serotonergic neurons in either sex.

4.5.1. Results

To determine whether reduced IIS in serotonergic neurons or diet influenced feeding behaviour male and female flies were transferred into vials (N=5, 10 flies per vial) containing DR, SY or FF food containing Brilliant Blue dye at a concentration of 2.5% w/v. The flies were left to feed for 30 minutes before their gut contents were extracted and aliquoted into a 96well plate for absorbance to be measured at 625nm. The data is shown as food ingested per fly (µg/mL).

As shown in Fig.13, reducing IIS in serotonergic neurons did not affect feeding behaviour in either sex on any diet. Furthermore, diet did not have consistent effects on feeding behaviour in either sex.

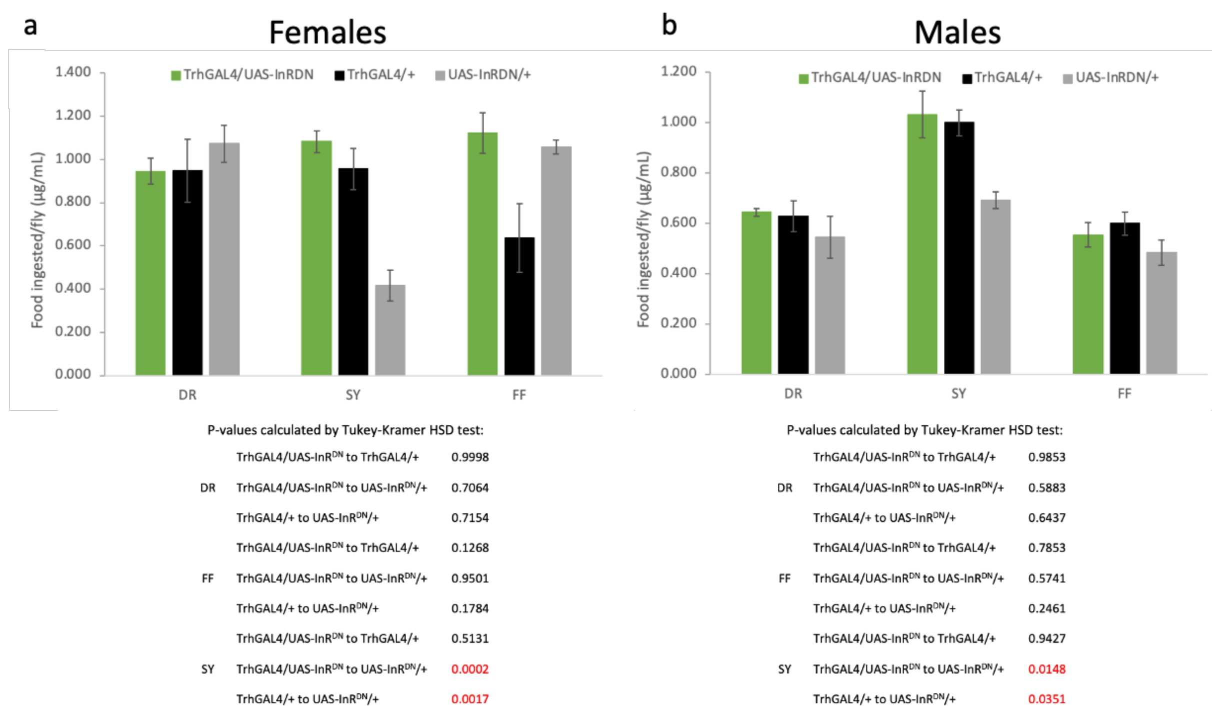


Figure 13: Feeding behaviour in female and male flies with reduced serotonergic IIS on SY, DR and FF diets. *a) Feeding behaviour of female flies in response to diet and reduced serotonergic IIS. b) Feeding behaviour of male flies in response to diet and reduced serotonergic IIS. Data are presented as mean ± SEM.*

4.5.2. Summary

Feeding behaviour was not affected by reduced serotonergic IIS in female or males. Additionally, feeding behaviour was not affected by diet in either sex.

4.6. Reducing IIS in serotonergic neurons has no effect on oxidative stress resistance in either sex.

4.6.1. Results

Male and female flies were maintained on DR and FF food for 17 days before being transferred to food containing H_2O_2 to test their resistance to oxidative stress (N=50). Female flies with reduced serotonergic IIS did not show changes in oxidative stress resistance when compared to controls on DR or FF food (Fig.14a). Similarly, there was no difference in the oxidative stress resistance of TrhGAL4/UAS-InR^{DN} males compared to controls on DR or FF (Fig.14b). Furthermore, dietary restriction had no effect on oxidative stress resistance in either sex.

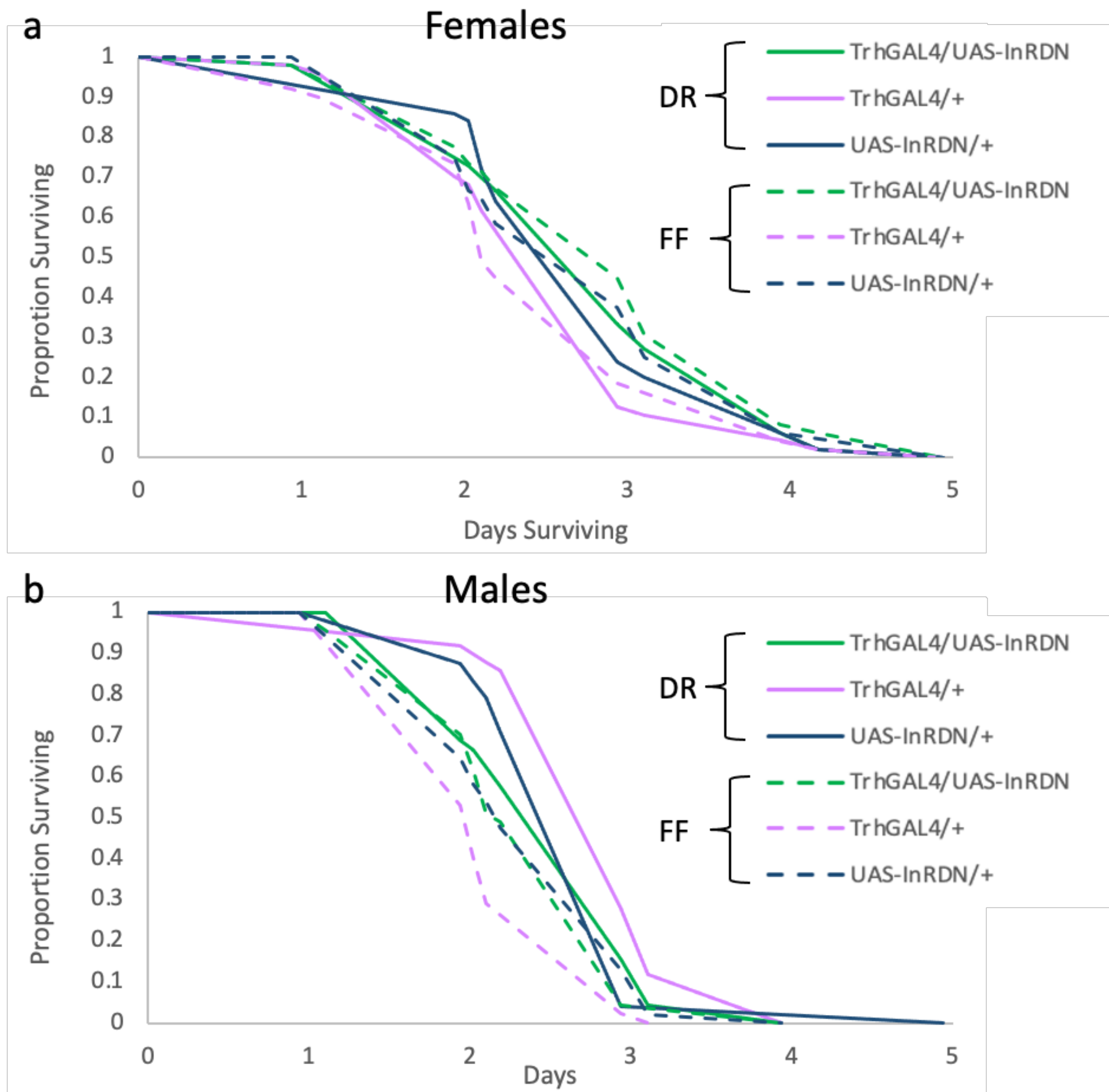


Figure 14. Survival of female and male flies with reduced serotonergic IIS compared with controls after exposure to oxidative stress. *Survival of female (a) and male (b) TrhGAL4/UAS-InR^{DN} flies compared to TrhGAL4/+ and UAS-InR^{DN} flies (N = 50). Data are presented as a proportion of surviving flies against time on H_2O_2 food. a) In females, genotype and food type had no statistically significant effect on oxidative stress resistance. Median lifespans: DR TrhGAL4/UAS-InR^{DN} = 2.94 days; DR TrhGAL4/+ = 2.94 days; DR UAS-InR^{DN}/+ = 2.94 days; FF TrhGAL4/UAS-InR^{DN} = 2.94 days; FF TrhGAL4/+ = 2.10 days; FF UAS-InR^{DN}/+ = 2.94 days. b) In males, TrhGAL4/+ flies were significantly less resistant to oxidative stress in comparison to the other two genotypes on FF food (TrhGAL4/+ to TrhGAL4/UAS-InR^{DN}: p = 0.0359; TrhGAL4/+ to UAS-InR^{DN}/+: p = 0.0227) and significantly more resistant to oxidative stress on DR (TrhGAL4/+ to TrhGAL4/UAS-InR^{DN}: p = 0.0056; TrhGAL4/+ to UAS-InR^{DN}/+: p = 0.0136). Median lifespans: DR TrhGAL4/UAS-InR^{DN} = 2.94 days; DR TrhGAL4/+ = 2.94 days; DR UAS-InR^{DN}/+ = 2.94 days; FF TrhGAL4/UAS-InR^{DN} = 2.19 days; FF TrhGAL4/+ = 2.02 days; FF UAS-InR^{DN}/+ = 2.19 days.*

4.6.2. Summary

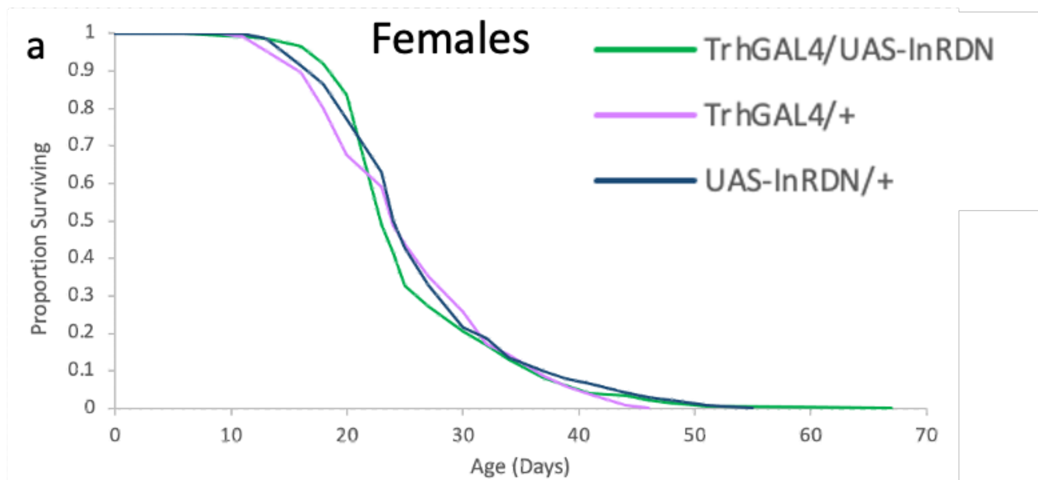
Reducing IIS in serotonergic neurons had no effect on oxidative stress resistance in either sex. Furthermore, no effect of dietary restriction was observed.

4.7. The effect of reducing IIS in serotonergic neurons on starvation resistance

4.7.1. Results

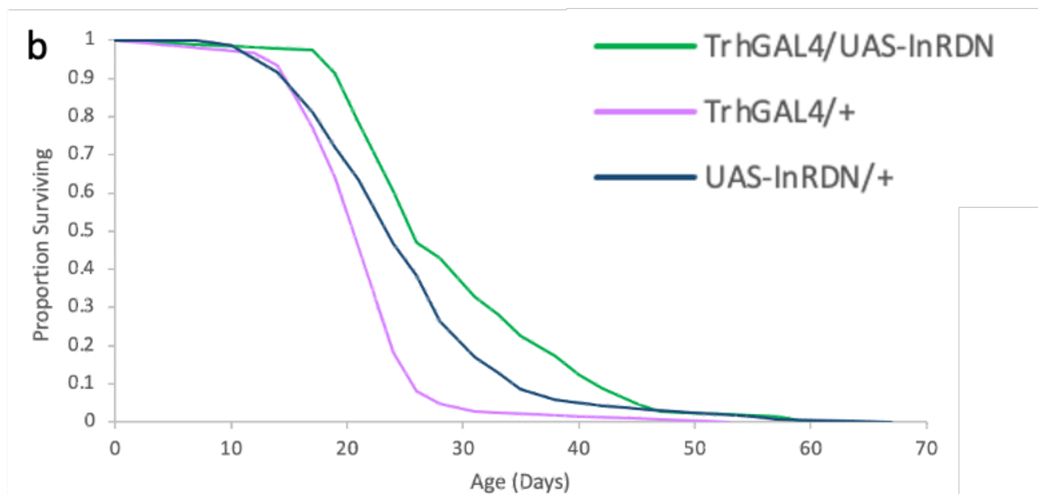
Two lifespan experiments were performed for female and male flies (N=150) where they were maintained on a low nutrient media containing yeast at a concentration of 10g/L (0.1). TrhGAL4/UAS-InR^{DN} flies were compared to controls (TrhGAL4/+ and UAS-InR^{DN}/+) to determine their resistance to prolonged starvation.

Female TrhGAL4/UAS-InR^{DN} flies were shown to have significantly extended lifespans in comparison to both controls in only one of the repeats (Fig.15). This suggests that reduced serotonergic IIS could improve starvation resistance in females, however the exact effect on starvation resistance remains unknown.



Chi-squared p-value for Log-Rank test on survival data:

TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	0.9101
TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	0.4623
TrhGAL4/+ to UAS-InR^{DN}/+	0.4887

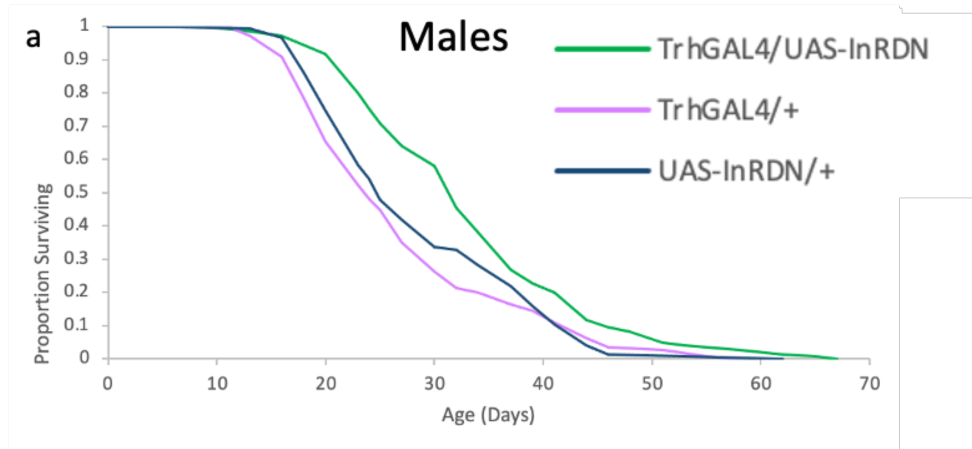


Chi-squared p-value for Log-Rank test on survival data:

TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	<0.0001
TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	0.0011
TrhGAL4/+ to UAS-InR^{DN}/+	<0.0001

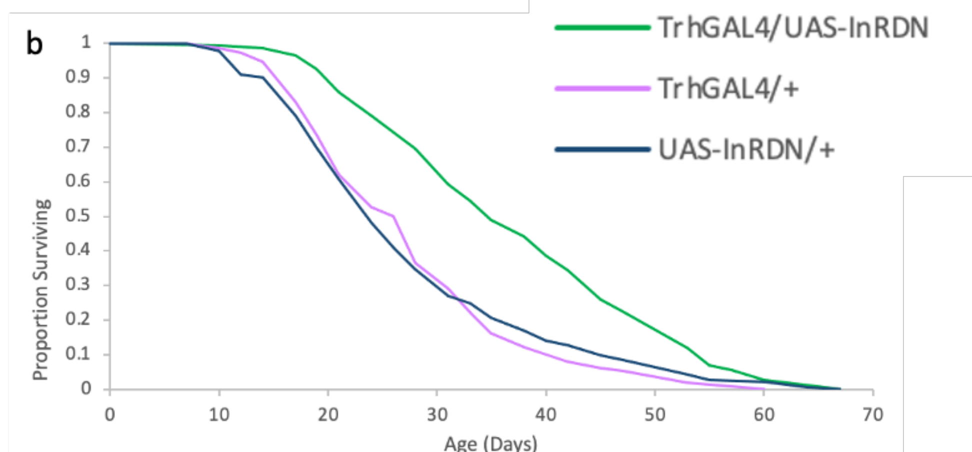
Figure 15. Survival of female flies with reduced IIS specific to serotonergic neurons compared with controls on 0.1 food. *Experimental flies with reduced serotonergic IIS are the TrhGAL4/UAS group, and the TrhGAL4/+ and UAS-InRDN/+ groups are the controls for the driver and transgene respectively (N=150). a) Median lifespans: TrhGAL4/UAS-InR^{DN} = 23 days; TrhGAL4/+ = 24 days; UAS-InR^{DN}/+ = 25 days. b) Median lifespans: TrhGAL4/UAS-InR^{DN} = 26 days; TrhGAL4/+ = 24 days; UAS-InR^{DN}/+ = 21 days.*

Male TrhGAL4/UAS-InR^{DN} flies were shown to have significantly extended lifespans in comparison to both controls (Fig.16). This suggests that reduced serotonergic IIS improves starvation resistance in males.



Chi-squared p-value for Log-Rank test on survival data:

TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	<0.0001
TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	0.0003
TrhGAL4/+ to UAS-InR^{DN}/+	0.3733

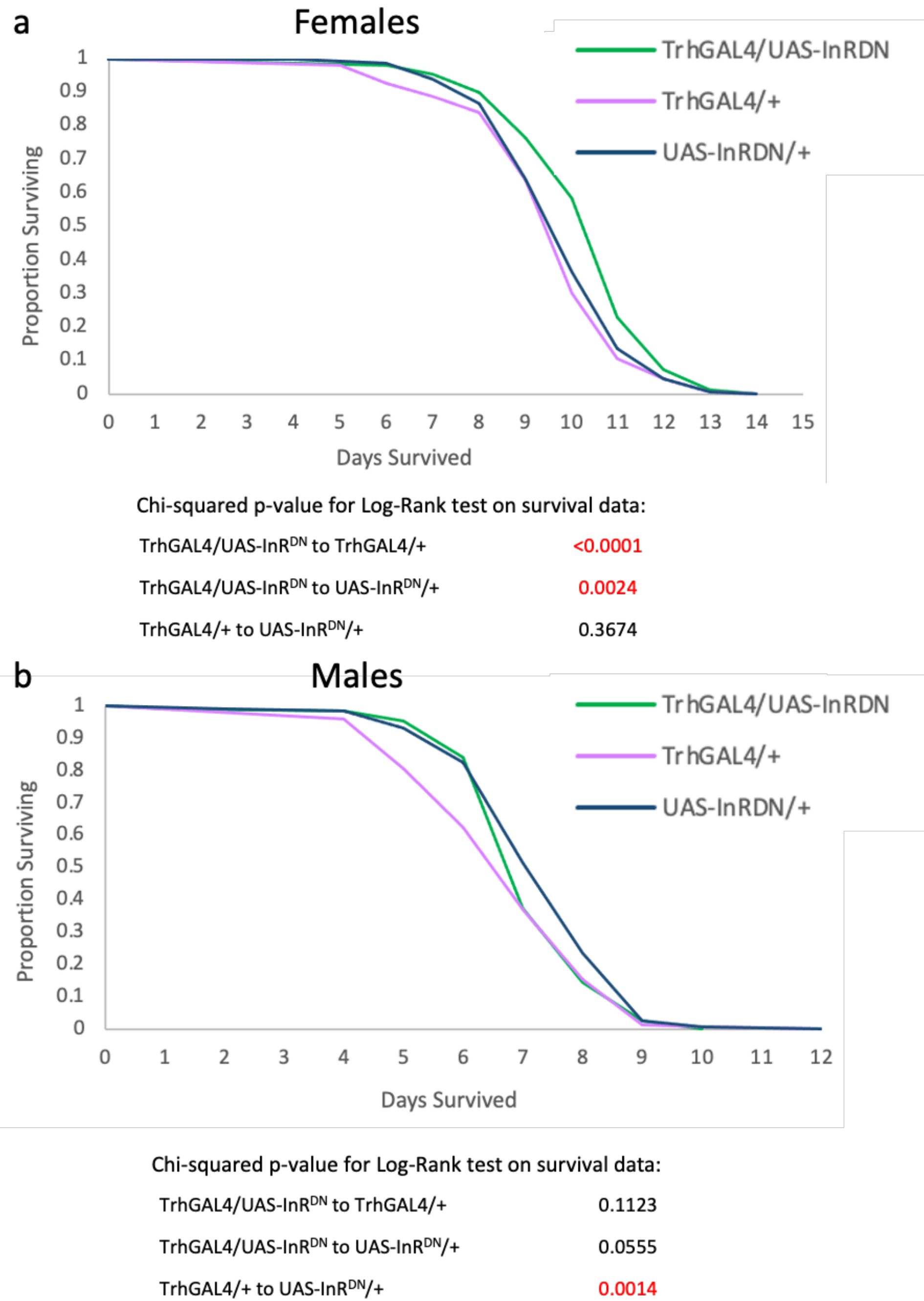


Chi-squared p-value for Log-Rank test on survival data:

TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	<0.0001
TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	<0.0001
TrhGAL4/+ to UAS-InR^{DN}/+	0.6577

Figure 16. Survival of male flies with reduced IIS specific to serotonergic neurons compared with controls on 0.1 food. *Experimental flies with reduced serotonergic IIS are the TrhGAL4/UAS group, and the TrhGAL4/+ and UAS-InRDN/+ groups are the controls for the driver and transgene respectively (N=150). a) Median lifespans: TrhGAL4/UAS-InR^{DN} = 32 days; TrhGAL4/+ = 24 days; UAS-InR^{DN}/+ = 25 days. b) Median lifespans: TrhGAL4/UAS-InR^{DN} = 35 days; TrhGAL4/+ = 27 days; UAS-InR^{DN}/+ = 24 days.*

In addition to the lifespans investigating the response of TrhGAL4/UAS-InR^{DN} flies to low nutrient conditions, further experiments were carried out to determine their response to complete starvation. To do this, male and female flies were maintained on SY food for 10 days before being transferred to food without sugar or yeast to test their resistance to starvation (N=100). As shown in Fig.17a, females with reduced serotonergic IIS had increased survival in response to complete starvation compared to controls. However, no difference in survival was observed between male TrhGAL4/UAS-InR^{DN} flies and controls (Fig.17b).



Chi-squared p-value for Log-Rank test on survival data (Females):

Comparison	p-value
TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	<0.0001
TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	0.0024
TrhGAL4/+ to UAS-InR^{DN}/+	0.3674

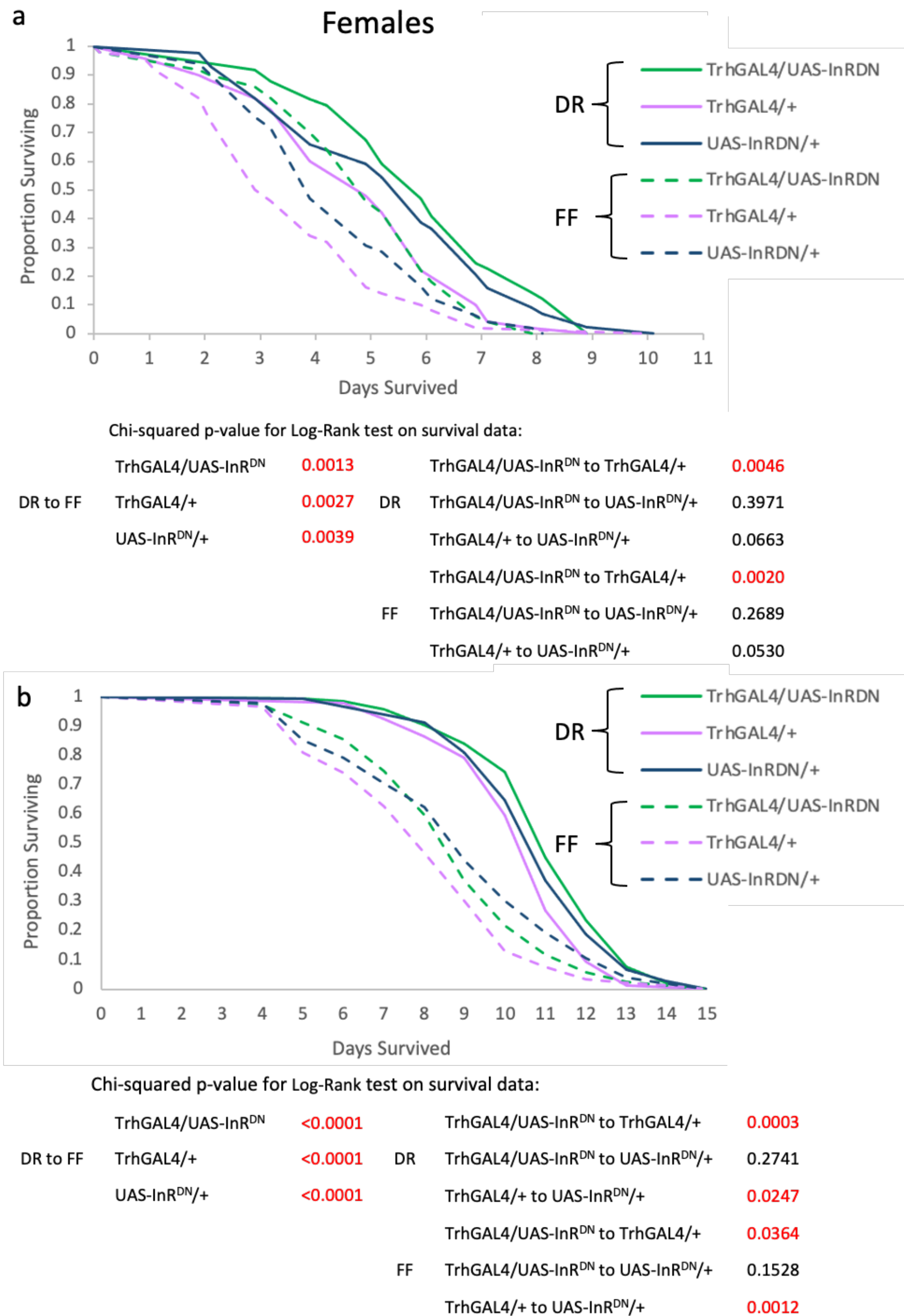
Chi-squared p-value for Log-Rank test on survival data (Males):

Comparison	p-value
TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	0.1123
TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	0.0555
TrhGAL4/+ to UAS-InR^{DN}/+	0.0014

Figure 17. Survival of flies with reduced serotonergic IIS in response to complete starvation. *a) Female survival and p-values. Median lifespans: SY TrhGAL4/UAS-InR^{DN} = 21 days; SY TrhGAL4/+ = 20 days; SY UAS-InR^{DN}/+ = 20 days. b) Male survival and p-values. Median lifespans: SY TrhGAL4/UAS-InR^{DN} = 17 days; SY TrhGAL4/+ = 17 days; SY UAS-InR^{DN}/+ = 18 days.*

The impact of dietary restriction on resistance to complete starvation in flies with reduced serotonergic IIS was also tested. The flies were maintained on DR and FF food for 17 days (Fig.18a and 19a, N=50), or 10 days (Fig.18b and 19b, N=100), before being transferred to media lacking sugar and yeast.

Females maintained on DR food before the switch onto starvation food showed improved resistance in comparison to those maintained on FF food across all genotypes and both experiments (Fig.18). However, there was no difference in the survival of TrhGAL4/UAS-InR^{DN} females compared to controls under DR or FF conditions.



Chi-squared p-value for Log-Rank test on survival data:

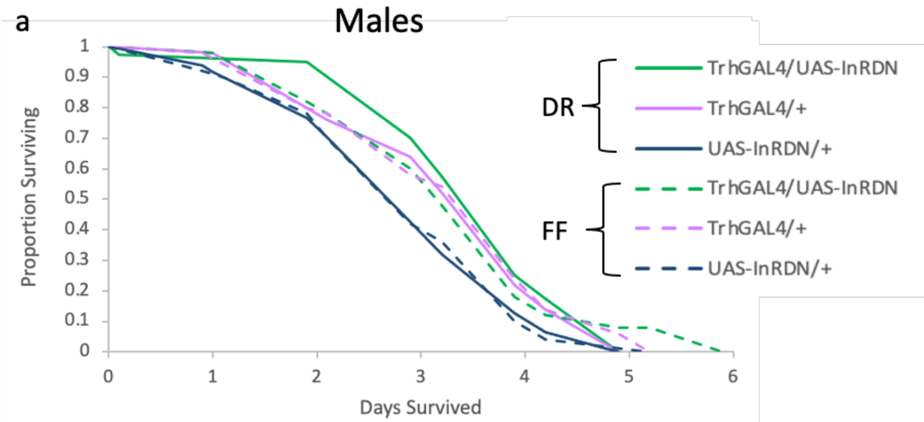
	TrhGAL4/UAS-InR^{DN}	0.0013		TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	0.0046
DR to FF	TrhGAL4/+	0.0027	DR	TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	0.3971
	UAS-InR^{DN}/+	0.0039		TrhGAL4/+ to UAS-InR^{DN}/+	0.0663
				TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	0.0020
			FF	TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	0.2689
				TrhGAL4/+ to UAS-InR^{DN}/+	0.0530

Chi-squared p-value for Log-Rank test on survival data:

	TrhGAL4/UAS-InR^{DN}	<0.0001		TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	0.0003
DR to FF	TrhGAL4/+	<0.0001	DR	TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	0.2741
	UAS-InR^{DN}/+	<0.0001		TrhGAL4/+ to UAS-InR^{DN}/+	0.0247
				TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	0.0364
			FF	TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	0.1528
				TrhGAL4/+ to UAS-InR^{DN}/+	0.0012

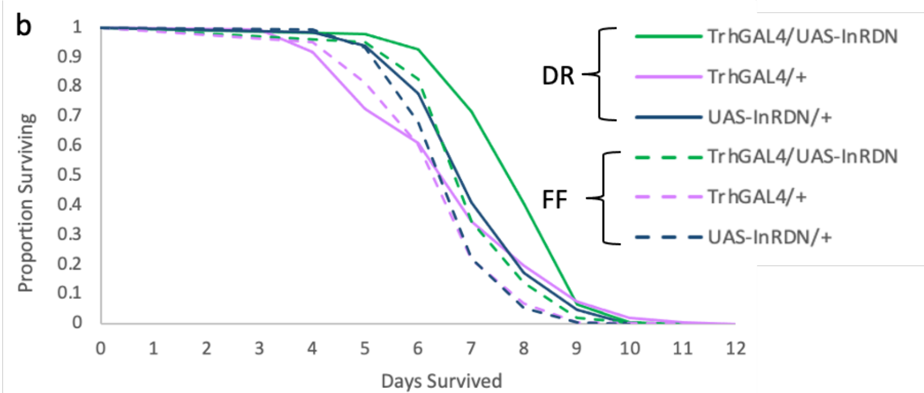
Figure 18. Survival of female flies with reduced IIS in serotonergic neurons compared to their controls after complete starvation. *Experimental flies with reduced serotonergic IIS are the TrhGAL4/UAS group, and the TrhGAL4/+ and UAS-InRDN/+ groups are the controls for the driver and transgene respectively (a: N=50; b: N=100). a) Median lifespans: DR TrhGAL4/UAS-InR^{DN} = 22.9 days; DR TrhGAL4/+ = 21.9 days; DR UAS-InR^{DN}/+ = 22.9 days; FF TrhGAL4/UAS-InR^{DN} = 21.9 days; FF TrhGAL4/+ = 20.1 days; FF UAS-InR^{DN}/+ = 20.9 days. b) Median lifespans: DR TrhGAL4/UAS-InR^{DN} = 21 days; DR TrhGAL4/+ = 21 days; DR UAS-InR^{DN}/+ = 21 days; FF TrhGAL4/UAS-InR^{DN} = 19 days; FF TrhGAL4/+ = 18 days; FF UAS-InR^{DN}/+ = 19 days.*

Effects of both food type and genotype on starvation resistance in males were inconsistent between experiments (Fig.19). The second lifespan, but not the first, showed an effect of food type on starvation resistance with DR flies showing more resistance to starvation than FF flies across all genotypes. Additionally, the second lifespan showed increased starvation resistance in TrhGAL4/UAS-InR^{DN} flies on DR and FF diets compared to controls, however this was also not seen in the first experiment.



Chi-squared p-value for Log-Rank test on survival data:

	TrhGAL4/UAS-InR^{DN}	0.4676		TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	0.3495
DR to FF	TrhGAL4/+	0.6306	DR	TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	0.0051
	UAS-InR^{DN}/+	0.8362		TrhGAL4/+ to UAS-InR^{DN}/+	0.0525
				TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	0.7810
			FF	TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	0.1131
				TrhGAL4/+ to UAS-InR^{DN}/+	0.0536



Chi-squared p-value for Log-Rank test on survival data:

	TrhGAL4/UAS-InR^{DN}	<0.0001		TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	<0.0001
DR to FF	TrhGAL4/+	0.0414	DR	TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	<0.0001
	UAS-InR^{DN}/+	0.0001		TrhGAL4/+ to UAS-InR^{DN}/+	0.2966
				TrhGAL4/UAS-InR^{DN} to TrhGAL4/+	0.0002
			FF	TrhGAL4/UAS-InR^{DN} to UAS-InR^{DN}/+	0.0007
				TrhGAL4/+ to UAS-InR^{DN}/+	0.3914

Figure 19. Survival of male flies with reduced IIS in serotonergic neurons compared to their controls after complete starvation. *Experimental flies with reduced serotonergic IIS are the TrhGAL4/UAS group, and the TrhGAL4/+ and UAS-InRDN/+ groups are the controls for the driver and transgene respectively (a: N=50; b: N=100). a) Median lifespans: DR TrhGAL4/UAS-InR^{DN} = 20.9 days; DR TrhGAL4/+ = 20.9 days; DR UAS-InR^{DN}/+ = 19.9 days; FF TrhGAL4/UAS-InR^{DN} = 20.2 days; FF TrhGAL4/+ = 20.9 days; FF UAS-InR^{DN}/+ = 19.9 days. b) Median lifespans: DR TrhGAL4/UAS-InR^{DN} = 18 days; DR TrhGAL4/+ = 17 days; DR UAS-InR^{DN}/+ = 17 days; FF TrhGAL4/UAS-InR^{DN} = 17 days; FF TrhGAL4/+ = 17 days; FF UAS-InR^{DN}/+ = 17 days.*

4.7.3. Summary

Under low nutrient conditions, reducing serotonergic IIS in male flies significantly extended lifespan when compared to controls across both repeats. However, female flies with reduced IIS in serotonergic neurons were long-lived in comparison to controls in only one of the two repeats carried out.

The effect of reduced serotonergic IIS on resistance to complete starvation was investigated on SY food. The results showed that reducing IIS in serotonergic neurons in females increased resistance to starvation. There was no effect of reducing serotonergic IIS observed in males.

The impact of dietary restriction on resistance to complete starvation was also investigated. Females maintained on DR food prior to complete starvation were more resistant to starvation than flies on FF food for all genotypes across two repeated experiments. Males also showed improved resistance to complete starvation after treatment with DR, however, this finding was only observed in one of the two repeats.

Reducing IIS in serotonergic neurons did not increase resistance to complete starvation in females under DR or FF conditions. Contrastingly, in males, reduced IIS in serotonergic neurons increased resistance to complete starvation in males under both DR and FF conditions in comparison to controls, however, this was only observed in one repeat. This suggests that reduced IIS in serotonergic neurons could alter the effect of dietary restriction on resistance to complete starvation. However, the findings are not consistent with those seen in flies fed SY food prior to starvation. In females, the increased resistance observed in TrhGAL4/UAS-InR^{DN} flies fed SY prior to starvation is absent when flies are kept under DR or FF conditions before starvation. In males, there was no increased resistance observed in TrhGAL4/UAS-InR^{DN} flies fed SY prior to starvation, however, when flies are kept under DR or FF conditions before starvation, they show improved resistance in one of the two repeats.

5. Results – The effect of reduced IIS in serotonergic neurons on *dilp* expression and serotonin levels in the *Drosophila* brain.

As shown in sections 4.1. and 4.2., reducing IIS in serotonergic neurons extends lifespan in females on standard food (SY) and further extends lifespan in female flies under dietary restriction (DR) conditions. The reason for the lifespan extension observed in female flies on SY and DR food in response to reduced serotonergic IIS is unknown. Previous research has identified a role for DILPs 2, 3 and 5 in lifespan extension and dietary restriction has been shown to decrease DILP5 expression at both the gene and protein level (Broughton *et al.*, 2005; Min *et al.*, 2008; Broughton *et al.*, 2010; Grönke *et al.*, 2010). Furthermore, RNA-induced knockdown of the 5-HT_{1A} receptor, which is present on the insulin-producing cells, has been shown to increase expression of *dilp2* and *dilp5* in flies indicating that serotonin signalling can influence *dilp* expression (Luo *et al.*, 2014). The first aim of the experiments reported in this section was to identify if reducing IIS in serotonergic neurons alters the effect of DR on DILP5 protein levels in the brain of female flies. The second aim was to determine if reducing serotonergic IIS alters the expression of serotonin in the brains of female flies. The third aim was to identify whether reduced serotonergic IIS changes in the expression of *dilp2, 3* and *5* in the heads of female flies.

To achieve these aims, we carried out immunofluorescent staining on the brains of female flies maintained under DR and compared them to the brains of females on fully fed (FF) food. The stained brains were then imaged using a confocal microscope and measures of integrated density were collected from the resulting z-stacks using FIJI software. We also used RT-qPCR to measure levels of *dilp2,3,5* transcripts in the heads of female flies across three food types, DR, SY and FF. The experimental flies with reduced IIS in the serotonergic neurons (TrhGAL4/UAS-InR^{DN}) were compared to the appropriate controls (TrhGAL4/+ and UAS-InR^{DN}/+) for all experiments.

5.1. Reduced IIS in serotonergic neurons increases levels of serotonin in the brain of females with no effect on DILP5 levels in IPCs.

5.1.1. Results

Brains of female flies were dissected out and stained using anti-DILP5 and anti-serotonin antibodies, followed by two secondary antibodies with fluorophores attached. Stained brains were mounted onto microscope slides and were imaged, and Z-stacks were generated using a Zeiss LSM880 confocal microscope. The images were then processed and analysed using FIJI (ImageJ) where measures of integrated density were taken. Serotonin was measured within the central brain region and DILP5 was measured specifically within the area of the IPCs (Fig.20). The expression pattern of the serotonin shown in Figure 20 is largely the same as that shown by Yao and Scott (2022), therefore we can be confident that the serotonin antibody is binding efficiently.

As shown in Fig.20b, serotonin levels were increased in TrhGAL4/UAS-InR^{DN} females compared to controls on both DR and FF diets. Generalised linear modelling found an effect of genotype ($p<0.0001$) but not diet. Further statistical analysis using Tukey-Kramer HSD testing found that TrhGAL4/UAS-InR^{DN} females on both diets had significantly higher serotonin levels compared to both of their controls (DR: to TrhGAL4/+ p=<0.0001; to UAS-InR^{DN}/+ $p=0.0004$. FF: to TrhGAL4/+ $p=0.0003$; to UAS-InR^{DN}/+ $p=0.0064$).

Fig.20c shows that DILP5 levels were lower, across all genotypes, in flies on a DR diet compared to those on an FF diet. Generalised linear modelling of the DILP5 levels found an effect of both genotype ($p<0.0001$) and food type ($p<0.0001$). Post-hoc testing using Tukey-Kramer HSD tests found that TrhGAL4/UAS-InR^{DN} flies had significantly lower levels of DILP5 than UAS-InR^{DN}/+ controls on both diets (DR $p=0.0140$; FF $p<0.0001$), however, there was no difference when compared to TrhGAL4/+ controls (DR $p=0.6887$; FF $p=0.1671$). The effect of diet was however consistent across all genotypes, with DILP5 levels being significantly lower in flies on DR food than on FF food (TrhGAL4/UAS-InR^{DN} $p=0.0020$; TrhGAL4/+ $p=0.0015$; UAS-InR^{DN}/+ $p<0.0001$).

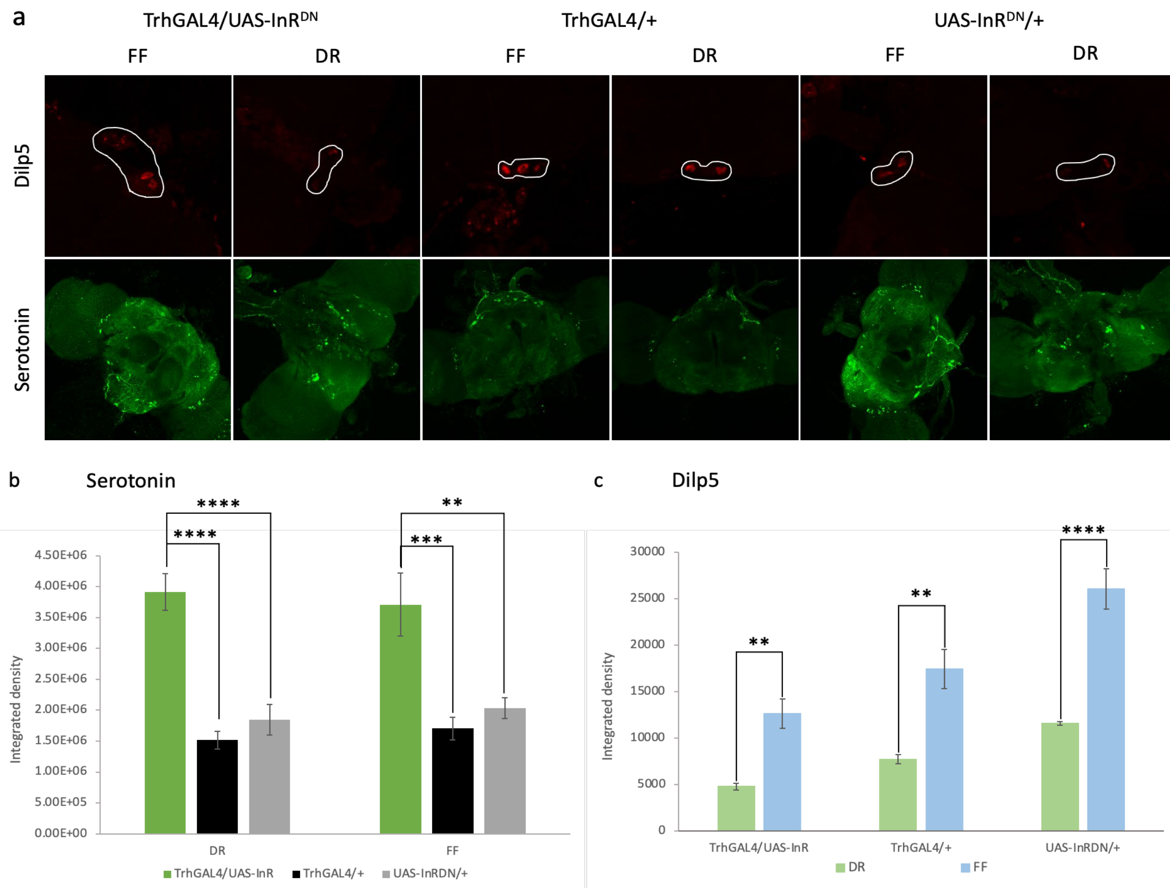


Figure 20. DILP5 and serotonin protein levels in female flies with reduced serotonergic IIS on DR and FF diets. *a) Confocal microscopy images of DILP5 and serotonin expression in female flies on DR and FF diets. Images were taken using a Zeiss LSM880 confocal microscope. The DILP5 producing IPCs are shown circled. b) TrhGAL4/UAS-InR^{DN} females produce significantly more serotonin than their controls on a DR diet (to TrhGAL4/+ p=<0.0001; to UAS-InR^{DN}/+ p<0.0001) and on an FF diet (to TrhGAL4/+ p=0.0003; to UAS-InR^{DN}/+ p=0.0064). c) UAS-InR^{DN}/+ females produced significantly more DILP5 than TrhGAL4/UAS-InR^{DN} females on both diets (DR p=0.0140; FF p<0.0001), but there is no significant difference between TrhGAL4/UAS-InR^{DN} females and TrhGAL4/+ females. Asterisks indicate significant differences: ****=p<0.0001, ***=p<0.001, **=p<0.01, *=p<0.05. The sample sizes for each group are as follows: Serotonin DR TrhGAL4/UAS-InR^{DN} N=5; Serotonin FF TrhGAL4/UAS-InR^{DN} N=4; Serotonin DR TrhGAL4/+ N=5; Serotonin FF TrhGAL4/+ N=6; Serotonin DR UAS-InR^{DN}/+ N=6; Serotonin FF UAS-InR^{DN}/+ N=4; DILP5 DR TrhGAL4/UAS-InR^{DN} N=7; DILP5 FF TrhGAL4/UAS-InR^{DN} N=6; DILP5 DR TrhGAL4/+ N=4; DILP5 FF TrhGAL4/+ N=5; DILP5 DR UAS-InR^{DN}/+ N=5; DILP5 FF UAS-InR^{DN}/+ N=5.)*

5.1.2. Summary

As shown in Fig.20, a higher protein content diet (FF) increases levels of DILP5 in the brains of female flies across all genotypes tested. However, reduced IIS in serotonergic neurons had no effect on DILP5 expression in comparison to controls on either food type.

Unlike DILP5, serotonin levels were not influenced by diet, however, there was a significant effect of reduced serotonergic IIS. Females with reduced IIS in serotonergic neurons (TrhGAL4/UAS-InR^{DN}) showed a significant increase in serotonin levels compared to their controls on both diets.

5.2. Diet, but not reduced IIS in serotonergic neurons, modulates *dilp5* mRNA levels in female heads.

5.2.1. Results

Levels of *dilp2, 3, 5* transcripts were measured by qPCR in 10-day old female fly heads after RNA extraction using Tri Reagent. As shown in Fig.22, transcript levels for *dilp2* and *dilp5* did not differ significantly between TrhGAL4/UAS-InR^{DN} females and controls on any food type. Generalised linear modelling found no effect of genotype or diet on *dilp2* expression. However, it did find an effect of diet on *dilp5* expression ($p<0.0001$). Post-hoc Tukey-Kramer tests found *dilp5* levels were significantly higher in flies on FF food in comparison to those on DR food for all three genotypes (TrhGAL4/UAS-InR^{DN} $p=0.0070$; TrhGAL4/+ $p=0.0318$; UAS-InR^{DN} $p=0.0133$), and while not significant, *dilp5* levels were also higher in flies on SY food compared to flies on DR food. No statistical analysis could be carried out on the *dilp3* data as N=1.

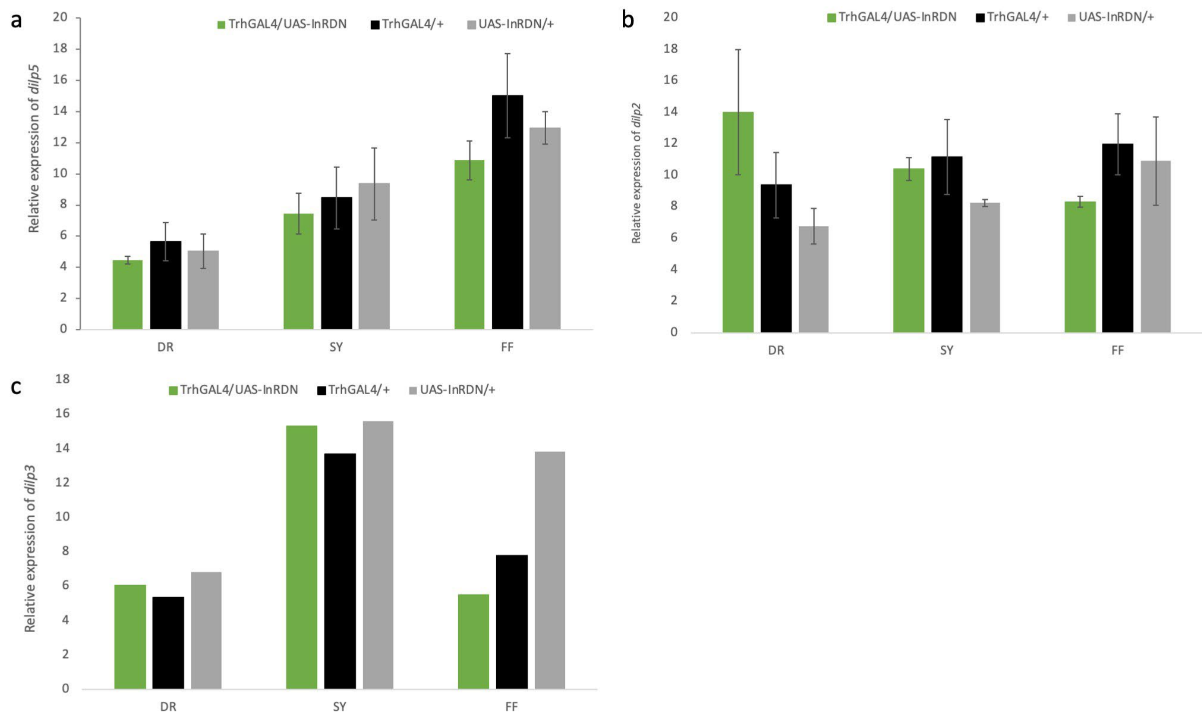


Figure 21. Relative expression of dilp2, 3, 5 in 10-day old female flies on DR, SY and FF food. *Female flies were snap frozen in liquid nitrogen at 10 days old and stored at -80°C. Each qPCR experiment used 20 heads to generate about 1000 ng RNA. TrhGAL4/UAS-InR^{DN} flies are the experimental group, and the controls are TrhGAL4/+ and UAS-InR^{DN}/+. Data are represented as mean relative expression ± SEM (except for graph 17c). a) The effect of reduced serotonergic IIS and food type on dilp5 expression in female heads. DR: TrhGAL4/UAS-InR^{DN} N=4, TrhGAL4/+ N=4, UAS-InR^{DN}/+ N=5; SY TrhGAL4/UAS-InR^{DN} N=3, TrhGAL4/+ N=3, UAS-InR^{DN}/+ N=3; FF TrhGAL4/UAS-InR^{DN} N=4, TrhGAL4/+ N=2, UAS-InR^{DN}/+ N=3. P-values for DR vs FF as follows: TrhGAL4/UAS-InR^{DN} p=0.0070; TrhGAL4/+ p=0.0318; UAS-InR^{DN} p=0.0133. b) The effect of reduced serotonergic IIS and food type on dilp2 expression in female heads. DR: TrhGAL4/UAS-InR^{DN} N=2, TrhGAL4/+ N=2, UAS-InR^{DN}/+ N=3; SY TrhGAL4/UAS-InR^{DN} N=2, TrhGAL4/+ N=2, UAS-InR^{DN}/+ N=3; FF TrhGAL4/UAS-InR^{DN} N=3, TrhGAL4/+ N=3, UAS-InR^{DN}/+ N=2. c) The effect of reduced serotonergic IIS and food type on dilp3 expression in female heads. DR: TrhGAL4/UAS-InR^{DN} N=1, TrhGAL4/+ N=1, UAS-InR^{DN}/+ N=1; SY TrhGAL4/UAS-InR^{DN} N=1, TrhGAL4/+ N=1, UAS-InR^{DN}/+ N=1; FF TrhGAL4/UAS-InR^{DN} N=1, TrhGAL4/+ N=1, UAS-InR^{DN}/+ N=1.*

5.2.2. Summary

No effect of diet or genotype was seen on levels of *dilp2*. However, diet did affect levels of *dilp5*. There was a significant decrease in *dilp5* expression in flies on a DR diet in comparison to an FF diet across all genotypes, and while not significant, *dilp5* expression was lower in flies under DR conditions than in flies under SY conditions.

6. Discussion

This project focused on the various phenotypic and molecular effects of reduced serotonergic IIS in combination with alterations in diet with the aim of determining whether lifespan is uncoupled from healthspan in these flies and what mechanism could underpin the responses observed. This project extended upon previous work which found that reduced serotonergic IIS extends female lifespan, but not male lifespan, and had no negative effect on locomotor senescence in either sex (Dravecz *et al.*, 2022). During this research, it was shown that reduced serotonergic IIS has varying effects on lifespan depending upon the dietary conditions the flies are exposed to. Specifically, females with reduced serotonergic IIS were long-lived under standard (SY) and dietary restriction (DR) conditions, however, they were not long-lived under fully fed (FF) conditions. Furthermore, the females under DR responded to reduced serotonergic IIS in addition to the expected response to DR when compared to females under FF conditions. Contrastingly, males with reduced serotonergic IIS were not long-lived under SY conditions and had varying responses to DR. However, they were long-lived under FF conditions. Additionally, reduced serotonergic IIS had no negative effects on stress resistance, negative geotaxis, or fecundity, indicating an uncoupling of lifespan and healthspan in these flies.

Levels of DILP5 were shown to decrease under DR conditions in the insulin-producing cells of female flies. Furthermore, reduced serotonergic IIS had no effect on *dilp2* or *dilp5* transcript levels. However, reducing IIS in serotonergic neurons did increase levels of serotonin in the central brain of female flies, irrespective of dietary conditions.

6.1. Effects of serotonergic IIS reduction on lifespan in response to DR.

In female *Drosophila*, reduced serotonergic IIS extended lifespan without influencing negative geotaxis or exploratory walking senescence and reduced pan-neural IIS has been shown to have no effect on fecundity in flies on standard food (SY) (Dravecz, 2020; Dravecz *et al.*, 2022). However, the impact of diet in combination with reduced serotonergic IIS on lifespan, fecundity, and negative geotaxis senescence has not been explored. A study using

chico-null homozygotes found that the mechanism of lifespan extension in *chico*-null mutants overlaps with the mechanism of lifespan extension by DR (Clancy *et al.*, 2002). Furthermore, *Drosophila* with ablated IPCs do not show the expected decrease in lifespan when food concentrations is increased, further supporting the idea that response to diet acts in part via IIS (Broughton *et al.*, 2010).

In females, proportional hazards analysis did not find a significant difference in survival between flies with reduced serotonergic IIS and the driver control. However, Kaplan-Meier estimates found a significant difference in lifespan between the females with reduced serotonergic IIS and both controls. Furthermore, previous experiments found that reducing IIS in serotonergic neurons extends female lifespan (Dravecz *et al.*, 2022). As a result, the differences in statistical findings could be explained by natural variation in lifespan. However, if previous research had not been completed on the effect of reduced serotonergic IIS in flies on a standard diet, this experiment would need to be repeated. This project did however confirm the finding that reducing serotonergic IIS does not extend lifespan in males on standard food (Dravecz *et al.*, 2022).

Reducing serotonergic IIS in females under DR conditions significantly increased lifespan compared to genetic controls and this extension occurred in addition to the normal response of increased lifespan in response to DR when compared to FF. This suggests that the effects of reducing serotonergic IIS and dietary restriction are additive. Therefore, it is likely that the mechanism of DR does not involve IIS in the serotonergic neurons. However, it may involve other tissues, cells, or receptors such as the IPCs (Broughton *et al.*, 2005).

In contrast to females, TrhGAL4/UAS-InR^{DN} males had extended lifespan under fully fed (FF) conditions. This suggests serotonergic IIS in males may be responsible for some of the decrease in lifespan observed in response to FF conditions, and that reducing serotonergic IIS can ameliorate some, but not all the negative effects of FF food on lifespan. This effect is similar to what is seen in flies with ablated IPCs (Broughton *et al.*, 2010). In one of the two lifespans carried out, TrhGAL4/UAS-InR^{DN} males on DR media had extended lifespans in comparison to their controls, however, the first lifespan found no significant effect. The extension of lifespan in response to DR is usually greater in females compared to males

which could explain the inconsistent responses to DR seen in the male lifespans and the large effect of DR seen within the female lifespans (Partridge *et al.*, 2005a). Repeats of the lifespan experiments should be completed to determine the effect of reduced serotonergic IIS on males fed a DR diet.

The changes in lifespan could be the result of alterations in fly behaviour resulting from the reduced serotonergic IIS. Serotonin is known to influence multiple behaviours including feeding behaviour, with systemic activation of serotonergic neurons being associated with reduced feeding (Albin *et al.*, 2015; Pooryasin and Fiala, 2015). Furthermore, serotonin has been shown to influence lifespan via sensory perception and nutrient choice, specifically by modulating protein intake (Ro *et al.*, 2016; Chakraborty *et al.*, 2019; Lyu *et al.*, 2021; Munneke *et al.*, 2022). In theory, this could all contribute to a protective effect against the negative impacts of an FF diet on lifespan in males. As a result, an assay to determine whether reduced serotonergic IIS affected feeding behaviour was carried out. This assay would simultaneously determine whether IIS in serotonin neurons is involved in the control of feeding behaviour.

The feeding behaviour assay found no effect of diet or reduced serotonergic IIS on feeding behaviour in either sex. This suggests that changes in feeding behaviour are not responsible for any of the lifespan phenotypes observed in flies with reduced IIS in serotonergic neurons. Furthermore, these findings indicate that IIS is not required in serotonergic neurons for the control of feeding behaviour. However, despite no noticeable visual differences in size, flies were not weighed before the feeding behaviour assay, and although the gut contents were isolated, measuring the concentration of food isolated by weight would have been more accurate than by measuring concentration by individual fly.

We also wanted to identify a potential mechanism which could explain why reduced IIS in serotonergic neurons extended lifespan in females. One theory is that reducing IIS in serotonergic neurons changes serotonin levels in the brain, and therefore alters expression of the *dilps* produced by the IPCs. This is because 5-HT$_{1A}$ receptors are present on the IPCs and 5-HT$_{1A}$-RNAi has been shown to increase *dilp2* and *dilp5* levels (Luo *et al.*, 2012; Luo *et al.,* 2014). The DILPs are a set of insulin-like peptides that are highly regulated throughout

the development of *Drosophila* and modulation of the DILPs via multiple mechanisms has been shown to affect growth and lifespan (Brogiolo *et al.*, 2001; Ikeya *et al.*, 2002; Broughton *et al.*, 2005; Grönke *et al.*, 2010). Specifically, loss of DILP2 has been shown to increase lifespan (Grönke *et al.*, 2010). DILP3 has also been shown to change in response to altered levels of DILP2 and DILP5 and vice versa. Levels of *dilp3* transcript were increased in *dilp2* and *dilp5* mutants and *dilp3* mutants had reduced levels of *dilp2* and *dilp5* transcripts suggesting DILP3 regulates *dilp2* and *dilp5* expression, however, *dilp3* mutants were not long-lived (Grönke *et al.*, 2010). DILPs have also been shown to respond to diet, specifically, *dilp5* transcripts decrease with dietary restriction and both transcript and protein increase when dietary yeast content is increased (Min *et al.*, 2008; Broughton *et al.*, 2010). The focus was placed on expression in female heads due to previous research which showed lifespan extension on SY food was specific to females and that levels of *dilp2* and *dilp5* increase in flies with RNAi-induced knockdown of 5-HT_{1A} receptors which are present on the IPCs (Luo *et al.*, 2014; Dravecz *et al.*, 2022). We also focused on *dilp2, 3, 5* transcripts in the heads because their combined role in lifespan is supported by studies showing the ablation of IPCs extends lifespan and time constraints would not allow for analysis of all DILPs (Broughton *et al.*, 2010). We analysed the protein expression of DILP5 in the brains of females due to the known effect of diet on its expression (Broughton *et al.*, 2010).

We found that reducing IIS in the serotonergic neurons of females had no effect on *dilp2* or *dilp5* transcript levels in the heads of female flies. Furthermore, levels of DILP5 protein were unaffected by reduced IIS in serotonergic neurons. This finding suggests that the lifespan extension observed in females is not in response to changes in *dilp* expression. The data from these experiments suggests that reducing serotonergic IIS does not alter expression of *dilp2* or *dilp5*, therefore it is unlikely to be influencing systemic levels of IIS which would be the proposed mechanism of lifespan extension if DILP expression was changing. Dietary restriction had the expected effect on DILP5 at both the gene and protein level, with lower levels of expression in females under DR (Broughton *et al.*, 2010). This shows that the response to DR in TrhGAL4/UAS-InR^{DN} flies is intact and likely mediated by the same mechanism as control flies.

Repeating the qPCR experiments is suggested to further clarify the effects of reduced serotonergic IIS on *dilp2*. Additionally, completing more experiments investigating the effect of diet and reduced serotonergic IIS on *dilp3* is necessary to ensure the effects on the IPCs are fully determined. Furthermore, it would be interesting to investigate the response of *dilp6* in the head and *dilps4, 6, 7* in the body to provide a more complete picture of the effects of reduced serotonergic IIS. This is because the individual DILPs have different spatiotemporal expression patterns and both redundancy and compensatory mechanisms in their function and expression have been observed (Grönke *et al.,* 2010; Nässel *et al*., 2015). DILP4 is expressed in the anterior midgut of larvae but shows very little expression in adults, DILP6 is expressed in the larval fat body, heart, salivary glands, and glial cells but only in the adult fat body, and DILP7 is expressed in the abdominal neuromeres of the ventral nerve chord in both larvae and adults (Nässel *et al.,* 2015). Grönke *et al.* (2010) investigated the function of DILPs1-7 and found that mutations within single *dilps* can have mild phenotypic effects on development, body weight, lifetime fecundity, and trehalose and lipid content. However, combinatorial *dilp* mutations can have stronger effects of the same phenotypes and others, including starvation resistance, oxidative stress resistance, lifespan, and glycogen content (Grönke *et al*., 2010). These findings show redundancy within the DILPs, however compensatory mechanisms also exist, including increased expression of DILP6 in *dilp2-3,5* mutants and DILPs 2 and 3 in *dilp5* mutants (Grönke *et al.,* 2010). It would also be beneficial to investigate the protein expression of DILP2 and DILP3 to further clarify the effects of reduced serotonergic IIS, both independently, and in combination with diet. These experiments should also be repeated in males due to the new findings that reduced serotonergic IIS can influence lifespan under FF conditions and potentially DR conditions.

In addition to investigating *DILPs 2, 3 and 5* transcript levels, protein levels of serotonin were also analysed in the brains of females on DR and FF diets. Serotonin has been shown to influence lifespan in *C. elegans* and *D. melanogaster*. In *C. elegans*, a deletion mutation in *ser-1* extends lifespan and further improves the lifespan of *eat-2* mutants (Murakami and Murakami, 2007). Serotonin receptor antagonists have also been shown to extend lifespan in *C. elegans* (Petrascheck *et al.*, 2007). Little research into the direct effect of serotonin signalling on lifespan in *Drosophila* has been completed, however, 5-HT$_{2A}$-null females have extended lifespan (Munneke *et al.*, 2022). This previous research indicates that serotonin

could be modulating the lifespan extension seen in females with reduced serotonergic IIS. Diet could also have a role in modulating serotonin levels. However, very little research has been carried out in flies. A study in *C. elegans* found that DR, but not reduced IIS, can ameliorate the age-related decline in serotonin levels, and considering the large amount of evolutionary conservation between many of the mechanisms in the two model organisms, this could be true in flies (Yin *et al.*, 2014).

This project found that reduced serotonergic IIS, but not diet, influences serotonin levels in female brains. Specifically, reduced serotonergic IIS increased serotonin levels. This finding supports the idea that serotonin is modulating the lifespan extension seen in females and suggests that IIS in serotonergic neurons could be modulating serotonin levels. However, the finding that reduced serotonergic IIS had no effect on feeding behaviour suggests that the role of serotonin in the lifespan extension seen in females is unrelated to the serotonergic control of feeding behaviour. Additionally, since only female brains were analysed in this project, analysis of male brains is still required to clarify whether serotonin expression could explain the lifespan extension seen in males under FF conditions. Furthermore, serotonin levels are known to decrease over the lifespan of *C. elegans*, *Drosophila* and humans (Rehman and Masson, 2001; Yin *et al.,* 2014; Liao *et al.,* 2017). Therefore, analysing levels of serotonin in TrhGAL4/UAS-InR^{DN} flies over the lifespan would be beneficial because if the decrease seen in *Drosophila* over lifespan is ameliorated, it would further support a role for serotonin in the modulation of lifespan.

Previous research in *C. elegans* and *Drosophila* indicates that reduced serotonergic signalling is associated with increases in longevity (Murakami and Murakami, 2007; Petrascheck *et al.*, 2007; Munneke *et al.*, 2022). This contradicts our finding that reduced serotonergic IIS increased expression of serotonin. However, different serotonin receptors can have opposing effects on behaviours. For example, 5-HT$_{1B}$ receptors suppress feeding, 5-HT$_{7}$ receptors enhance feeding, and signalling via 5-HT$_{1A}$ receptors enhances male aggression whereas 5-HT$_{2A}$ receptors decreases male aggression (Johnson *et al.*, 2009; Banu *et al.*, 2023). Furthermore, activation of specific subsets of serotonergic neurons can differentially affect behaviour. For example, activation of R50H05 neurons induces feeding in sated flies, however, systemic activation of serotonergic neurons reduced feeding in starved and sated

flies (Albin *et al.*, 2015). This indicates that the increased serotonin levels could be responsible for the lifespan extension, however, the extension seen may be the result of the sum of effects on numerous processes downstream of serotonin signalling. Further experiments are required determine if the increase in serotonin is required for the lifespan extension seen in females with reduced serotonergic IIS. One potential experiment which could be carried out is a survival assay on females with reduced serotonergic IIS in conjunction with knockout of *Trhn*, the gene responsible for producing the rate-limiting enzyme in neuronal serotonin synthesis (Kasture *et al.,* 2018).

6.2. A sexually dimorphic response to dietary restriction in flies with reduced IIS in serotonergic neurons

This project found a difference in the response to diet between males and females with reduced IIS in serotonergic neurons. In males, reducing serotonergic IIS extended lifespan on FF food, similarly to IPC-ablated flies (Broughton *et al.,* 2010). However, this did not occur in females. In females, the extension of lifespan in flies with reduced serotonergic IIS is additive to DR and therefore unlikely to be the result of a modulation of the DILPs (Broughton *et al.,* 2010). It could involve other neuropeptides secreted by the IPCs, such as DSK (drosulfakinin), DH44 (diuretic hormone 44), and DMS (dromyosuppressin), however, very little research has been done into their effects on lifespan (Nässel and Zandawala., 2020).

The sexual dimorphism observed in the response of lifespan is not unexpected. Numerous examples of sexually dimorphic responses to reduced IIS exist within *Drosophila*. For example, *chico*-null females are long-lived, whereas *chico*-null males are slightly short-lived (Clancy *et al.*, 2001). Furthermore, heterozygous *chico* males show a smaller lifespan extension in comparison to heterozygous females (Clancy *et al.*, 2001). Male *InR* mutants also show a smaller lifespan extension than their female counterparts (Tatar *et al.*, 2001). Reducing pan-neural IIS and reducing IIS in serotonergic neurons also did not extend lifespan of males in previous experiments (Ismail *et al.*, 2015; Dravecz *et al.*, 2022). There is also a sexually dimorphic response to DR in flies with the lifespan extension seen in females being

larger than in males (Magwere *et al.*, 2004). However, we do not know why there is a sexually dimorphic response to diet and reduced IIS.

6.3. The effect of dietary restriction in combination with reduced serotonergic IIS on female fecundity.

In addition to effects on lifespan, diet also influences fecundity, with DR reducing fecundity of females (Partridge *et al.*, 2005a). Reduced IIS can also reduce fecundity (Clancy *et al.*, 2001; Broughton *et al.*, 2005). However, unlike diet, time- and tissue-specific reductions in IIS do not always reduce fecundity (Hwangbo *et al.*, 2004; Giannakou *et al.*, 2007; Dravecz, 2020). This suggests that reduced serotonergic IIS may not impact fecundity, however alterations in diet should.

As expected, there was no consistent effect of reduced serotonergic IIS on fecundity throughout lifespan. There were some significant differences observed, however they were not repeated which could suggest the differences were caused by natural variation within the samples. Despite this, previous research suggests that reduced serotonergic IIS is unlikely to impact fecundity. This is because fecundity and lifespan are not always coupled and pan-neural reduction in IIS did not alter fecundity (Ismail *et al.*, 2015). However, in agreement with previous research, diet did impact fecundity (Partridge *et al*., 2005a). Females on FF media laid significantly more eggs on average than females on DR across all genotypes and age points.

6.4. Effects of reducing IIS in serotonergic neurons on negative geotaxis.

With respect to negative geotaxis, reduced systemic IIS has been shown to attenuate age-related declines in locomotor senescence (Martin and Grotewiel, 2006; Ismail *et al.*, 2015). Contrastingly, reduced pan-neural (Ismail *et al.*, 2015; Dravecz *et al.*, 2022) and serotonergic IIS (shown here) do not slow locomotor senescence in flies on a standard diet. Dietary restriction has also been shown to have little to no impact on age-related declines in negative geotaxis (Bhandari *et al.*, 2007; Bazzell *et al.*, 2013).

The experiments reported within this project show that negative geotaxis senescence is not affected by diet or reduced serotonergic IIS. All treatments across both sexes showed no significant impact of diet or genotype on age-related declines in negative geotaxis performance. This agrees with previous research into the effect of reduced pan-neural and reduced serotonergic IIS and the effect of DR (Bhandari *et al.*, 2007; Ismail *et al.*, 2015; Dravecz *et al.*, 2022). Furthermore, it suggests that the role of serotonergic neurons in the control of walking speed does not require IIS (Howard *et al.*, 2019).

6.5. The effects of reduced IIS in serotonergic neurons on oxidative stress resistance and starvation resistance.

It is well-known that IIS pathway is involved in the response to stress and has links to other pathways which also influence stress resistance, including the mTOR and JNK pathways (Broughton and Partridge, 2009). Flies with reduced IIS are often resistant to oxidative stress and starvation (Clancy *et al.*, 2001; Hwangbo *et al.*, 2004; Broughton *et al.*, 2005). However, this study found that reducing IIS in serotonergic neurons had no effect on oxidative stress resistance and neither did diet. This suggests that reduced serotonergic IIS is not responsible for the oxidative stress resistance observed in other IIS models that show extended lifespan and improved oxidative stress resistance. However, the sample size for this experiment was 50 flies per treatment. This is a low sample size for a survival experiment because the standard sample size is between 100-150 (Piper and Partridge, 2016). As a result, it would be beneficial to repeat this experiment with larger sample sizes to confirm that oxidative stress resistance is not affected by diet or reduced serotonergic IIS. Despite this, the lack of effect of diet partially supported by previous research which found that DR had no effect of oxidative stress resistance in young flies, however, it reduced oxidative stress resistance at older ages (Burger *et al.*, 2007).

The effect of diet and reduced serotonergic IIS on starvation resistance were tested using two different experimental designs. The first followed the design of a classic lifespan experiment, like the ones carried out using the DR, SY and FF media. The flies were sorted

into vials containing a low nutrient food (0.1) and maintained throughout their lifespan with deaths being counted every 2-3 days. The second design mimicked the oxidative stress resistance assay, whereby flies were maintained on either DR, SY, or FF food for 10 or more days before being transferred onto food containing no sugar or yeast (0xSY), after which deaths were scored one or more times a day.

The starvation lifespan experiment using 0.1 food found that reducing serotonergic IIS in males extends lifespan under low nutrient conditions. Furthermore, reducing serotonergic IIS in females extended lifespan in one of the two repeats carried out. This suggests that reducing IIS in serotonergic neurons can improve starvation resistance, resulting in extended lifespans in males under low nutrient conditions, and can potentially do the same in females. If this is the case, it further supports the finding that dietary restriction does not act via serotonergic IIS because, if it did, flies would act as if they were already experiencing dietary restriction and therefore would have decreased lifespans in comparison to controls.
As a result, the experiment would need to be repeated to determine the exact effect.

In contrast, the starvation resistance experiment found varying results. Females showed improved resistance when fed DR food prior to starvation. Males also showed improved starvation resistance when fed DR prior to starvation, however this was only shown in one of the two experiments, which could be explained by the response to DR in males usually being smaller and therefore potentially less likely to appear as statistically significant. These findings are supported by previous research which found that DR improves starvation resistance, and that the effect is usually stronger in females than in males (Chippindale *et al.*, 1993; Kapahi *et al.*, 2004). Reduced serotonergic IIS also improved starvation resistance in males fed DR and FF diets before starvation, however, this result was also only shown in one of the two experiments. These varying results could be due to the difference in the age at which flies were transferred onto the starvation media. The flies in the first experiment were transferred at 17 days whereas the flies in the second experiment were transferred at 10 days and it has been reported that DR increases starvation resistance in young flies but reduces resistance at older ages (Burger *et al.*, 2007). This could explain why the significant differences were observed in males in the second experiment. As a result, the starvation resistance experiments should be repeated using a consistent experimental method.

One potential mechanism underlying the effects of reduced serotonergic IIS on starvation resistance could be altered metabolism. IIS is known to be involved in metabolic homeostasis across numerous organisms, including flies and humans, and in flies it has been linked to the control of feeding behaviour, digestion, and energy storage (Chowański *et al.*, 2021). Reducing systemic IIS by ablating the IPCs increased energy stores in the form of whole-body levels of trehalose, glycogen, and lipids, alters the levels of circulating glucose and trehalose, and increases lifespan (Broughton *et al.*, 2005). Conversely, pan-neural reduction of IIS had no impact on haemolymph glucose concentration and increased IIS in the fat body is associated with increased triglyceride storage (DiAngelo and Birnbaum, 2009; Dravecz, 2020). This indicates that IIS can have varying effects on energy storage, and therefore metabolic homeostasis depending on the tissues in which it is acting. Furthermore, reducing serotonergic IIS may also impact changes in activity levels in response to starvation. Starved flies exhibit hyperactivity and reduced sleep (Keene *et al.*, 2010). Furthermore, systemic reduction of IIS increases daytime activity and extends lifespan (Metaxakis *et al.*, 2014), however, constitutive reduction in pan-neural IIS has no impact on activity levels and extends female lifespan (Dravecz, 2020). This indicates that IIS and starvation can both impact activity levels, however, the effects of IIS are likely tissue specific. As a result, measuring levels of trehalose and glucose in haemolymph, changes in stored energy in the form of triglycerides and glycogen, and activity levels in flies with reduced serotonergic IIS is recommended due to the varying effects of reduced IIS on metabolism and activity.

6.6. Summary of findings and where they fit into future work.

This project found that reduced serotonergic IIS can interact with diet and sex to produce varying effects on lifespan. In females, reduced serotonergic IIS was able to extend lifespan under standard conditions, dietary restriction conditions, and potentially, very low nutrient conditions. In males, reduced serotonergic IIS was able to extend lifespan under FF and very low nutrient conditions, and potentially under DR. Furthermore, this project supports previous findings that reduced serotonergic IIS has little to no effect on healthspan, including negative geotaxis senescence and oxidative stress, even when diet is altered.

The results of the immunohistochemistry and qPCR experiments suggest that the response to DR in females with reduced serotonergic IIS is, at least in part, due to reduced levels of DILP5. However, reducing IIS in serotonergic neurons had no direct effect on expression of *dilp2* or *dilp5*. Reduced IIS in serotonergic neurons was associated with an increase in serotonin in the brains of female flies. This suggests that reducing IIS in serotonergic neurons does not extend lifespan by modulating *dilp* expression in the IPCs, however, serotonin may be modulating the lifespan increase observed in females. These findings further confirm the notion that modulating IIS in specific neuronal subtypes has varying effects on lifespan and behaviour and that lifespan and healthspan are not always coupled.

The main aim of ageing research is to contribute to finding a target capable of extending healthspan of humans by reducing the risk of age-related diseases. Identifying mechanisms capable of extending lifespan with no detrimental effects on lifespan supports this research by confirming that lifespan and healthspan can be uncoupled via time- or tissue-specific modulations of signalling pathways, in this case the IIS pathway. Additionally, the results of this project support a role for serotonin in the control of lifespan. However, the mechanism by which reduced IIS in serotonergic IIS affects levels of serotonin is not known and elucidating the mechanism behind this interaction may identify new targets with positive effects on healthspan. As a result, further research is necessary.

6.7. Limitations of this work.

The main limitation for this work was time constraints which meant that the repeats needed to clarify the results of some experiments could not be completed. For example, due to the variable results shown in the male DR lifespans, the female 0.1 lifespans, and the starvation resistance experiments, repeats should be completed. Furthermore, only one *dilp3* qPCR reaction was carried out using female heads so more experiments are needed to draw conclusions about any changes which may occur in *dilp3* transcript levels.

Another limitation is the differing experimental designs of the two starvation resistance experiments. Previous research has shown that age can impact the effect of DR on starvation

resistance (Burger *et al.*, 2007). As a result, the experiment should be repeated, and experimental methods should be kept consistent.

Additionally, in the experiments carried out during this project, flies under dietary restriction (DR) were compared to flies under fully-fed (FF) conditions. Furthermore, FF food contains a higher quantity of yeast than standard food (SY) which has been shown to have some negative effects on lifespan (Bass *et al*., 2007). As a result, the effects of DR can only be discussed in relation to FF conditions. One suggestion to rectify this could be to repeat the lifespan experiments on SY, DR and FF food in parallel and directly compare flies from all three conditions.

6.8. Future directions for this work.

Further research is required to determine the exact mechanism by which reduced serotonergic IIS extends lifespan. While this project found that DILP2 and DILP5 are not responsible for the extension seen in females, further qPCR would be necessary to determine whether any changes occur in *dilp3* and other *dilps* including *dilp6* which has been shown have links to lifespan (Bai *et al.*, 2012; Dravecz, 2020). Additionally, this project investigated the effect of diet alongside reduced serotonergic IIS and found sexually dimorphic responses, with females showing improved survival under DR and males showing improved survival on FF food. As a result, it would be beneficial to carry out the immunohistochemistry and qPCR experiments on males. Completing the immunohistochemistry analysis of brains at multiple timepoints across the lifespan is also advisable because serotonin levels are known to decrease with age in *Drosophila* (Liao *et al.*, 2017; El Husseiny *et al.*, 2022), and if they consistently remain higher in flies with reduced serotonergic IIS it would further support the role of increased levels of serotonin in the lifespan extension seen in these flies.

In combination, the results of the immunohistochemistry and qPCR experiments suggest that serotonin levels, but not DILP2, 5 levels are affected by reduced serotonergic IIS, at least in female flies. This suggests that the control of lifespan is occurring via serotonin signalling, and while serotonin signalling can directly impact lifespan, it could also be modulating the

secretion of other neuropeptides from the median neurosecretory cells (mNSCs) which include the IPCs from which DILPs are secreted. These include DSK (drosulfakinin), DH44 (diuretic hormone 44), and DMS (dromyosuppressin) (Nässel and Zandawala, 2020). However, very little research has been completed on the role of these neuropeptides in lifespan. Furthermore, the response may not be occurring via the mNSCs but via another set of neurosecretory cells which receive signals from the serotonergic neurons. RNA sequencing in the heads of females with reduced serotonergic IIS could be used to identify potential targets, including other neuropeptides.

It would also be interesting to investigate whether serotonin is necessary for the lifespan extension seen in these flies, potentially via knockdown or knockout of *Trhn*, the gene which encodes the enzyme responsible for the rate-limiting step in serotonin synthesis (Kasture *et al.*, 2018). This could be further expanded upon by carrying out knockdown or knockout experiments of serotonin receptors known to be involved in the control of lifespan via serotonin, such as the 5-HT_{2A} receptor (Munneke *et al.*, 2022).

6.9. Conclusions.

To conclude, this project found that reduced serotonergic IIS produces sexually dimorphic changes in lifespan in response to diet, with females showing increased lifespans on SY and DR, and potentially 0.1 diets and males only showing increased lifespans when on 0.1 or FF diets. These findings are important because they show that reduced serotonergic IIS is capable of modulating lifespan across multiple diets, however, at least in females, the response to DR does not occur via serotonergic IIS.

Additionally, diet and reduced serotonergic IIS had no effect on negative geotaxis or oxidative stress, but they may have had an effect of starvation resistance. Furthermore, diet but not reduced serotonergic IIS did influence female fecundity. These results indicate that reduced serotonergic IIS has very little effect on the behaviours stated above. This suggests that reduced serotonergic IIS is does not damage the neural circuitry involved in negative geotaxis. It also indicates that the lifespan extensions observed may not be linked to changes in stress resistance or fecundity, which is often assumed due to previous research showing

improved stress resistance and reduced fecundity in flies with reduced IIS. This project also found no impact of reduced serotonergic IIS or diet on feeding behaviour. This indicates that the lifespan extensions observed are not simply the result of reduced food intake and self-inflicted DR.

Finally, the immunohistochemistry and qPCR data suggest that serotonin, but not DILPs 2 and 5, is responsible for the lifespan extensions observed in females. This finding goes against the hypothesis that reduced IIS in serotonergic neurons extends lifespan by decreasing DILP expression via interactions between the serotonergic neurons and the IPCs. However, this finding requires further research as not all DILPs were investigated, and it is not known whether serotonin is necessary for the phenotypes observed.

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