Insulin/IGF-like Signalling and Brain Ageing in *Drosophila melanogaster*

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I declare that this thesis is my own work and has not been submitted in substantially the same form for the award of higher degree elsewhere.

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Abstract

Human life expectancy has been steadily increasing since the mid-nineteenth century in developed countries, mainly due to improved public health and lifestyle changes, which has led to the increasing prevalence of age-related diseases. Understanding the biological mechanisms of ageing is essential to improve human health at older ages and extend health-span. Reduced Insulin/IGF-like signalling (IIS) improves longevity and some measures of health-span in model organisms, such as *C. elegans*, *Drosophila melanogaster* and *Mus musculus*, suggesting an evolutionarily conserved role. Recent studies, however, have found a disconnection between lifespan extension and behavioural health-span. It was recently shown that selective reduction of IIS in *Drosophila* neurons extended female lifespan but did not improve negative geotaxis senescence and had a detrimental effect on exploratory walking senescence in both sexes. This project addresses the following two hypotheses: (1) the negative effects of reduced IIS on behavioural senescence may be due to detrimental effects on neuronal function at older ages that outweigh any positive effects of reduced IIS on neuronal ageing; and/or (2) individual neuronal subtypes respond differently to IIS changes, thus the behavioural outcomes of pan-neuronal IIS reduction are the sum of a mixture of positive, negative and neutral functional effects. We found that adult-specific pan-neural IIS reduction is sufficient to extend female lifespan and result in detrimental but reversible effects on behavioural senescence. The data suggest that the detrimental behavioural effects of reduced pan-neuronal IIS are likely due to a reduction in neuronal function and are not due to accelerated neuronal ageing. Altered *Drosophia* Insulin-like peptide (*dilp*) expression observed in response to adult pan-neuronal IIS reduction in females may suggest an endocrine mechanism of lifespan extension involving modulation of *dilps* from the brain insulin producing cells and fat body. IIS reduction in specific neuronal subtypes either does not affect or has detrimental effects on lifespan and health-span suggesting that individual neuronal subtypes do respond differently to IIS changes. We did not find evidence that the ageing of neurons is altered by reduced IIS and further work is needed to elucidate the molecular mechanisms involved in lifespan extension and reduced neuronal function due to reduced pan-neuronal IIS.

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Abbreviations

AMPK – AMP-dependent protein kinase

ChAT – Choline O-acetyltransferase

CNS – Central Nervous System

CR – Calorie Restriction

DAM – *Drosophila* Activity Monitor

DEPC – Diethyl pyrocarbonate

DILP – *Drosophila* Insulin-like Peptide

dInR – *Drosophila* Insulin Receptor

DN – Dominant Negative

DR – Dietary Restriction

FOXO – Forkhead Box-O transcription factor

GABA – Gamma-Aminobutyric acid

Gad1 – Glutamate decarboxylase 1

GFP – Green Fluorescent Protein

GLM – General Linear Modelling

GS – GeneSwitch

hsp70 – Heat Shock Protein 70

IGF – Insulin- like Growth Factor

IIS – Insulin/IGF like signalling

InR – Insulin Receptor

IPC – Insulin Producing Cells

JNK – c-Jun N-terminal Kinase

Nrf – Nuclear respiratory factor

PBS – Phosphate-buffered saline

PDK1 – Phosphoinositide-dependent kinase-1

PFA – Paraformaldehyde

PI3K – Phosphoinositide 3-kinase

PIP3 – Phosphatidylinositol (3,4,5)-trisphosphate

PTEN – Phosphatase and tensin

qPCR – Quantitative Polymerase Chain Reaction

S6K – S6 kinase

SEM – Standard Error of Means

SIRT – Sirtuins

SY – Sugar/Yeast

TFEB – Transcription factor EB

Th – Tyrosine hydroxylase

TNT - Tris-NaCl-Tween buffer

TOR – Target of Rapamycin

TSC2 – Tuberous Sclerosis Complex 2

UAS – Upstream Activation Sequence

Vglut – Vesicular Glutamate Transporter

w^{Dah} – $white^{Dahomey}$

Chapter 1: Introduction

1.1: Human lifespan extension in the last 150 years

Life expectancy has increased significantly worldwide since the mid nineteenth century due to improved public health, medicine and changes in lifestyle and nutrition (Partridge, 2010). Up until the 1930s, the main cause of extended survival rates was an improvement in hygiene such as the availability of clean water and better sewage treatment and waste management (Cutler and Miller, 2005). Improved treatments for infectious diseases have also played a major role. The first vaccine was made by Edward Jenner in 1798 against smallpox, and by the end of the 19th century, vaccines against rabies, typhoid, cholera and plague were developed. Through the 1920s-30s more vaccines became available to prevent often deadly infections, such as diphtheria, yellow fever, pertussis, tetanus, influenza and rickettsia (Plotkin, 2014). The improvement of virology and the ability to grow viruses in laboratories led to the development of numerous vaccines in the second half of the 20th century, including polio (1955), measles (1963), mumps (1967) and rubella (1969) (Plotkin, 2014). Along with vaccines, antibiotics were also developed in the mid-20th century. These advances led to a rapid increase in human life expectancy as mortality rates among the youth and middle-aged population dropped (Wilmoth, 2000).

The leading cause of death in the United States was heart disease since 1921, and stroke was on the third place since 1938. Between 1950-1996, the death rates of cardiovascular diseases decreased by more than half, and the reduction of cardiovascular disease mortality is responsible for an estimated 73% of the total death rate decline over that time period (Wilmoth, 2000). The decline of an other major cause of death, cancer has only started to decline since 1980-90 (Wilmoth, 2000). **Figure 1** compares how some of the major causes of death changed between 1908, 1948 and 2010 in the UK.

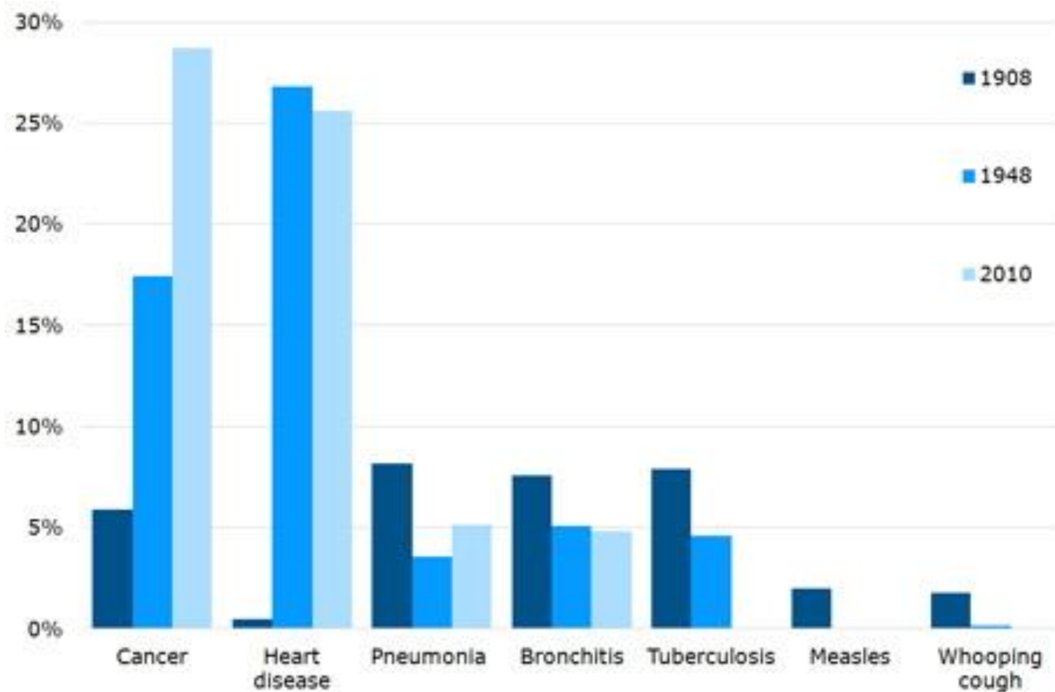


Figure 1 - Changes in major causes of death since 1908 in England and Wales
The figure shows, that the major causes of deaths in 1908 were infectious diseases and the prevalence of cancer and cardiovascular diseases were low. Better hygiene, vaccination and antibiotics helped to reduce deadly infection, therefore people could live long enough to die from age related diseases, such as cancer and heart diseases. With the development of blood pressure drugs and heart surgery, by 2010 cancer became the major cause of death (Thompson, et al. 2012)

According to the Office of National Statistics, life expectancy at birth in the UK was 79.3 years for males and 82.9 for females between 2016-2018 (Ons.gov.uk, 2019). According to Public Health England (2017), death rates due to heart disease and stroke have halved since 2001 in both genders, but the death from dementia and Alzheimer's have increased by 60% in males and by 50% in females. The major cause of death in 2015 was cancer, and if all forms of cancer are grouped together, they were responsible for 24.8% of all death in females and 30% of all deaths in males. For males, the second major cause of death was ischaemic heart disease being accountable for 14.2% of all death, followed by dementia and Alzheimer's disease with 8%. For females, dementia and Alzheimer's were the second major cause of death by 15.3% followed by heart disease with 8.8%. The major causes of death under the age of 35 years are external causes, such as accidents and suicide (GOV.UK, 2019).

Increased life expectancy changes the age structure of the population leading to concerning socio-economic issues. According to the Office for National Statistics, the 65 and over age group is predicted to grow 5 times faster than the working age

population in England. By 2024 the working age population is likely to grow by 3.6% while the predicted growth of the age group 65 and over is 20.4%. The increased proportion of the 65+ age group leads to an increase in the old-age dependency ratio, which means the retirement age needs to increase gradually. (Ons.gov.uk, 2019).

So far, the increased lifespan in the developed world has been due to reduced baseline mortality rates, but the rate of ageing has not declined (Partridge, 2010). Even though the overall health at a given age has improved, there has been no change in the underlying process of ageing (Partridge, 2010). Together with increasing population size, these factors mean that more people are living long enough to suffer from age related diseases or loss of functions. Currently, age-related diseases are considered as separate medical problems, and each disease is treated one-by-one. The ultimate goal of the ageing research is to gain a better understanding of the biological causes and mechanism of ageing in order to develop a broad-spectrum, preventative intervention for age related diseases to improve health and function at older ages (Partridge, 2010).

1.2: Why do we age?

Biologically, ageing can be defined as an increased likelihood of death and reduced fecundity throughout adulthood caused by intrinsic functional decline in cells and tissues (Partridge, 2010). Ageing is a deleterious trait, so why did evolution not get rid of it? This question has been addressed by numerous studies since the 1930s and during the second half of the 20th century, when the three main classical evolutionary theories of ageing were formed. They are all based on the recognition that in nature organisms die from extrinsic causes, such as predation, accidents or infections (Partridge, 2010). Therefore, from an evolutionary point of view, later life periods are less important, simply because organisms in nature do not live long enough to die from intrinsic causes. Late acting genetic variations are less affected by natural selection, because many of their bearers will die from extrinsic causes at the same rate as non-bearers before the variant would show its phenotypic effect and affect the fitness of the organism (Flatt and Partridge, 2018 and Reichard, 2017). The classical theories only apply to organisms, where there is a difference between parents and their offspring, if the parent is identical to the offspring, natural selection cannot distinguish between them (Flatt and Partridge, 2018). Therefore, the traditional ageing theories mainly apply

to the ageing of animals with complex bodies that show distinct developmental stages until they reach adulthood. Recent theoretical and empirical studies (summarised and reviewed in Reichard, 2017) have found numerous deviations from the classical theories and shown that ageing and senescence is not apparent in all species, and constant or even decreasing mortality risk over age may be possible.

The three main classical evolutionary theories of ageing are the mutation accumulation theory, antagonistic pleiotropy and the disposable soma theory which provide complementary explanations of why ageing occurs, and all are based on the idea that due to extrinsic mortality, the strength of natural selection decreases with age.

The first theory was developed by Medawar (1952) in his famous book An Unsolved Problem of Biology. The mutation accumulation theory states that harmful mutations can accumulate in the genome if they are only expressed later in life when most individuals have died from extrinsic causes. Therefore, senescence occurs, because natural selection cannot effectively clear late acting harmful mutations from the population. The antagonistic pleiotropy theory originates from Williams (1957) who suggested that genes can have age-specific pleiotropic effects and natural selection promotes the spread of a gene that increase the fitness of the organism at early-life, even if it has detrimental effects later in life. Due to extrinsic mortality, natural selection has a stronger effect on early life events, therefore pleiotropic genes providing early-life advantages experience positive selection. The disposable soma theory of Kirkwood (1977) distinguishes between the germline and the somatic cells. It states that the somatic cells are designed to form a body that protects and helps propagate the germline. While the somatic cells accumulate mutations and their function declines with age, germ cells are protected from somatic mutations, and they contain the whole set of genetic information to build up the body from generation to generation. It is possible to repair the body, but it is too costly to keep the whole organism at the original state, therefore it is more efficient in the long term to invest in reproduction than investing in the maintenance of the current copy of the organism (Reichard (2017).

Ageing is thus a non-adaptive side effect of evolution, based on the reduced effect of natural selection at older ages to maintain fitness. Therefore, ageing did not evolve, survival did (Flatt and Partridge, 2018). There are no known genes that have the role of causing or regulating ageing. Unlike development, there is no regulation on the pattern of senescence and no genes are responsible for ensuring that age related decline happens in the right tissues at the correct order and speed. Thus, ageing happens in an unregulated manner as cells and tissues accumulate damage and fail

to maintain function at older ages. The evolutionary theories of ageing suggest that ageing has to be a complex process and a highly polygenic trait as there are numerous genes that promote survival and fecundity (Partridge, 2010).

1.3: What is Ageing?

While there are numerous theories describing why ageing happens, the exact mechanisms of ageing are still unclear. During ageing multiple types of damage accumulate in single tissues and different types of tissues experience different spectrum of changes. Furthermore, the exact phenotype of ageing differs between individuals (Finch & Kirkwood 1999). Thus, there is no single process that regulates ageing, rather, it is caused by the accumulation of various independent damages that occur in parallel with little or no common cause (Partridge, 2010). Due to the huge complexity and variability of the ageing process, it is considered medically intractable as fixing a single age-related damage would only have little effect on the overall health and ageing of the whole organism (Partridge, 2010). Currently, specific age-related diseases, such as cancer, cardiovascular diseases and dementia are considered medically more tractable than the ageing process itself, therefore medical research is focusing on the treatment of these diseases individually (Partridge, 2010).

As ageing is a polygenic trait considered a side effect of evolution, it was assumed that single-gene mutations would be unlikely to affect the rate of ageing. Moreover, as organisms live very different lifestyle in different environments encountering different sources of damage, the existence of evolutionarily conserved mechanisms of ageing was considered unlikely (Partridge & Gems 2002). Research in laboratory model organisms, however, identified evolutionarily conserved interventions and mutations that can modulate the lifespan of an organism. These are currently being widely studied in the hope of finding therapeutic interventions to ‘treat’ ageing and to promote health-span in humans (Partridge, 2010).

1.4: The study of ageing in model organisms

1.4.1: Dietary restriction

The first environmental intervention that was found to extend lifespan was calorie restriction (CR – reduced calorie intake without causing malnutrition) or dietary restriction (DR – reduction of nutrient intake without causing malnutrition) (Lian, et al. 2015). McCay, Crowell and Maynard (1935) found that rats under CR had a longer lifespan and since then there has been much interest in the lifespan extending effect and mechanism of action of CR or DR. Maroso (2005) studied the effects of CR on rodents and found that if started early in life, it could extend the maximum lifespan by 30-60%. If CR was started in adulthood (at the age of 1 year) in rodents, it increased the maximum lifespan by 10-20% (Weindruch and Walford, 1988). CR has also been shown to improve health and ameliorate age-related diseases in rodents. Maroso (2005) found that CR prevented diabetes, autoimmune and respiratory diseases, while Mattson (2005) showed that CR reduced neurodegeneration and β-amyloid deposition in mouse models of Alzheimer disease. With the help of CR, the incidence of chronic diseases has also been reduced, such as cancer, which is the main cause of death among laboratory rodents (Longo and Fontana 2010).

DR has been shown to extend the lifespan of various model organisms, such as *Saccharomyces cerevisiae* (yeast), *Caenorhabditis elegans* (nematode worms) and *Drosophila melanogaster* (fruit flies) (Partridge, 2010). The key mediator of lifespan extension is not the calorie intake alone, but the protein/amino acid and individual nutrient intake (Mirzaei, Suarez and Longo, 2014). **Figure 2** shows the sex specific effect of DR on fruit flies as an example. DR is achieved in flies by feeding them with agar media containing reduced amounts of yeast. The figure shows that males have a lower response to DR, while the female lifespan is increased by 60% and they have higher optimal DR food concentration (Magwere, Chapman and Partridge, 2004).

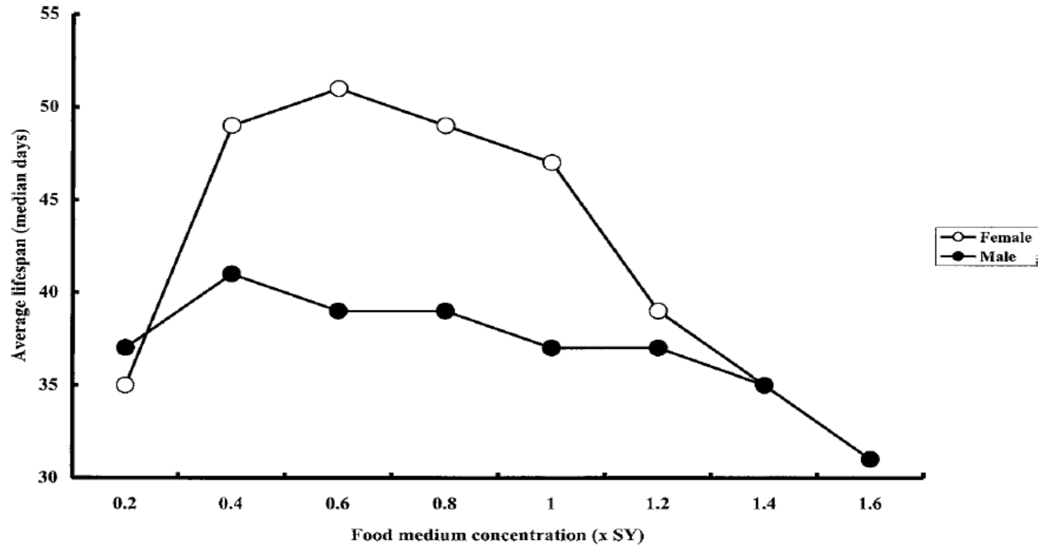


Figure 2 - Average lifespan of male and female Drosophila in response to various food concentrations
DR females show a 60% increase in average lifespan compared to the fully fed flies, while the increase was only 30% in males compared to fully fed flies (1.6 SY). Females have the highest average lifespan at 0.6x Sugar/Yeast (SY) content, while the optimum food concentration for males is 0.4x SY (Source: Magwere, Chapman and Partridge, 2004).

Increasing evidence shows that the lifespan extending effect of DR is mediated by individual nutrient content of the food. In particular, the reduction in protein/amino acid content, and not the reduced calorie content (de Marte and Enesco, 1986, Miller, et al. 2005, Mair, Piper and Partridge, 2005, Grandison, Piper and Partridge, 2009). Mair, Piper and Partridge (2005) showed that in fruit flies, protein restriction has a more important role in increasing lifespan than reduced calorie intake. Grandison, Piper and Partridge, (2009) further investigated which nutrients are responsible for the lifespan extension and reduced fecundity in response to DR using *Drosophila*. This study found that adding back vitamins, lipids or carbohydrates to the DR food did not have any effect on the lifespan or fecundity. When non-essential amino acids were added back to the diet, flies had slightly shortened lifespan with no effect on fecundity. However, adding back essential amino acids shortened lifespan and increased fecundity to the level of full feeding, and adding even more essential amino acids to the diet further increased fecundity and reduced lifespan. When essential amino acids were added back individually, none of them reduced lifespan and only methionine increased fecundity. Therefore, an imbalance of the amino acid content of the diet is responsible for the lifespan extending effects and reduced fecundity of DR in fruit flies. In 1986, de Marte and Enesco found that a low tryptophan diet increased survival and reduced organ growth in mice. Lifespan extension was also achieved in mice by a methionine-

deficient diet which also slowed down the age-related decline of the immune system, liver function and increased stress resistance (Miller, et al. 2005).

1.4.2: Single gene mutations

One of the major breakthroughs of ageing research was the discovery of single gene mutations that can increase the longevity of laboratory model organisms. As a result of systematic chemical mutagenesis screening, Klass (1983) identified eight long lived *C. elegans* mutants. Three of those had a lifespan extending mutation in their *age-1* gene (Friedman and Johnson (1988), encoding the catalytic subunit of PI3K, which is part of the insulin signalling pathway (Morris, Tissenbaum and Ruvkun, 1996). Kenyon, et al. (1993) found that mutation of *daf-2* in *C. elegans* doubles the lifespan of the worms in the presence of active *daf-16,* which is required for lifespan extension. The *daf-2* mutation did not only increase the lifespan of the worms, it also improved their health-span. Later it was found that *daf-2* codes for the *C. elegans* insulin receptor, while *daf-16* codes for the worm FOXO which are both part of the insulin/insulin-like growth-factor signalling (IIS) pathway (Lin, et al. 1997).

Numerous other lifespan-extending mutations have since been found in various other laboratory model organisms in the IIS pathway as well as in the TOR pathway, and in other genes involved in nutrient sensing (Partridge, 2010). By the end of the 20th century it was clear that single gene mutations can extend the lifespan of laboratory model organisms and hope arose that several aspects of the age-related decline can be ameliorated by a single mutation or intervention (Partridge, 2010). As a few example, over-expressing *SIR2* in yeast *Saccharomyces cerevisiae*, which is a gene coding for a protein deacetylase, promotes longevity of the yeast mother cells (Kaeberlein, McVey and Guarente, 1999). The Ames dwarf mice and the Snell dwarf mice have mutations in genes involved in the development of the pituitary gland, therefore they are both deficient in growth hormone and thyroid-stimulating hormone due to different mutation, but both have significantly extended lifespan (Brown-Borg et al. 1996). The *chico* gene mutation in *Drosophila melanogaster* strains also produces dwarf animals and increases the lifespan of homozygous mutants flies by 48% and 36% in heterozygotes. *Chico* encodes for the *Drosophila* insulin receptor substrate, which is an other subunit of the IIS (Clancy, et al. 2001).

1.5: Evolutionary conserved nutrient signalling pathways modulate ageing

Finding environmental interventions and numerous single gene mutations that extend the lifespan of laboratory model organisms was a breakthrough in ageing research, since despite the previous belief, it was possible to extend lifespan and health-span of an organism by a single intervention. However, it was still unknown at the end of the 20th century if the findings in model organisms would have any relevance to human ageing. In research, model organisms are useful tools to understand various processes in the human body due to evolutionary conservation of genes and their functions. As ageing is considered as a side effect of evolution and is a maladaptive trait, it was questioned if there is any evolutionary conservation in the ageing processes (Partridge, 2010).

The finding that various model organisms with different physiology and environment, such as *S. cerevisiae, C. elegans and D. melanogaster* and mice all respond to DR with increased longevity was promising, as it suggested evolutionary conservation (Partridge, 2010). However, the exact mechanism of lifespan extension with DR is still not fully understood, there is still no proof of evolutionary conservation, as it could also be an evolutionary convergence (Mair and Dillin, 2008). The relevance of DR to human lifespan extension is still under debate. There were a few DR experiments in rhesus monkeys, that also responded to DR (Mattison, et al. 2012). The long-term longitudinal study of Colman, et al. (2009) showed that DR in rhesus monkeys delayed the onset of age-related diseases and reduced the incidence of age-related death. Evidence in humans is very limited, some short-term DR studies in humans showed functional improvements, such as reduced obesity, insulin resistance, inflammation, increased oxidative stress, and improved heart function, however is not yet clear from those short-term studies of the positive effects are due to DR or just a showing recovery from an unhealthy diet (Holloszy and Fontana, 2007, Fontana, et al. 2016). The exact mechanism of action of lifespan extension in response to DR is still not fully understood, but there are numerous nutrient sensing and signalling pathways that are thought to play a key role in modulating lifespan in response to DR, such as insulin/IGF signalling (IIS) pathway, target of rapamycin (TOR), AMP-dependent protein kinase (AMPK) and SIRT(sirtuin) (reviewed by Lian, et al. 2015).

Lifespan extending single gene mutations were first identified in *C. elegans* and first it was believed that reduced IIS increases the lifespan of worms only, as its mechanism of action involves activating genes related to the developmental arrest (dauer) in adult worms, which normally acts as a response of larvae to low food availability and overcrowded environment (Kenyon, et al. 1993, McElwee, Bubb and Thomas, 2003, McElwee et al., 2004). The evolutionary conservation of lifespan extension by reduced IIS was doubted, as other model organisms and humans lack this type of developmental arrest, therefore they do not have the mechanism extend lifespan by reduced IIS (Partridge, 2010).

In the early 2000s mutation in various subunits of the *Drosophila* IIS pathway extended the lifespan of fruit flies and provided more and more evidence of the evolutionarily conserved role of IIS in modulating ageing. Flies with mutated insulin receptor (Tatar, et al. 2001) or insulin receptor substrate called *chico* (Clancy, et al. 2001) were long lived. More supporting evidence came from the experiments of Broughton, et al. (2005) showing that the ablation of the neurosecretory cells in the fly brain, which are responsible for the secretion of insulin like peptides extended the lifespan of the flies and increased their starvation and oxidative stress resistance.

Reducing IIS in invertebrates with open circulatory system was shown to promote longevity, so the next question was whether reduced IIS has any beneficial effects on the lifespan of mammals, as they are more sensitive to blood sugar level changes and reduction in their insulin production or insulin resistance leads to diabetes (Partridge, 2010). Bluher, Kahn and Kahn (2003) used fat-specific insulin receptor knockout mice to reduce IIS which extended their lifespan. In the same year, Holzenberger et al. (2003) also induced lifespan extension in mice using a mutated Igf-1 receptor. Directly studying the effect of genetic interventions on human lifespan is unethical and it would be time consuming, therefore the search for candidate longevity genes in humans is performed by population based genetic association studies. Willcox, et al. (2008) found strong association between FOXO3A genetic variants on human lifespan and health span. Based on the strong evidence for its evolutionarily conserved role in ageing, in this review I focus on the IIS/TOR nutrient sensing signalling network and discuss its role in modulating lifespan and health-span.

Another nutrient sensing pathway that modulates ageing is the target of rapamycin (TOR) pathway, which has a role in regulating cell growth and protein synthesis and degradation, and it responds to amino acids. TOR is essential for lifespan extension by DR and it acts upstream of transcriptional factors, such as TFEB,

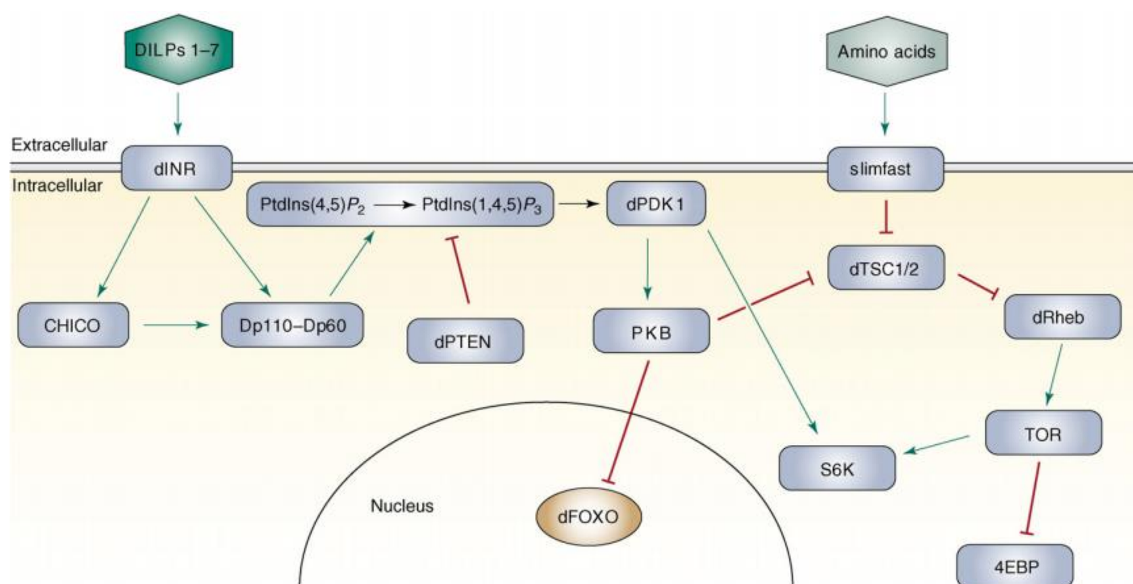
FOXO and Nrf, which have role in maintaining a pool of amino acids and regulating protein synthesis, controlling autophagy, which is the process of clearing excess proteins and non-functional organelles and also in reducing oxidative stress (Antikainen, et al. 2017). It is interlinked with the IIS pathway as IIS activates both of the TOR complexes by Akt/PKB and PIP3 (**Figure 3**). The TOR complex 1 negatively regulates IIS by activating Grb10, which is an IIS inhibitor and promoting the degradation of insulin substrate. The IIS and TOR signalling pathways also share transcriptional factors that modulate longevity, such as Nrf and FOXO (Antikainen, et al. 2017). Decreased TOR signalling increases lifespan in various model organisms. In yeast, TOR and Sch9, the homologue of Akt extends lifespan and increases stress resistance (Fabrizio, et al. 2001 and Kaeberlein, et, al. 2005). Reduced TOR also increases the lifespan of *C. elegans* (Hansen, et al. 2007) and in *Drosophila* using rapamycin, which requires the downregulation of S6K and activation of autophagy (Kapahi, et al. 2004, Bjedov, et al. 2010 and Partridge et al. 2011). Harrison, et al. (2009) showed that rapamycin-fed mice with reduced TOR signalling have extended lifespan and the deletion of S6K1 in mice also promotes longevity and improves health-span (Selman, et al. 2009). Rapamycin also inhibits the development or slows down cancer growth in mice, which is the most common cause of death in laboratory mice (Wilkinson, et al. 2012).

AMP-activated protein kinase (AMPK) functions as an energy sensor, and it was found to play a role in the DR induced lifespan extension (Fukuyama, et al. 2012). In response to DR in *C. elegans*, AMPK is activated and promotes *daf16* (the worm FOXO) dependent transcription which increases lifespan and stress resistance (Greer et al. 2007). Over-expressing AMPK by increasing the AMP:ATP and ADP:ATP ratio in flies promotes longevity in a DR unrelated manner (Stenesen et al., 2013).

Sirtuins (SIRT) are nicotinamide adenine dinucleotide-dependent protein deacetylases and the protein family is responsible to mediate processes, such as metabolism, protein acetylation and they have a role in age related loss of cognitive function. The mammalian SIRT family consist of seven members (SIRT 1-7) while *Drosophila* has 5 of them (Sir2, Sirt2, Sirt4, Sirt6 and Sirt7) (Lian, et al. 2015). In *Drosophila*, Sir2 is involved in the lifespan extension due to DR, since increased Sir2 extends lifespan, while reduced Sir2 disables lifespan extension in response to DR (Rogina and Helfand, 2004). Banerjee et al. (2012) showed that modification of *Drosophila* Sir2 in the adult fat body affects the lifespan of the flies in a diet-dependent manner. The knockdown of Sir2 in the fat body blocks lifespan extension by DR while its overexpression shows similar lifespan extension to DR. Hoffmann et al. (2013)

found that 3-fold overexpression of Sir2 in *Drosophila* in the fat body extends the lifespan of male and female flies by about 13%. They compered the transcriptional profiles of fat bodies after DR and Sir2 overexpression and found that the profiles are different, suggesting that Sir2 extends lifespan independently from DR.

Of the numerous pathways and genes identified that modulate longevity, the IIS pathway is the most widely studied with the strongest evidence for an evolutionarily conserved role. Our experiments focused on the role of IIS in lifespan and health-span using *Drosophila* as the model organism.



<u>Figure 3</u> - The IIS / TOR pathway in Drosophila
The TOR nutrient sensing pathway responds to amino acids and regulates cells growth, protein synthesis and degradation. It is interlinked with the IIS pathway via Akt/PKB, which phosphorylates TSC2, which is a negative regulator for TOR. Figure adapted from Giannakou and Partridge, (2007), the green arrows indicate activation while the red lines show inhibition.

1.5: The IIS pathway and ageing

The IIS pathway has been widely studied in various laboratory model organisms and it has the strongest evidence for having an evolutionarily conserved role in modulating longevity. **Figure 4** compares the IIS pathway of worms, flies and mice. Even though the pathway shows similarities in different organisms, there are some striking differences as well. While *C. elegans* have 40 insulin like peptide ligands (Zheng, et al. 2018), fruit flies only have eight and mice (and other mammals) have three. On the other hand, while mice (and mammals) have three types of receptors that the ligands are able to bind to, worms and flies only have one. Similarly, mice have four types of insulin receptor substrates while worms and flies have one (Broughton and Partridge 2009).

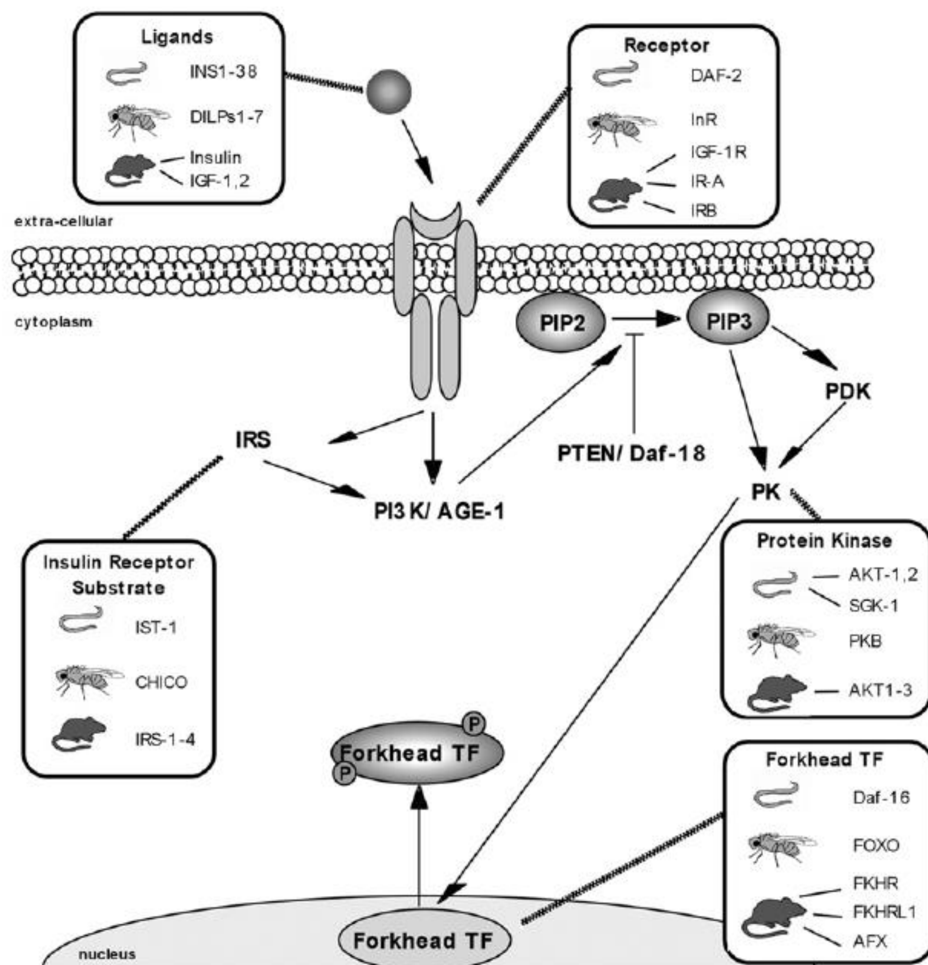


Figure 4 - Comparison of the IIS pathway in worms, flies and mice
In worms and flies, the regulation of the pathway is mainly happening at the level of the ligands binding to a receptor, as worms and flies have a lot more insulin like peptides, while mice only have 3. On the other hand, mice have 3 types of insulin receptors that are able to form heterodimers, therefore increasing the variety and 4 types of receptor substrates, while flies and worms only have one. IIS promotes the phosphorylation of FOXO transcription factor, worm and flies only have one FOXO variant, while mice have 3 (Broughton and Partridge 2009).

The signalling through the IIS pathway is initiated by insulin or insulin-like peptides binding to the insulin receptor on cell surfaces. The insulin receptor is a tyrosine kinase receptor, which goes under dimerization and autophosphorylation in response to binding to insulin. The signal is then transduced to PI3K, that converts PIP2 to PIP3. This activates a protein kinase cascade through Akt/PKB which leads to the phosphorylation of FOXO, which is a transcription factor regulating genes that promote longevity. When FOXO is phosphorylated, it is transported out from the nucleus. If IIS is reduced, FOXO is not phosphorylated, therefore it stays in the nucleus and it can promote the transcription of pro-longevity genes (Mathew, Pal Bhadra and Bhadra, 2017). The IIS pathway interacts with numerous other pathways forming a complex network. Two of these are the evolutionarily conserved TOR and the JNK pathways. TOR is an other nutrient sensing pathway activated by to amino acids and growth factors, and it regulates protein synthesis, autophagy and growth. Downregulation of TOR can extend lifespan and promote health at older ages in animal models. The JNK pathway is activated in response to stresses, such as oxidative stress and UV radiation and increased JNK signalling extends lifespan (Broughton and Partridge 2009).

FOXO proteins have a conserved (from *C. elegans* to mammals) DNA binding domain called Forkhead box (FOX) and they belong to the Forkhead family transcription factors. The human Forkhead family consists of more than 100 members. Invertebrates have only one FOXO gene while mice have four (Martins, Lithgow and Link, 2015). FOXO proteins are one of the main transcriptional effectors of the IIS pathway. FOXO is activated in times of nutrient deficiency and its role is to promote adaptation to food shortage and stresses. IIS acts as an inhibitor of FOXO by promoting its movement out from the nucleus. Reduced IIS or DR promotes the translocation of FOXO into the nucleus where it promotes pro-longevity genes responsible for DNA repair, stress resistance, cell cycle arrest and it can also induce apoptosis, thus it protects against age-related pathologies such as neurodegeneration and cancer (Martins, Lithgow and Link, 2015). FOXO also increases insulin sensitivity by promoting the expression of insulin receptor and its substrate, therefore it protects against diabetes (Mathew, Pal Bhadra and Bhadra, 2017). FOXO also promotes longevity by the maintenance of protein homeostasis by regulating autophagy and the ubiquitin-proteasome system (Webb and Brunet, 2014). Ageing is associated with reduced proteasomal activity leading to the accumulation of damaged proteins and neurodegenerative disorders, such as Parkinson's or Alzheimer's (Martins, Lithgow

and Link, 2015). The accumulation of damage caused by reactive oxygen species (ROS) promotes ageing and FOXO increases lifespan by increasing the antioxidant capacity of cells (Kops et al., 2002). FOXO can activate the expression of important detoxification enzymes, such as catalase, manganese superoxide dismutase and GADD45 (Kops et al., 2002 and Nemoto & Finkel, 2002). On the other hand, inactivation of FOXO leads to the intracellular accumulation of ROS (Tothova et al., 2007). FOXO has shown to play a role in in stem cell biology and tissue homeostasis. Old mice with deleted FOXO3a have decreased regenerative potential (Miyamoto et al., 2007). During ageing, adult stem cells show reduced regenerative potential causing an imbalance between cell removal and regeneration (Martins, Lithgow and Link, 2015).

1.6: Drosophila as a model organism

Studying the biological mechanism of ageing is difficult in humans due to their long lifespan and ethical obstacles, therefore ageing research needs to rely on using laboratory model organisms. As discussed before, findings in model organisms can be relevant to human ageing due to the existence of evolutionarily conserved pathways in the ageing process. Commonly used models for ageing are the fruit fly *Drosophila melanogaster*, nematode *C. elegans* and mouse *Mus musculus*, all having their advantages and disadvantages (Groteweil, et al., 2005).

Fruit flies have been used in ageing research for more than a century. Flies show physiological signs of ageing and it is possible to observe and measure various markers for age-related loss of function, such as altered metabolism, behaviour (such as reduced courtship or exploration and increased sleep fragmentation), decreased stress resistance, fecundity, impaired learning and memory, reduced physical activity (like negative geotaxis) (Piper and Partridge, 2018). Many of the mammalian tissues, such as the heart or kidney have their equivalent in fruit flies, but not in *C. elegans*. Moreover, 77% of the human genes associated with age-related diseases are expressed in the equivalent fly tissues (Piper and Partridge, 2018). One of the main advantages of invertebrate models for ageing research is their short lifespan, which makes them an effective pipeline for studying the evolutionarily conserved interventions that promote longevity and enable more repeats for each experiment. As

a comparation, worms live about 3 weeks, flies about 3 months, killifish has a lifespan on 6-8 month and mice and rats live about 3 years (Piper and Partridge, 2018).

Other advantage of fruit flies is that their rearing and maintenance is cheap, there are no ethical restriction for their experimental use, they have well defined dietary requirements and it is easy to generate large populations of flies for experiments. Furthermore, their tissues can be dissected relatively easily and there are a large variety of genetic tools already available for their genetic manipulation. One main disadvantage of *Drosophila* as a model of ageing is that it is not yet known what flies die of (Piper and Partridge, 2018).

1.7: The Drosophila IIS pathway

The fruit fly IIS consists of a single insulin/IGF receptor (dInR), insulin receptor substrate (chico), PI3K and its target protein PKB and they have only one type of FOXO (**Figure 3**). Flies have eight ligands for the dInR, called *Drosophila* insulin-like peptides or DILPs, each of their genes having a unique, cell and developmental stage specific expression pattern (Mathew, Pal Bhadra and Bhadra, 2017). DILP1,2,3 and 5 are expressed in Insulin Producing Cells (IPCs) in the brain in larvae and adult flies, the expression of DILP1 is transient in adults. The IPCs are two clusters of median neurosecretory cells located on the dorso-medial protocerebrum, in the pars intercerabalis in the adult brain, which is analogous region of the mammalian hypothalamus. The axons project to other locations inside the fly brain and to the heart of the fly as well (Nässel, Liu and Luo, 2015). DILP3 is also expressed in the adult midgut muscle cells, and DILP5 in the renal tubes and in the follicle cells of the ovary of female flies (Nässel, Liu and Luo, 2015). DILP4 is expressed mainly in the embryonic midgut and mesoderm (Brogiolo et al., 2001). DILP6 is mainly expressed in the fat body of adult flies and in the adipose cell, salivary glands, heart and the glial cells of the central nervous system (CNS) of the larvae (Nässel, Liu and Luo, 2015). DILP7 is expressed in about 20 neurons in the abdominal ganglia of larvae and adult flies, some of their axons terminate in the hindgut, in the sub esophageal ganglion of the brain and in the female reproductive tract (Yang, et al. 2008). Finally, DILP8 is expressed in the imaginal discs of larvae and in adult ovaries (Colombani, Andersen and Leopold, 2012; Garelli et al., 2012).

All DILPs have the ability to regulate growth as agonists of the dInR with DILP2 having the strongest effect (Ikeya, et al. 2002). All single *dilp* null mutant flies are homozygous viable and apart from *dilp2* null mutants, which have extended lifespan, all other single *dilp* mutants have normal lifespan (Grönke, et al. 2010). The ablation of the median neurosecretory cells, which produce dilp2,3 and 5 in adult flies, extends the lifespan of both males and females (Broughton, et al. 2005). The overexpression of *dilp6* in the fat body of adult flies also promotes longevity and reduces the expression of *dilp2* and *5* in the brain as well as DILP2 release into the haemolymph (Bai, Kang and Tatar, 2012).

Genetically altering various units of the IIS pathway in order to reduce the signalling through it promotes longevity. Downregulation of positive regulators of IIS, therefore negative regulators of dFOXO, such as *dilp*s, *dInR* or *chico* increases lifespan, so does the upregulation of the negative regulators of IIS, such as dPTEN and dFOXO (Mathew, Pal Bhadra and Bhadra, 2017).

The alteration of IIS in specific tissues, such as head, fat body or muscles is sufficient to increase lifespan (Mathew, Pal Bhadra and Bhadra, 2017). The activation of dFOXO selectively in the pericerebral fat body reduces *dilp2* expression and promotes longevity. Furthermore, it also reduces endogenous IIS in the peripheral fat body, thus dFOXO in the brain fat body affects ageing both cell-autonomously and non-autonomously (Hwangbo, et al. 2004). The muscle ageing of the flies is due to the accumulation of protein aggregates impairing the function of the tissue. The removal of the damaged proteins is regulated by dFOXO and its target, 4E-BP by autophagy. Overexpression of dFOXO in muscles extends lifespan and improves physical function at older ages, while its downregulation leads to defective proteostasis. Muscle specific dFOXO overexpression showes systemic activation of 4E-BP expression and regulates the release of DILP2 and 5 and the food intake of the flies (Demontis and Perrimon 2010). Partial ablation of the IPCs in the fly brain in adult flies only extends lifespan, increases starvation resistance and reduces the fecundity of females (Haselton et al. 2010). The overexpression of dFOXO in the adult fat body increased female lifespan by 20-50% and reduced their fecundity by 50% along with increasing oxidative stress resistance, however, male lifespan was not affected (Giannakou, et al. 2004). Hwangbo et al. (2004) found that dFOXO overexpressed in the adult head fat body extended lifespan without reducing fecundity. Giannakou, et al. (2007) used and inducible system to overexpress *dFOXO* in the fat body and found that, while continuous overexpression of *dFOXO* in the adult fat body lowered the mortality rate, reversing the IIS status early in adulthood led to a complete switch to mortality rates

with normal IIS levels. Restoring IIS levels later showed incomplete switch of morality rates, up until the age of 4 weeks, where the switch had no effect anymore.

All these examples demonstrate, that reducing IIS in specific tissues during adulthood can extend lifespan and there is no need for altering the IIS pathway constitutively in the whole body. The hope of the research is that reducing IIS at the right place and time could promote longevity with no detrimental pleiotropic effects (Dillin, Crawford and Kenyon, 2002, Broughton and Partridge 2009).

1.9: Reduced IIS – effects on lifespan versus health-span

Ageing research most commonly uses lifespan or mortality rates to measure the success of interventions promoting longevity. However, lifespan on its own does not provide information about the health of the organism and the ultimate goal of ageing research is not just extending lifespan on its own but to promote health and function at older ages. Some experiments measured improved function with age in response to reduced IIS. For example, the long lived *chico* (insulin receptor substrate) mutant flies show improved locomotor performance with age in the form of slower decline of negative geotaxis with age (Martin and Grotewiel, 2006), so do other mutations to the subunits of the insulin signalling pathway in flies, such as *pdk-1* (phosphoinositi-dependent kinase-1), *Dp110* (the catalytic subunit of the PI3 kinase) and *Akt* (protein kinase B) (Jones et al., 2009). Reduced IIS in the full body of the flies by expressing a dominant negative insulin receptor or by the ablation of the insulin producing cells in the fly brain also extends lifespan and slows down negative geotaxis decline as well as improves some measures of the exploratory walking behaviour (Ismail, et al. 2015).

Overexpression of *dFOXO* and its target *4E-BP* in muscles also extends the lifespan of flies and delay muscle functional decay, while mutation in *dFOXO* leads to dysfunctional proteostasis (Demontis and Perrimon 2010). The decline of cardiac function with age is also slowed down by IIS reduction. Wessells et al. (2004) measured the changes of heart function with age in *Drosophila* and found that resting heart rate decreases, and the rate of stress-induced heart failure increases as the flies are getting older. When IIS was reduced by a mutated *dInR* or *chico*, the age-related changes of the fly heart were minimised or absent in the long-lived flies. Furthermore, overexpression *dPTEN* (negative regulator of IIS) or *dFOXO* in the fly heart also prevented the decline of cardiac function with age. Wessells et al. (2009) later found

that protection of the heart function by IIS reduction is mediated through 4E-BP. In *C. elegans*, longevity-promoting mutations of the IIS can inhibit tumour growth (Pinkston et al. 2006 and Pinkston-Gosse & Kenyon 2007). In mice, Alzheimer's disease models show reduced accumulation of amyloid-ß and protection against premature death in response to IGF-1 resistance in the neurons (Freude et al. 2009), and amyloid-deposition was reduced along with behavioural deficit due to the deletion of Irs2 (Killick et al. 2009). This shows that alterations in the IIS pathway can provide some protection against age-related diseases. In flies, reduced activity through the IIS/TOR pathway, such as PI3K, Akt, TOR or JNK could reduce the neurotoxicity of proteins related to Alzheimer's or Parkinson's disease in the fly model of these diseases (Hirth, 2010).

While all these findings look promising, there are more and more experiments show a disconnection between lifespan and health-span. The most commonly observed negative side effect of lifespan extension by reduced IIS is reduced fecundity (Friedman and Johnson, 1988; Kenyon, 2011; Clancy et al., 2001; Tatar et al., 2001, Broughton, et al., 2005; Grönke, et. al. 2010). However, studies in worms and flies show that increased lifespan can be uncoupled from reduced fecundity (Toivonen and Partridge, 2009). In *C. elegans*, the knockdown of DAF-2 (the worm insulin receptor) during development decreases fecundity, while the adult-specific knockdown of DAF-2 increases lifespan without affecting fecundity (Dillin et al., 2002). In flies, the overexpression of dFOXO in the adult fat body extends lifespan without reducing fecundity (Hwangbo et al., 2004; Giannakou et al. 2004, 2007). Therefore, it is possible to extend lifespan without reduction in fecundity. On the other hand, Haselton, et al., (2010) found that while adult-specific ablation of the IPCs extend lifespan without causing insulin resistance in *Drosophila*, female flies have reduced fecundity. Thus, to avoid fecundity reduction by reduced IIS, the tissue specificity of the expression is also important, not just the timing of the knockdown.

The relationship between longevity, reproduction and nutrient sensing pathways are widely studied. Both reproduction and somatic maintenance are energetically expensive processes and nutrient sensing pathways are responsible for interpreting nutrient levels and allocating energy for reproduction, growth and maintenance (reviewed by Templeman and Murphy, (2017)). Lifespan can be extended by artificial selection for late-life reproduction in *Drosophila*, which reduces early-life fecundity (Luckinbill et al., 1984) and selection for extended lifespan correlated with decreased overall reproduction (Zwaan, Bijlsma and Hoekstra, 1995). There's also mechanistic connection between the reproductive system and life expectancy, as the ablation of *C. elegans* germline stem cells extend lifespan in the presence of DAF-16 (worm FOXO)

and signals from the somatic gonad can influence longevity in a DAF-2 (worm InR) dependent manner (Hsin and Kenyon, 1999). However, infertility alone does not necessarily promote longevity, as the ablation of the somatic gonad (not just the germ cells) does not extend lifespan (Hsin and Kenyon, 1999), neither does the genetic prevention of oocyte or sperm formation in worms (Arantes-Oliveira et al., 2002). Eliminating germ cells in *Drosophila* promotes longevity and modulates IIS, as long-lived germ-line-less flies show the characteristics of IIS impedance, such as the upregulation of dFOXO (Flatt et al., 2008). The transplantation of younger ovaries into old female mice extends the remaining lifespan of the host (Cargill, et al., 2003), and castration in humans is also associated with increased lifespan (Min, et al., 2012), therefore the gonadal regulation of ageing is evolutionarily conserved and related to IIS.

IIS play an important role in controlling the body and organ size throughout the development of *Drosophila* by regulating the nutritional conditions during organ formation (Brogiolo, et al, 2001, Shingleton, et al. 2005 and Kramer et al. 2003). Kramer, et al. (2003) found that the constitutive activation of dFOXO during the first and second instar of larval development lead to developmental arrest and altered feeding behaviour, which was reversible by discontinuing the expression of dFOXO. Constitutive expression of dFOXO during the third larval instar causes reduced body size in adult flies. Analysis of the wings and eyes of these dwarf flies showed that the reduction in size was due to smaller cell size as well as lower cell number (Kramer, et al. 2003). Shingleton, et al. (2005) showed that dInR activity is required throughout development in a time- and organ specific manner. Reduced IIS by a mutated dInR before the larvae reach the critical size to commit to metamorphosis increases total developmental time but has no effect on the adult body or organ size. After larvae reach the critical size, the dInR mutation can no longer affect development time but reduces organ and adult body size. The adult body size is reduced by dInR mutation from reaching the critical size until the pupariation, while the organ size can be affected from the critical size to early pupal development. The cell size and cell number in the fly wings were affected differently based on the activity of the dInR. (Shingleton, et al. 2005). Dillin, Crawford and Kenyon, (2002) showed that in *C. elegans* IIS influenced ageing during adulthood while it regulated dauer (developmental arrest) during early larval development. Grönke, et al. (2010) found that loss of insulin like peptides in *Drosophila* can affect developmental time and adult body size. The loss of *dilp*2 and *dilp*6 caused several hours of delay in egg to adult development. *Dilp*1-4 mutants showed about a day developmental delay while *dilp*2,3,5 mutant flies had severe

developmental delay of 8-17 days. This delay happened at the larval or pupal stage, the *dilp*2,3,5 embryos developed into first instar larvae at the same rate as wild type flies. These flies also eclosed over a much longer period (over almost 10 days) compared to wild type flies (within a day) (Grönke, et al, 2010). Grönke, et al. (2010) measured the body weight of adult *dilp*-null mutant flies finding that the loss of *dilp1*, *dilp2* and *dilp6* reduces growth. The loss of *dilp1* reduced adult body weight but did not affect egg to adult developmental time showing that the growth defects are not necessarily coupled to developmental delay. Among the single *dilp* mutants, the loss of dilp6 had the biggest effect on body weight, suggesting a role in promoting growth during larval-pupal development. *Dilp*2,3,5 mutants as well as *dilp1*-4,5 mutants showed severe (about 50%) reduction in body weight (Grönke, et al. 2010).

IIS also plays a role in the development and correct function of the nervous system. The insulin receptors in the *Drosophila* brain are required for the development of the visual system, since the InR functions as guiding the photoreceptor cell axons from the retina to the brain (Song et al., 2003). IIS/TOR plays a role in neuronal differentiation (reviewed by Bateman, 2015) and InR is required for the development of the peripheral nervous system (Dutriaux, et al., 2013). The overactivation of IIS by the loss of its repressors, such as PTEN causes precocious appearance of cell fate markers, while the reduction of IIS/TOR pathways by loss of InR, PI3K, S6K or TOR delays the acquisition of cell fate markers in *Drosophila* (Bateman and McNeill, 2004 and 2006). Later on, McNeill, Craig and Bateman (2008) showed that the loss of S6K delays differentiation while the loss of eIF4E (also downstream of TOR) inhibits growth without affecting the timing of differentiation. Furthermore, there is crosstalk between the IIS/TOR pathway and the epidermal growth factor receptor signalling (McNeill, Craig and Bateman, 2008). Avet-Rochex et al. (2014) identified the gene *unkempt*, a component of the TOR signalling pathway that regulates neuronal differentiation. As the IIS/TOR pathway plays a role in regulating body and organ size, developmental time and it is also involved in axonal guidance, neurogenesis and neuronal differentiation, it is possible that IIS reduction before adulthood negatively affects the health span of the flies.

Several studies showed that reducing IIS can lead to accelerated cognitive/behavioural decline. Reducing IIS in worms had detrimental effects on associative learning, whereas increased IIS ameliorated their learning ability (Vellai, et al. 2006). While DAF-2 mutation in worms improved memory and learning ability in young worms, it did not improve long term memory at old age (Tomioka, et al. 2006). CNS-specific removal of IRS-2 showed a reduction in NMDA receptor-dependent

synaptic plasticity in the hippocampus of young mice (Costello, et al. 2012). Measuring negative geotaxis and odour avoidance in DR flies showed that, while the flies are long lived, DR does not ameliorate their behavioural ageing (Bhandari, et al. 2007). While reduction of IIS in flies can slower the decline of negative geotaxis and walking speed, the experiments of Ismail, et al. (2015) showed that due to reduced IIS by the expression of a dominant negative insulin receptor or by the ablation of median neurosecretory cells improves negative geotaxis and some measures of exploratory walking, the improvement is due to improved muscle function, the cognitive measures of exploratory walking behaviour did not improve in response to reduced IIS.

The variable effect of reduced IIS on the health of the organism suggests that the role of IIS in health-span depend on how IIS effects the function of different tissues. Little is known about the role of IIS on neuronal function and ageing but based on the numerous cell types in the CNS which are likely to have different sensitivity to IIS changes, manipulating the IIS/TOR network will have diverse effect on the ageing and/or function of the various cell types.

1.8: The UAS-GAL4 system

Our studies require a system that allows the spatial and potentially temporal control over the expression of a transgene. Fortunately, there are systems like that already developed in fruit flies. The heat shock (*hsp70*) promoter is used to induce temporal expression of a transgene, but it is unable to control spatial expression, as *hsp70* is expressed in essentially all cells (Roman, et al. 2001). The UAS-GAL4 system (shown on **Figure 5**) was developed to provide spatial control of a transgene. The GAL4 gene is a yeast transcription factor, which is placed near a promoter or an enhancer in the fly genome. The promoter or enhancer can be specific to a cell type and drives the expression of GAL4 only on those cells. The upstream activating sequence (UAS) is present in all cells, and it is linked to the transgene, in our experiments, it is the dominant negative insulin receptor. The transgene is only expressed when GAL4 binds to the UAS sites, which only happens in the specific cells that express GAL4 (Roman, et al. 2001). This system is suitable for spatial control of transgene expression, but it is a constitutive system that does not allow temporal control.

Spatial and temporal transgene expression can be achieved by the tetracycline-regulated transactivator system. The tetracycline transactivator binds to the

tetracycline operator in the absence of tetracycline, while the reverse transactivator binds to the operator in the presence of tetracycline. The system uses a tetracycline analogue, doxycycline, which regulates the expression of the transgene near tetracycline operator (Roman, et al. 2001). The expression of the transgene is only turned on in the presence of doxycycline, and since it has negative effect on the health of the flies, this system is not suitable for lifespan and health-span studies (Roman, et al. 2001).

Another system that offers both spatial and temporal expression of a transgene is the GeneSwitch system. It is based on a chimeric gene encoding for the GAL4 DNA-binding domain along with the human progesterone receptor-ligand-binding domain and the activation domain from the human protein, p65. The system is similar to the UAS-GAL4 system, but in this case this chimeric molecule only binds to the UAS binding sites in the presence of the antiprogestin, RU486. The spatial control is determined by the promoter before the chimeric molecule and the temporal control is regulated by the oral administration of RU486 (Roman, et al. 2001). This is the chosen system in our experiments to control the expression of the dominant negative insulin receptor temporally.

Temporal control of the GAL4 expression can also be done by using $GAL80^{ts}$, which is a temperature sensitive repressor of GAL4. $GAL80^{ts}$ is active at 18°C but it fails to repress GAL4 at or above 29°C, therefore the transgenes are only expressed at higher temperatures. This is unfortunately unsuitable for longevity studies as the lifespan of the flies are heavily affected by the temperature (Suster et al., 2004).

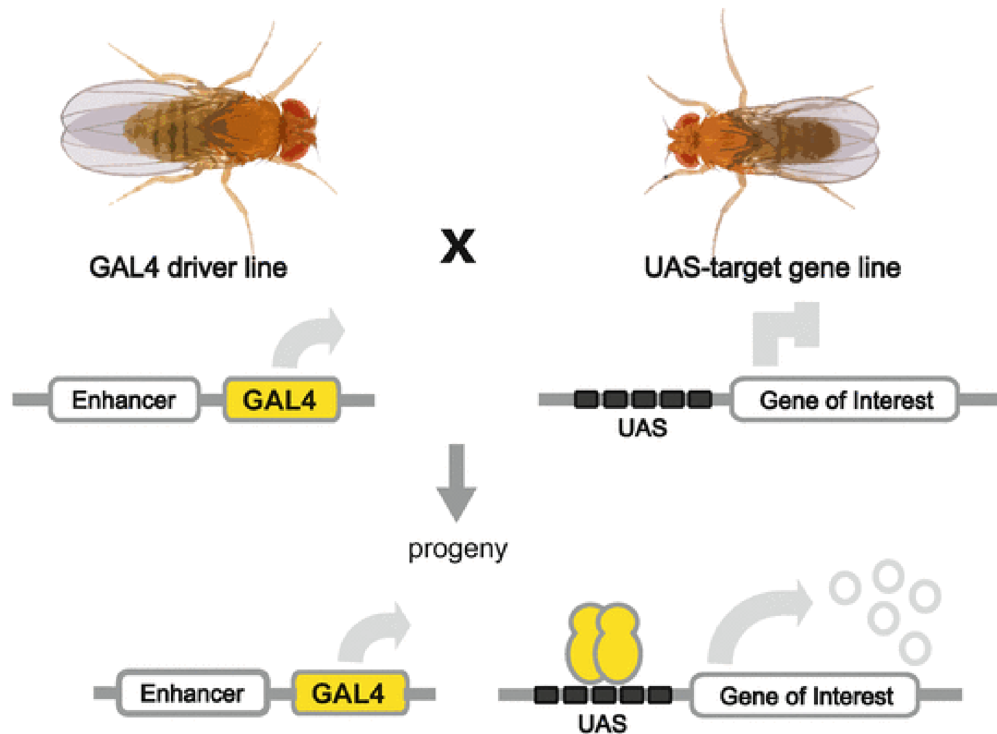


Figure 5 - Diagram of the UAS-GAL4 system
The expression of the transgene (here Gene of interest) is only active when UAS binds to GAL4, which is only present in specific cells determined by the promoter before GAL4. The UAS and GAL4 stocks are bred separately and crossed for experiments. The offspring of the cross will express the transgene at a spatially restricted manner (Southall, Elliott and Brand, 2008).

1.10: IIS and the central nervous system

The central nervous system (CNS) is responsible for regulating physiology and behaviour, so not surprisingly, it is also involved in the IIS reduction mediated lifespan extension. While the CNS of worms, flies and mice have very different structure, organisation and complexity, one thing is common. The neuronal tissue in all those model organisms secretes factors that can directly or indirectly alter IIS in distant tissues, therefore playing and endocrine role (Broughton and Partridge, 2009). In *Drosophila*, the CNS secretes some of the insulin-like peptides (DILPs) from the IPCs, therefore acting as a positive regulator of IIS and promoting ageing (Broughton, et al. 2005). The ablation of the median neurosecretory cells, which is the location of DILP2,3 and 5 production extends the lifespan of fruit flies (Broughton, et al. 2005).

IIS has a controversial role in the CNS, since on one hand it is producing DILPs therefore increasing IIS, which promotes ageing. On the other hand, IIS has a

neuroprotective effect and reduced signalling through the pathway can be harmful for the integrity of the CNS, but still extending lifespan (Broughton and Partridge 2009). The role of IIS in the CNS is not well understood yet. The endocrine role of the CNS in IIS and longevity is well supported; however, it is not clear if the CNS also has a cell-autonomous effect on survival. IIS play an important role in the development of CNS, neuronal function and survival, meaning that reducing IIS may affect neuronal health detrimentally (Broughton and Partridge 2009).

Chambers et al. (2015) found that IIS is involved in both learning and memory, using the olfactory learning and long-term memory model in *Drosophila*. Disruption of IIS in the ellipsoid body of the fly brain impaired long-term memory in flies and IIS is required in the mushroom body in adults for learning and memory formation. Furthermore, IIS also plays a role in regulating sleep in flies. Cong, et al. (2015) found that *dilp* mutant flies (except of *dilp4*) decreased total sleep, while upregulation of *dilp*s or *dInR* in the nervous system increased sleep, showing that IIS is an important regulator of sleep in flies.

PI3K, which is part of the IIS pathway was shown to regulate synapse number in both larval and adult fly neurons. Overexpression of PI3K promotes synaptogenesis even in aged adult neurons, and the newly formed synapses are functional and affect fly behaviour and continuous PI3K expression is required for synapse maintenance (Martin-Pena, et al. 2006). In flies, age-related memory impairment is correlated with PKA activity in the mushroom bodies to form olfactory memories and can be suppressed by reduced PKA activity (Yamazaki, et al. 2010). The age-related memory impairment is caused by glial disfunction due to increased pyruvate carboxylase activity with ageing, and PKA reduces the activity of pyruvate carboxylase (Yamazaki, et al. 2014).

These variable, often negative effects of IIS suggest that the pathway plays a diverse role in the CNS and reduced IIS can have positive or negative effects on the survival and function of the neurons, depending on the different cell types. As each component of the CNS have individual sensitivity to IIS, altering the pathway in different cell types is likely to have different effect on the behaviour and health of the organism (Ismail, et al. 2015).

1.11: Preliminary data

In order to seek for further evidence for the disconnection between lifespan and health-span, and to investigate the role and importance of IIS in neuronal function and ageing, Ismail et al. (2015) studied the effects of reduced ubiquitous and neuron-specific IIS on two locomotor behaviours, negative geotaxis and exploratory walking in *Drosophila*. They used two models for systemic IIS reduction, one of them expresses a dominant negative insulin receptor in all cells by the *daughterless* GAL4 driver (daGAL4/UAS-InR^{DN}), whereas the other has ablated insulin-like peptide producing cells in its brain (d2GAL/UAS-rpr). To reduce IIS specifically in the neurons, they used the elavGAL4 driver to insert the dominant negative insulin receptor in the neurons (elavGAL4/UAS-InR^{DN}).

The results on lifespan showed that ablation of the IPCs in the fly brain extends the lifespan of both males and females, while the systemic expression of InR^{DN} only increases female lifespan, so did the neuron-specific expression of InR^{DN}, the males had normal lifespan. Negative geotaxis improved at older ages in both males and females in response to IPC ablation, and systemic IIS reduction by InR^{DN} slowed down the decline of female negative geotaxis but males were unaffected. Neuron specific IIS reduction via InR^{DN} did not improve negative geotaxis. These results suggest that amelioration of negative geotaxis is caused by peripheral effects, such as muscle strength, rather than improved brain function (Ismail, et al. 2015). These results are shown in **Figure 6**.

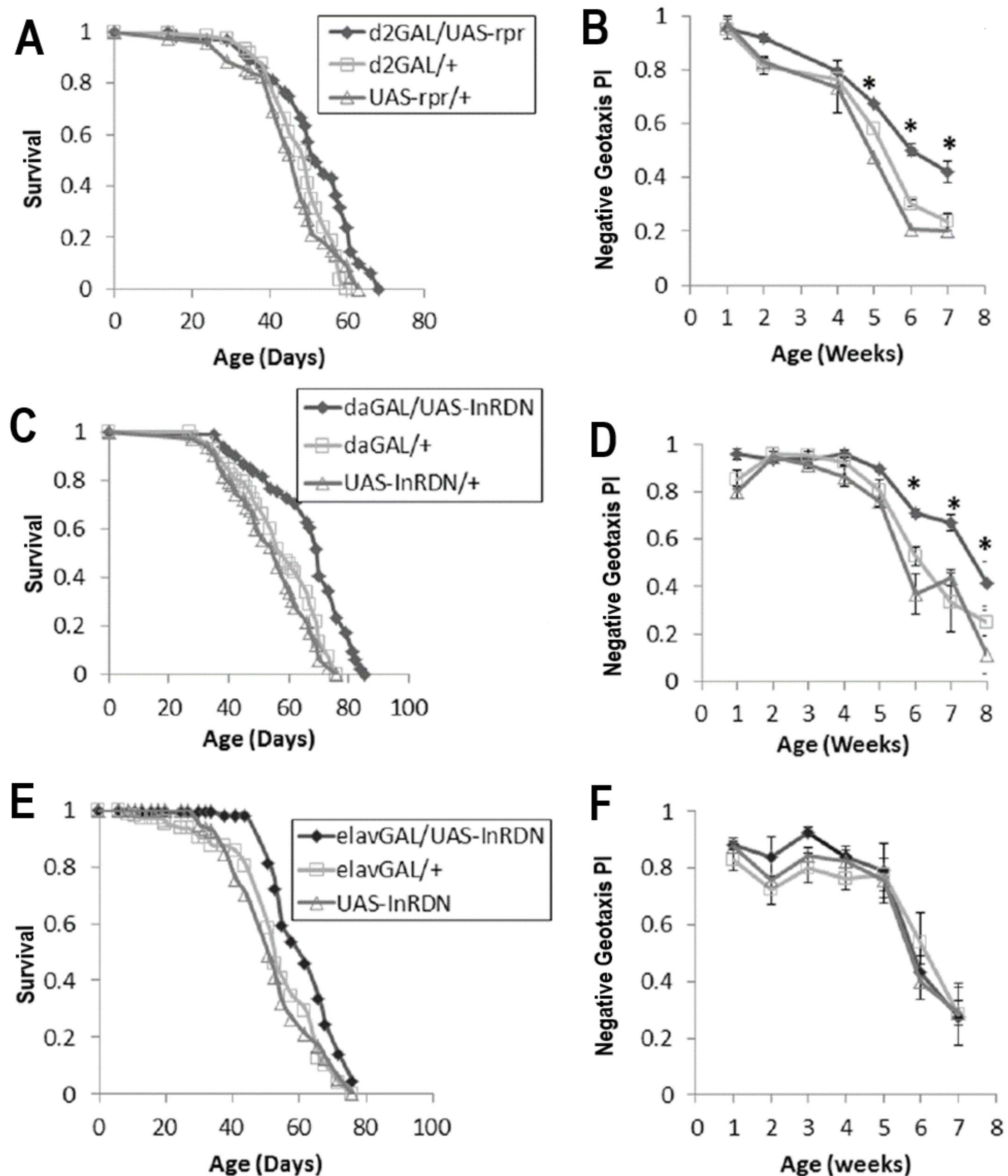


Figure 6 - **Lifespan and negative geotaxis senescence of female flies with ubiquitous (d2GAL/UAS-rpr and daGAL4/UAS-InRDN) or neuron specific (elavGAL4/UAS-InR^{DN}) IIS reductions**

A-B: d2GAL/UAS-rpr lifespan, negative geotaxis (in performance index). C-D: daGAL4/UAS-InR^{DN} flies lifespan, negative geotaxis (in performance index). E-F: elavGAL4/UAS-InR^{DN} flies lifespan, negative geotaxis (in performance index). Significant difference ($p<0.05$) is indicated by an asterisk (*) at a specific age point (Ismail, et al. 2015).

Reduced IIS by the ablation of IPCs did not affect the exploratory walking behaviour and it had a little positive effect by the systemic expression of InR^{DN}, slightly delaying the decline of total distance walked and walking speed in females, which are both parameters based on muscle strength, not cognitive function, none of the

parameters that represent decision making (such as rotation frequency) were affected. Neuron-specific IIS reduction had detrimental effects on exploratory walking parameters showing muscle strength and decision making in both males and females. This further supports the idea that systemic IIS reduction improves muscle function, but it is not beneficial for cognitive function (Ismail, et al. 2015). Some of the female exploratory walking results are shown on **Figure 7**.

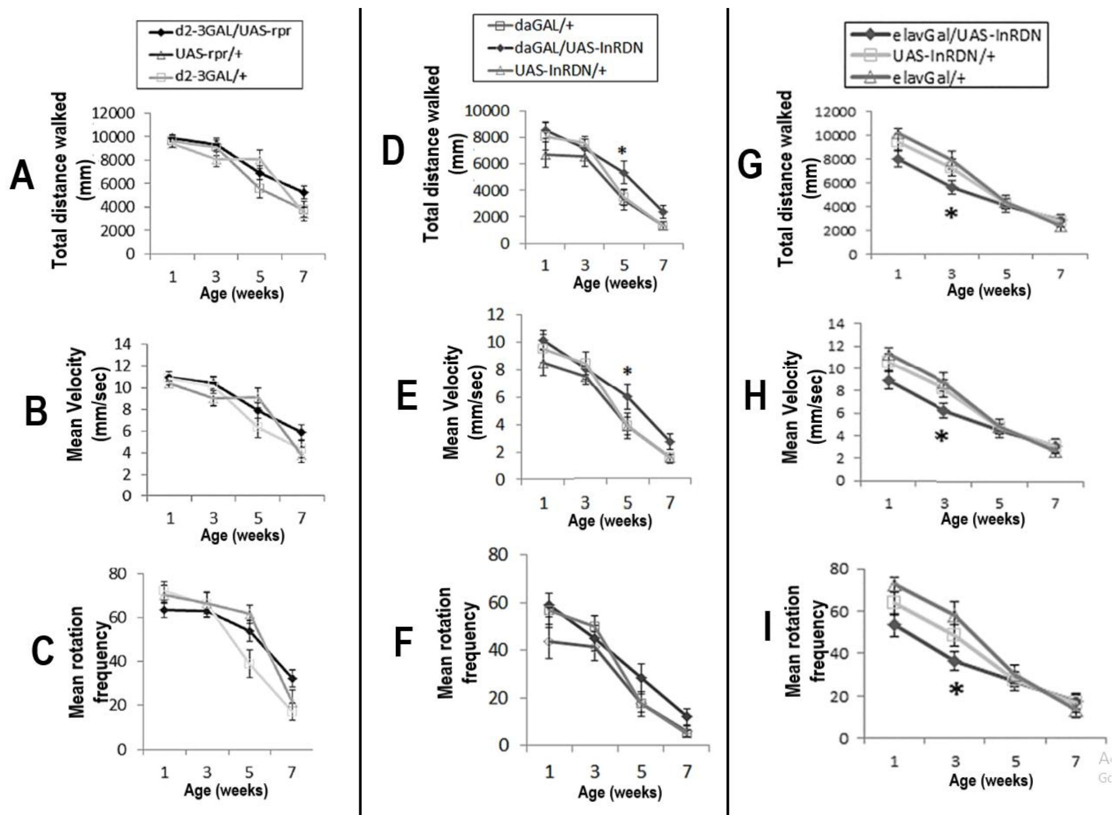


Figure 7 - Exploratory walking of female flies with ubiquitous (d2GAL/UAS-rpr and daGAL4/UAS-InRDN) or neuron specific (elavGAL4/UAS-InR^{DN}) IIS reductions
A-C: d2GAL/UAS-rpr – total distance walked, velocity and rotation frequency. D-F: daGAL4/UAS-InR^{DN} – total distance walked, velocity and rotation frequency. G-I: elavGAL4/UAS-InR^{DN} flies – total distance walked, velocity and rotation frequency. Significant difference ($p<0.05$) is indicated by (*) at a specific age point (Ismail, et al. 2015).

These results show that while reduced IIS promotes longevity, it can detrimentally affect the function of the CNS. Amelioration of negative geotaxis and some exploratory walking parameters in response to systemic IIS reduction are caused by non-neural tissues and the reduction of IIS in the CNS is generally not beneficial (Ismail, et al. 2015).

These results support that disconnection between lifespan and health-span, however the cause of the disconnection is not yet known. The exact role of IIS in the

CNS is still not clear, and these results do not show if the detrimental effects of pan-neural IIS reduction on exploratory walking is caused by accelerated neuronal ageing or reduced neuronal function.

Aims and Objectives

The aim of this project is to investigate the role of the Insulin-IGF-like Signalling (IIS) pathway in the *Drosophila* central nervous system (CNS) during ageing and study the effects of IIS reduction in neurons or in specific neuronal subtypes on lifespan and behavioural senescence. Furthermore, the project is aiming to identify endocrine changes due to reduced IIS in the neurons.

Hypothesis

Previous studies (Vellai, et al. 2006, Tomioka, et al. 2006, Costello, et al. 2012 and Bhandari, et al. 2007) and our preliminary data (Ismail, et al. 2015) *show* a disconnection between lifespan and health-span with IIS modulations. We have two hypotheses for this disconnection that are investigated in this study.

(1) The negative effects seen on behavioural decline when IIS is reduced in the neurons may be caused by detrimental effects on the function of the neurons that outweighs the beneficial effects of reducing IIS on the ageing of the neurons AND/OR

(2) Individual neuronal subtypes show a different response to reduction in IIS and the outcomes of IIS reduction in all neurons on behavioural decline is the sum of the positive, negative and neutral effects on each neuronal subtype.

Research questions:

Based on these two hypotheses, this project addresses three specific questions:

(1) Are the detrimental effects of pan-neural IIS reduction caused by reduced neuronal function or accelerated neuronal ageing?

(2) How does pan-neural IIS reduction extend lifespan and what endocrine effects does it cause?

(3) Which neuronal subtypes play a role in modulating lifespan and behavioural senescence in response to altered IIS?

Objective 1

To determine if pan-neural IIS reduction causes any reversible or irreversible changes in neurons that result in declines in neural function or neuronal damage.

Research Design

This objective addresses specific question (1) by determining if reduced neuronal IIS during development caused the detrimental effects of pan-neural IIS reduction on behaviour in previous studies and then determining if flies can recover from reduced neuronal IIS in adulthood. If reduced neuronal IIS causes accelerated neuronal ageing, then restoring IIS to normal levels before behavioural testing should not result in any amelioration of behavioural function compared to flies with continued neuronal IIS reduction. If reduced neuronal IIS does not accelerate neuronal ageing, but instead compromises neuronal function, restoration of IIS levels should result in an improvement in behavioural function compared to flies with continued neuronal IIS reduction.

The inducible elavGS-GAL4 driver (Sofola et al, 2010) was used to target the UAS-InR^{DN} transgene (Ismail et al. 2015) to neurons at specific times throughout their life. Firstly, to determine if the detrimental effects of pan-neural IIS reduction on behaviour shown in the Preliminary data (Chapter 1.11) were caused by detrimental effects on neurons during the development of the fly, IIS was reduced only from adulthood. Lifespan and behavioural measurements were compared to the results with constitutive IIS reduction in neurons using elavGAL4 driver (Ismail, et al. 2015) (Chapter 4 and 5). To study if the detrimental effects on behavioural decline due to reduced pan-neural IIS in the adult flies are reversible, elavGS/UAS-InR^{DN} flies were treated with the inducing drug RU486 from 3 days old adult flies and experimental flies were allowed 3 day and 7 day recovery time off RU486 before each behavioural measurement (Chapter 5). To investigate if reducing IIS in the neurons causes any permanent changes to the CNS such cell death, apoptotic cells in the fly brain of elavGAL4/UAS-InR^{DN} and elavGS/UAS-InR^{DN} genotypes were visualised using the TUNEL apoptosis assay and counted (Chapter 7.2.7)

Objective 2

To study the endocrine effects of pan-neural IIS reduction and identify what downstream signalling components are affected when IIS is reduced in the neurons of *Drosophila melanogaster*.

Research Design

This will address specific question (2). Real time qPCR was used to measure the expression of Drosophila Insulin-like peptides (DILPs) in fly heads and bodies in response to constitutive and adult specific IIS reduction in the neurons. To investigate some endocrine effects of pan-neural IIS reduction, fecundity, haemolymph glucose content, starvation and oxidative stress resistance was measured (Chapter 7).

Objective 3

To determine the role of neuronal subtypes in modulating the lifespan and behavioural decline of flies in response to reduced IIS.

Research Design

This will address specific question (3). To reduce insulin signalling in specific neuronal subtypes, the UAS-InR^{DN} transgene was targeted to the GAL4 drivers of these neuronal subtypes that are currently available in research labs or at stock centres, namely Th-GAL4 (dopaminergic neurons), Vglut-GAL4 (glutamatergic neurons), Chat-GAL4 (cholinergic neurons), and Gad1-GAL4 (GABAergic neurons). The lifespan, sleep (activity pattern), fecundity, negative geotaxis and exploratory walking senescence of male and female flies were recorded every 10 days throughout their lifespan as in Ismail, et al. 2015.

Chapter 2: Materials and Methods

2.1: Genetic background and maintenance of *Drosophila melanogaster* stocks

The wild type *Drosophila melanogaster* strain that was used throughout the experiment is called the white Dahomey (w^{Dah}). The original Dahomey strain was captured in Benin (Africa) in the 1970. To keep the stain out-bred, they were kept in large cages with overlapping generations (Puijk and de Jong, 1972). The original strain had red eyes, the white eyed mutant was created in the Partridge Lab by repeated backcrossing of the mutant *white* gene, from the w^{1118} *Drosophila* strain into the wild-type Dahomey strain (Ziehm, et al. 2013). To modulate insulin signalling in the fly neurons or in specific neuronal subtypes, the UAS-GAL4 and GeneSwitch (GS) systems were used. All of the genetically modified stocks were backcrossed five times to the wild type w^{Dah} strain. **Table 1** shows a summary of all the genetically modified *Drosophila* lines that were used throughout the experiments.

Table 1 - The list of the UAS, GAL4 and GS Drosophila lines used during the experiments
Stock number refers to Bloomington Stock Centre. The dominant negative activity of the InR^{DN} transgene was achieved by an amino acid substitution of the R1409A kinase domain in the Drosophila insulin receptor (Ismail, et.al. 2015). MARCM set, GFP labels the cell surface (mouse CD8 is a transmembrane protein), highly concentrated in neuronal processes, L.L. Viable P insertion, but stock is segregating CyO, K.C. 1/00

Type	Name	Acronym	Stock number	References (where available)
UAS	Dominant negative insulin receptor	InR^{DN}	15635	Ismail et al. (2015)
	GFP	MCD8-GFP	5137	Kolodziejczyk, et al. (2008)
GAL4	Dopaminergic	ThGAL4	8848	Friggi-Grelin, et al. (2003)
	Glutamatergic	VglutGAL4	26160	Shao, et al. (2011)
	Cholinergic	ChAT-GAL4	6798	Salvaterra, et al. (2001)
	GABAergic	Gad1-GAL4	51630	Ng, et al. (2002)
	Pan-neuronal	elavGAL4	25750	Ismail, et al. (2015)
	Daughterless (ubiquitous)	daGAL4	13991	Ikeya, et, al. (2009) and Ismail, et al. (2015)
GS	Pan-neuronal	elavGS	-	Sofola et al (2010)

All wild-type and genetically modified stocks were kept in disposable Drosophila bottles plugged with sponge bungs. The stocks were fed by standard sugar-yeast (SY) media (Bass et al. 2007) and stored at room temperature in natural light. Stocks were transferred into fresh bottles every 3 weeks to allow overlapping generations of flies to mate. To maintain the genetic variability of the wild type w^{Dah} background, they were maintained in approximately 8 bottles and mixed every time they were transferred into fresh bottles. The fly stocks are kept at room temperature while flies for experiments were maintained at 25ºC, 70% humidity and 12:12 light-dark cycle.

2.2: Collection of virgin female flies

Female flies can store sperm after copulation; thus, the exact genotype of their offspring is unpredictable. In order to generate the desired offspring, virgin female flies were collected and used for genetic crosses. Young females are unreceptive to male courtship until about 6-8 hours after eclosion at 25ºC and they can be identified as they have less pigmentation compared to older flies. To maximise the efficiency of virgin collection, bottles with eclosing flies were kept at 25ºC during daytime and fresh virgins were collected twice or three times a day 4-6 hours apart. Virgins were stored at 10 fly/vial at 25ºC and the vials were checked for any larvae after 2 days, to make sure no mated females were present in the vials.

2.3: Genetic backcrosses

The genetic background of an organism can affect its life expectancy and behavioural patterns and the genetic background can modulate some phenotypic effects of a mutation. To ensure that the results we see are caused by the examined mutation and not due to a difference in the genetic background of the control and experimental groups, all the genetically modified UAS, GAL4 and GS lines were backcrossed to the wild type w^{Dah} background 5 times.

The backcrossing was carried out in batch crosses in 3 stages, each cross was carried out in vials with 10 virgin females and 5-10 males, 10 vials per cross. At the first stage, virgin w^{Dah} females were crossed with males from the homozygous UAS, GAL4

or GS lines with red eyes. All the offspring was heterozygous and had pale red eyes. At the second stage, virgin females from the first cross were collected and crossed with *w*^{Dah} males. Their offspring were either homozygous wild type with white eyes or heterozygous with pale red eyes. This stage was repeated three more times using heterozygous virgin females with pale red eyes from the previous cross. At the third stage heterozygous virgin females were crossed with heterozygous males producing wild type (white eyed), heterozygous (pale red eyes) and homozygous (dark red eyes). The homozygous UAS, GAL4 or GS flies were identified by their dark red eye colour and virgin females and males were collected to set up a stock in bottles (**Figure 9B**). **Figure 8** summarises the backcrossing process.

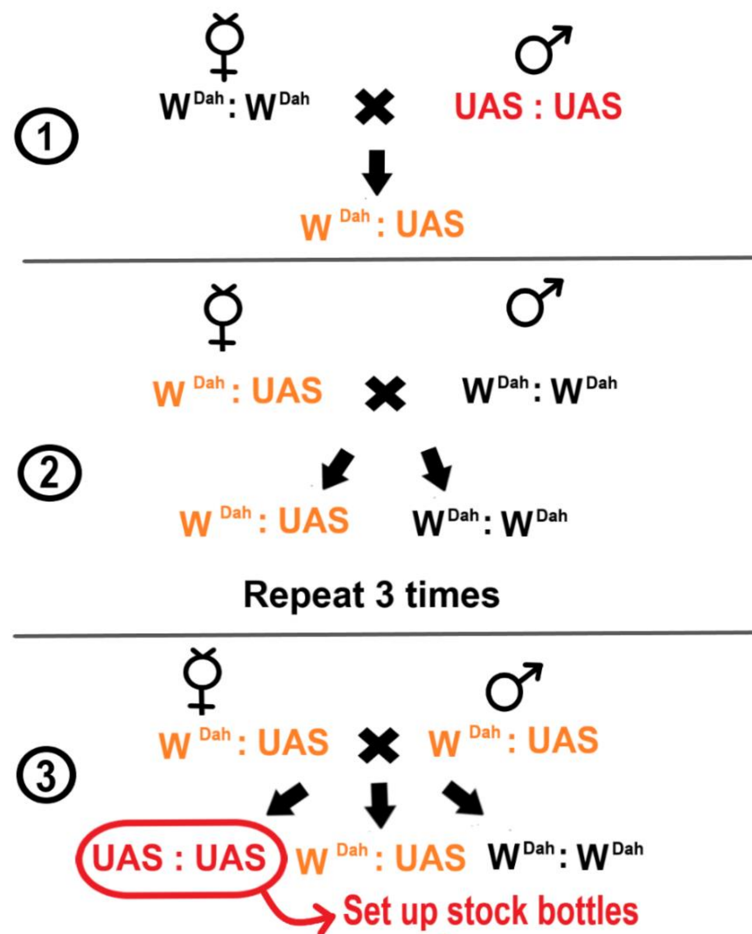


Figure 8 - The 3 stages of backcrossing
All batch crosses were carried out in vials, 10 virgin females and 5-10 males per vial, 10 vial per cross. The figure only shows the backcrossing of the UAS lines for simplicity, but the GAL4 and GS lines were backcrossed exactly the same way. Red text colour represents the dark red eyes, the orange text shows pale red eyes, while the black text show white eye colour.

2.4: Generation of flies for experiments

Young male and virgin female flies were collected from the appropriate background and kept in vials until the age of 3-4 days. The UAS-GAL4 and UAS-GS crosses were set up as shown in **Table 2**. Approximately 50 virgin females and 30 males from the correct background were transferred into *Drosophila* cages (**Figure 9A**) with red grape juice agar plates (recipe in **Table 3**) with live yeast as a food source. Cages were kept at 25ºC 70% humidity covered from light to promote egg laying. Eggs were collected 24 hours and 48 hours later, the flies were transferred onto fresh grape juice plate with live yeast after the first collection. The egg collection was done based on the method of Clancy and Kennington (2001). Eggs were washed off from the plate using Phosphate Saline Buffer (PBS) into a 50 μL Falcon tube. Eggs were allowed to settle and sucked up from the bottom using a widened 200 μL micropipette tip and dispensed into bottles with standard food in 30 μL aliquots aiming for about 500 eggs/bottle density. Bottles with the eggs were kept at the 25ºC incubator and they eclosed in 10 days. Adult, already mated flies at the age of 3-4 days were anaesthetised by CO_2 and sorted into *Drosophila* vials as 10 flies per vial, males and females separately.

Table 2 - UAS-GAL4 and UAS-GS crosses
Except the elavGAL4 x UAS-InR^{DN} cross, as elavGAL4 is on the X chromosome, so the virgin females had to come from the elavGAL4 stain. For the first lifespan experiment, the reciprocal crosses were treated separately. Since there was no difference between the lifespan or behaviour of the reciprocal crosses, they were mixed for further experiments.

Virgin females (~50)		Males (~30)	
UAS-InR^{DN}	X	GAL4 or GS	**Experimental**
GAL4 or GS	X	UAS-InR^{DN}	
w^{Dah}	X	GAL4 or GS	**Control**
w^{Dah}	X	UAS-InR^{DN}	

2.5: Drosophila maintenance during experiment

Experimental flies were kept in disposable *Drosophila* vials (**Figure 9C**), each containing 10 male or female flies. Vials were plugged with clean cotton balls and maintained at 25°C, 70% humidity with a 12 h dark/light cycle. Vials were laid flat on

their side to protect the old flies from getting stuck in the agar media and the flies were transferred into vials with fresh food approximately every 3 days.

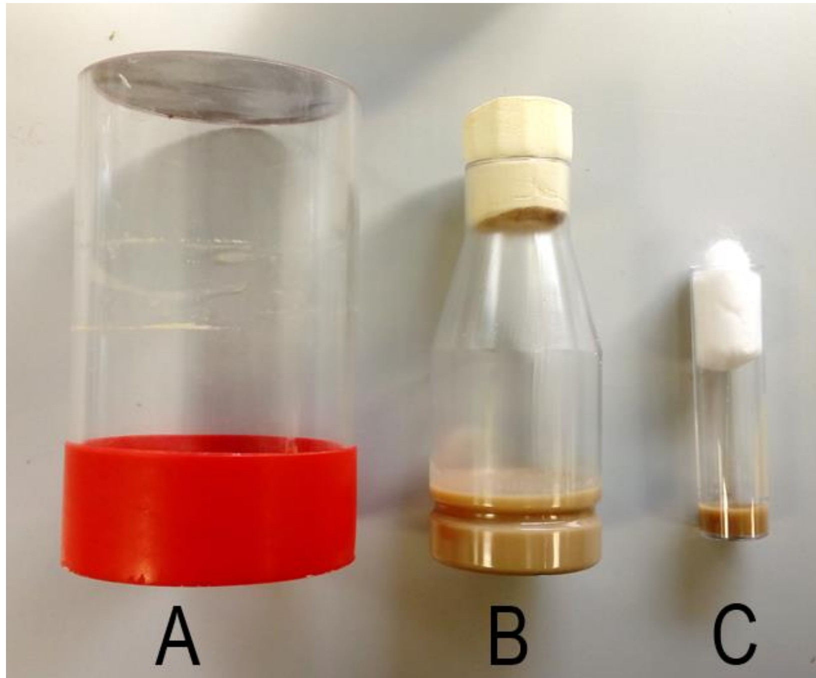


Figure 9 - Fly containers used throughout the experiments
A: cage used to generate flies for experiments, B: bottle (from FL Plastics) for maintaining fly stocks. C: Vial (from Regina Industries Ltd.) for keeping flies during experiments.

2.6: Drosophila media

All flies were kept on standard 0.5 x sugar/ 1x yeast food containing 50g/l sugar and 100g/l yeast unless otherwise stated (Bass et al. 2007). **Table 3** summarises the types of media used throughout the project with their ingredients.

To make fly media, the agar powder was added to hot water (or grape juice) and mixed thoroughly. The mixture was brought to the boil to ensure that the agar powder had dissolved completely. During constant stirring, the sugar-yeast mixture was added to the agar solution and was brought to the boil again. Once boiling, the mixture was removed from the heat and the cold water was added to speed up the cooling down. It was left to cool down with occasional stirring to stop the agar setting at the sides and on the top. Once the mixture was below 60°C the nipagin (10% dissolved in 100% Ethanol) and the propionic acid was added to inhibit mould growth. Then the media

was poured into bottles or vials (or petri dishes for the grape juice food) and it was left to set for a day at room temperature covered by breathable fabric.

When making the 200 mM RU486 food, 85.9 mg of RU486 (Mifepristone – by TCI) was dissolved in 5 ml of 100% ethanol per litre of food. The RU486 solution was added to the food after the nipagin and the proprionic acid. To the food of the control group of the RU486 experiments the extra 5 ml of ethanol was added without the RU486. The oxidative stress resistance experiment used a media containing 30% H_20_2, which was added once the media cooled down to 60°C.

Table 3 - Recipes of fly media

Ingredient \ Diet	Standard 0.5xS/1.0 xY	Starvation 0xSY	RU486 food	RU486 control food	Grape juice plates	H_2O_2 food
Water (ml)	700	1000	700	700	1000 (grape juice)	160
Agar (g)	15	15	15	15	16	3
Sugar - sucrose (g)	50	0	50	50	0	10
Yeast - MP Biomedicals (g)	100	0	100	100	0	0
Water at the end (ml)	170	0	170	170	0	6.7
Nipagin – 10% in 100% ethanol (ml)	30	30	30	30	0	0
Propionic Acid (ml)	3	3	3	3	0	0
100% Ethanol (ml)	0	0	5	5	0	0
RU486 (mg)	0	0	85.9	0	0	0
30% H_20_2 (ml)	0	0	0	0	0	33.3

2.7: Weighing flies

The genetic crosses were done as described in the *2.4: Generation of flies for experiments* section. 3 days old flies were sorted into vials using CO_2 as 10 flies/vial,

males and females separately (N=60). The vials were frozen upside down after 48 hours and the flies were weighed 10 at a time and their average weight was calculated.

2.8: Dissecting and fixing brains for GFP analysis

CO_2 anaesthetised flies were dipped in 70% ethanol for 2 minutes, then transferred to PBS for dissection. Dissected brains were kept on ice in PBS protected from direct sunlight. 4% paraformaldehyde (PFA) was made freshly from frozen 20% PFA stock for fixing the fly brains. After 20 minutes in the 4% PFA at room temperature with gentle shaking, the brains were washed 3 times for 10 minutes in PBS. Then they were washed 3 times in TNT for 10 minutes at room temperature with gentle shaking, followed by another 3 washes in PBS for 10 minutes. The brains were then soaked in 2% n-propylgallate and stored in dark at 4°C. The brains were mounted onto microscope slides right before they were examined by confocal microscopy. Ingredients of the reagents are shown in **Table 4**.

Table 4 - PBS, TNT and 2% n-propylgallate ingredients

PBS (1×)	TNT	2% n-propylgallate
137 mM NaCl	0.1M Tris HCl (pH8)	80% glycerol
2.7 mM KCl	0.3M NaCl	20% PBS
10 mM Na_2HPO	0.5% Triton X-100	2% n-propylgallate powder

2.9: Survival analysis

Flies of each genotype were sorted by CO_2 anaesthesia into vials with the appropriate food at the age of 3-4 days at 10 flies/vial, males and females separately (N=100 or 150). They were transferred into fresh vials every 3 days and the dead flies were counted during every transfer. The survival data are presented as a proportion of surviving flies over time.

2.10: Fecundity

Female fecundity was measured by counting the number of eggs laid over a 24 hour period. Mated females were kept as 10 fly/vials (starting N=100) and the egg counting was repeated every 10 days throughout their life. Flies were kept on standard food or RU486 food in the vial depending on the experiment. The data are presented as the mean number of eggs laid per day per female.

2.11: Negative Geotaxis

Flies kept as 10 flies/vial were transferred into serological pipettes (25 cm long, 1.5 cm diameter) using a funnel (N=3 for each group). The pipettes were banged down one at a time hard enough so that the flies fall to the bottom of the tube and were examined by eye for 45 seconds (

Figure 10). The number of flies staying at the bottom (i.e. not climbing more than 1 cm) and the ones reaching the 'top' (climbing more than 10 cm) were noted down. Flies that reached the top once but fell down to the bottom again were counted as reaching the top. Each measurement was repeated 3 times, and their performance index ($^1/_2 * \frac{N_{total}+N_{top}-N_{bottom}}{N_{total}}$) was calculated (Kerr et al, 2011). The experiment was carried out every 10 days throughout their lifespan, each experiment starting at the same time of the day to avoid differences caused by different daily activity level.

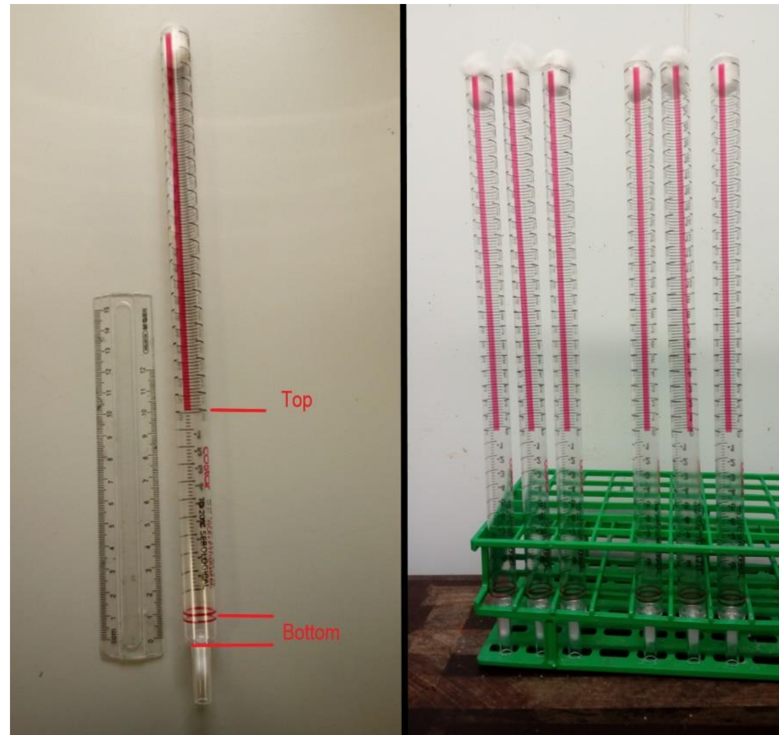


Figure 10 - Serological pipettes used for negative geotaxis
Flies staying in the bottom 1 cm of the tube were counted as staying on the bottom, flies climbing over the 10 cm red line were counted as reaching the 'top'. Tubes with flies were kept upright throughout the experiment and they were banged down and monitored by eye for 45 seconds one by one.

2.12: Exploratory Walking

Exploratory walking analyses were carried out every 10 days throughout the lifespan of the flies, with every experiment started at the same time of the day. Individual flies were sampled from a population of flies (N=16) and aspirated into a 40 mm diameter 10 mm height circular arena containing a 2% agar gel as a base (N=16) (**Figure 11A**). Each fly was used once for exploratory walking experiment and discarded after the measurement. Their activity was recorded for 15 minutes and the videos were analysed using EthoVision XT video tracking software (Noldus). The arena settings are shown in **Figure 11B**. The analysis data from EthoVision were exported to Microsoft Excel. Data are presented as the average performance of the 16 individual flies at each timepoint. Parameters used are total distance walked, duration in central zone, walking duration, number of movement bouts, rotation frequency, first rotation time and velocity. Some of the parameters are be correlated, as the velocity depends on the total distance walked and the walking duration.

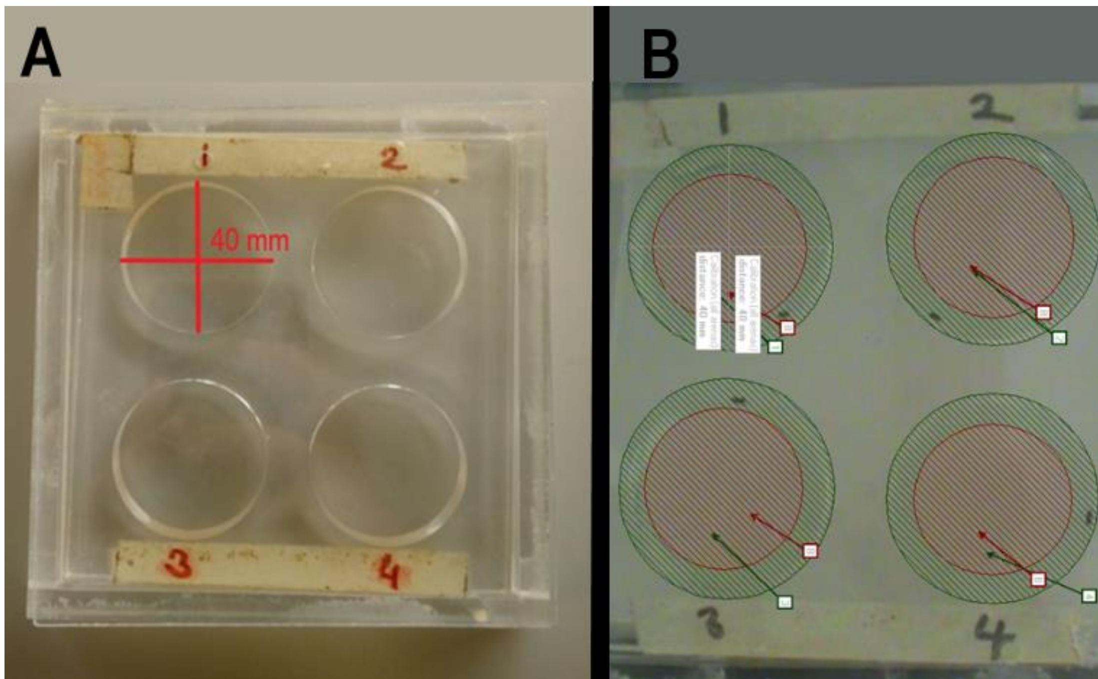


Figure 11 - **The exploratory walking arenas**
A) Individual flies were aspirated into each chamber and their movement was videoed for 15 minutes. Videos were analysed using EthoVision XT video tracking software (Noldus) with the arena settings as shown in B). Green represents the full arena and red shows the central zone.

2.13: Sleep Analysis

Sleep analysis was carried out every 10 days throughout the lifespan of the flies. Flies were briefly knocked out by CO_2 and individually transferred into Trikinetics Drosophila Activity Monitor tubes containing the appropriate food (**Figure 12**). Their activity was monitored in 1-minute bins for at least 4 days. The raw sleep data was processed using the DrosoSleep software (**Figure 13**).

DrosoSleep software was created by Gabor Nyiro in order to replace the commonly used BeFly! excel add-in, which is not functioning reliably on the latest versions of Microsoft Office. The current version of DrosoSleep analyses sleep behaviour similarly to BeFly!, however it does not analyse circadian rhythm. The Monitor text files created by the DAMs can be imported into the software and it measures daily and hourly activity based on the experimental settings. In our experiments, we used 1-minute bin length and 5 consecutive 1 minute bins counted as sleep (Shaw, et al. 2000). We used 0 movement as our inactivity threshold and flies were considered dead if they showed less than 100 mins of activity per day. The

software creates a Microsoft Excel spreadsheet of the analysed data, which is processed and presented as the average performance of the 15 individual flies at each timepoint. In this research we used the daily parameters of total activity (number of minutes spent active), total activity level (number of times the fly crossed the infrared beam), total sleep in dark, total sleep in light, number of sleep bouts in dark, number of sleep bouts in light, average length of sleep bouts in dark and average length of sleep bouts In light. Parameters can be correlated, as the total daily activity depends on the total sleep in dark and total sleep in light. Similarly, the number and the length of the sleep bouts are also correlated. Sleep fragmentation occurs when the number of sleep bouts increases while the average sleep bout length shortens without changing the amount of total sleep.

Figure 12 - Drosophila Activity Monitor
The Drosophila activity monitor (Trikinetics) Flies were kept individually in the tubes. Each tube is 6.5 cm long, containing about 0.5 cm of the appropriate fly media.

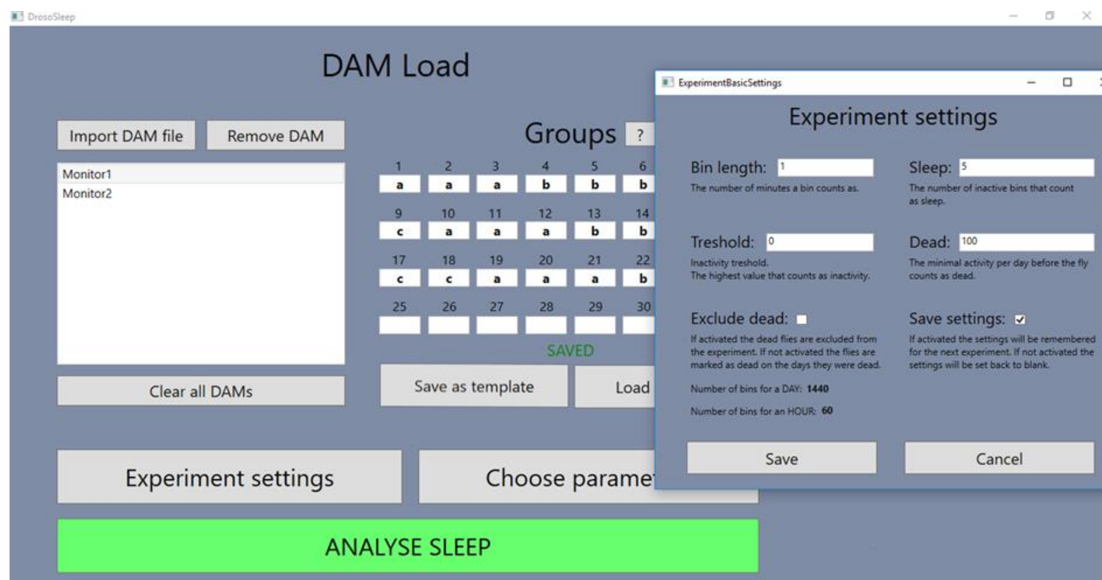


Figure 13 - The DrosoSleep software
The new software was created to analyse the data produced by the DAMs

2.14: Oxidative stress resistance - H_2O_2

At the age of 3-4 days, adult flies were sorted into vials containing H_2O_2 food made as described in **Table 3**. The number of dead flies were counted twice a day and the survival data are presented as a proportion of surviving flies over time.

2.15: Haemolymph glucose assay

Haemolymph was collected from adult flies and glucose levels measured using the method described in Broughton et al. (2008). To collect haemolymph, shortened pipette tips with inserts were made prior to the experiment as described in **Figure 14** Young, 3-4 days old flies were knocked out on ice and decapitated by clearly cutting the neck using a blade. Four fly bodies were placed neck down into the pre-made tips with inserts and three of these tips with inserts were placed into an Eppendorf tube on ice. Flies in the tubes were then spun at 4000 rpm (1,500 x g) for 15 minutes at 4°C. 12 flies can produce approximately 1-3 µL of haemolymph. Immediately after spinning, the Eppendorf tubes with the haemolymph were frozen at -20°C.

150 µL of Thermo Scientific Infinity^{TM} Glucose affinity reagent was pipetted into each well of a Costar 96-well flat-bottomed cell culture plate and was warmed up at 37°C for 5 minutes. 1 µL of haemolymph or standard was added to the reagent in each well. The concentration of glucose in each sample was calculated using a glucose standard curve. 20 mM glucose solution in MilliQ water was the starting point of the 2-fold serial dilution used as the standard curve. The absorbance of each sample at 340 nm was measured after a 10-minute incubation at 37°C using TECAN Infinite M200 PRO plate reader.

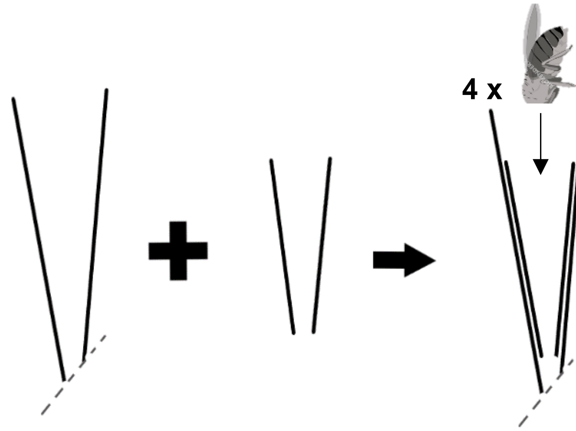


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Figure 14 - Heamolymph collecting tips with inserts
200 µl micropipette tips were used for both parts. The first one was cut at the 200 µL line and its end was cut diagonally. The second one was cut approximately in half. The smaller second tip was slid into the larger first one with the diagonal cut. 4 decapitated flies were inserted into the insert neck down.

2.16: TUNEL apoptosis assay and confocal microscopy

CO_2 anaesthetised flies were soaked in 70% ethanol for 2 minutes and they were transferred to and partially dissected in 1% PBS so that the brain was revealed but it was still attached to the body. Dissected flies were then stored in PBS on ice in Eppendorf tubes, each containing about 5-10 flies with exposed brains. To tag the apoptotic cells in the brains, the Millipore ApopTag® Red in situ apoptosis detection kit was used. The brains were fixed in 500 µL 4% PFA (made freshly from 20% PFA stock) for 10 minutes at room temperature with gentle shaking. Then the brains were washed in PBS 3 times for 5 minutes and post-fixed in ethanol:acetic acid (2:1) at -20°C for 5 minutes. The brains were washed 3 times for 5 minutes in PBS again and 60 µL Equilibration buffer was applied for 10 seconds, then removed. Next, 60µL working strength TdT enzyme was added and incubated at 37°C for an hour covered from light. Then the samples were agitated for 15 seconds in working strength Stop/Wash Buffer, then incubated for 10 minutes at room temperature. Samples were then washed in 500 µL PBS 3 times for 1 minute. In the meantime, working strength Anti-Digoxigenin Conjugate was made and warmed up to room temperature in the dark. After the washes, 60 µL of working strength Anti-Digoxigenin Conjugate was added to the samples and incubated for 30 minutes at room temperature in the dark. The sample preparation was finished off with 4 more washes in 500 µL PBS for 2 minutes and were stored in 80% glycerol in PBS at -20°C. The recipes of the working strength solutions are in **Table 5**.

Before examining the samples using confocal microscopy, they were post dissected to remove the bodies attached to the brains. The cleaned-up brains were mounted onto microscopy slides using DAPI Vectashield and visualised under a ZEISS LSM 880 confocal microscope using 20x objective. Nuclei was stained with DAPI and was excited at 358 nm and emitting blue light, while apoptotic cells were stained with Propidium Iodide (PI) and 488 nm argon laser was used to emit red light. The apoptotic cells were identified by the overlap of the DAPI and PI stains. Z-stacks were recorded and analysed using ImageJ (NIH, Bethesda, MD, USA) using the analyse particle function (**Figure *15***). Data are presented as the average number of fluorescent cells in fly brains (N=5-6).

Table 5 - ApopTag® Red in situ apoptosis detection kit working strength solutions

TdT enzyme	Stop/Wash Buffer	Anti-Digoxigenin Conjugate
77 µL reaction buffer	1 mL Stop/Wash Buffer	68 µL Blocking Solution
33 µL TdT	34 mL of distilled H_2O	62 µL Anti-Digoxigenin Conjugate

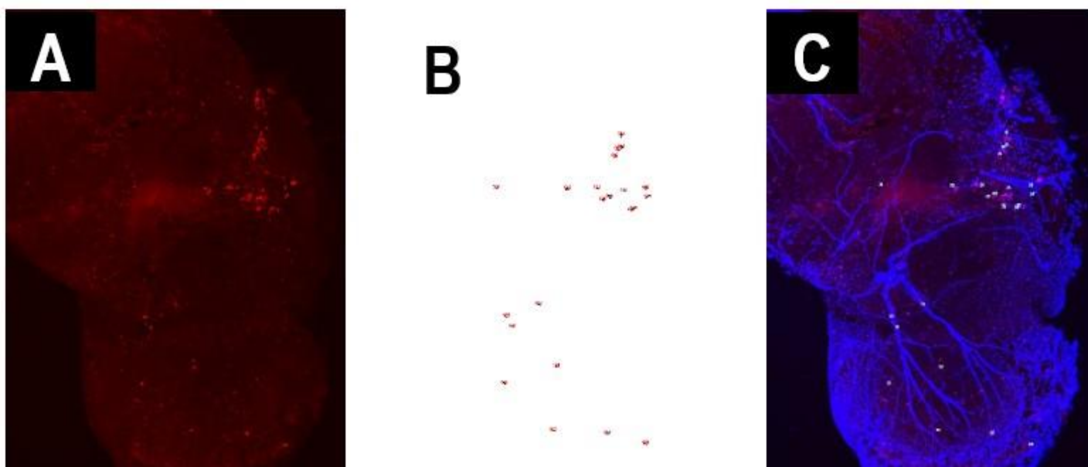


Figure 15 – Apoptotic cell detection in brain images using ImageJ
A) Z-stack image of a brain sample with red stain only, representing apoptotic cells. B) Apoptotic cells identified by ImageJ based on the overlapping red and blue fluorescence. C) Z-stack image of red (apoptotic cells) and blue (nuclei stained by DAPI) combined fluorescence.

2.17: Trizol RNA extraction from fly heads and bodies

Flies from the appropriate genotype were separated by sex and sorted into Eppendorf tubes using CO_2 at 20 flies per tube. Eppendorfs with the flies were immediately dropped into liquid nitrogen. Once frozen, tubes were removed from the liquid nitrogen using forceps and stored at -80°C. To separate fly heads from bodies, the Eppendorf tubes were vigorously banged down one by one immediately after taking them out from the freezer. The contents of the tubes were emptied onto a clean filter paper and 20 heads or 10 bodies were counted and added into 1.7mm Zirconium Bead Ribolyser tubes (OPS Diagnostics) containing 1 mL ice-cold Tri Reagent® (Sigma). Fly heads and bodies were homogenised using a ribolyser at 6.5 m/s for 20 seconds then left at room temperature for 5 minutes. Then 200 µL chloroform was added to the samples and were shaken for about 15 seconds. After a 3-minute incubation at room temperature, samples were centrifuged for 15 minutes at 12,000 rpm (13,300 x g) at 4°C. Next, 450µL the upper aqueous layer was carefully transferred to a clean Eppendorf tube and 450 µL of Isopropyl alcohol and 45 µL of 3M Sodium acetate (NaOAc) was added and incubated for 40 minutes to overnight at -80°C.

After incubation, the samples were defrosted and spun for 15 minutes at 12,000 rpm (13,300 x g) at 4°C. The waste was carefully removed keeping the pellet intact and washed in 500 µL ice-cold 70% Ethanol in DEPC water. Samples were placed in the centrifuge and spun for 10 minutes at 10,000 rpm (9,200 x g) at 4°C. The DEPC-ethanol wash was repeated two more times and after the third wash the ethanol was removed, pellets were briefly air-dried on ice and dissolved in 10 µL of DEPC water for heads and 20 µL for bodies. The RNA content and purity of the samples were measured at 260 nm using Nanodrop and the samples were stored at -80°C.

2.18: cDNA generation

RNA samples were defrosted on ice and cDNA was generated from about 500 ng of RNA in heads and about 1000 ng RNA in bodies using either the SuperScript III SuperMix kit or the SuperScript III enzyme and components bought separately.

2.18.1: SuperScript III System (Invitrogen):

The following ingredients were added to 0.2 ml PCR tubes on ice: X µL RNA, 1 µL of 50 µM Oligo $(dT)_{20}$, 1 µL of dNTP mix and 11-X µL DEPC treated water. The samples were heated at 65°C for 5 minutes then chilled on ice for at least 1 minute. After pulse centrifugation the following components were added: 4 µL of 5x RT buffer, 1 µL 0.1 M DDT, 1 µL RNaseOut Recombinant RNase Inhibitor and 1 µL SuperScript III RT enzyme (200 units/µL). Contents were mixed by gentle pipetting and incubated for 50 minutes at 50°C. The reaction is terminated at 70°C for 15 minutes and samples were kept on ice or stored at -20°C.

2.18.2: SuperScript III First strand synthesis SuperMix kit (Invitrogen):

The following ingredients were combined in 0.2 ml PCR tubes on ice: 10 µL 2x RT Reaction Mix, 2 µL RT enzyme, X µL RNA, 8-X µL DEPC treated water. Components were gently mixed and incubated at 25°C for 10 minutes, then the temperature was increased to 50°C for 30 minutes. Reaction was terminated at 85°C for 5 minutes and samples were chilled on ice while adding 1 µL of *E.coli* RNase H. Samples were then incubated at 37°C for 20 minutes then kept on ice or stored at -20°C.

2.19: Quantitative polymerase chain reaction (QPCR)

The cDNA samples were defrosted, briefly centrifuged to collect content at the bottom and diluted with 30 µl ice-cold Distilled water then kept on ice until qPCR plates got ready for loading. Primer master mixes were made for each primer pair. *β-actin*, *Tubulin* and *Rpl32* primers were used for reference genes (Ponton, et al. 2011) and for *Dilp2, 3, 4, 5, 6* were tested for fly heads and *Dilp4, 5, 6, 7* were tested for fly bodies in both males and females. All primers are from Invitrogen. *Dilp* primer sequences were supplied by Dr Susan Broughton (Broughton, et al. 2005).

B- Actin Primers:

Forward primer: CACACCAAATCTTACAAAA
Reverse primer: AATCCGGCCTTGCACATG

Tubulin Primers:

Forward primer: TGTCGCGTGTGAAACACTTC
Reverse primer: AGCAGGCGTTTCCAATCTG

Rpl32 Primers:

Forward primer: ATGCTAAGCTGTCGCACAAATG
Reverse primer: GTTCGATCCGTAACCGATGT

Dilp2 Primers:

Forward primer: ATGGTGTGCGAGGAGTATAATCC
Reverse primer: TCGGCACCGGGCATG

Dilp3 Primers:

Forward primer: AGAGAACTTTGGACCCCGTGAA
Reverse primer: TGAACCGAACTATCACTCAACAGTCT

Dilp4 Primers:

Forward primer: GCGGAGCAGTCGTCTAAGGA
Reverse primer: TCATCCGGCTGCTGTAGCTT

Dilp5 Primers:

Forward primer: GAGGCACCTTGGGCCTATTC
Reverse primer: CATGTGGTGAGATTCGGAGCTA

Dilp6 Primers:

Forward primer: CGATGTATTTCCCAACAGTTTCG
Reverse primer: AAATCGGTTACGTTCTGCAAGTC

Dilp7 Primers:

Forward primer: CAAAAAGAGGACGGGCAATG
Reverse primer: GCCATCAGGTTCCGTGGTT

The total reaction volume was 20 µL containing 10 µL 2x SYBR Green Master Mix (Sigma Aldrich), 7µL of cold distilled water, 1 µL of 10 µM Forward Primer. 1 µL of 10 µM Reverse Primer and 1 µL of cDNA. The samples were loaded into a BioRad Hard-Shell 96-well PCR plate and run on a Bio-Rad CFX96 Real Time System C1000 Thermal Cycler machine using the following protocol, based on Broughton, et al. (2005).

- 94°C for 3 minutes
- 94°C for 30 seconds ← Repeat 39 more times
- 55°C for 30 seconds
- 72°C for 1 minute. 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 Bio-Rad CFX Manager software Version 3.0.

2.20: Statistical analysis

The statistical analysis of the raw data was carried out using JMP Version 14.3.0 (SAS Institute, Cary, NC 27513, USA) statistical analysis Software or Microsoft Excel 365 ProPlus. Lifespan data were analysed by survival analysis using Log Rank tests in Excel. Statistical analysis for the rest of the data was tested for normality using the Shapiro-Wilk W test on studentised residuals (Sokal and Rohlf, 1995) and transformed if needed. For the behavioural experiments (negative geotaxis, exploratory walking, sleep) the effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype of age*genotype has a significant effect, post hoc pairwise comparison was carried out. Student's t-test was used to compare 2 groups or Tukey-Kramer HSD test for 3 groups at each timepoint. For the recovery experiments Dunnett's Method was used comparing the elavGS/InR^{DN} control group to the other three groups. Significant difference was determined as $p<0.05$. All graphs were generated using Excel and the data are shown as the average of the raw data with error bars representing +/- SEM.

Chapter 3: Backcrossing and Validation of *Drosophila* Stocks

3.1: Introduction

Before experimental analysis, all fly stocks were backcrossed into the wild type w^{Dah} background as described in Chapter 2.3. Flies from different genetic backgrounds may have different life expectancy or could show different rates of behavioural declines (Grotewiel et al 2005). Furthermore, genetic mutations can show varying phenotypic effects depending on the genetic background (Ziehm, et al. 2013). Therefore, it is necessary to backcross any genetically modified fly stock onto the genetic background used for the control groups, otherwise the experimental and control results would not be comparable. After backcrossing, the *Drosophila* lines were validated to ensure they contain the desired mutation. The following GAL4 lines were ordered from the Bloomington Drosophila Stock Centre, and therefore needed backcrossing 5 times to the w^{Dah} background: Dopaminergic (ThGAL4), Glutamatergic (VglutGAL4), Cholinergic (ChAT-GAL4) and GABAergic (Gad1-GAL4). The Stock numbers are shown in **Table 1**. The elavGS and the UAS-InR^{DN} line was kindly provided by Nazif Alic. The elavGAL4 and daGAL4 lines were previously backcrossed to w^{Dah} and validated as described in Ismail, et al. (2015). These stocks, therefore, underwent a shorter backcrossing process where stage 2 was only repeated once (**Figure 8**) prior to testing.

3.1.2: Aims

To confirm that the newly backcrossed UAS-InR^{DN} line acted to reduce insulin/IGF-like signalling (IIS).

To ensure that the newly ordered and backcrossed GAL4 lines showed the appropriate neuronal subtype expression patterns, as previously published.

3.1.3: Research design

In order to confirm the expected activity of the UAS- InR^{DN} line to reduce IIS the daGAL4 driver was used to achieve ubiquitous expression. As insulin works as a growth hormone, two of the side effects of the systemic reduction of IIS are smaller body size and reduced female fecundity compared to flies with normal IIS. The difference in size was measured by comparing the weight of the control and experimental groups, while female fecundity was measured by counting the number of eggs laid over 24 hours.

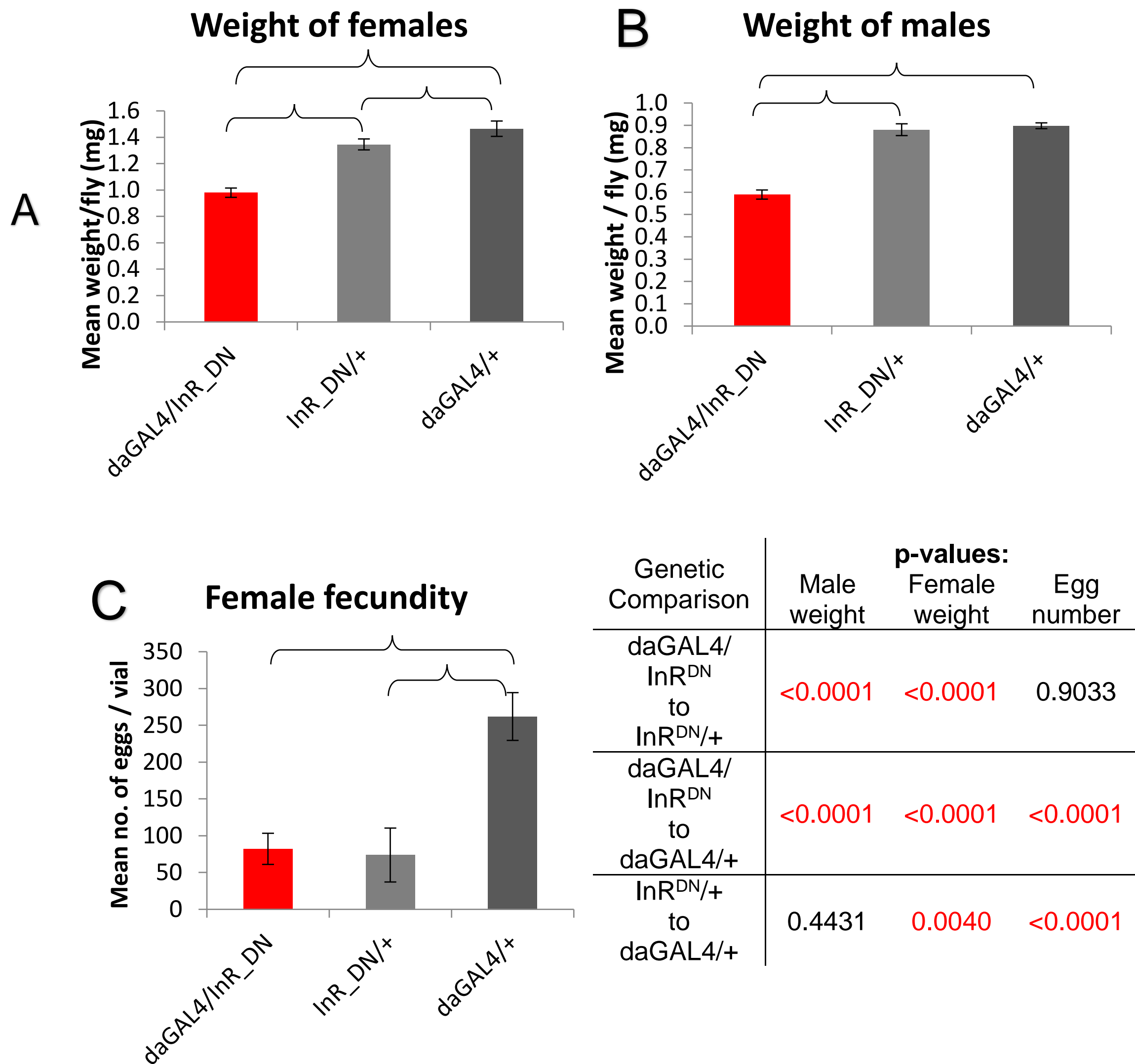
To confirm that the GAL4 drivers expressed GAL4 in the appropriate neuronal subtype, GAL4 virgin females were crossed with UAS-MCD8-GFP transgenic males to tag the surfaces of GAL4 expressing neurons with GFP. The brains of the offspring flies were dissected and visualised under confocal microscopy.

3.2: Results

3.2.1: Validation of UAS-InR^{DN} stock

After the UAS-InR^{DN} line was backcrossed into w^{Dah} for 5 generations, UAS-InR^{DN} virgins were crossed with daGAL4 males. The resulting offspring, which expressed the dominant negative insulin receptor ubiquitously, were sorted by gender into vials as 10 flies/vial at the age of 3 days and frozen at -20ºC at the age of 5 days. Flies were generated and weighed as described in Chapter 2.7 and the eggs of the female flies were counted as described in Chapter 2.10. The results of the weight measurements are visualised in **Figure 16A** and **Figure 16B** showing that both daGAL4/UAS-InR^{DN} females and males weighed significantly less compared to the control groups (daGAL4/+ and UAS-InR^{DN}/+). These data show that there is indeed systemic IIS reduction in the experimental flies, and thus the UAS-InR^{DN} line is functional. The weight of the UAS-InR^{DN}/+ control group was slightly lower than that of the daGAL4/+ control group in females suggesting that the transgene itself can cause slight changes to the weight. The female fecundity data on **Figure 16C** supports this as the experimental daGAL4/UAS-InR^{DN} flies laid significantly fewer eggs compared to the

daGAL4/+ control group, however the fecundity of the UAS-InR^{DN}/+ group was also affected.



Genetic Comparison	p-values: Male weight	Female weight	Egg number
daGAL4/ InR^{DN} to InR^{DN}/+	<0.0001	<0.0001	0.9033
daGAL4/ InR^{DN} to daGAL4/+	<0.0001	<0.0001	<0.0001
InR^{DN}/+ to daGAL4/+	0.4431	0.0040	<0.0001

Figure 16 - Validation of UAS-InR^{DN} line
A: Mean weight of individual 5 days old female flies (N=50) with ubiquitous IIS reduction (daGAL4/InR^{DN}) and control groups (daGAL4/+ and InR^{DN}/+)
B: Mean weight of individual 5 days old male flies (N=50) with ubiquitous IIS reduction (daGAL4/InR^{DN}) and control groups (daGAL4/+ and InR^{DN}/+)
C: Number of eggs laid by 10 flies in each vial over 24 h period (N=50). with ubiquitous IIS reduction (daGAL4/InR^{DN}) and control groups (daGAL4/+ and InR^{DN}/+).
Columns connected by braces indicate significant difference ($p<0.05$)
The table shows the Tukey-Kramer HSD comparisons of means of the male and female fly weight and number of eggs laid by females. Red font colour indicates significant difference ($p<0.05$)

3.2.2: Validation of the GAL4 lines

After each GAL4 line was backcrossed into w^{Dah} background for 5 generations, virgins from the appropriate GAL4 stocks were crossed with UAS-MCD8-GFP males and offspring collected which expressed GFP on the cell surface of the targeted neurons. The brains were then dissected, fixed and confocal microscopy was used to examine the GFP expression pattern. The confocal images created from Z-stacks are shown in **Figure 17**. Dopaminergic, glutamatergic, cholinergic and GABAergic GAL4 lines were tested and their expression patterns matched the published patterns (Hsu and Bhandawat, 2016, Meissner,et al. 2019 dopaminergic: White, et al. 2010, glutamatergic: Sinakevitch-Pean, 2001, cholinergic: Thany, Tricoire-Leignel and Lapied, 2010, GABAerigic: Enell, et al. 2007).

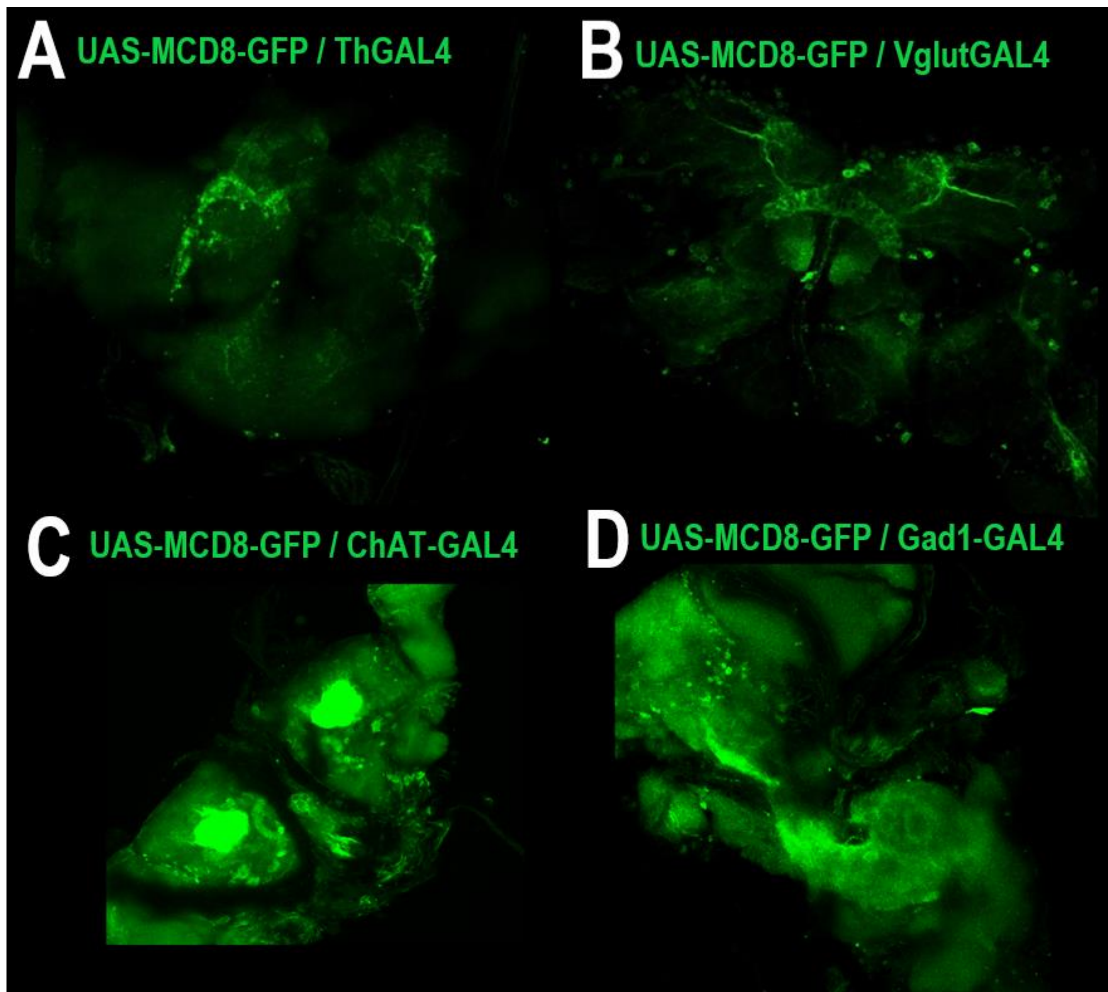


Figure 17 - UAS-MCD8-GFP / GAL4 expression patterns
A: dopaminergic, B: glutamatergic, C: cholinergic, D: GABAergic. All four are sufficiently similar to the expected expression pattern to start their experimental usage.

3.3: Summary

In this chapter we tested if the newly backcrossed UAS-InR^{DN} line acted to reduce IIS. We expressed the UAS-InR^{DN} transgene using the ubiquitous daGAL4 driver and measured the weight of adult flies and the fecundity of females. Two well documented side effects of systemic IIS reduction are smaller body size (therefore reduced weight) and lower female fecundity compared to wild type flies. We found that systemic expression of UAS-InR^{DN} significantly lowered the weight of males and females and the fecundity of females compared to flies without the UAS-InR^{DN} transgene, therefore the transgene is acting to reduce IIS. Other, more costly and labour-intensive ways to test if IIS is knocked down would be to measure the expression of genes that code for the units of the IIS pathway downstream of InR. Gronke, et al. (2010) estimated the activity of IIS by measuring the transcript levels of 4E-BP, which is a translational regulator a directly regulated by dFOXO and expressed when dFOXO is activated in response to reduction in IIS. Measuring AKT phosphorylation using Western blotting can also indicate the activity of the IIS pathway, as done by Ikeya, et al. (2009). The expression of the dominant negative insulin receptor transgene in the flies can be directly measured using qPCR, as done by Ismail, et al. (2015).

We also visualised the expression patterns of the newly ordered and backcrossed GAL4 lines by crossing them with UAS-MCD8-GFP transgenic flies, therefore expressing GFP where GAL4 is also expressed. GFP was then visualised in the dissected fly brains using confocal microscopy and the expression patterns matched the previously published expression patterns of the appropriate neuronal subtypes. Thus, the dopaminergic (ThGAL4), glutamatergic (VglutGAL4), cholinergic (ChAT-GAL4) and GABAergic (Gad1-GAL4) lines express GAL4 in the appropriate types of neurons, therefore suitable for constitutive IIS reduction in specific neuronal subtypes when crossed with UAS-InR^{DN}.

Chapter 4: The role of constitutive and adult specific pan-neural IIS reduction on lifespan

4.1: Introduction

The insulin/IGF-like signalling (IIS) pathway is an evolutionarily conserved nutrient sensing pathway having the strongest evidence for its role in modulating longevity, therefore, it has been widely studied in various model organisms. *Drosophila* lifespan can be extended by altering various parts of the IIS pathway. Systemic downregulation of the *Drosophila* Insulin receptor (Tatar et al. 2001) or its substrate, CHICO (Clancy et al. 2001) extends lifespan of the flies, so does upregulation of negative regulators of IIS, such as dPTEN (Hwangbo et al., 2004) and dFOXO in fat body (Hwangbo et al., 2004, Giannakou et al., 2004). While the IIS pathway is evolutionary conserved and there are similarities between the fly and mammalian IIS pathway, they are not the same. Flies have a single insulin receptor and we do not distinguish between their different types of insulin like peptides. On the other hand, there are distinctions between insulin and IGFs and their receptors in mammals (Broughton and Partridge 2009).

Lifespan extension can be achieved by tissue or time specific reduction of IIS in the fly heads, fat bodies or in muscles (Mathew, et al. 2017). The fat body and the muscles were shown to have an important role in lifespan extension by IIS and selective reduction of the signalling pathway in those tissues can extend lifespan. Increased expression of dFOXO in the adult fat body of the flies increased lifespan and resistance to paraquat in females and reduced female fecundity but did not affect male flies (Giannakou et al., 2004). Overexpressing dFOXO in the fat body later in adulthood from the age of 14 or 21 days still extends the lifespan of the flies, but in a smaller extent. Overexpressing dFOXO only in early adulthood at the age of 3-14 or 3-21 days in the fat body also extends lifespan (Giannakou et al., 2007). Increased dFOXO/4E-BP signalling in fly muscles extends lifespan and improves negative geotaxis behaviour. Muscle ageing in flies is due to the accumulation of protein aggregates and FOXO and its target 4E-BP delays age related muscle functional decline by removing damaged proteins (proteostasis). FOXO/4E-BP signalling in muscles not only regulates proteostasis in muscles, increased signalling can promote the proteostasis

systemically by regulating feeding behaviour and the release of insulin like peptides (Demontis and Perrimon, 2010).

Alic, et al. (2014) studied the possible cell non-autonomous longevity promoting effect of FOXO and found that *dfoxo* to *dfoxo* signalling is not required for the antiaging effect of elevated *dfoxo* levels in the fat body, as both wild type and *dfoxo*-null mutant flies have improved negative geotaxis in response to increased *dfoxo* expression in the gut and fat body. Increased *dfoxo* expression in the gut/fat body and in the neuroendocrine cells promotes healthy ageing by signalling to various other factors in the various tissues, which process is not fully understood yet. As an example, increased *dfoxo* signalling in the gut/fat body alters the expression of *dilp6* (Bai, et al. 2012) and neuropeptide-like precursor 4 (Alic, et al. 2014).

The transcription factor FOXO is essential for lifespan extending effect of reduced IIS signalling. In both *C. elegans* and *Drosophila*, removal of DAF-16 or dFOXO blocks the lifespan extending effect of IIS reduction (Kenyon et al., 1993, Slack, et al. 2011). In worms, the numerous other phenotypes produced by IIS reduction, such as reduced fecundity, smaller body size and increased oxidative stress are suppressed in the absence of DAF-16, suggesting they are under a common regulator (Dillin et al., 2002 Larsen, 1995; Honda & Honda, 1999). In contrast, the phenotypic effects of reduced IIS, such as reduced growth, fecundity and stress resistance are still present in the lack of dFOXO in *Drosophila*, even though the flies are not long lived, suggesting that there are additional factors that create the long-lived fly phenotype with reduced IIS, that are independent of dFOXO (Slack, et al. 2011). As a transcription factor, FOXO promotes longevity by changing the expression of various genes via secondary transcriptional regulators. However, the identity and role in lifespan extension of these secondary transcriptional regulators are still unclear. Alic, et al. (2014) identified a second-tier transcription factor called *Anterior open*, which is directly regulated by dFOXO in the adult fruit fly gut, which can extend lifespan if overexpressed in the adipose tissue. *Anterior open* also provides protection from the lifespan shortening effects of the co-activation of *dfoxo* and *Pointed* transcription factors. This study shows the complexity of interactions between FOXO and its downstream transcription factor and emphasises the importance to gain a better understanding of the tissue-specific transcriptional network around FOXO in order to understand its role and method of promoting longevity (Alic, et al. 2014).

While reduced IIS is beneficial in the fat body and in muscles, lower IIS may affect the ageing and the function of various tissue types differently. Some tissues require IIS

for their function, therefore, reducing IIS could affect those tissues detrimentally. As the brain is an insulin responsive tissue, it is important to investigate if reduced IIS is beneficial or detrimental for the ageing and function of neurons. Ubiquitous or systemic reduction of IIS via ablation of insulin-producing cells (IPCs) (d2GAL4/UAS-rpr) or ubiquitous expression of a dominant negative insulin receptor (daGAL4/UAS-InR^{DN}) has been shown to extend the lifespan of flies (Broughton et al, 2005). Previous studies in our lab showed that constitutive reduction of IIS only in neurons (elavGAL4/ UAS-InR^{DN}) extended the lifespan of female flies, although male lifespan was not increased, confirming the role of the nervous system in flies in modulating lifespan (Ismail, et al. 2015). The sexually dimorphic effect of reduced IIS is commonly seen with systemic IIS reductions, as males often show smaller, if any, lifespan extension, however its reason is still poorly understood (Ismail, et al. 2015, Ikeya, et al. 2009). Ismail, et al. (2015) found disconnection between the lifespan and the health-span of flies with pan-neural IIS reduction. While systemic IIS reduction improved negative geotaxis and some exploratory walking parameters, neuron specific IIS reduction did not improve negative geotaxis and had detrimental effects on exploratory walking. Chambers, et al. (2015) found that IIS in the mushroom body is required for learning and long-term memory in flies and disruption of IIS in the ellipsoid body has detrimental effect on long-term memory.

This study aimed to determine whether constitutive pan-neural reduction of IIS was necessary to achieve lifespan extension, or if it is sufficient to reduce IIS in the neurons of adult flies and still promote longevity. If the detrimental effects of reduced IIS on fly behaviour are caused by reduced IIS negatively affecting the development of neurons, adult specific IIS reduction could eliminate those negative cognitive/behavioural effects. Adult specific reduction of IIS in neurons was achieved using the GeneSwitch elavGS transgene to drive expression of UAS-InR^{DN} in neurons during the adult period. The effect of this adult specific reduction on lifespan was compared to that of constitutive pan-neural IIS reduction (elavGAL4/ UAS-InR^{DN}).

4.1.1: Aims

To investigate if adult specific reduction of IIS in neurons is sufficient to extend the lifespan of flies using the inducible elavGS/UAS-InR^{DN} system.

4.1.2: Research design

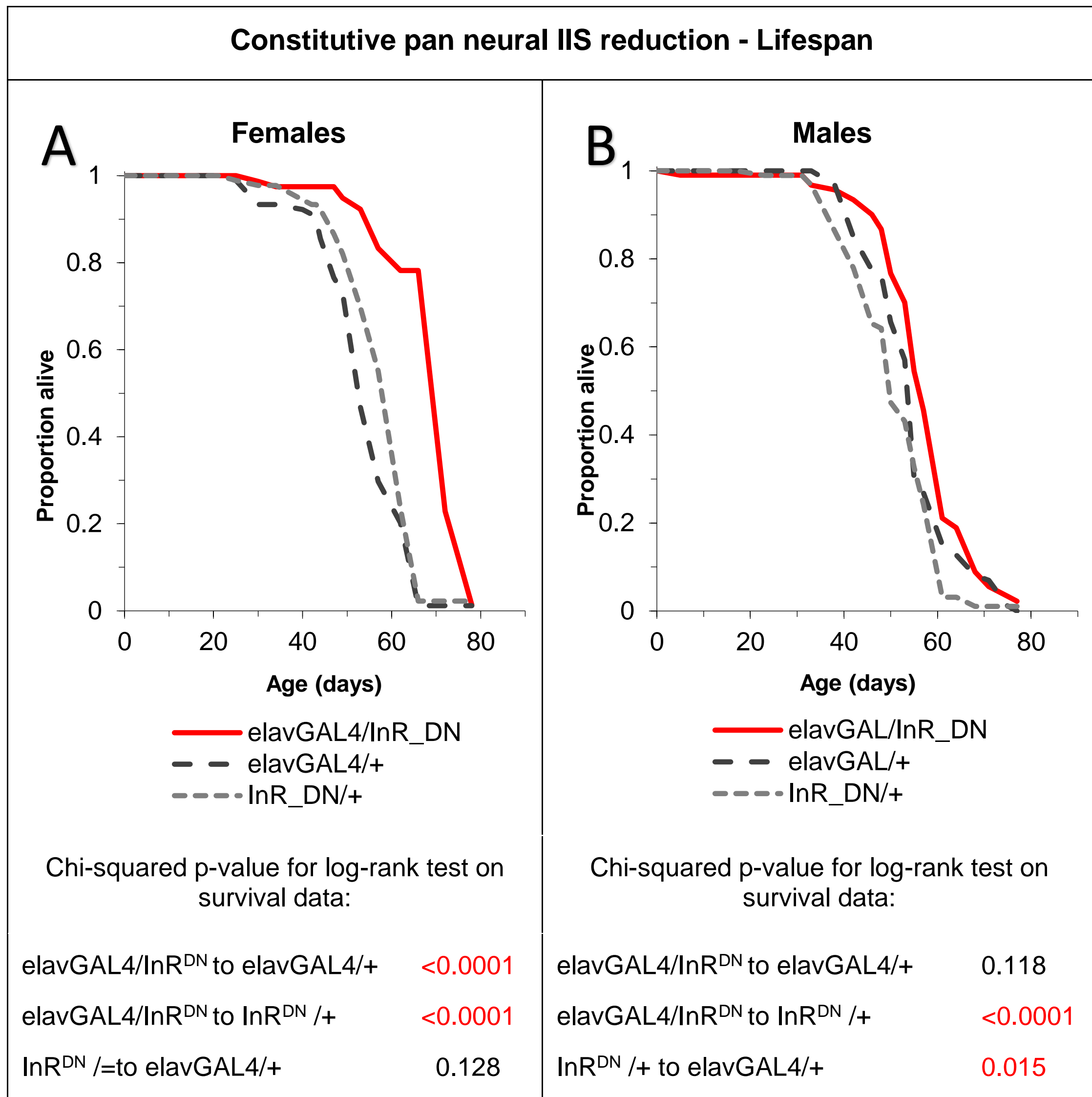
For constitutive reduction of IIS in neurons, elavGAL4/UAS-InR^{DN} (experimental) and elavGAL4/+ and UAS-InR^{DN}/+ (control) crosses were generated as described in Chapter 2.4. At the age of 3 days, flies were sorted by CO_2 anaesthesia and separated by gender. Male and female flies were transferred into standard food vials (10 per vial) and maintained under standard conditions (25°C, 70% humidity with a 12 h dark/light cycle) (N=100 for each group) throughout the lifespan.

For adult specific pan-neural IIS reduction, crosses to generate elavGS/UAS-InR^{DN} and elavGS/+ flies were set up as described in Chapter 2. At the age of 3 days flies were sorted by CO_2 anaesthesia and separated by gender. Half of the elavGS/UAS-InR^{DN} and half of the elavGS/+ flies were transferred into vials of standard food containing 200mM RU486 (+RU486) while the other half were transferred onto standard food containing 5 ml / L of 100% ethanol (-RU486) (N=100 for each group). The elavGS/UAS-InR^{DN} + RU486 group was compared to its control group, elavGS/UAS-InR^{DN} - RU486 to determine the effects of adult specific IIS reduction in neurons on lifespan. The elavGS/w^{Dah} + RU486 group was compared to elavGS/w^{Dah} - RU486 to control for any effects of RU486 itself on lifespan.

4.2: Results

4.2.1: Constitutive reduction of IIS in the neurons extends lifespan in females but not in males

In support of previous studies (Ismail et al. 2015), female flies with reduced IIS in neurons (elavGAL4/UAS-InR^{DN}) were long lived compared to both control groups ($p<0.0001$), while the lifespan of male elavGAL4/UAS-InR^{DN} flies did not increase significantly compared to the elavGAL4/+ control ($p=0.118$) (**Figure 18**). Thus, reduced IIS in neurons extends female lifespan.



Females		Males	
elavGAL4/InR^{DN} to elavGAL4/+	<0.0001	elavGAL4/InR^{DN} to elavGAL4/+	0.118
elavGAL4/InR^{DN} to InR^{DN} /+	<0.0001	elavGAL4/InR^{DN} to InR^{DN} /+	<0.0001
InR^{DN} /=to elavGAL4/+	0.128	InR^{DN} /+ to elavGAL4/+	0.015

Figure 18 - Lifespan of male and female flies with constitutive pan-neural IIS reduction
A) Survival of elavGAL4/UAS-InR^{DN} once mated female flies compared to elavGAL4/+ and UAS-InR^{DN}/+ controls. Median lifespans and sample sizes were: elavGAL4/UAS-InR^{DN} = 69 days, N=99; elavGAL4/+ = 51 days, N=104; and UAS-InR^{DN}/+ = 59.5 days, N=101. elavGAL4/UAS-InR^{DN} females showed an increased survival compared to both controls (P<0.0001)
B) Survival of elavGAL4/UAS-InR^{DN} male flies compared to elavGAL4/+ and UAS-InR^{DN}/+ controls. Median lifespans and sample sizes were: elavGAL4/UAS-InR^{DN} = 56 days, N=98; elavGAL4/+ = 54 days, N=100; and UAS-InR^{DN}/+ = 49 days, N=99.
Survival curves were compared using nonparametric log rank tests and p values are shown under the graphs, with significant differences highlighted in red (p<0.05).

4.2.2: Adult specific reduction of IIS in the fly neurons extends female lifespan, but slightly reduces male lifespan

The elavGS/InR^{DN} + RU486 and elavGS/w^{Dah} + RU486 groups were fed on standard food containing 200 mM RU486 from the age of 3 days throughout their lifespan, while the elavGS/InR^{DN} and elavGS/w^{Dah} control groups were kept on standard food.

Reduced IIS in adult female neurons in the elavGS/InR^{DN}+RU486 group resulted in extension of lifespan compared to the control elavGS/InR^{DN} -RU486 group (p<0.0001) (**Figure 19A)** In contrast, male elavGS/InR^{DN}+RU486 flies showed a small reduction in lifespan (p=0.006) (**Figure 19B**). To determine if there was any effect of RU486 itself on lifespan, the survival of the elavGS/+ control group with or without RU486 was measured (**Figure 19C** and **D**). The results show that RU486 does not affect female lifespan significantly (p=0.398), but it significantly reduced the lifespan of male flies (p<0.0001). These experiments were repeated giving similar results: The elavGS/InR^{DN} + RU486 females were long-lived (p<0.0001) compared to elavGS/InR^{DN} - RU486, while the male elavGS/InR^{DN} + RU486 lifespan was not significantly different from its control group (p=0.053). In the repeat experiment, there was no significant effect of RU486 on elavGS/+ lifespan in either sex (p=0.32 for females and p=0.0688 for males). Together, these data show that reduced IIS in adult neurons extends female, but not male lifespan.

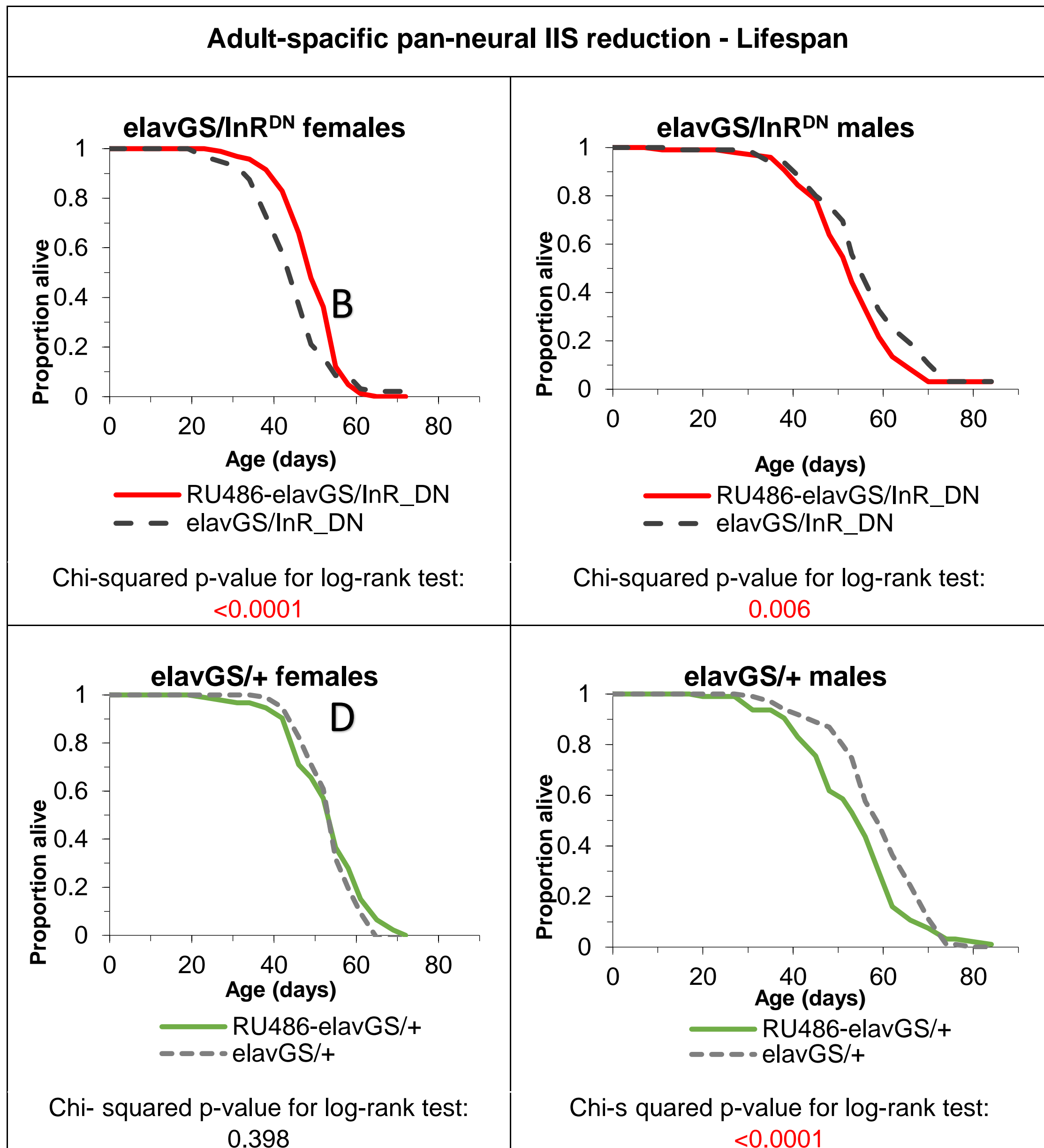


Figure 19 - Lifespan of male and female flies with adult specific pan-neural IIS reduction

A) Survival of RU486-elavGS/UAS-InR^{DN} once mated female flies compared to elavGS/ UAS-InR^{DN} control (without RU486). Median lifespans and sample sizes were: RU486-elavGS/UAS-InR^{DN} = 47.5 days, N=100; elavGAL4/+ = 44 days, N=98. RU486-elavGS/UAS-InR^{DN} females showed an increased survival compared to the control (P<0.0001)

B) Survival of RU486-elavGS/UAS-InR^{DN} male flies compared to elavGS/ UAS-InR^{DN} control (without RU486). Median lifespans and sample sizes were: RU486-elavGS/UAS-InR^{DN} = 52 days, N=97; elavGAL4/+ = 54.5 days, N=97. RU486-elavGS/UAS-InR^{DN} males showed reduced survival compared to the control (P<0.006)

C) Survival of RU486-elavGS/+ once mated female flies compared to elavGS/+ control (without RU486). Median lifespans and sample sizes were: RU486-elavGS/UAS-InR^{DN} = 53.5 days, N=100; elavGAL4/+ = 53.5 days, N=101. There is no significant difference between the two goups (P<0.398)

D) Survival of RU486-elavGS/+ male flies compared to elavGS/+ control (without RU486). Median lifespans and sample sizes were: RU486-elavGS/+ = 54.5 days, N=99; elavGAL4/+ = 57.5 days, N=101. RU486-elavGS/+ males showed reduced survival compared to the control (P<0.0001)

Survival curves were compared using nonparametric log rank tests and p values are shown under the graphs, with significant differences highlighted in red (p<0.05).

4.3: Discussion

The effect of constitutive pan-neural IIS reduction on lifespan were the same as previously published experiments (Ismail et al. 2015) (**Figure 18**), confirming that constitutive reduction of IIS in the neurons only extends lifespan in females and has no effect on male lifespan. When IIS was reduced in adult neurons using the inducible GeneSwitch system, the females were similarly long lived compared to constitutive IIS reduction and RU486 had no significant effect on female lifespan. In the first experiment the male experimental group showed significant reduction in lifespan, however RU486 itself without the transgene also caused significant decrease in longevity suggesting that the lifespan reduction in experimental males was caused by RU486 itself, and not by the reduction of IIS in their neurons (**Figure 19**). Therefore, males with reduced pan-neural IIS from adulthood are likely to have no effect on lifespan, similarly to the results seen with the constitutive driver. In fact, when the experiment was repeated, neither the experimental males nor the RU486 control showed a significant decline in life expectancy.

The results show that pan-neural IIS reduction in adulthood results in similar effects to constitutive pan-neural IIS reduction, and therefore it is not necessary to reduce IIS in neurons throughout the development of flies to achieve the lifespan extending effect in females. Male lifespan, however, consistently does not respond to neuronal IIS reduction. The reason for the sexually dimorphic effect of reduced IIS is yet unknown. In Chapter 7 some potential endocrine effects of reduced IIS are studied, such as the expression of insulin like peptides in fly heads and bodies, which may provide some possible clues on the difference between the sexes.

The variable effect of RU486 itself on male lifespan is also interesting and should be taken as a warning sign. It was recently shown that RU486 can have tissue-specific effects on gene expression and function (Robles-Murguia et al, 2019). The data presented here add further support to the continued and consistent use of the elavGS/+ control with and without RU486 in the analyses of all phenotypes.

Chapter 5: The effect of adult specific pan-neural IIS reduction on negative geotaxis and exploratory walking senescence

5.1: Introduction

The role of IIS in central nervous system (CNS) ageing is controversial. On one hand, the CNS is responsible for secreting DILPs from the insulin producing cells (IPCs), and thus, is an endocrine positive regulator of IIS promoting ageing. The ablation of the IPCs in the fruit fly brain extends lifespan (Broughton, et al. 2005). On the other hand, reduction of IIS can be harmful to the integrity of the CNS, playing a role in the development, function and survival of neurons (Broughton and Partridge 2009).

There are numerous studies showing detrimental effects of reduced IIS on brain function despite increased lifespan of the organism. In worms, reducing IIS can cause learning defects, while increased IIS due to a loss of function mutation in *daf-18* (worm PTEN) can enhance their performance in chemotaxis learning assay compared to wild type flies (Vellai, et al. 2006). Long lived worms with *daf2* mutation show improvement in their memory and learning ability at young age however their memory was not improved at old age (Tomioka, et al. 2006). Measuring negative geotaxis and odour avoidance in long lived DR flies showed that DR does not ameliorate their behavioural ageing (Bhandari, et al. 2007). Removing insulin receptor substrate 2 from the mouse CNS negatively affected the synaptic plasticity in the hippocampus of young mice (Costello, et al. 2012).

A previous study in our lab (Ismail et. al, 2015) investigated the effect of pan-neural IIS signalling reduction on locomotive and cognitive behavioural decline in fruit flies. Two behavioural measurements were used, namely exploratory walking and negative geotaxis, and the effects of pan-neural IIS reduction (with elavGAL4/UAS-InR^{DN}) were compared to two fly models with systemic IIS reduction (daGAL4/UAS-InR^{DN} and d2GAL4/UAS-rpr). The results showed that systemic IIS reduction in both models improved the decline of negative geotaxis, however IIS reduction in the neurons did not affect negative geotaxis. Systemic IIS reduction delayed the senescence of walking distance and velocity during the exploratory walking experiment

but had no effect on the cognitive parameters, suggesting that systemic IIS reduction improves peripheral function, not brain function. Pan-neural IIS reduction caused accelerated decline in both locomotive and cognitive parameters (**Figure 7**). The UAS-InR^{DN} transgene showed lower expression in the brain using the daGAL4 driver compared to elavGAL4/UAS-InR^{DN} brains, which could explain why the daGAL4/UAS-InRDN background did not suffer from negative effects on brain function in response to ubiquitous IIS reduction (Ismail, et al. 2015).

Together these results show that it is possible to increase life span without improving cognitive and behavioural health. One hypothesis to explain the lifespan extension is that the beneficial effects of IIS reduction on peripheral organs (e.g. muscles) and/or neurons outweigh its negative effects on brain function (Broughton and Partridge, 2009).

To determine if constitutive reduction of IIS in neurons causes any negative developmental effects that could lead to the detrimental effects on exploratory walking seen in Ismail et al. (2015), we repeated the locomotor behavioural experiments (exploratory walking, negative geotaxis) using the elavGS/InR^{DN} inducible system to reduce IIS in fly neurons only during the adult period. We repeated the experiments once again with the inducible system, this time allowing recovery from reduced IIS before each behavioural measurement to determine whether or not the negative effects on the behavioural declines are reversible.

5.1.1: Aims

To investigate if the negative effects of pan-neural IIS reduction on behavioural decline are caused by developmental effects due to constitutive IIS reduction.

To determine if the detrimental effects of adult specific pan-neural IIS reduction on behavioural decline are reversible after recovery from reduced IIS.

5.1.2: Research design

To determine if constitutive reduction of IIS in neurons has a negative effect throughout the development of the flies that affects their behavioural decline later in

adult life, we repeated the negative geotaxis and exploratory walking experiments from Ismail et. all (2015), but this time using the inducible elavGS line instead of the constitutive elavGAL4. The experimental flies were maintained on food containing 200 mM RU486 from the age of 3 days. Flies were sampled from the population about every 10 days throughout their life and their performance of negative geotaxis and exploratory walking was measured. The last timepoints were measured when the flies were between the age of 50-60 day. In general, our elavGS fly populations started to die rapidly from around the age of 40 days. At the age of 50 days, 50-60% of the flies were still alive and by the age of 60 days the proportion of the surviving flies was reduced to 25-35%. Experiments were performed at the same time of the day to avoid the effects of their varying daily activity.

To investigate if the effects of pan-neural IIS reduction on behavioural decline are reversible, we repeated the exploratory walking and negative geotaxis experiments with the inducible elavGS system, but this time allowing 3, 4 or 7 days (depending on the experiment) recovery time off the RU486 food before each behavioural measurement. Based on Giannakou, et al. (2007), who found that RU486 induced expression of dFOXO returns to normal levels by 5 days following removal of the inducer, we expected that upon one week recovery from RU486 induced UAS-InR^{DN} expression, the InR function will fully recover.

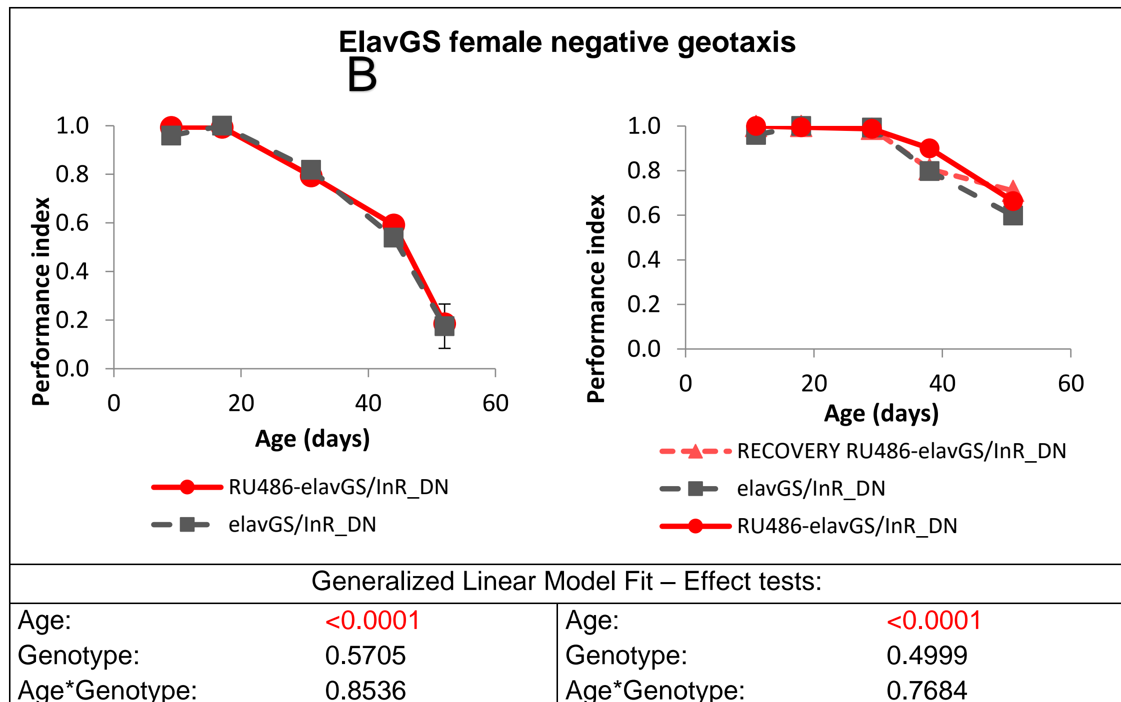
5.2.1: Pan-neural reduction of IIS in adult flies does not affect the senescence of negative geotaxis

Flies for the negative geotaxis experiment were sorted onto the appropriate food at the age of 3 days at 10 flies per vial, males and females separately. The experimental flies were fed with a media containing 200mM RU486. The 'Recovery' flies were transferred to standard food 4 days before each negative geotaxis experiment to recover from the effects of reduced IIS in their neurons. The negative geotaxis experiment was started at 2 pm for each timepoint. The experiment was carried out as described in Chapter 2.11 using the serological pipettes shown in **Figure 10**. At 1:30 pm the flies were tipped into the tubes using a funnel and the allowed to calm down until 2 pm when the first negative geotaxis measurement was started. Their performance index was calculated and shown in **Figure 20** and **Figure 21**.

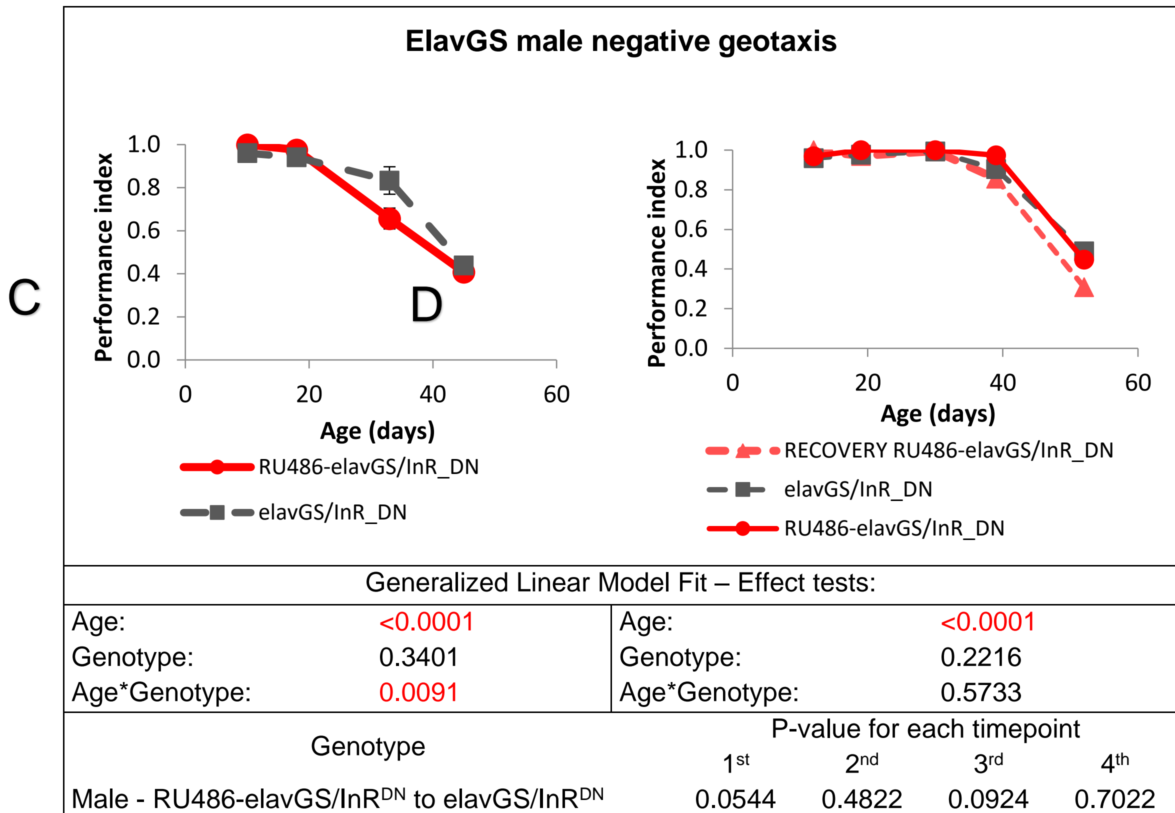
The experiment investigated the effect of adult specific pan-neural IIS reduction on the negative geotaxis senescence with RU486-elavGS/InR^{DN} having reduced IIS in their neurons from the age of 3 days. The elavGS/InR^{DN} group was maintained on standard food so the UAS-InR^{DN} transgene was inactive due to the lack of RU486. The Recovery RU486-elavGS/InR^{DN} group were given 4 days of recovery time off the RU486 food before the negative geotaxis measurement.

The elavGS/+ control groups were included to determine if the chemical RU486 itself had any effect on the negative geotaxis behaviour of the flies. The RU486-elavGS/+ group carried no UAS transgene, such that any change in the behaviour of that group compared to the elavGS/+ group on normal food was caused by RU486. There was also a Recovery RU486-elavGS/+ group to check if any potential damage caused by RU486 can be reversed with 4 day recovery off the drug.

As shown in **Figure 20**, all groups showed an age-related decline in their ability to perform negative geotaxis. None of the experimental manipulations, namely adult specific pan-neural expression of UAS-InR^{DN} (**Figure 20A** and **C**), RU486 alone (**Figure 21**) or 4 day recovery (**Figure 20B** and **D**) had any significant effect on the rate or age of onset of the decline.



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.5705	Genotype:	0.4999
Age*Genotype:	0.8536	Age*Genotype:	0.7684



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.3401	Genotype:	0.2216
Age*Genotype:	0.0091	Age*Genotype:	0.5733

Genotype	P-value for each timepoint			
	1st	2nd	3rd	4th
Male - RU486-elavGS/InR^{DN} to elavGS/InR^{DN}	0.0544	0.4822	0.0924	0.7022

Figure 20 - Effect of pan-neural IIS reduction from the age of 3 days on negative geotaxis senescence

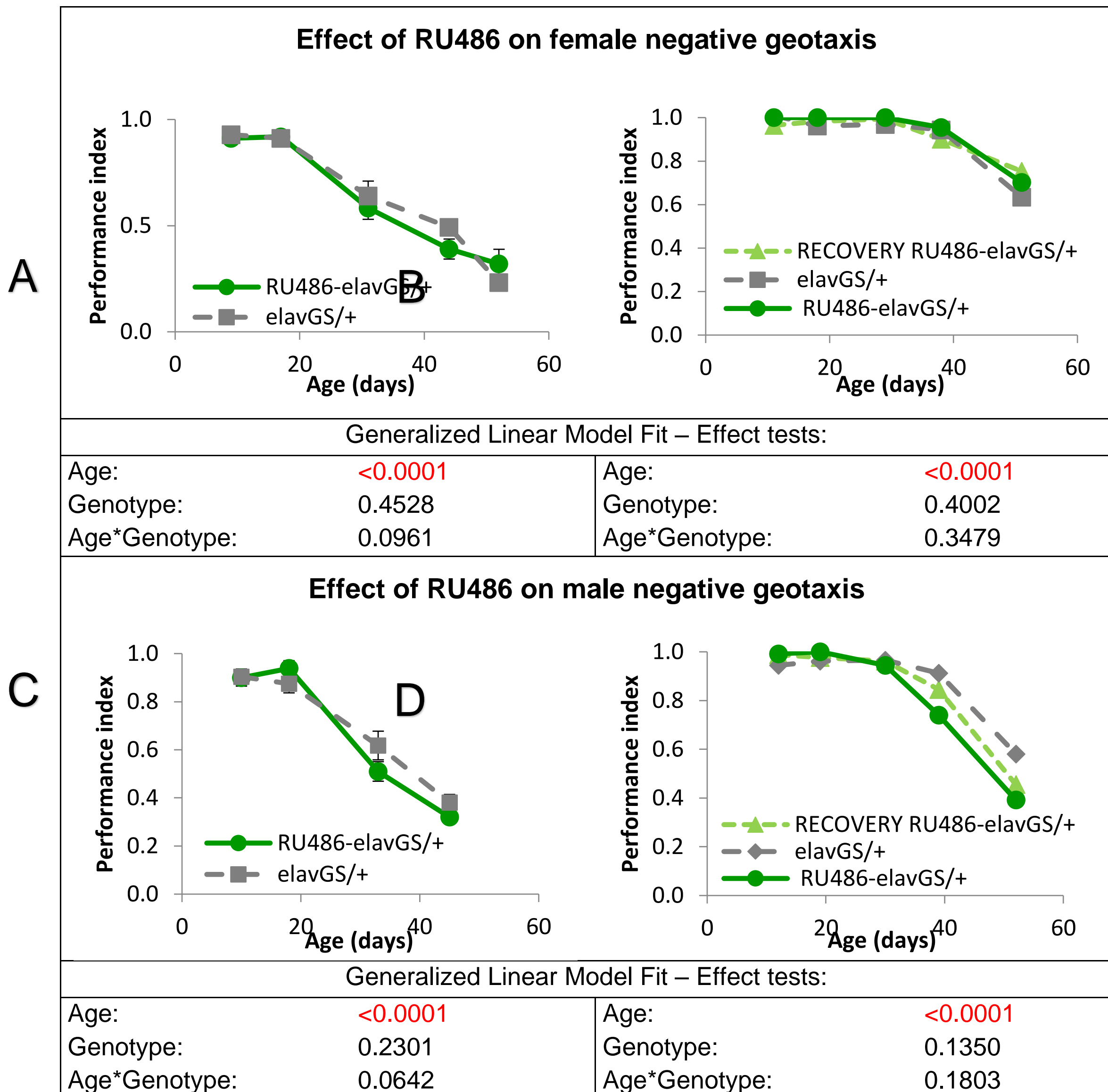
Negative geotaxis performance index of flies over the lifespan. N=3 (group of 10 flies) for each measurement. Error bars represent +/- SEM. Data was analysed by JMP statistical software. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype has a significant effect, post hoc pairwise comparison was carried out using Student's t-test for 2 groups or Tukey-Kramer HSD test for 3 groups at each timepoint. Significant difference is highlighted with red text colour ($p<0.05$)

A) RU486-elavGS/UAS-InR^{DN} female flies compared to elavGS/UAS-InR^{DN} controls without RU486 in their media.

B) RU486-elavGS/UAS-InR^{DN} female flies compared to RECOVERY RU486-elavGS/UAS-InR^{DN} with 4 days of recovery time off the media containing RU486 before each measurement and elavGS/UAS-InR^{DN} controls without RU486 in their media.

C) RU486-elavGS/UAS-InR^{DN} male flies compared to elavGS/UAS-InR^{DN} controls without RU486 in their media.

D) RU486-elavGS/UAS-InR^{DN} male flies compared to RECOVERY RU486-elavGS/UAS-InR^{DN} with 4 days of recovery time off the media containing RU486 before each measurement and elavGS/UAS-InR^{DN} controls without RU486 in their media.



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.4528	Genotype:	0.4002
Age*Genotype:	0.0961	Age*Genotype:	0.3479

Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.2301	Genotype:	0.1350
Age*Genotype:	0.0642	Age*Genotype:	0.1803

Figure 21 - Effect of RU486 on negative geotaxis senescence

Negative geotaxis performance index of flies over the lifespan. N=3 (group of 10 flies) for each measurement. Error bars represent +/- SEM. Data was analysed by JMP statistical software. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype has a significant effect, post hoc pairwise comparison was carried out using Student's t-test for 2 groups or Tukey-Kramer HSD test for 3 groups at each timepoint. Significant difference is highlighted with red text colour ($p<0.05$)

A) RU486-elavGS/+ female flies compared to elavGS/+ controls without RU486 in their media.

B) RU486-elavGS/+ female flies compared to RECOVERY RU486-elavGS/+ with 4 days of recovery time off the media containing RU486 before each measurement and elavGS/+ controls without RU486 in their media.

C) RU486-elavGS/+ male flies compared to elavGS/+ controls without RU486 in their media.

D) RU486-elavGS/+ male flies compared to RECOVERY RU486-elavGS/+ with 4 days of recovery time off the media containing RU486 before each measurement and elavGS/+ controls without RU486 in their media.

5.2.2: Adult specific pan neural IIS reduction has detrimental effects on exploratory walking decline

The exploratory walking experiment was carried out as described in Chapter 2.12. ElavGS/UAS-InR^{DN} and elavGS/+ flies were separated by gender and sorted onto the appropriate food (with or without 200mM RU486) at 10 flies per vial at the age of 3 days. The exploratory walking experiment was started at 12 pm on each timepoint. The videos were analysed using EthoVision XT video tracking software (Nodus) and the results are shown in **Figure 22** and **Figure 23**.

The experiment investigated the effect of adult specific pan-neural IIS reduction on exploratory walking senescence. The RU486-elavGS/UAS-InR^{DN} group had reduced IIS in its neurons from the age of 3 days throughout their life, while the elavGS/UAS-InR^{DN} control group was maintained on standard food and had no IIS reduction. The elavGS/+ control groups were included to determine if the chemical RU486 itself had any effect on exploratory walking senescence. The RU486-elavGS/+ group shows if the drug itself cause any behavioural changes compared to the elavGS/+ group.

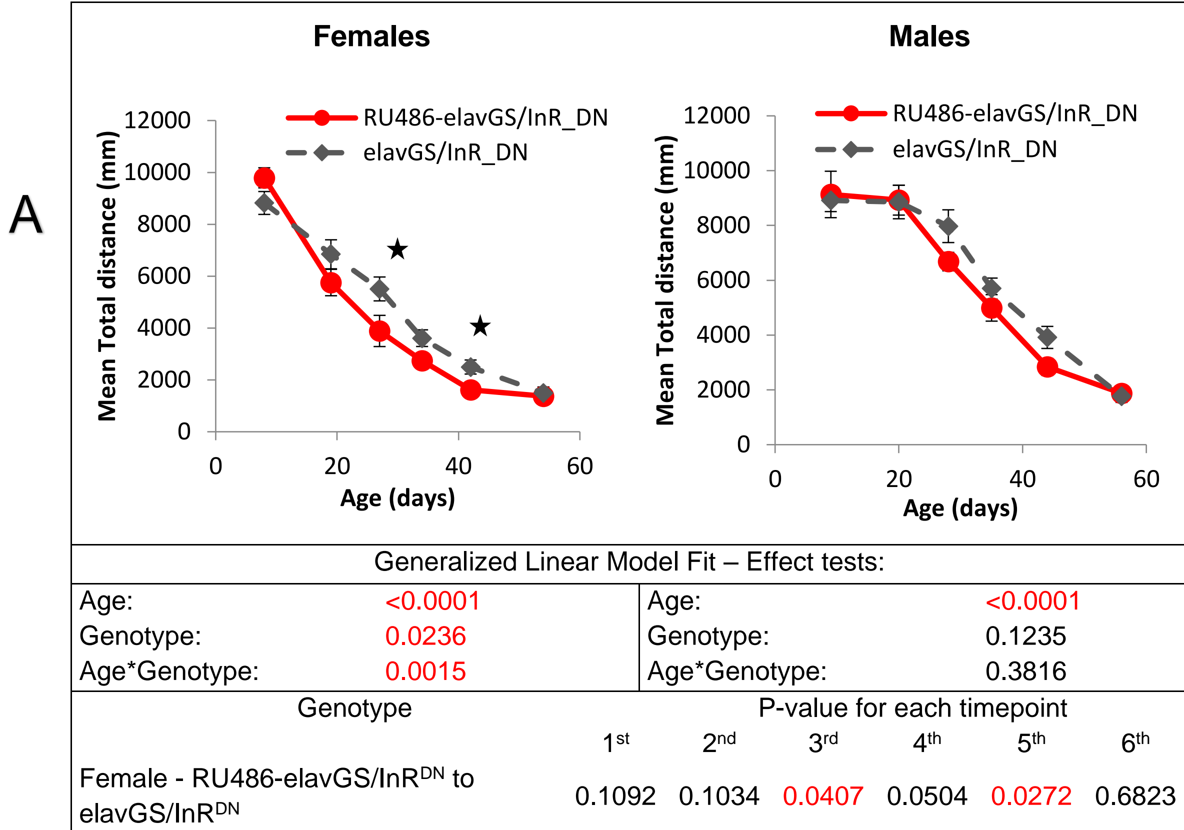
The data in **Figure 22** show that reducing IIS in adult female fly neurons causes detrimental effects on some, but not all exploratory walking parameters, and has no positive effects. It significantly decreased the total distance walked from the age of 27 days along with the walking duration from the age of 19 days. Reduced IIS in adult neurons resulted in detrimental effects on the decline of some parameters such as a lower rotation frequency at 27 days and reduced walking speed. The lack of effect of RU486 on the elavGS/+ control group (**Figure 23**) shows that the changes observed in the elavGS/InR^{DN} groups are due to the expression of the InR^{DN} in adult neurons.

The exploratory walking of the males with adult specific IIS reduction in their neurons (**Figure 22**) was less affected than the behaviour of the females. However, males did show some negative effects on their behaviour at older ages, and similarly to females, no amelioration in the decline of exploratory walking senescence was observed. Around the age of 44 days, males performed total distance walked, duration in central zone, rotation frequency, first rotation time and velocity significantly less well than flies with normal IIS in their neurons. Surprisingly, the drug RU486 itself had some significant effects on male walking behaviour, improving some of the parameters (**Figure 23**). Around the age of 46 days, RU486 improved the following parameters: total distance, walking duration, rotation frequency and velocity. The positive effects of RU486 appear around the same age when the negative effect of IIS reduction become

significant, which suggests that the detrimental effect of reduced IIS in neurons in males is somewhat masked by the positive effect of RU486 on exploratory walking behaviour.

Adult specific pan-neural IIS reduction

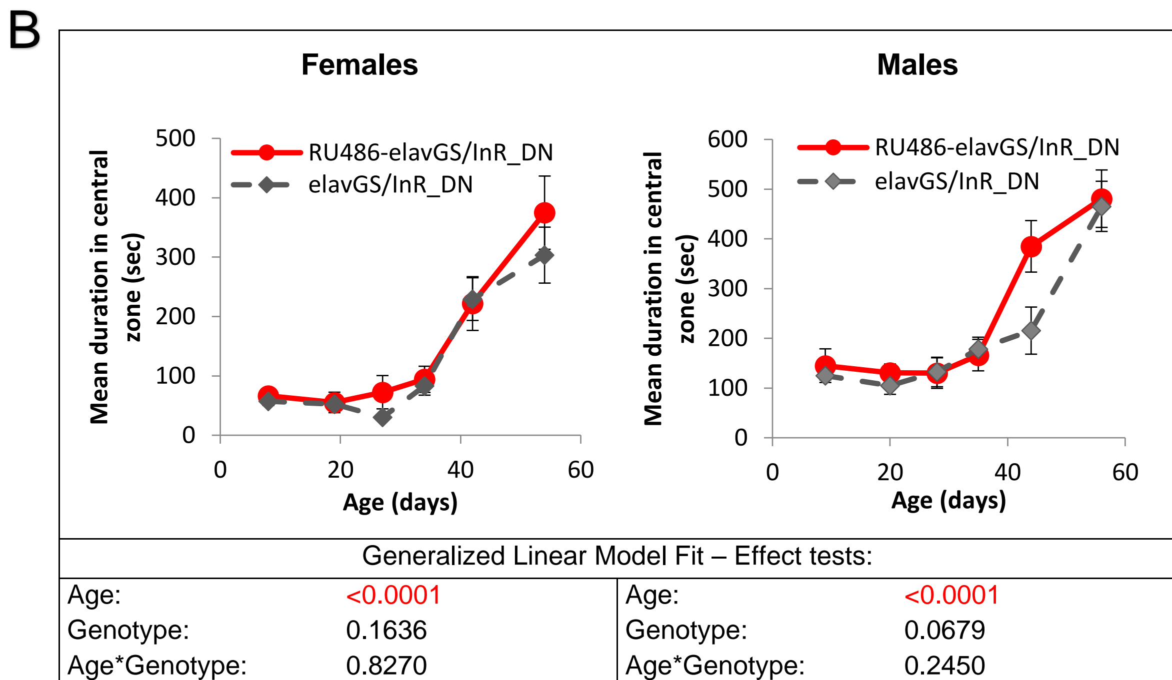
A



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0236	Genotype:	0.1235
Age*Genotype:	0.0015	Age*Genotype:	0.3816

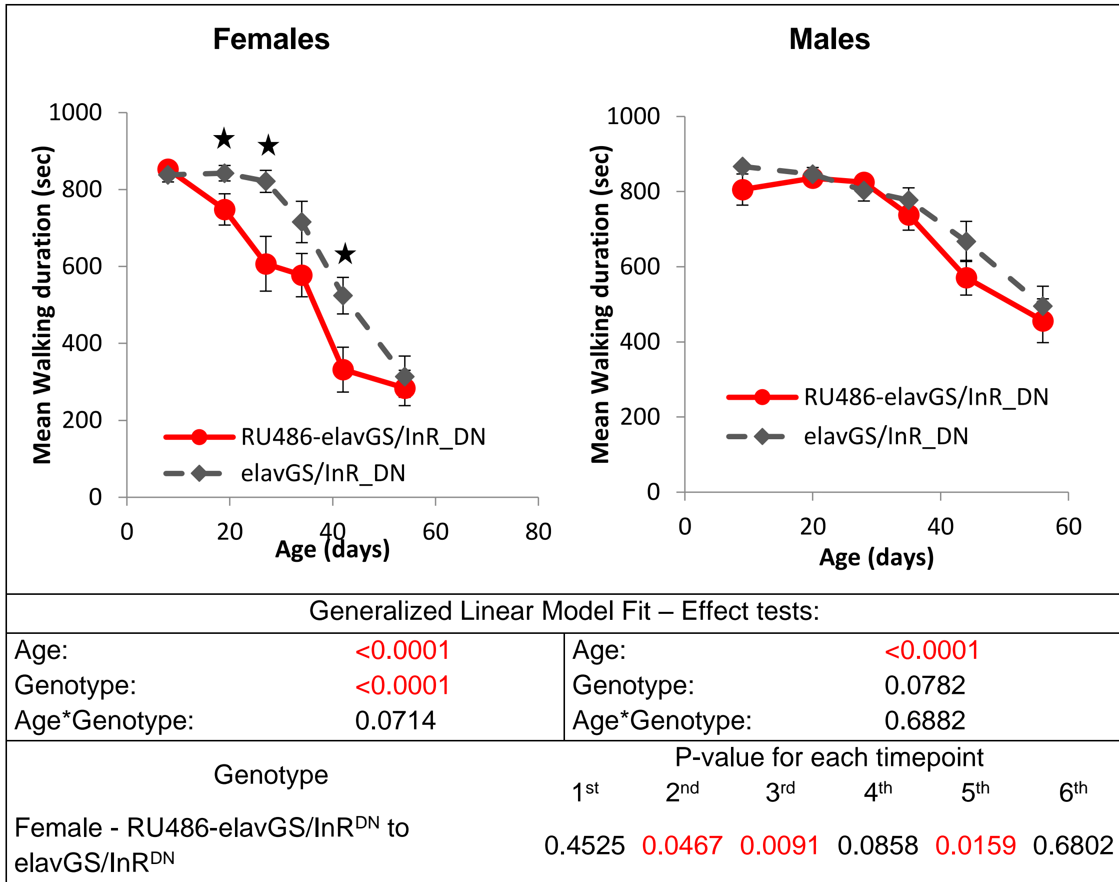
Genotype	P-value for each timepoint					
	1st	2nd	3rd	4th	5th	6th
Female - RU486-elavGS/InR^{DN} to elavGS/InR^{DN}	0.1092	0.1034	0.0407	0.0504	0.0272	0.6823

B



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.1636	Genotype:	0.0679
Age*Genotype:	0.8270	Age*Genotype:	0.2450

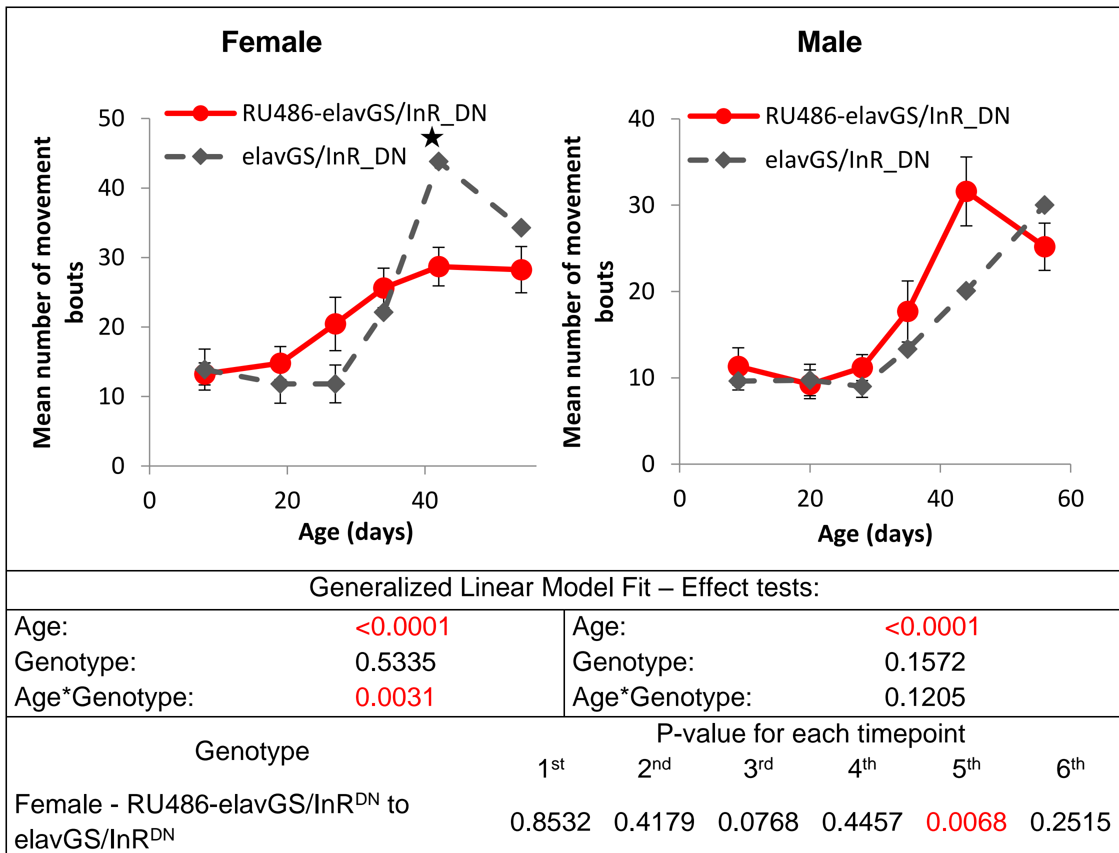
C



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	<0.0001	Genotype:	0.0782
Age*Genotype:	0.0714	Age*Genotype:	0.6882

Genotype	P-value for each timepoint 1st	2nd	3rd	4th	5th	6th
Female - RU486-elavGS/InR^{DN} to elavGS/InR^{DN}	0.4525	0.0467	0.0091	0.0858	0.0159	0.6802

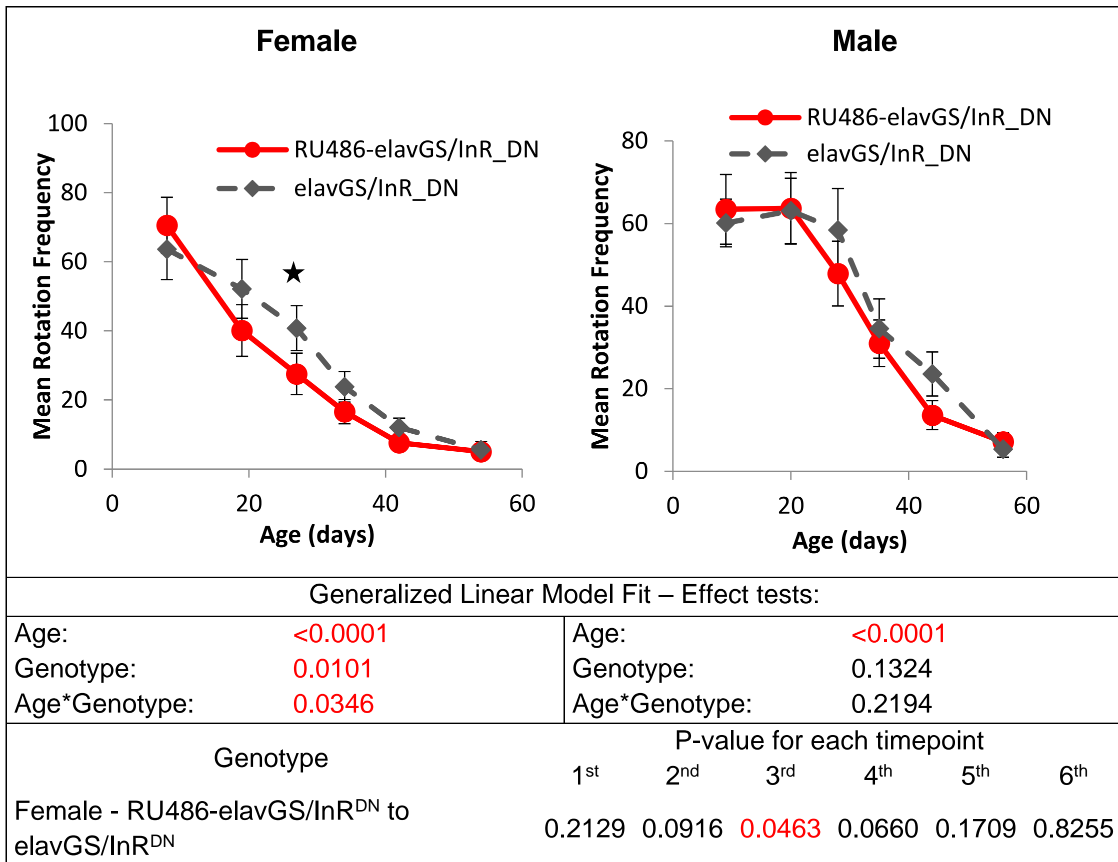
D



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.5335	Genotype:	0.1572
Age*Genotype:	0.0031	Age*Genotype:	0.1205

Genotype	P-value for each timepoint 1st	2nd	3rd	4th	5th	6th
Female - RU486-elavGS/InR^{DN} to elavGS/InR^{DN}	0.8532	0.4179	0.0768	0.4457	0.0068	0.2515

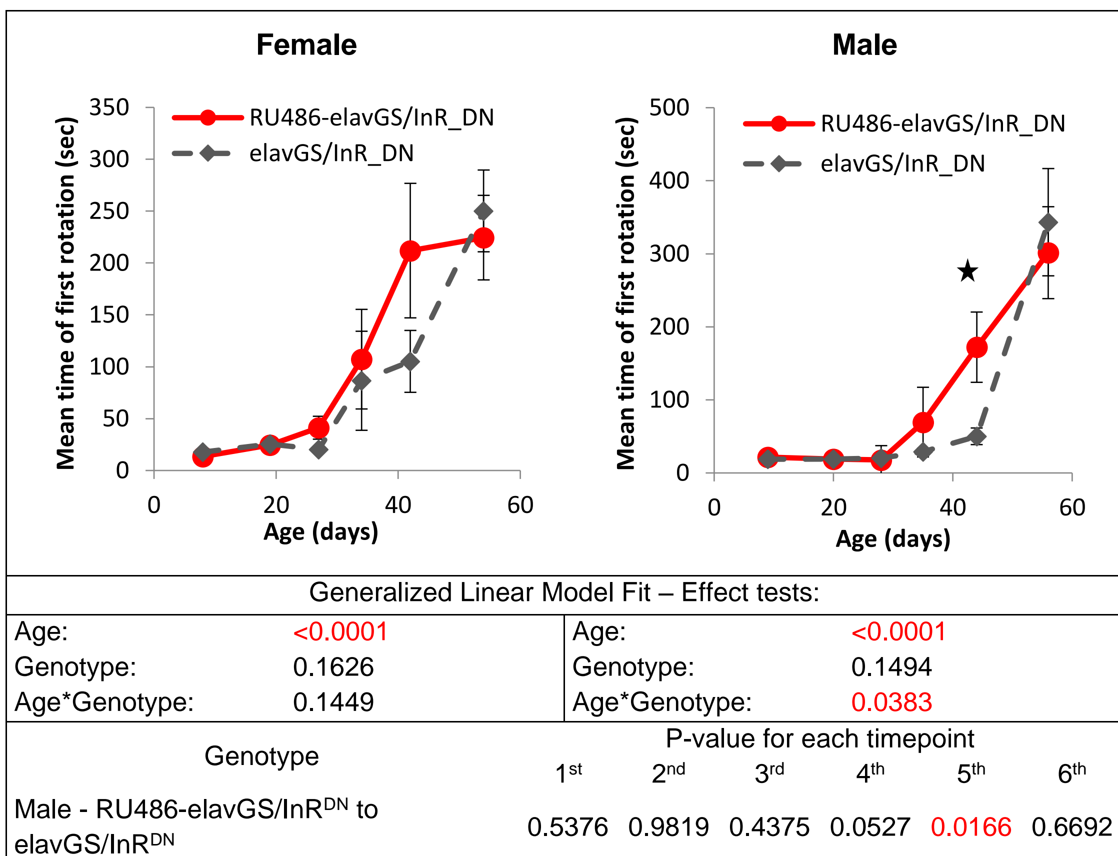
E



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0101	Genotype:	0.1324
Age*Genotype:	0.0346	Age*Genotype:	0.2194

Genotype	P-value for each timepoint 1st	2nd	3rd	4th	5th	6th
Female - RU486-elavGS/InR^{DN} to elavGS/InR^{DN}	0.2129	0.0916	0.0463	0.0660	0.1709	0.8255

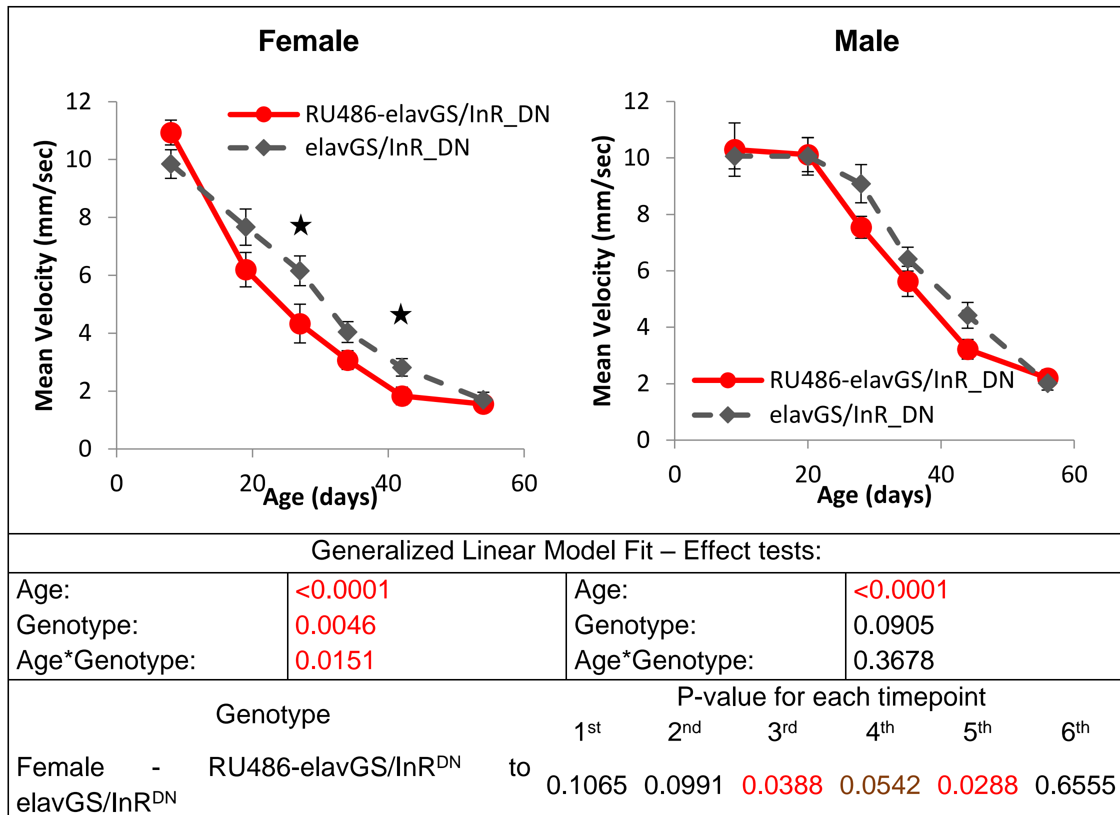
F



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.1626	Genotype:	0.1494
Age*Genotype:	0.1449	Age*Genotype:	0.0383

Genotype	P-value for each timepoint 1st	2nd	3rd	4th	5th	6th
Male - RU486-elavGS/InR^{DN} to elavGS/InR^{DN}	0.5376	0.9819	0.4375	0.0527	0.0166	0.6692

G



Generalized Linear Model Fit – Effect tests:			
Age: Genotype: Age*Genotype:	<0.0001 0.0046 0.0151	Age: Genotype: Age*Genotype:	<0.0001 0.0905 0.3678

Genotype	P-value for each timepoint 1st	2nd	3rd	4th	5th	6th
Female - RU486-elavGS/InR^{DN} to elavGS/InR^{DN}	0.1065	0.0991	0.0388	0.0542	0.0288	0.6555

Figure 22 - The exploratory walking senescence of male and female and flies with pan-neural IIS reduction from the age of 3 days

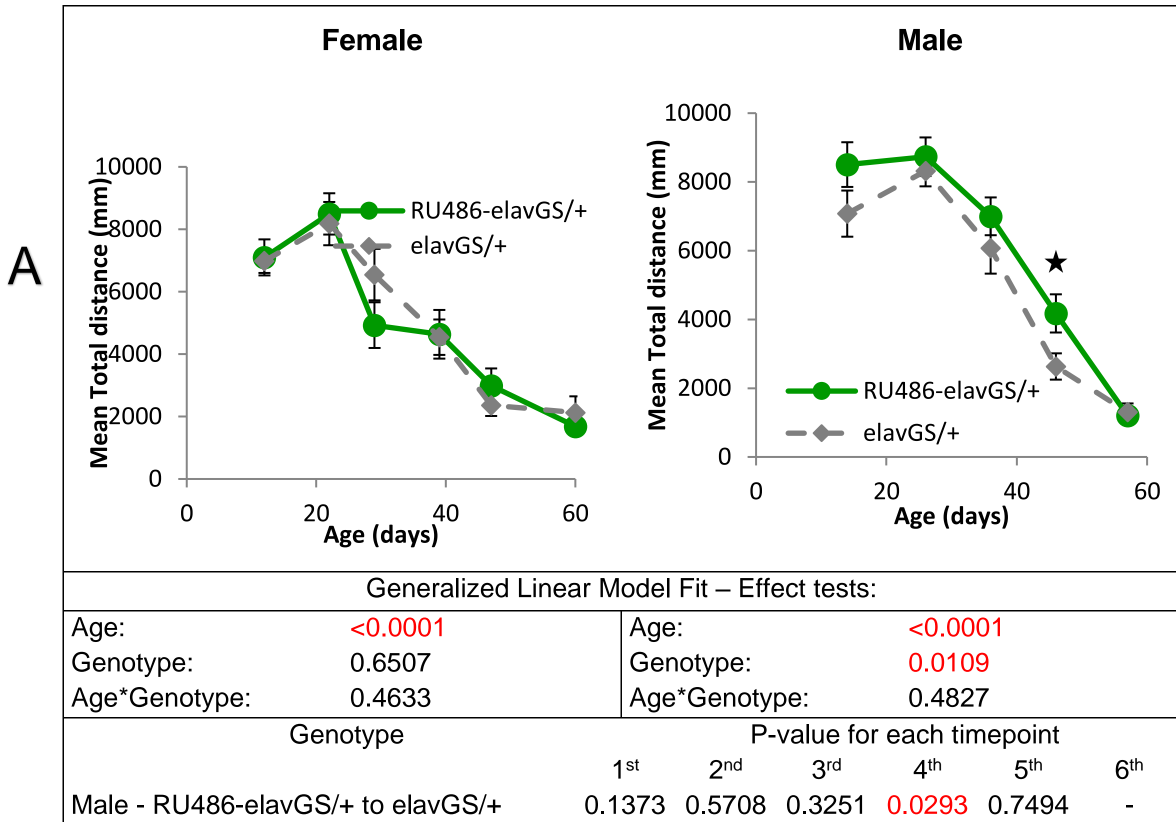
RU486-elavGS/UAS-InR^{DN} group has reduced IIS induced by RU486 from the age of 3 days. The elavGS/UAS-InR^{DN} control group had no RU486 in their media at all. Female data: left column, male data: right column. N=16 for each genotype. Error bars represent +/- SEM.

The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype had a significant effect, post hoc pairwise comparison was carried out using Student's t-test. at each timepoint. Significant difference is highlighted with red text colour ($p<0.05$). On the graphs the black star (★) shows significant difference.

A) Total distance walked over the lifespan **B)** Duration in central zone over the lifespan **C)** Walking duration over the lifespan **D)** Number of movement bouts over the lifespan **E)** Rotation frequency over the lifespan **F)** First rotation time over the lifespan **G)** Velocity over the lifespan

Effect of RU486 on walking behaviour

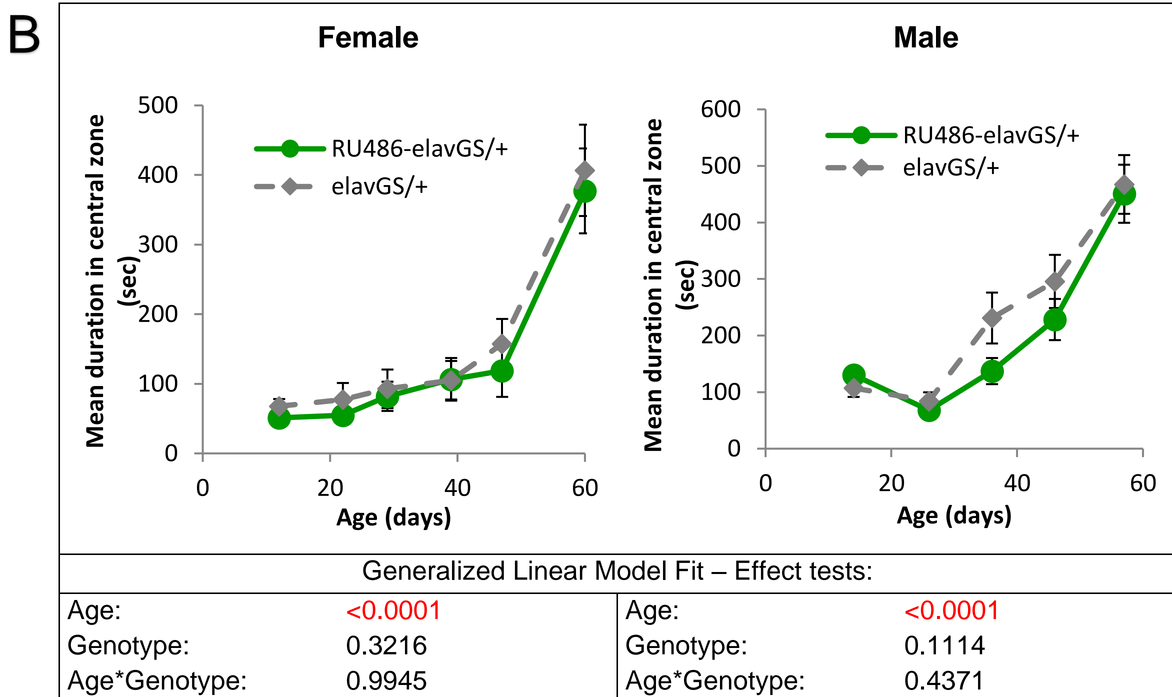
A



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.6507	Genotype:	0.0109
Age*Genotype:	0.4633	Age*Genotype:	0.4827

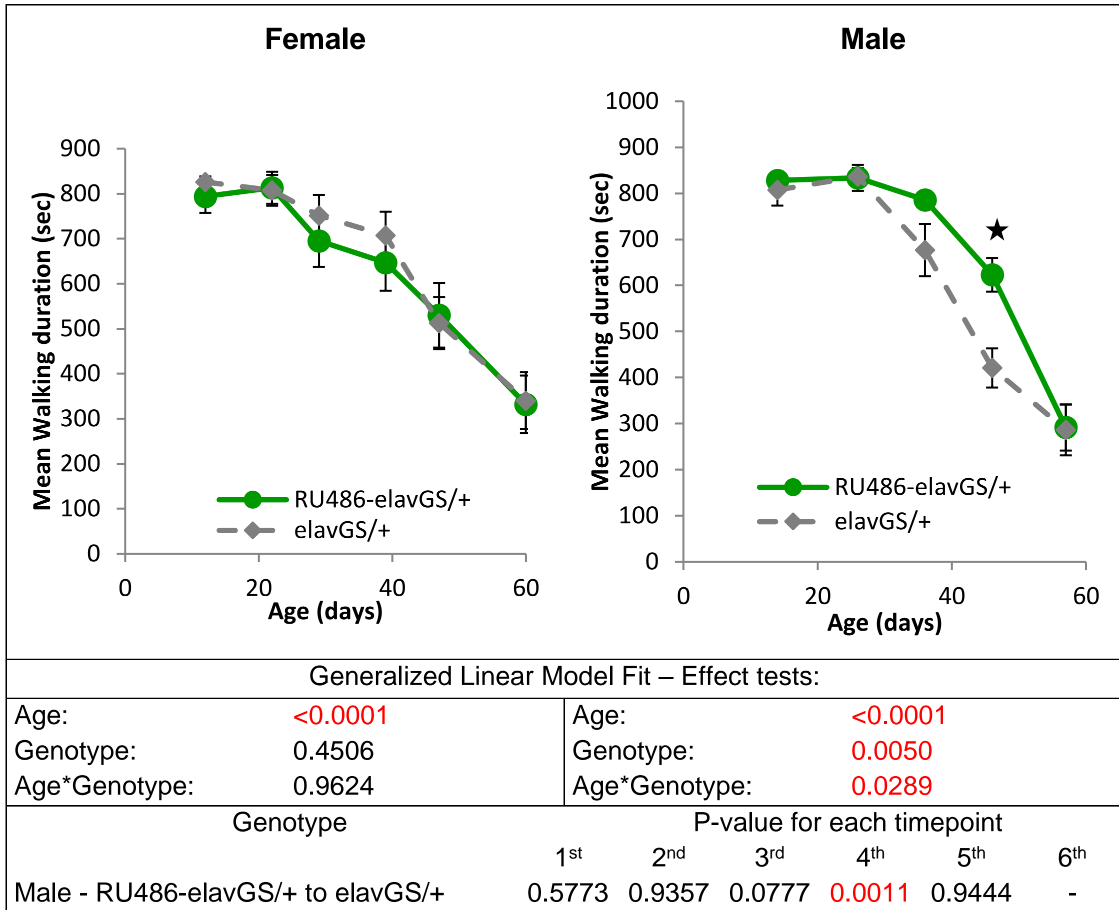
Genotype	P-value for each timepoint					
	1st	2nd	3rd	4th	5th	6th
Male - RU486-elavGS/+ to elavGS/+	0.1373	0.5708	0.3251	0.0293	0.7494	-

B



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.3216	Genotype:	0.1114
Age*Genotype:	0.9945	Age*Genotype:	0.4371

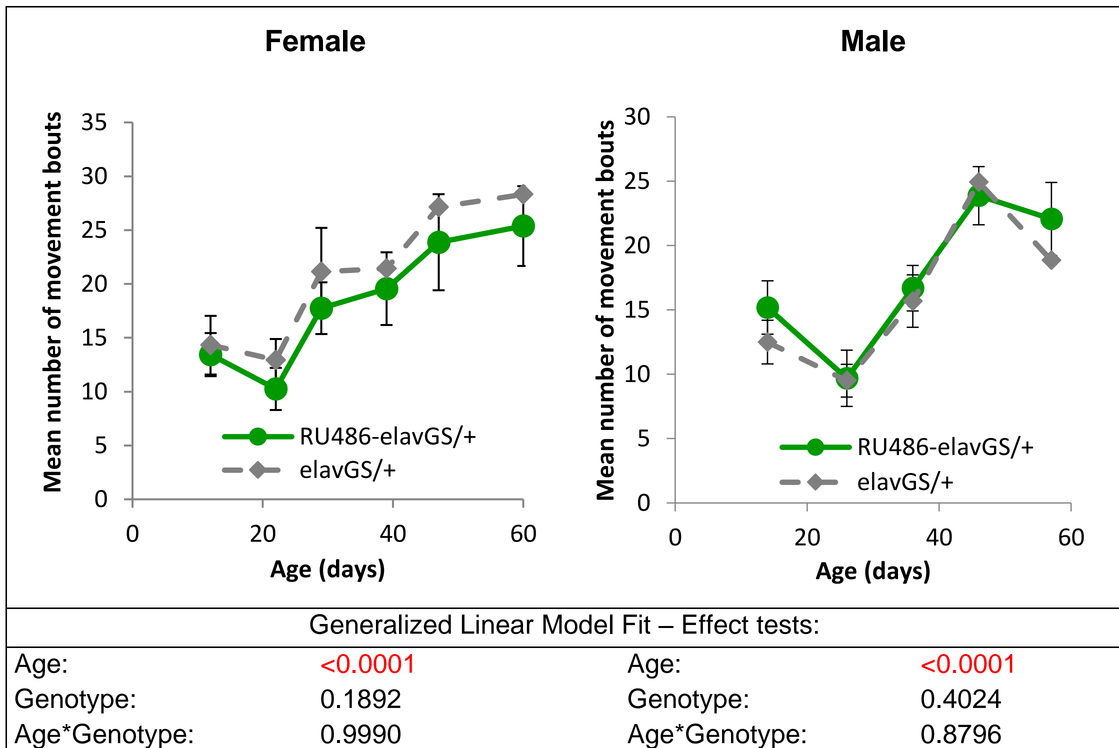
C



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.4506	Genotype:	0.0050
Age*Genotype:	0.9624	Age*Genotype:	0.0289

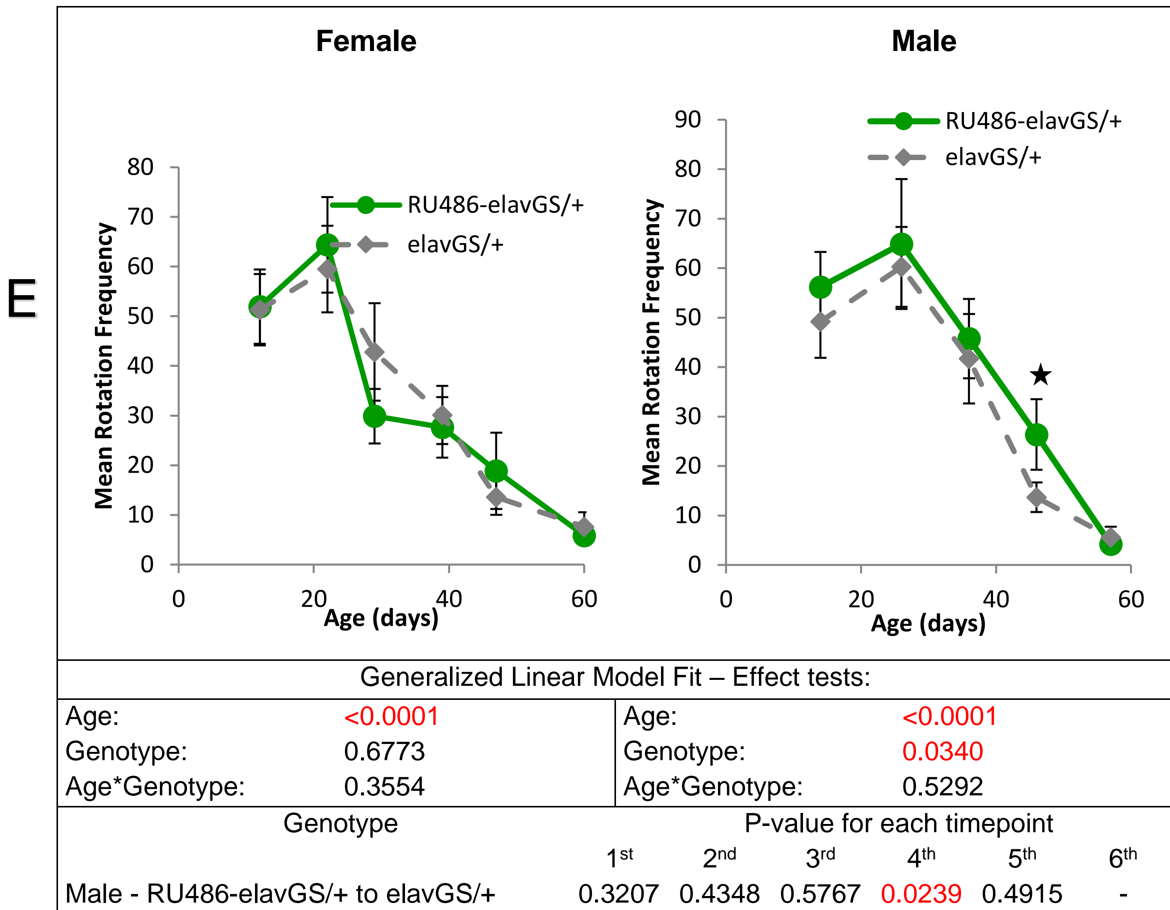
Genotype	P-value for each timepoint					
	1st	2nd	3rd	4th	5th	6th
Male - RU486-elavGS/+ to elavGS/+	0.5773	0.9357	0.0777	0.0011	0.9444	-

D



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.1892	Genotype:	0.4024
Age*Genotype:	0.9990	Age*Genotype:	0.8796

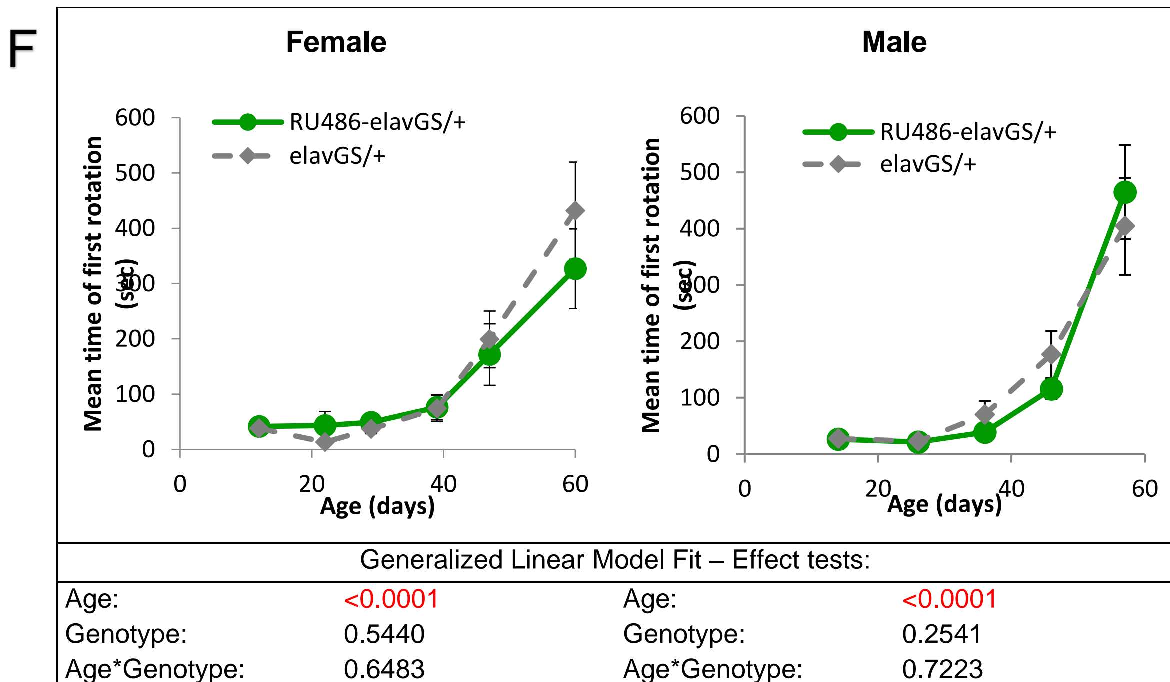
E



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.6773	Genotype:	0.0340
Age*Genotype:	0.3554	Age*Genotype:	0.5292

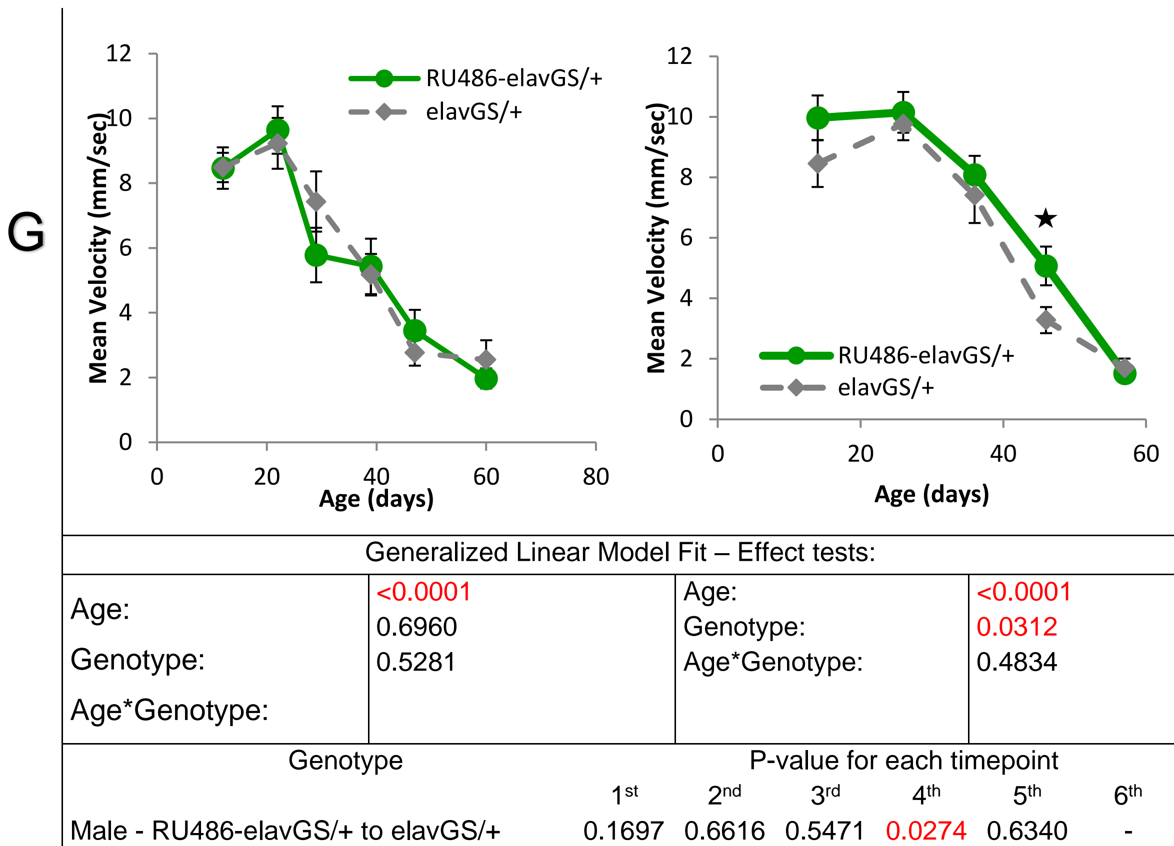
Genotype	P-value for each timepoint					
	1st	2nd	3rd	4th	5th	6th
Male - RU486-elavGS/+ to elavGS/+	0.3207	0.4348	0.5767	0.0239	0.4915	-

F



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.5440	Genotype:	0.2541
Age*Genotype:	0.6483	Age*Genotype:	0.7223

Female Male



Generalized Linear Model Fit – Effect tests:			
Age: Genotype: Age*Genotype:	<0.0001 0.6960 0.5281	Age: Genotype: Age*Genotype:	<0.0001 0.0312 0.4834

Genotype	P-value for each timepoint					
	1st	2nd	3rd	4th	5th	6th
Male - RU486-elavGS/+ to elavGS/+	0.1697	0.6616	0.5471	0.0274	0.6340	-

Figure 23 - The effect of RU486 on the exploratory walking senescence of female and male flies

RU486-elavGS/+ group has reduced IIS induced by RU486 from the age of 3 days. The elavGS/+ control group had no RU486 in their media at all. Female data: left column, male data: right column. N=16 for each genotype. Error bars represent +/- SEM.

The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype had a significant effect, post hoc pairwise comparison was carried out using Student's t-test. at each timepoint. Significant difference is highlighted with red text colour ($p<0.05$). On the graphs the black star (★) shows significant difference.

A) Total distance walked over the lifespan **B)** Duration in central zone over the lifespan **C)** Walking duration over the lifespan **D)** Number of movement bouts over the lifespan **E)** Rotation frequency over the lifespan **F)** First rotation time over the lifespan **G)** Velocity over the lifespan

5.2.3: The senescence of exploratory walking in female flies can recover from the detrimental effects of reduced pan-neural IIS

The exploratory walking experiment was repeated introducing two recovery groups to determine if flies can recover from the negative effects of reduced IIS in their neurons. The RU486-elavGS/UAS-InR^{DN} group was maintained on media containing 200mM RU486. The recovery groups were transferred to standard food 3 or 7 days before each exploratory walking timepoint. The elavGS/UAS-InR^{DN} control group was maintained on standard food throughout their whole life. The exploratory walking

experiment was carried out the same way as previously described, starting the first video at 12 pm on each timepoint. The RU486-elavGS/+ group was again included to determine if the drug itself caused any behavioural changes compared to the elavGS/+ group.

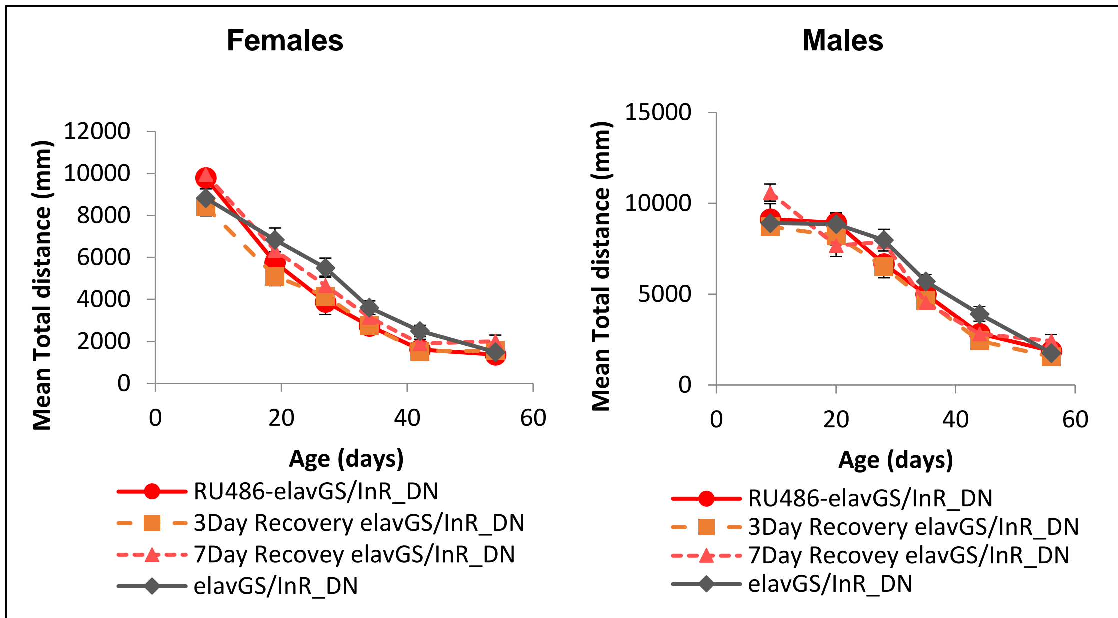
The statistical analysis used the Generalised Linear Model fit to identify significant effects of Genotype and/or Age. Post hoc pairwise comparisons of means were carried out using Dunnett's Method for each timepoint, in order to compare the control groups (elavGS/UAS-InR^{DN} or elavGS/+ without RU486) to each RU486 treated group.

The previous experiment showed that pan-neural adult IIS reduction caused significantly worsened decline in female flies in the total distance walked, walking duration, rotation frequency and velocity parameters. In this experiment, the walking duration and velocity parameters showed significant difference between RU486-elavGS/UAS-InR^{DN} and the elavGS/ UAS-InR^{DN} control, and close to significant effects for total distance ($p=0.0520$ at the age of 27 days and $p=0.0507$ at 42 days) and rotation frequency ($p=0.0646$ at the age of 27 days) (**Figure 24**). RU486-elavGS/UAS-InR^{DN} females removed from RU486 treatment for 7 days prior to each behavioural test time-point showed no significant difference to the elavGS/UAS-InR^{DN} control in some of their exploratory walking behaviour, indicating that females can recover from the detrimental effects of InR^{DN} expression. Female flies allowed to recover for only 3 days from RU486 treatment, however, showed some significant differences to the elavGS/UAS-InR^{DN} control, indicating that 3 days was not sufficient time to recover.

RU486-elavGS/UAS-InR^{DN} males showed detrimental effects for total distance, rotation frequency and velocity compared to the elavGS/UAS-InR^{DN} control, but unlike females, male flies removed from RU486 treatment did not recover behavioural function (**Figure 24**). The recovery control experiment with the elavGS/+ flies showed no significant effect on any of the time points apart from male walking duration at age 46 days, where RU486 improved the function of the flies and the improvement was lost after 3 or 7 days of recovery (**Figure 25**).

Adult specific pan-neural IIS reduction with recovery

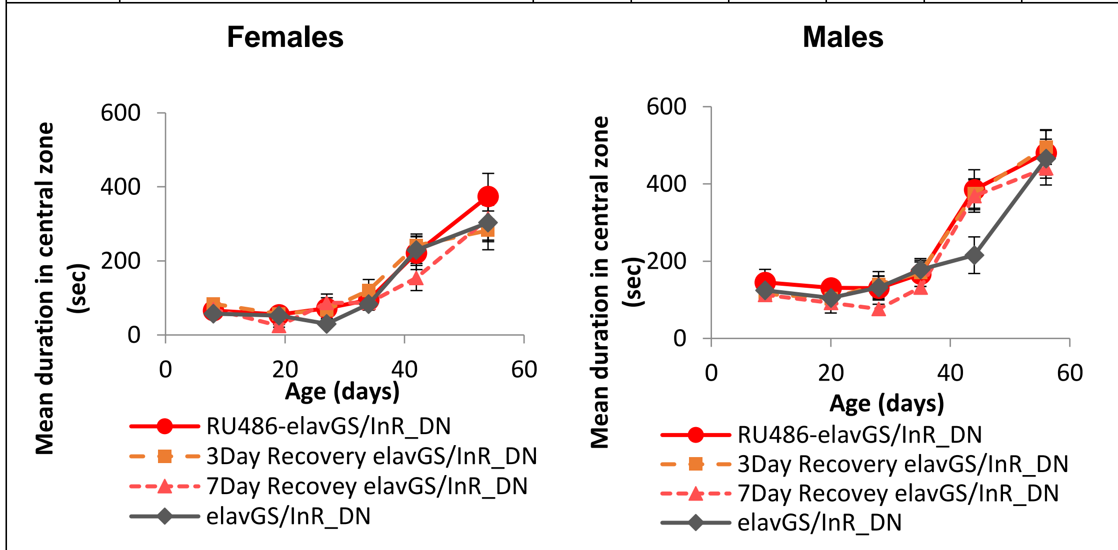
A



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	<0.0001	Genotype:	0.0074
Age*Genotype:	0.0866	Age*Genotype:	0.0452

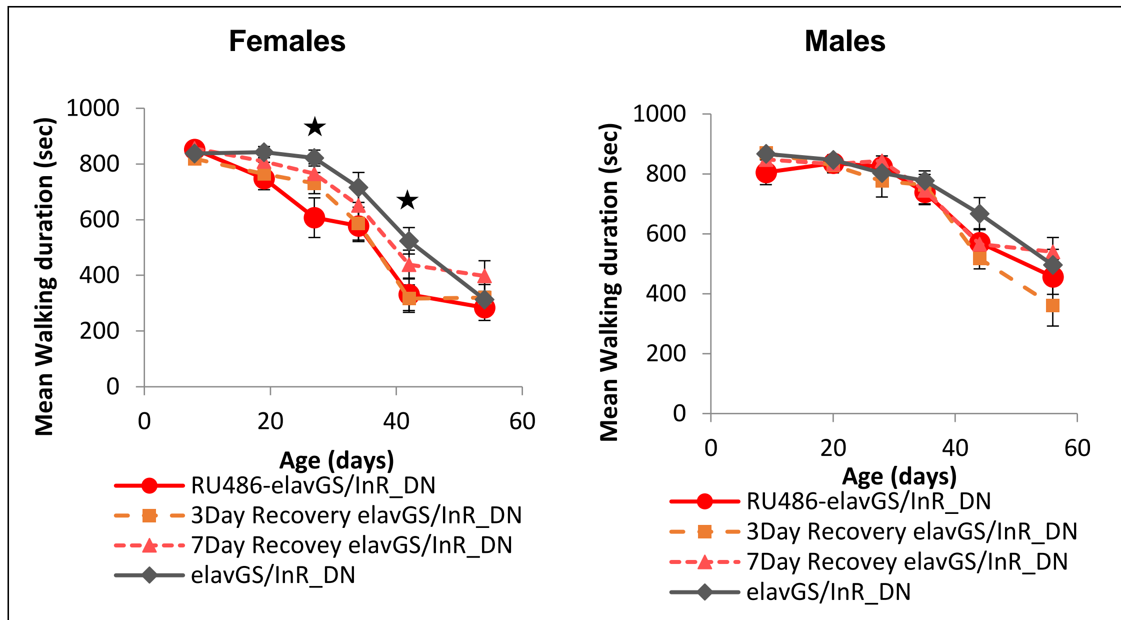
Dunnett's Method using elavGS/InR^{DN} as control		P-value for each timepoint 1st	2nd	3rd	4th	5th	6th
Female	To 7 day Recovery elavGS/InR^{DN}	0.1003	0.8296	0.4661	0.6262	0.2500	0.2766
	To 3 day Recovery elavGS/InR^{DN}	0.7927	0.0566	0.1253	0.1537	0.0319	0.9972
	To RU486 elavGS/InR^{DN}	0.1860	0.1986	0.0520	0.1524	0.0507	0.9660
Male	To 7 day Recovery elavGS/InR^{DN}	0.0946	0.3710	0.9977	0.1019	0.0581	0.2584
	To 3 day Recovery elavGS/InR^{DN}	0.9883	0.7778	0.0955	0.1822	0.0060	0.9195
	To RU486 elavGS/InR^{DN}	0.9854	0.9996	0.1572	0.4517	0.0678	0.9869

B



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.3379	Genotype:	0.1095
Age*Genotype:	0.7353	Age*Genotype:	0.2587

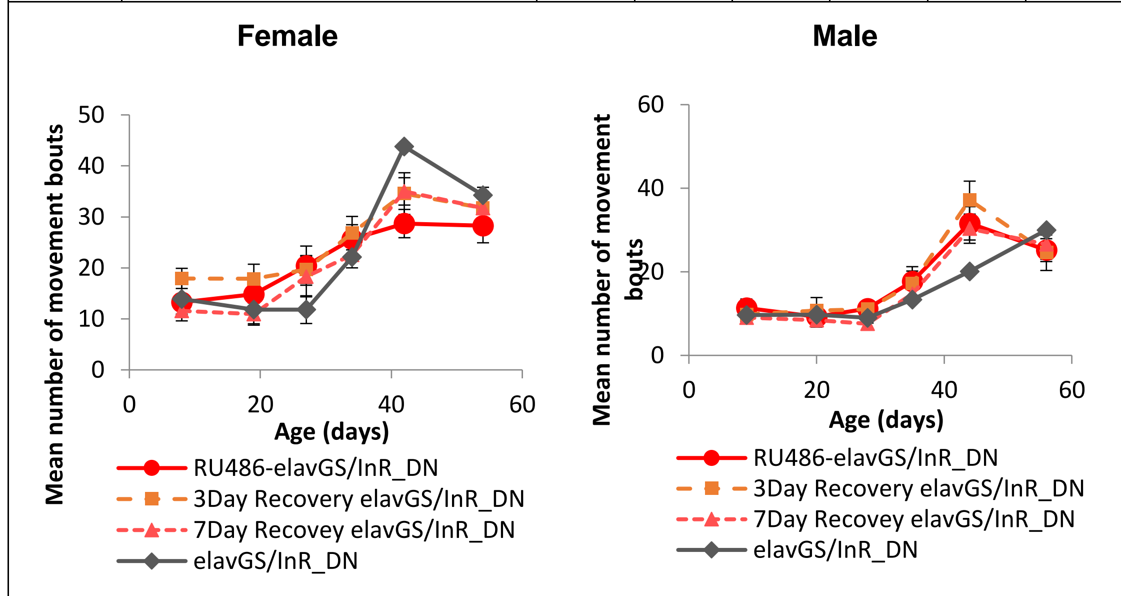
C



Generalized Linear Model Fit – Effect tests:			
Age: Genotype: Age*Genotype:	<0.0001 <0.0001 0.3939	Age: Genotype: Age*Genotype:	<0.0001 0.0462 0.3192

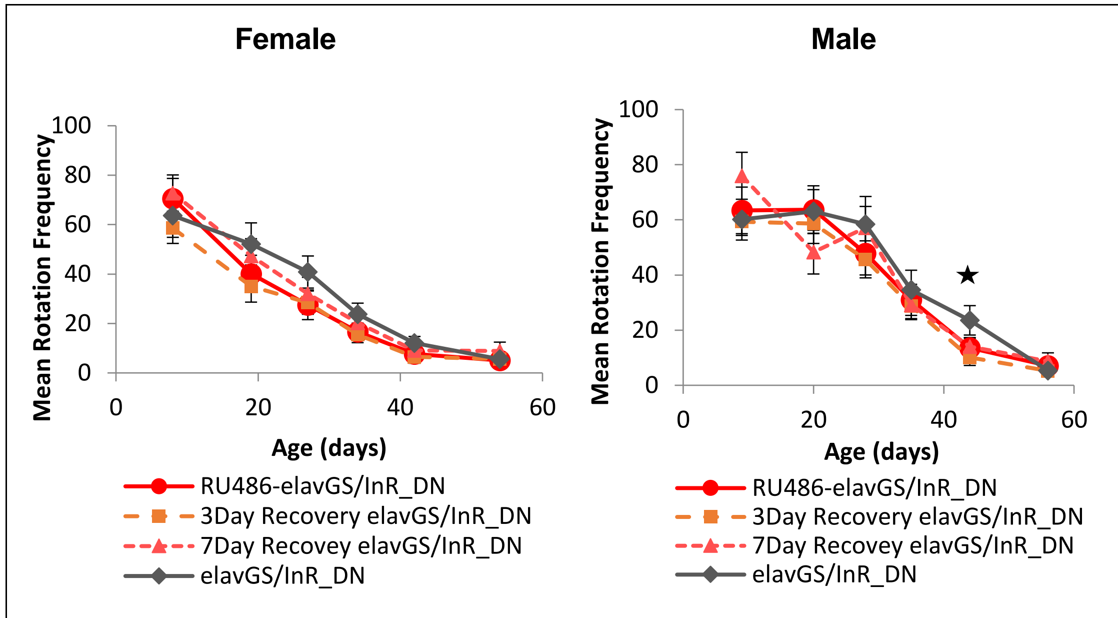
Dunnett's Method using elavGS/InR^{DN} as control		P-value for each timepoint					
		1st	2nd	3rd	4th	5th	6th
Female	To 7 day Recovery elavGS/InR^{DN}	0.6589	0.8606	0.7255	0.7678	0.5158	0.5293
	To 3 day Recovery elavGS/InR^{DN}	0.5908	0.3314	0.3834	0.2651	0.0172	0.9994
	To RU486 elavGS/InR^{DN}	0.7677	0.1988	0.0050	0.3316	0.0296	0.9592
Male	To 7 day Recovery elavGS/InR^{DN}	0.8986	0.9436	0.6933	0.8880	0.2836	0.9085
	To 3 day Recovery elavGS/InR^{DN}	0.9994	0.8762	0.8776	0.9906	0.0664	0.2529
	To RU486 elavGS/InR^{DN}	0.1664	0.9673	0.9304	0.8228	0.3401	0.9363

D



Generalized Linear Model Fit – Effect tests:			
Age: Genotype: Age*Genotype:	<0.0001 0.2638 0.0908	Age: Genotype: Age*Genotype:	<0.0001 0.2260 0.1004

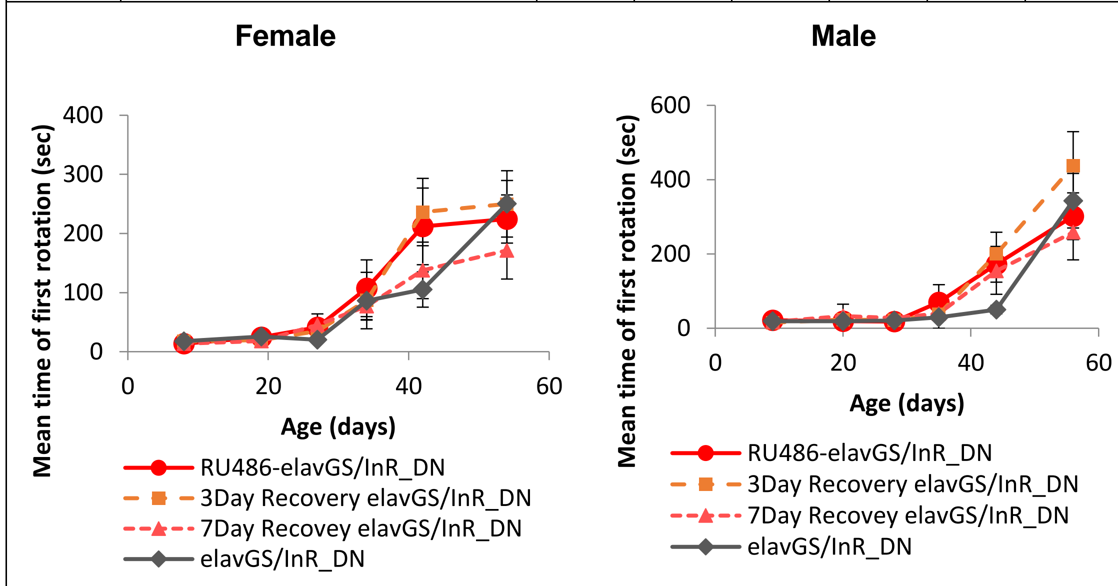
E



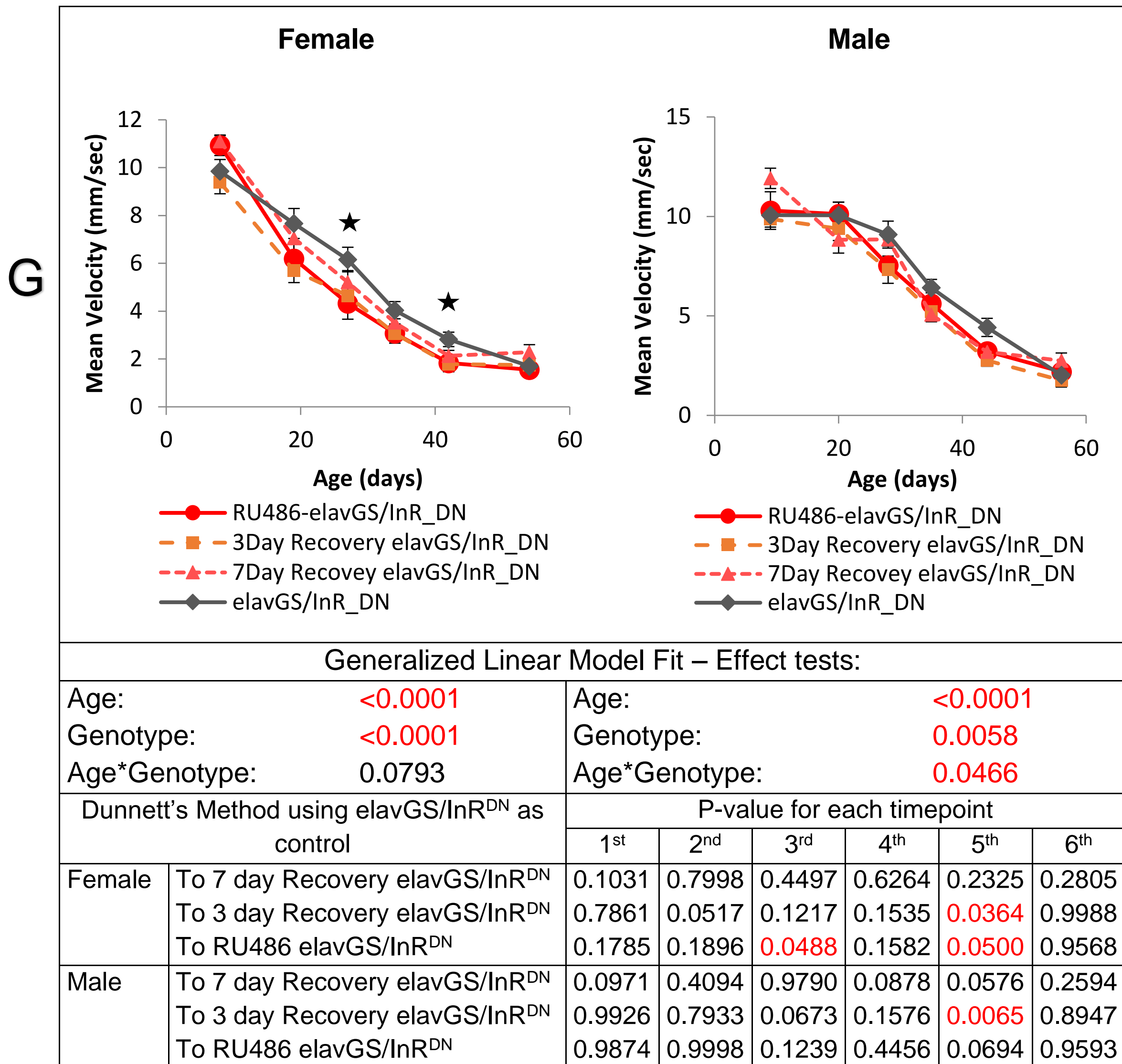
Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	<0.0001	Genotype:	0.0138
Age*Genotype:	0.0905	Age*Genotype:	0.0023

Dunnett's Method using elavGS/InR^{DN} as control		P-value for each timepoint 1st	2nd	3rd	4th	5th	6th
Female	To 7 day Recovery elavGS/InR^{DN}	0.1737	0.7885	0.3047	0.6685	0.6183	0.4426
	To 3 day Recovery elavGS/InR^{DN}	0.6155	0.0256	0.0908	0.0861	0.1658	1.0000
	To RU486 elavGS/InR^{DN}	0.3720	0.1582	0.0646	0.1633	0.3153	0.9950
Male	To 7 day Recovery elavGS/InR^{DN}	0.0267	0.1137	0.9911	0.5132	0.0312	0.4295
	To 3 day Recovery elavGS/InR^{DN}	0.9982	0.8766	0.0694	0.2438	0.0015	0.9999
	To RU486 elavGS/InR^{DN}	0.9008	0.9995	0.1586	0.7858	0.0127	0.8337

F



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.1414	Genotype:	0.1892
Age*Genotype:	0.3876	Age*Genotype:	0.1566



Generalized Linear Model Fit – Effect tests:								
Age:	<0.0001		Age:			<0.0001		
Genotype:	<0.0001		Genotype:			0.0058		
Age*Genotype:	0.0793		Age*Genotype:			0.0466		
Dunnett's Method using elavGS/InR^{DN} as control			P-value for each timepoint					
			1st	2nd	3rd	4th	5th	6th
Female	To 7 day Recovery elavGS/InR^{DN}		0.1031	0.7998	0.4497	0.6264	0.2325	0.2805
	To 3 day Recovery elavGS/InR^{DN}		0.7861	0.0517	0.1217	0.1535	0.0364	0.9988
	To RU486 elavGS/InR^{DN}		0.1785	0.1896	0.0488	0.1582	0.0500	0.9568
Male	To 7 day Recovery elavGS/InR^{DN}		0.0971	0.4094	0.9790	0.0878	0.0576	0.2594
	To 3 day Recovery elavGS/InR^{DN}		0.9926	0.7933	0.0673	0.1576	0.0065	0.8947
	To RU486 elavGS/InR^{DN}		0.9874	0.9998	0.1239	0.4456	0.0694	0.9593

Figure 24 - The effect of inducible pan-neural IIS reduction on male and female flies with 3- and 7-day recovery time from reduced IIS

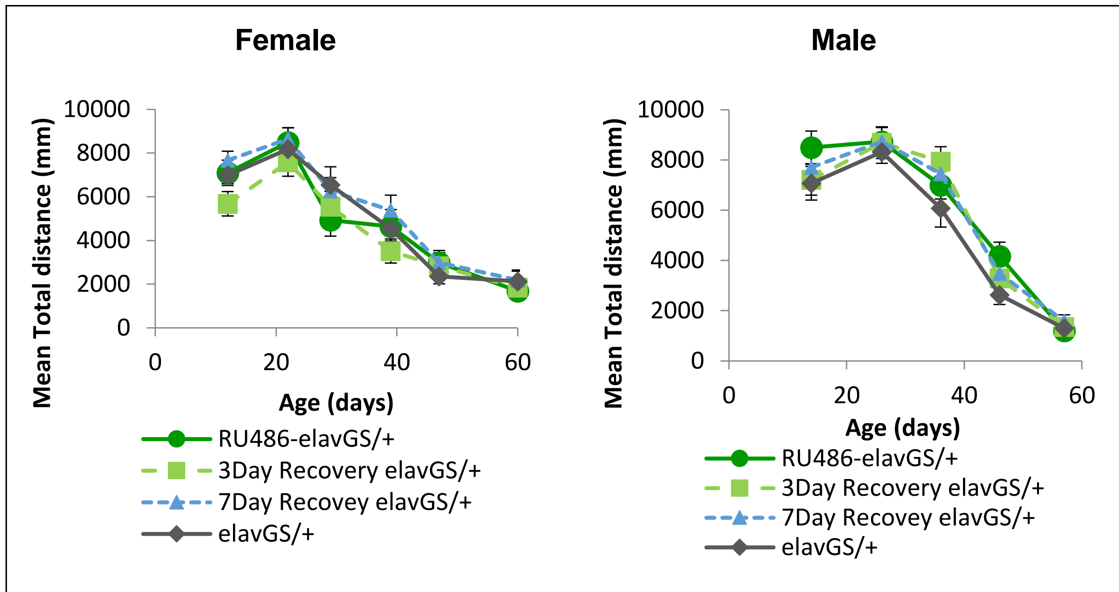
RU486-elavGS/UAS-InR^{DN} group had reduced IIS induced by RU486 from the age of 3 days. The 3 and 7 day recovery flies were removed from the RU486 food 3 or 7 days before the experiment and kept on standard food. The elavGS/UAS-InR^{DN} control group had no RU486 in their media at all. Female data: left column, male data: right column. N=16 for each genotype. Error bars represent +/- SEM.

The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype had a significant effect, the elavGS/InR^{DN} control group was compared to the other 3 groups using Dunnett's Method at each timepoint. Significant difference is highlighted with red text colour ($p<0.05$). On the graphs the black star (★) shows significant difference

A) Total distance walked over the lifespan **B)** Duration in central zone over the lifespan **C)** Walking duration over the lifespan **D)** Number of movement bouts over the lifespan **E)** Rotation frequency over the lifespan **F)** First rotation time over the lifespan **G)** Velocity over the lifespan

Effect of RU486 on walking behaviour with recovery

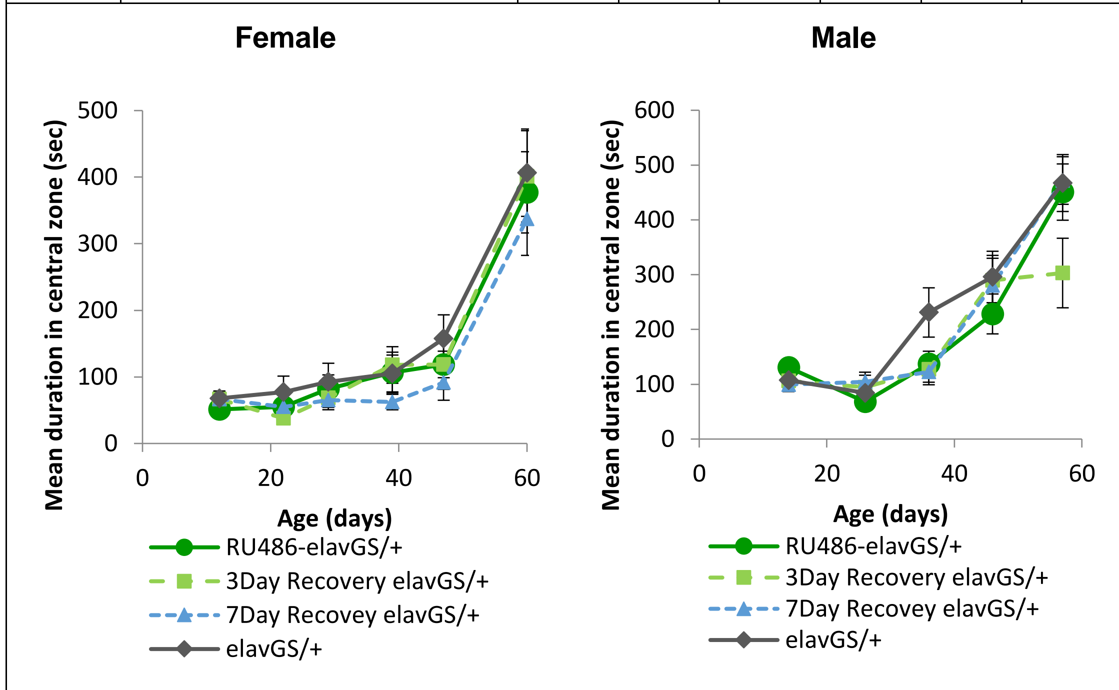
A



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0171	Genotype:	0.0580
Age*Genotype:	0.7769	Age*Genotype:	0.7212

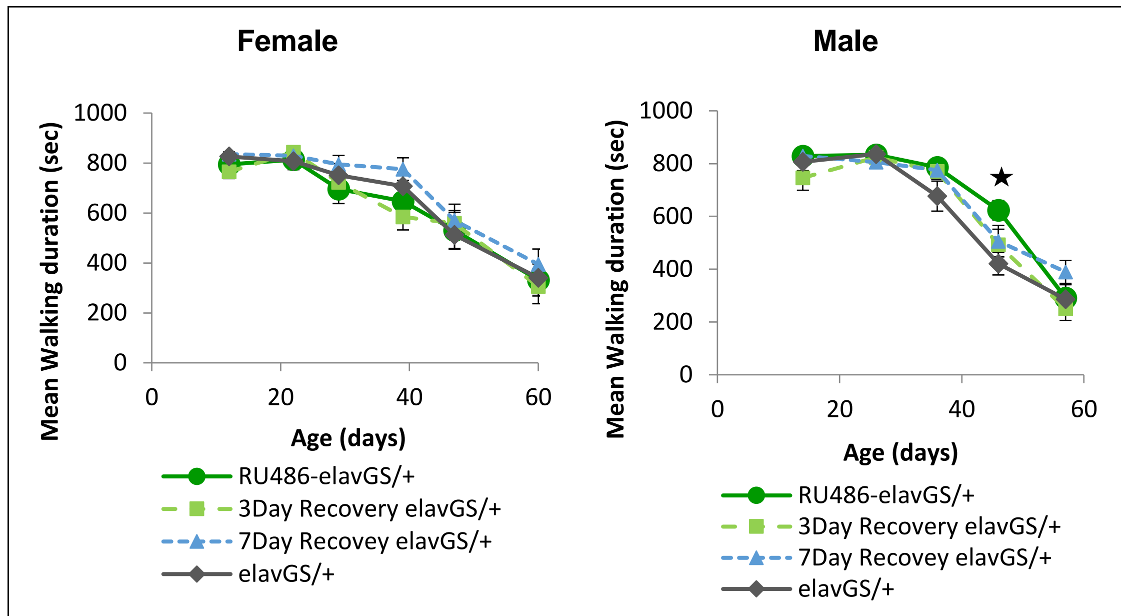
Dunnett's Method using elavGS/+ as control		P-value for each timepoint					
		1st	2nd	3rd	4th	5th	6th
Female	To 7 day Recovery elavGS/+	0.6422	0.9160	0.9850	0.6922	0.6616	0.9993
	To 3 day Recovery elavGS/+	0.1612	0.8409	0.6444	0.5523	0.8175	0.9275
	To RU486 elavGS/+	0.9968	0.9721	0.2732	0.9993	0.6543	0.7867

B



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.2162	Genotype:	0.1041
Age*Genotype:	0.9975	Age*Genotype:	0.1168

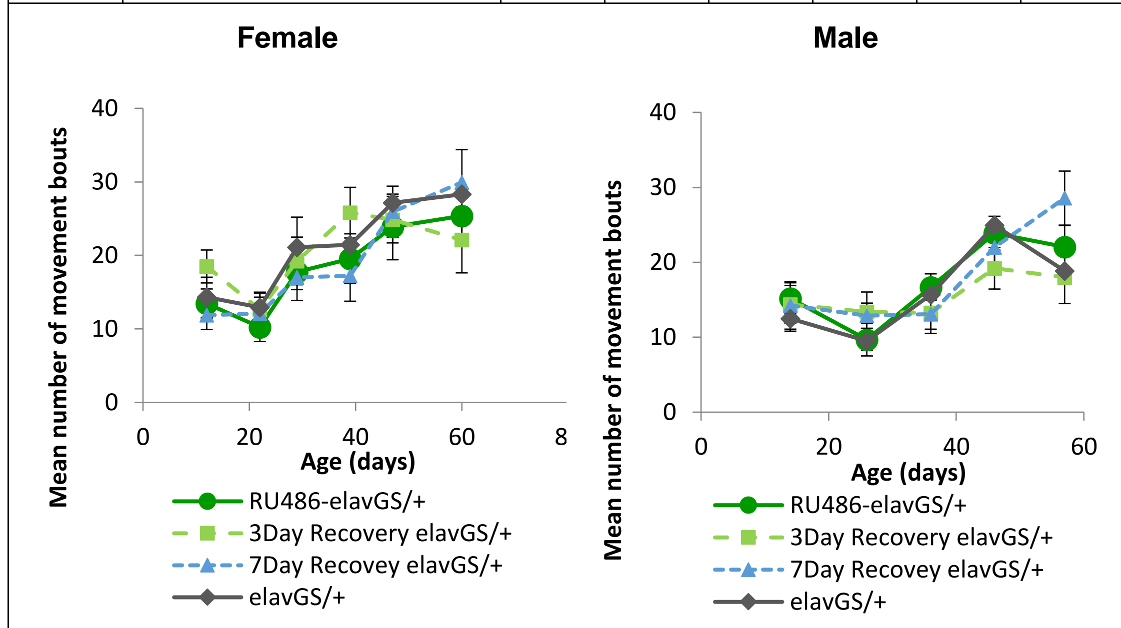
C



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0474	Genotype:	0.0128
Age*Genotype:	0.9409	Age*Genotype:	0.0910

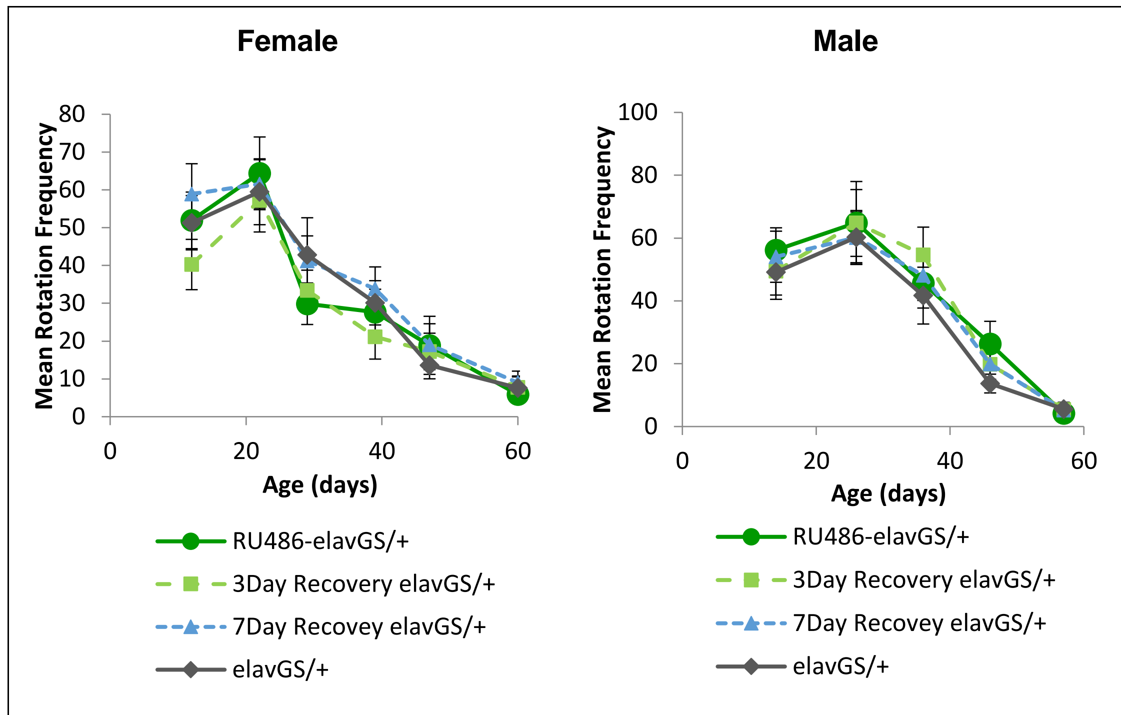
Dunnett's Method using elavGS/+ as control		P-value for each timepoint 1st	2nd	3rd	4th	5th	6th
Female	To 7 day Recovery elavGS/+	0.9835	0.9072	0.8560	0.8575	0.6938	0.8816
	To 3 day Recovery elavGS/+	0.1812	0.6840	0.9526	0.9239	0.2605	0.9669
	To RU486 elavGS/+	0.6752	0.9979	0.7366	0.9947	0.7702	0.9995
Male	To 7 day Recovery elavGS/+	0.9325	0.6027	0.1641	0.4881	0.3491	
	To 3 day Recovery elavGS/+	0.4098	0.9551	0.1947	0.6270	0.9356	-
	To RU486 elavGS/+	0.9382	0.9994	0.1080	0.0163	0.9991	

D



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.4794	Genotype:	0.2186
Age*Genotype:	0.8798	Age*Genotype:	0.0985

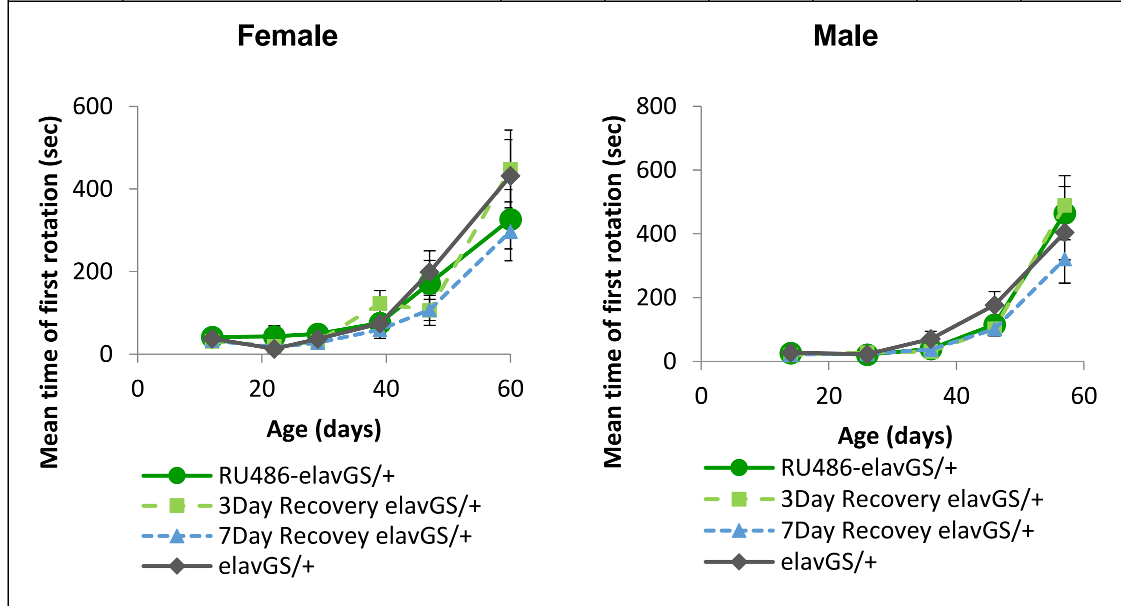
E



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0170	Genotype:	0.1653
Age*Genotype:	0.5551	Age*Genotype:	0.7239

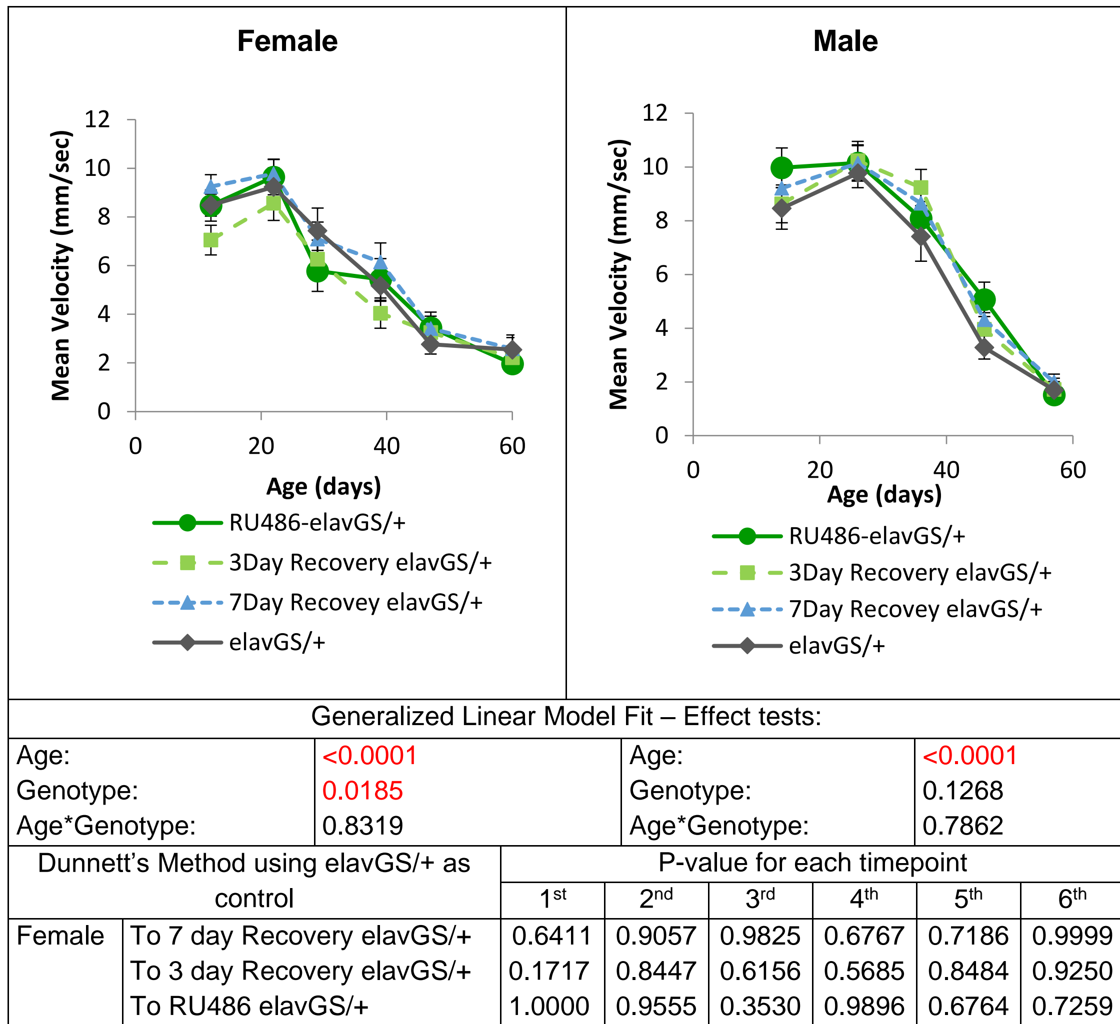
Dunnett's Method using elavGS/+ as control		P-value for each timepoint					
		1st	2nd	3rd	4th	5th	6th
Female	To 7 day Recovery elavGS/+	0.4707	0.9824	0.9925	0.9015	0.6489	0.9527
	To 3 day Recovery elavGS/+	0.1845	0.9756	0.4439	0.3955	0.8544	1.0000
	To RU486 elavGS/+	0.9992	0.8253	0.2003	0.9654	0.6644	0.9099

F



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.3542	Genotype:	0.2021
Age*Genotype:	0.5599	Age*Genotype:	0.9606

G



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0185	Genotype:	0.1268
Age*Genotype:	0.8319	Age*Genotype:	0.7862

Dunnett's Method using elavGS/+ as control		P-value for each timepoint					
		1st	2nd	3rd	4th	5th	6th
Female	To 7 day Recovery elavGS/+	0.6411	0.9057	0.9825	0.6767	0.7186	0.9999
	To 3 day Recovery elavGS/+	0.1717	0.8447	0.6156	0.5685	0.8484	0.9250
	To RU486 elavGS/+	1.0000	0.9555	0.3530	0.9896	0.6764	0.7259

Figure 25 - The effect RU486 on male and female flies with 3 and 7 day recovery time off RU486

RU486-elavGS/+ group had reduced IIS induced by RU486 from the age of 3 days. The 3 and 7 day recovery flies were removed from the RU486 food 3 or 7 days before the experiment and kept on standard food. The elavGS/+ control group had no RU486 in their media at all. Female data: left column, male data: right column. N=16 for each genotype. Error bars represent +/- SEM.

The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype had a significant effect, the elavGS/+ control group was compared to the other 3 groups using Dunnett's Method at each timepoint. Significant difference is highlighted with red text colour ($p<0.05$). On the graphs the black star (★) shows significant difference

A) Total distance walked over the lifespan **B)** Duration in central zone over the lifespan **C)** Walking duration over the lifespan **D)** Number of movement bouts over the lifespan **E)** Rotation frequency over the lifespan **F)** First rotation time over the lifespan **G)** Velocity over the lifespan

5.3: Discussion

A major question arising from Ismail et al. (2015) is what causes the detrimental effects of reduced IIS in neurons on exploratory walking senescence and the lack of effect of reduced IIS in neurons on negative geotaxis senescence. The aims of the experiments presented here using the inducible elavGS driver were to: (1) determine if the detrimental effects on functional decline due to reduced pan-neural IIS observed by Ismail et al. (2015) were due to effects on the adult CNS or developmental effects due to expression of the constitutive elavGAL4 transgene during the larval and pupal stages; and (2) to determine whether the detrimental effects of reduced IIS in neurons are reversible.

Adult specific pan neural IIS reduction had no effect on the senescence of negative geotaxis, similarly to the effects seen in Ismail et al. (2015) using the constitutive elavGAL4 driver (**Figure 26**). Reducing IIS systemically not only improved negative geotaxis, it also showed some positive effects on some of the exploratory walking parameters which are largely influenced by peripheral tissue health (e.g. muscles). Systemic IIS reduction failed to improve any of the decision-making parameters (Ismail et.al, 2015). When Ismail, et al. (2015) reduced IIS constitutively only in neurons using elavGAL4 driver, some detrimental effects in some of the locomotion and decision-making parameters were found. Our experiments with the adult specific IIS reduction in neurons using the elavGS driver showed similar detrimental effects on exploratory walking (**Figure 27** and **Figure 28**).

These results show that reducing IIS in the brain is not beneficial for the fly behaviour and reducing IIS in the adult flies is sufficient to induce the detrimental effects of IIS on brain function. Therefore, the negative effects of constitutive IIS reduction on fly behaviour were not due to developmental effects induced by reduced IIS.

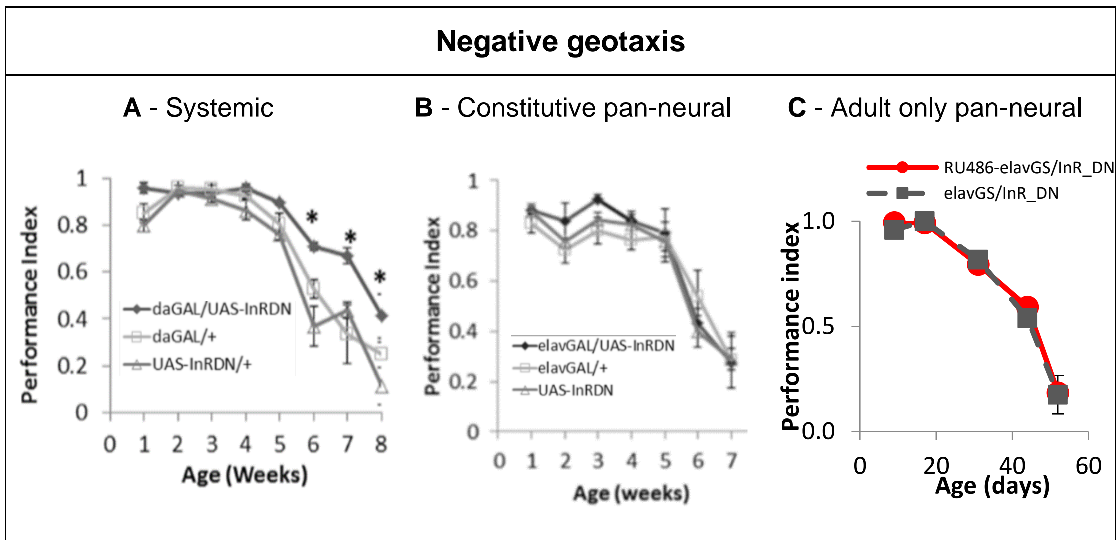


Figure 26 - **The effect of full body and pan-neural IIS reduction on negative geotaxis in females**

A) full body IIS reduction using the daGAL4 driver improves negative geotaxis at older ages (Ismail et al. (2015)). **B)** Constitutive neuron specific IIS reduction has no effect on negative geotaxis (Ismail et al. (2015)). **C)** Adult specific IIS reduction in the fly neurons from the age of 3 days show no effect on negative geotaxis.

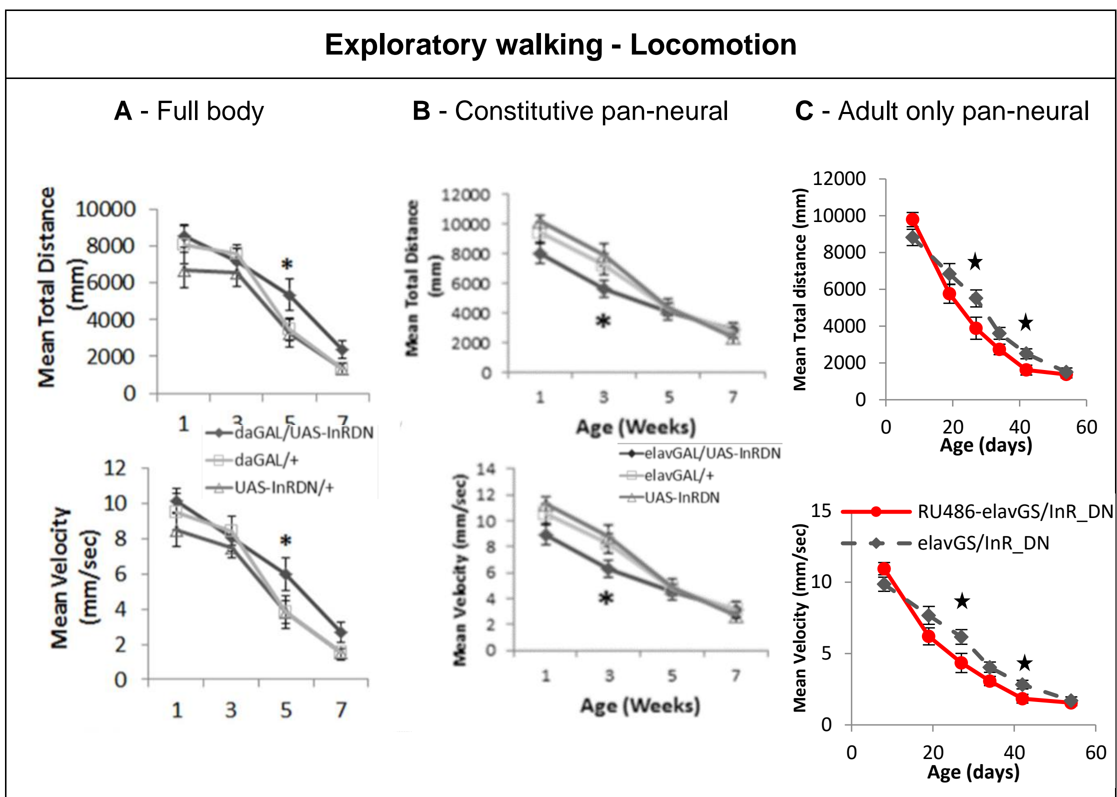


Figure 27 - **The effect of full body and pan-neural IIS reduction on exploratory walking locomotion parameters (female data)**

A) Full body IIS reduction using the daGAL4 driver delays the decline of total distance walked and velocity (Ismail et al. (2015)). **B)** Constitutive neuron specific IIS reduction speeds up the decline of total distance walked and velocity (Ismail et al. (2015)). **C)** Adult specific IIS reduction in the fly neurons from the age of 3 days also speeds up the decline of total distance walked and velocity.

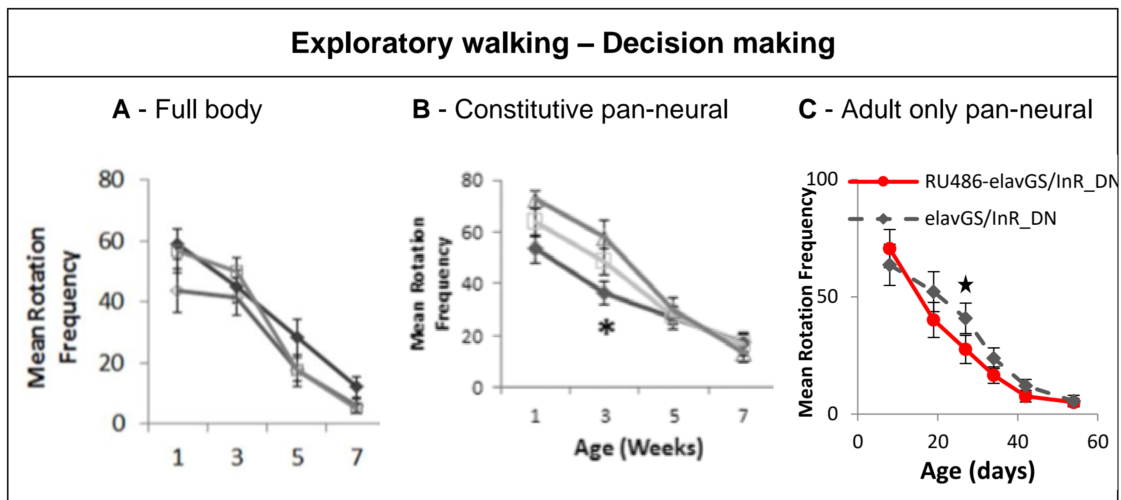
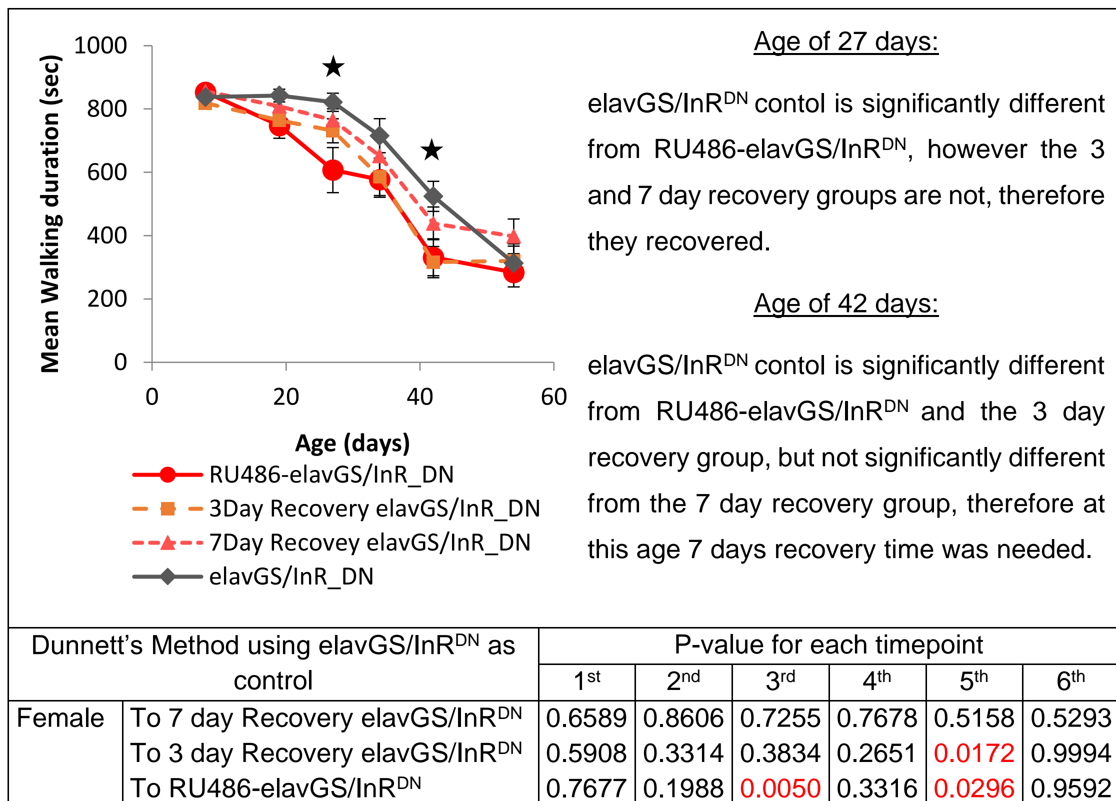


Figure 28 - The effect of full body and pan-neural IIS reduction on one of the decision-making parameters of exploratory walking – rotation frequency (female data)
A) Full body IIS reduction using the daGAL4 driver has no effect on rotation frequency (Ismail et al. (2015)). **B)** Constitutive neuron specific IIS reduction speeds up the decline of rotation frequency (Ismail et al. (2015)). **C)** Adult specific IIS reduction in the fly neurons from the age of 3 days also speeds up the decline of rotation frequency.

The recovery experiments presented here investigated if the negative effects of reduced IIS are permanent, or if they can be reversed and possibly improved after a short recovery period. The question of reversibility is important because detrimental effects of reduced IIS could be due to acute effects on neuronal function or accelerated neuronal ageing. Given the role of reduced IIS to slow ageing, it is unlikely that reduced IIS in neurons would result in accelerated ageing of neurons. This experiment therefore tests our first hypothesis, which states that the negative effects on behavioural decline due to reduced IIS in neurons are caused by detrimental effects on the function of the neurons that outweigh any beneficial effects of reducing IIS on neuronal ageing.

The experiment used the inducible elavGS driver and the UAS-InR^{DN} transgene to induce IIS reduction in neurons when RU486 is present. Flies were kept on RU486 food from the age of 3 days then transferred to standard food 7 or 3 days before each exploratory walking measurement. Our results show that functional loss can be recovered from, suggesting that reduced IIS does not accelerate neuronal ageing, and instead causes reversible detrimental functional effects. **Figure 29** shows an example of exploratory walking recovery. However, the fact that performance of any behaviour did not improve compared to flies with normal IIS after the recovery period, raises the possibility that our hypothesis that slowed neuronal ageing is masked by detrimental functional effects is incorrect. The data, as presented, instead suggest that IIS either does not influence neuronal ageing or results in some permanent negative functional

effects which still mask any slowed ageing. However, further experiments are needed to make conclusions about the role of IIS in neuronal ageing. First of all, we would need to confirm if InR function has fully recovered after 7 days without RU486 induction. This could be done by measuring AKT phosphorylation levels using Western blotting, but this is technically challenging because of the lack of sensitivity of the assay on only a subset of cells in the brain which have reduced IIS. Such experiments also did not fit into the time-frame and budget of this project, but future experiments are planned measuring AKT phosphorylation in isolated neurons. It is possible that reducing IIS causes permanent detrimental effects in the neurons, as reducing IIS promotes FOXO localisation in the nucleus and FOXO has a role in cell survival and apoptosis. More about this in Chapter 7.



Dunnett's Method using elavGS/InR^{DN} as control		P-value for each timepoint					
		1st	2nd	3rd	4th	5th	6th
Female	To 7 day Recovery elavGS/InR^{DN}	0.6589	0.8606	0.7255	0.7678	0.5158	0.5293
	To 3 day Recovery elavGS/InR^{DN}	0.5908	0.3314	0.3834	0.2651	0.0172	0.9994
	To RU486-elavGS/InR^{DN}	0.7677	0.1988	0.0050	0.3316	0.0296	0.9592

Figure 29 - An example of exploratory walking recovery
Female walking duration taken from **Figure 24** to highlight the effects of 3 and 7 day recovery.

In summary, the experiments in this chapter showed that reducing IIS in adult fly neurons is sufficient to induce detrimental behavioural effects indicating that the negative effects of constitutive pan-neural IIS signalling were not due to developmental effects. The data showing recovery from the detrimental effects of adult pan-neural IIS reduction, indicate that the exacerbated behavioural declines are due to effects on neuronal function and not due to an acceleration of neuronal ageing.

It is worth noting that while RU486 itself did not have any significant effect on female behaviour, it did affect some of the male exploratory walking parameters significantly. It is, therefore, difficult to draw conclusions from the male experiments, as we cannot separate the effect of RU486 from the effect of reduced IIS.

Chapter 6: The effects of pan-neural IIS reduction on sleep behaviour

6.1: Introduction

Previous studies have shown that similarly to humans, the sleep pattern of *Drosophila melanogaster* shows characteristic age-related changes such as sleep fragmentation (Koh et al. 2006, Metaxakis et al. 2014). The role of IIS in this sleep senescence has also been investigated (Metaxakis et al. 2014, Cong, et al. 2015). Cong, et al. (2015) showed that DILPs play an important role in regulating sleep, as all of the *dilp* mutant flies except *dilp4* and the *dInR* mutant flies have reduced total sleep, while the upregulation of *dilp*2 and *dInR* increases sleep. *Dilp2* was found to be expressed in the Clock Neurons in the brain and its expression is reduced in response to starvation (Cong, et al. 2015). This further indicates the role of DILPs in sleep regulation and the reduced *dilp2* expression could explain how starvation inhibits sleep (Keene, et al. 2010).

Metaxakis et al. (2014) showed that increased sleep fragmentation with age in *Drosophila*, involving increased day sleep, reduced night sleep, increased number of sleep bouts at day and night and decreased night sleep bout duration, is influenced by IIS. To investigate the effects of ubiquitous IIS reduction on sleep, Metaxakis et al. (2014) used virgin females from two models of reduced IIS: the *dilp2-3,5* mutant compared to the w^{Dah} control and daGAL4UAS-InR^{DN} flies compared to daGAL4/+ and UAS-InR^{DN}/+ controls. The dilp2-3,5 mutant flies showed significantly less increase in all aspects of sleep fragmentation with age, while the daGAL4/UAS-InR^{DN} flies did not show any changes in daytime behaviour compared to controls, age related night sleep fragmentation was ameliorated. Therefore, the experiments of Metaxakis et al. (2014) suggest that systemic reduction of IIS can ameliorate age-related sleep fragmentation in *Drosophila*.

However, *dilp2-3,5* mutation affected sleep pattern at young age, as young, 10 days old mutant flies already showed increased daytime activity and reduced daytime sleep. Therefore, it is not clear if reduced IIS ameliorates age related sleep

fragmentation, as flies already show altered activity at young age (Metaxakis, et, al. 2014). Metaxakis et. al (2014) also investigated the role of dFOXO in sleep pattern changes in response to reduced IIS and found that loss of dFOXO does not affect sleep in wild type flies, while loss of dFOXO in *dInR^DN^* mutant flies reduced daytime activity without affecting night-time sleep. Thus, reduced IIS affects daytime activity and sleep through dFOXO, but sleep at night is altered through a different pathway. Increased daytime activity is also mediated by AKH signalling, as the loss of AKH receptor abrogated the elevated daytime activity in IIS mutant flies, with no effect on sleep or activity at night. Furthermore, increased AKH release increases daytime activity and reduces daytime sleep in wild type flies, but not in *dilp2-3,5*, *dfoxo*, or *Akh receptor* mutants (Metaxakis, et, al. 2014). The increased activity throughout the day is also related to octopaminergic signalling, as the inhibition of octopaminergic signalling also abrogated the increased daytime activity of IIS mutant flies without affecting night sleep or activity, while inhibited octopaminergic signalling has no effect on the sleep of wild type flies (Metaxakis, et al. 2014). On the other hand, the effects of IIS on night sleep is mediated by TOR and S6K signalling. Disruption of TOR signalling using rapamycin increases night sleep duration and reduced night sleep fragmentation without affecting daytime sleep in wild type flies but did not affect night sleep in IIS mutant flies. Feeding of rapamycin to old flies also increased night-time sleep duration and reduced sleep fragmentation with no effect on daytime sleep (Metaxakis, et, al. 2014). The systemic expression of constitutively active S6K suppresses the effect of rapamycin on sleep, therefore, rapamycin rescue sleep fragmentation and increase night sleep duration through S6K activity (Metaxakis, et, al. 2014).

Although IIS in neurons does not appear to play a role in the regulation of sleep at young age (Erion et al, 2012), it is not known how IIS in neurons effects age-related sleep fragmentation. We hypothesized that, similarly to the effect of reduced IIS on exploratory walking senescence (Ismail et al, 2015, Chapter 5), reduction of IIS in neurons may cause detrimental effects on neuronal function with age that could influence sleep. To investigate this, we measured sleep throughout life in flies with constitutively reduced neuronal IIS (elavGAL4/InR^{DN}) and in flies with adult specifically reduced neuronal IIS (elavGS/InR^{DN}). We repeated the sleep experiments once again with the inducible system, this time allowing recovery from reduced IIS before each behavioural measurement to determine whether or not any negative effects on sleep at each age are reversible.

6.1.1: Aims

To investigate the effects of constitutive pan-neural IIS reduction on daily activity and sleep fragmentation.

To investigate the effects of pan-neural IIS reduction specifically in adulthood on daily activity and sleep fragmentation.

To determine if recovery time from pan-neural adult-specific IIS reduction before sleep experiments altered the sleep behaviour of flies compared to those with reduced IIS in neurons throughout adulthood.

6.1.2: Research design

For constitutive reduction of IIS in neurons, crosses to generate elavGAL4/UAS-InR^{DN} (experimental) and elavGAL4/+ and UAS-InR^{DN}/+ (control) flies were set up as described in Chapter 2.4. At the age of 3 days, flies were sorted by CO_2 anaesthesia and separated by gender. Male and female flies were transferred into standard food vials (10 per vial) and maintained under standard conditions (25°C, 70% humidity with a 12 h dark/light cycle) (N=100 for each group) throughout the lifespan.

For adult specific pan-neural IIS reduction, crosses to generate elavGS/UAS-InRDN and elavGS/+ flies were set up as described in Chapter 2.4. Flies were sorted onto food containing 200 mM RU486 at the age of 3 days and kept on it throughout their life (RU486-elavGS/InR^{DN} group), while the control flies were maintained on standard food (elavGS/InR^{DN} group). All flies were sorted as 10 flies per vials separated by gender and kept under standard experimental conditions (25ºC, 70% humidity, 12 h dark/light cycle).

To measure the daily activity of flies with reduced pan-neural IIS, we used Trikinetics *Drosophila* Activity Monitors (DAMs) as shown on **Figure 12**. About every 10 days, flies were sampled from the population and individual flies (N=15) were transferred into DAM tubes and kept under standard conditions for 4 days. The data were recorded in one minute bins and 'sleep' was defined as a minimum of 5 minutes with no activity (Shaw, et al. 2000). If a fly showed less than 100 min activity per day, it was considered dead.

This study measured 8 parameters of fly activity to analyse sleep behaviour. Total activity is the number of active minutes throughout a 24 h period, while Total activity level shows the total number of times the fly crossed the infrared beam in that 24 hour period. Total sleep in dark or in light shows the total number of minutes the flies spent asleep (being inactive for 5 or more minutes) in a 12 h period with or without light. The number of sleep bouts in dark or light shows the number of uninterrupted sleep sections in a 12 h dark or light period. More sleep bouts mean that the sleep of the flies is more fragmented. Mean sleep bout length in the dark or light shows the average length of the sleep bouts, where shorter sleep bouts suggest more fragmented sleep.

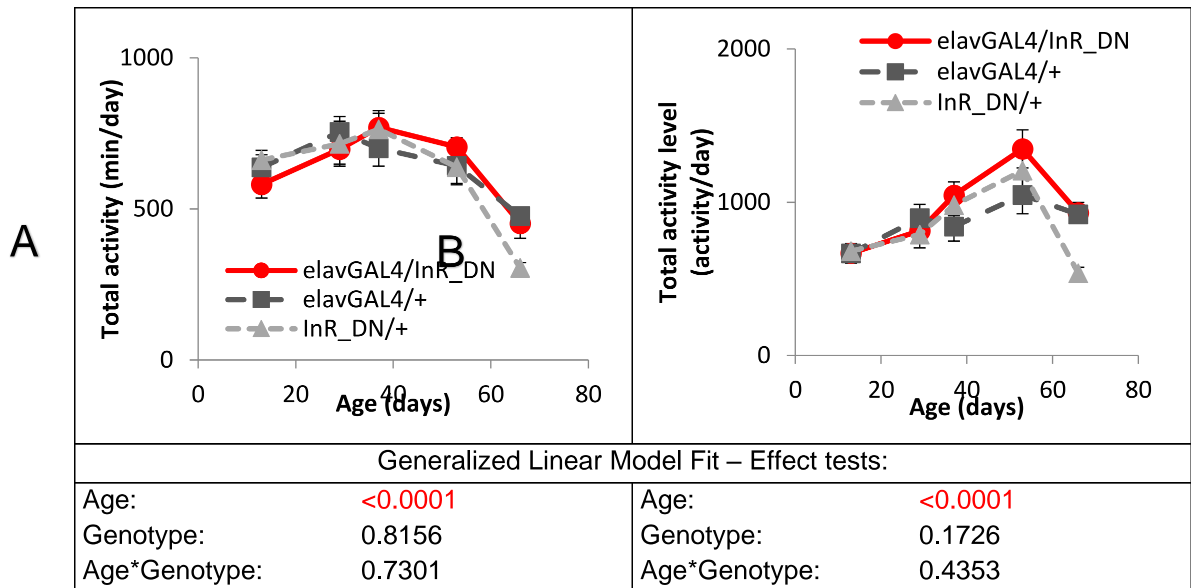
6.2: Results

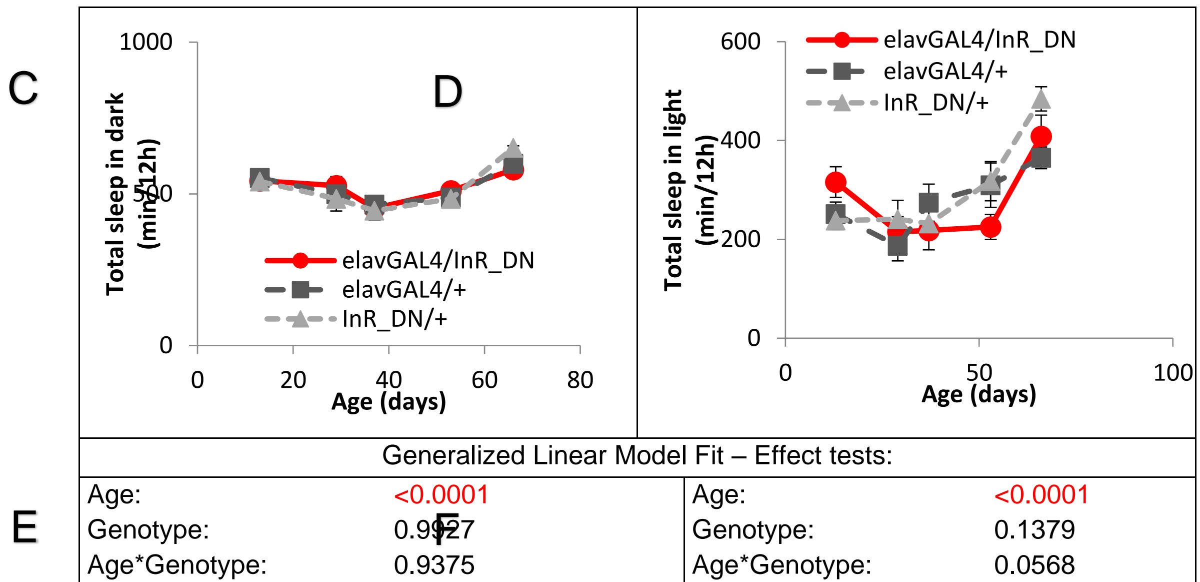
6.2.1: Constitutive pan-neural IIS reduction does not affect sleep behaviour in flies

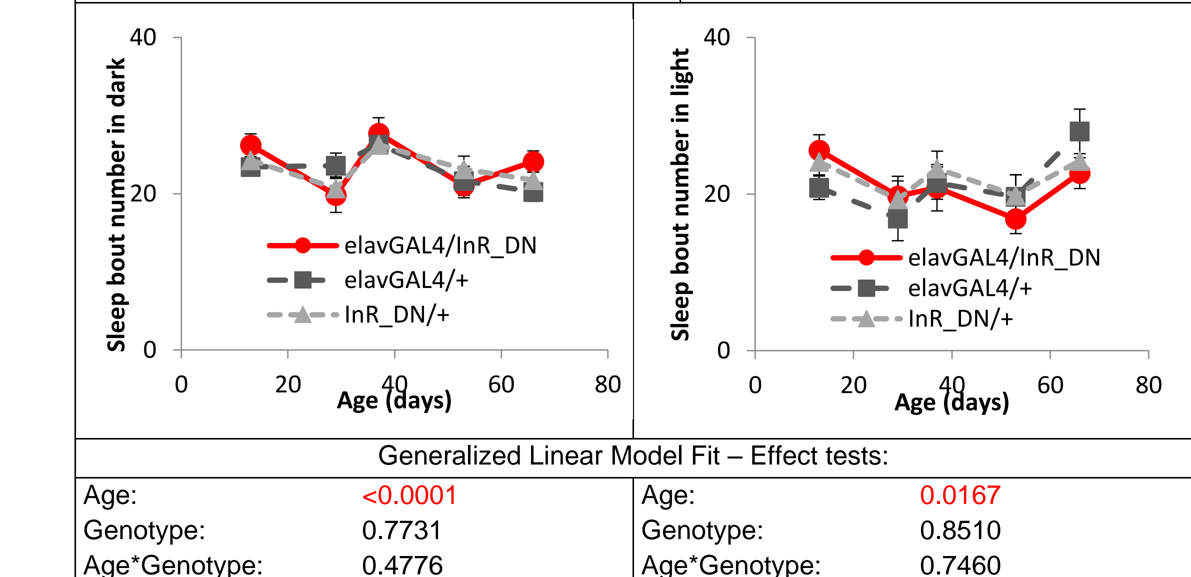
Flies with constitutive IIS reduction in neurons (elavGAL4/InR^{DN}) were compared to the elavGAL4/+ and InR^{DN}/+control groups. The data was initially analysed using General Linear Modelling to determine any significant effects of age, genotype and age*genotype interaction. When significant ($p<0.005$) effects were found, post hoc pairwise comparisons of means were carried out using the Tukey-Kramer HSD test.

In general, the sleep data did not show such a consistent change over age, in contrast to the robust age-related declines seen in the exploratory walking and negative geotaxis behaviours. In the sleep experiments presented here age was a significant effect in females (**Figure 30**) but not in males (**Figure 31**), which did not show significant age effects in total activity, total sleep in light and mean bout length in light. **Figure 30** and **Figure 31** show that reduced IIS in neurons had no significant effect on male or female sleep behaviour at all ages.

Constitutive pan-neural IIS reduction - Females







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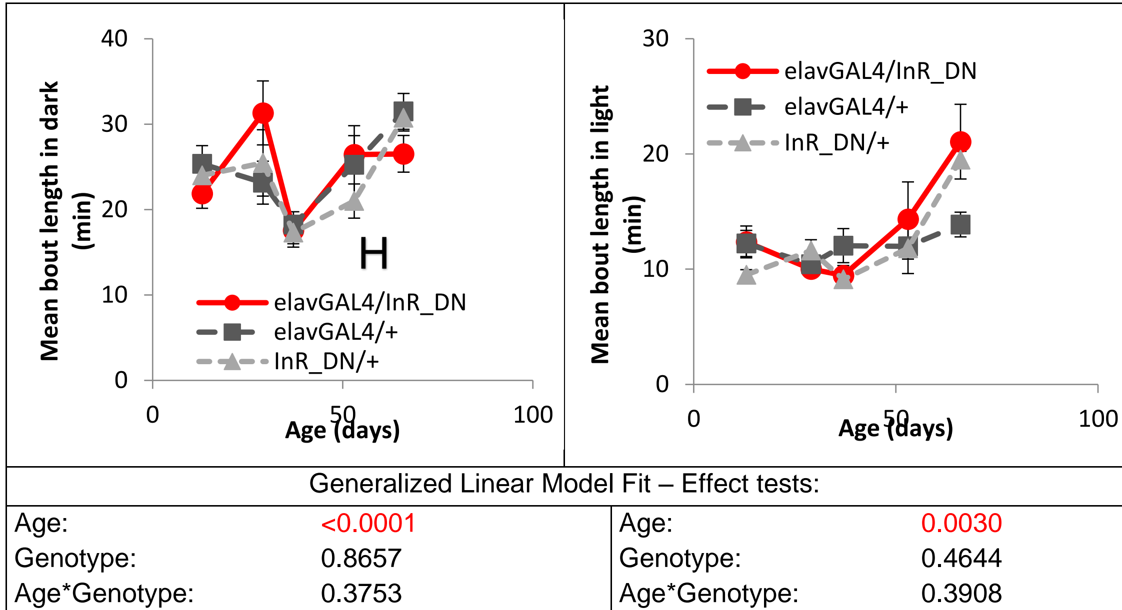


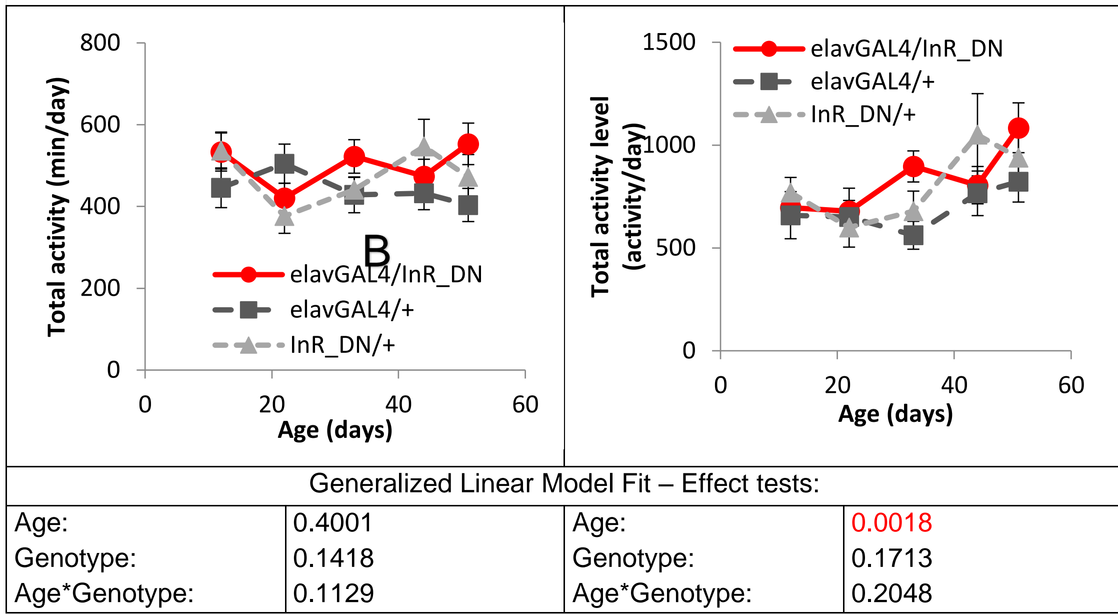
Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.0030
Genotype:	0.8657	Genotype:	0.4644
Age*Genotype:	0.3753	Age*Genotype:	0.3908

Figure 30 - Effect of constitutive pan-neural IIS reduction on the sleep behaviour of female flies

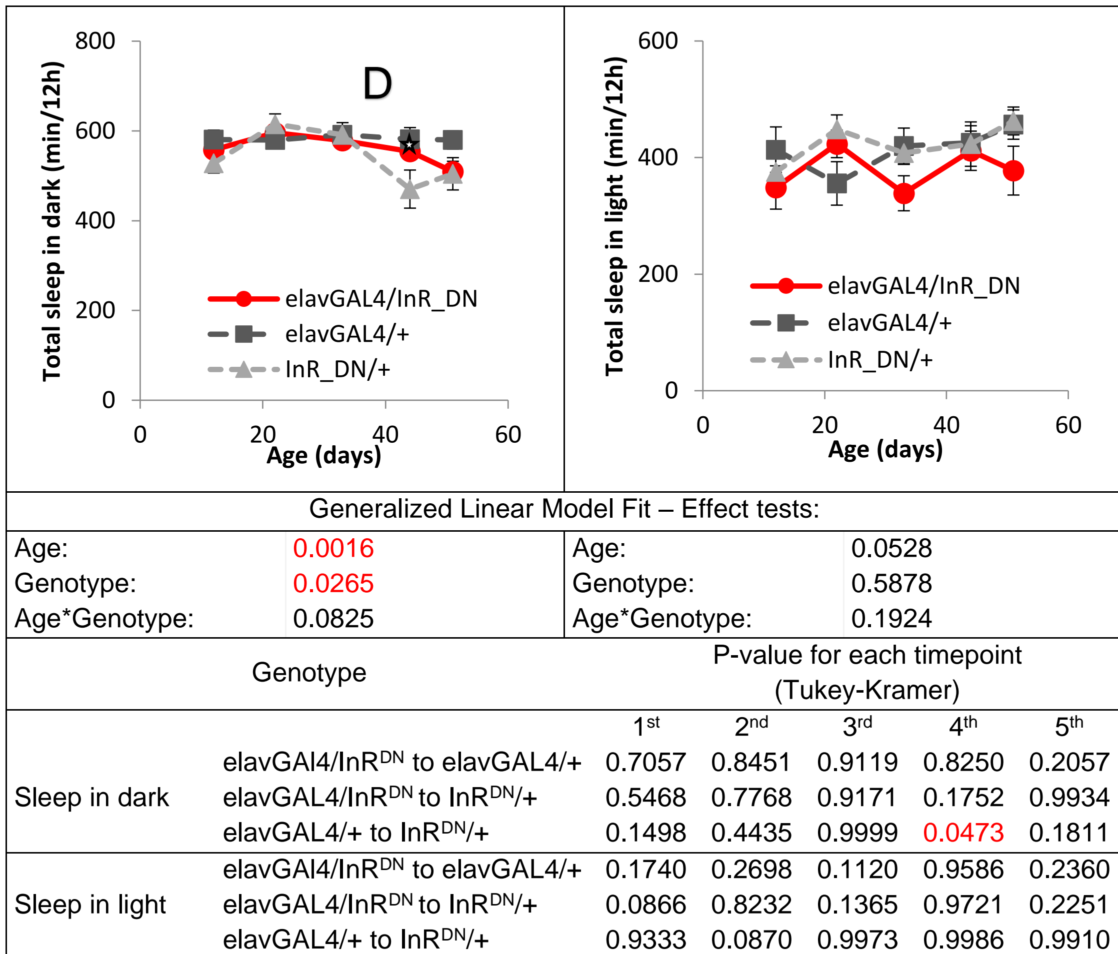
The activity of the mated female flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as 'sleep'. Flies counted as 'dead' if they show less than 100 min activity per day, N=15 for each group and timepoint. The experimental elavGAL4/UAS-InR^{DN} group with constitutive pan-neural IIS reduction was compared to elavGAL4/+ and UAS-InR^{DN}/+ control groups. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. As there was no significant ($p<0.05$) genotype or age*genotype effect in this experiment, there was no need for a post hoc test pairwise comparation.

A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

Constitutive pan-neural IIS reduction - Males



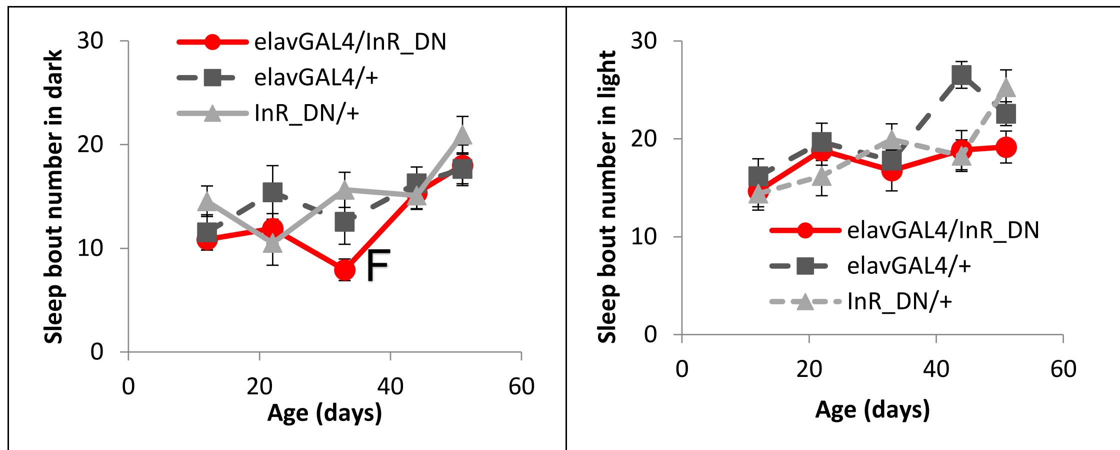
Generalized Linear Model Fit – Effect tests:			
Age: Genotype: Age*Genotype:	0.4001 0.1418 0.1129	Age: Genotype: Age*Genotype:	0.0018 0.1713 0.2048



Generalized Linear Model Fit – Effect tests:			
Age: Genotype: Age*Genotype:	0.0016 0.0265 0.0825	Age: Genotype: Age*Genotype:	0.0528 0.5878 0.1924

Genotype		P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Sleep in dark	elavGAl4/InR^{DN} to elavGAL4/+	0.7057	0.8451	0.9119	0.8250	0.2057
	elavGAL4/InR^{DN} to InR^{DN}/+	0.5468	0.7768	0.9171	0.1752	0.9934
	elavGAL4/+ to InR^{DN}/+	0.1498	0.4435	0.9999	0.0473	0.1811
Sleep in light	elavGAl4/InR^{DN} to elavGAL4/+	0.1740	0.2698	0.1120	0.9586	0.2360
	elavGAL4/InR^{DN} to InR^{DN}/+	0.0866	0.8232	0.1365	0.9721	0.2251
	elavGAL4/+ to InR^{DN}/+	0.9333	0.0870	0.9973	0.9986	0.9910

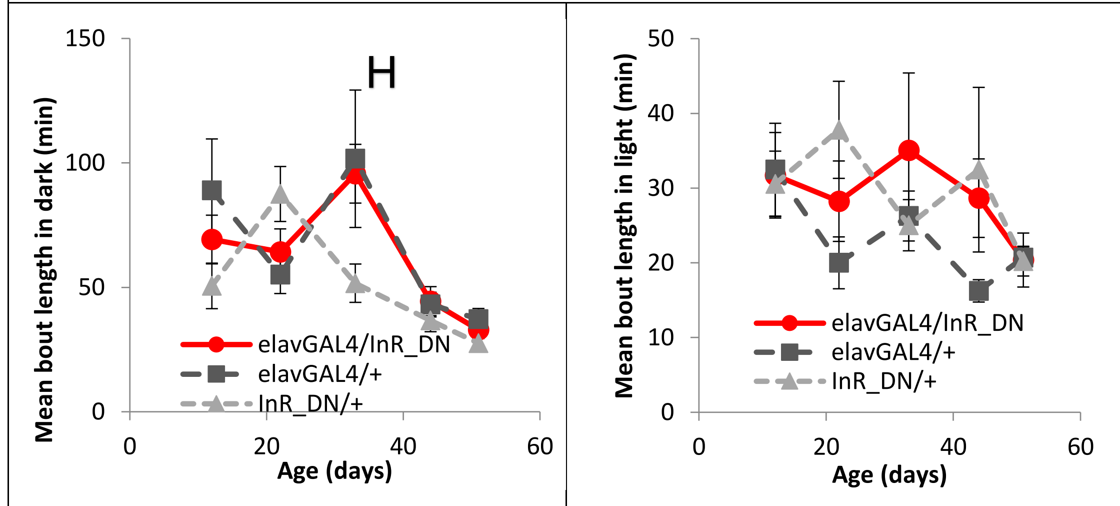
E



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0604	Genotype:	0.0164
Age*Genotype:	0.0962	Age*Genotype:	0.0230

	Genotype	P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Sleep bout number in light	elavGAl4/InR^{DN} to elavGAL4/+	0.8202	0.7933	0.9100	0.0104	0.3249
	elavGAL4/InR^{DN} to InR^{DN}/+	0.9929	0.5637	0.3959	0.9726	0.0490
	elavGAL4/+ to InR^{DN}/+	0.7551	0. 2338	0.6225	0.0048	0.5043

G



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.1850
Genotype:	0.1400	Genotype:	0.1652
Age*Genotype:	0.0349	Age*Genotype:	0.4566

	Genotype	P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Bout length in dark	elavGAl4/InR^{DN} to elavGAL4/+	0.6110	0.7979	0.9723	0.9820	0.7401
	elavGAL4/InR^{DN} to InR^{DN}/+	0.6537	0.2177	0.2363	0.5762	0.6547
	elavGAL4/+ to InR^{DN}/+	0.1665	0.0647	0.1386	0.6793	0.2432

Figure 31 - Effect of constitutive pan-neural IIS reduction on the sleep behaviour of male flies

The activity of the male flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as ‘sleep’. Flies counted as ‘dead’ if they show less than 100 min activity per day, N=15 for each

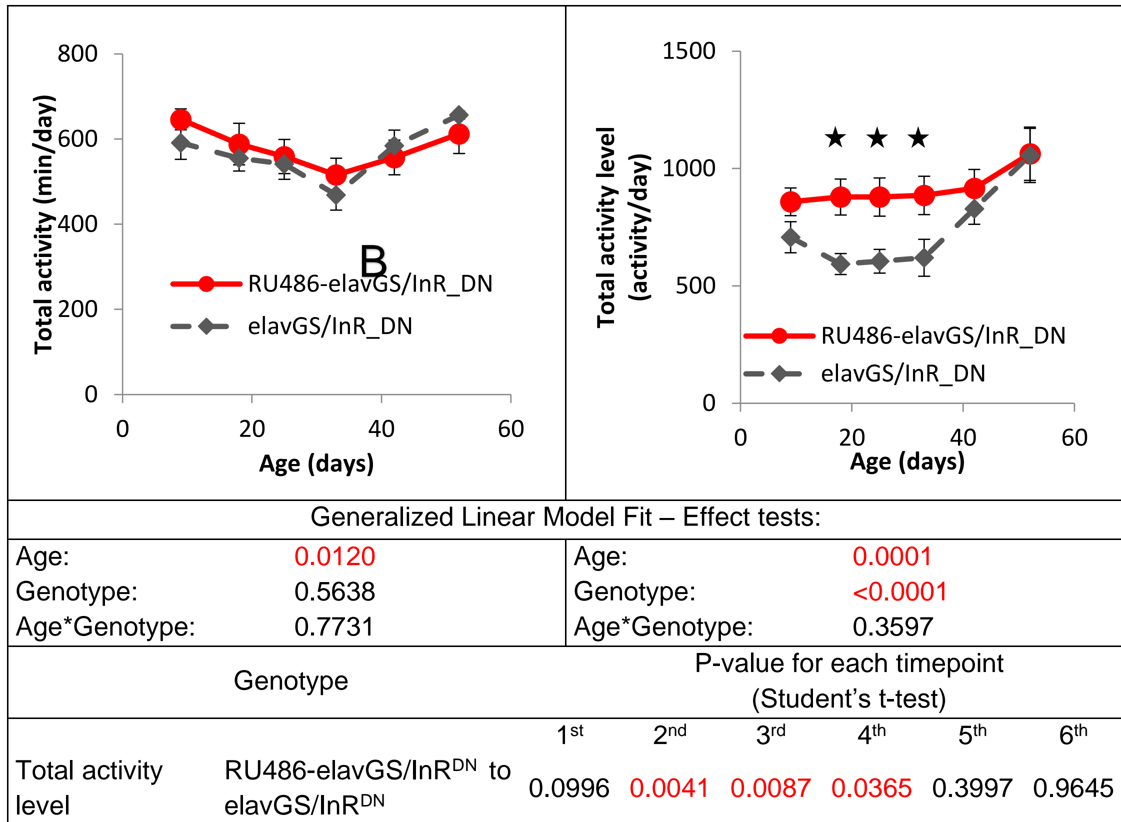
group and timepoint. The experimental elavGAL4/UAS-InR^{DN} group with constitutive pan-neural IIS reduction was compared to elavGAL4/+ and UAS-InR^{DN}/+ control groups. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. If there was significant (p<0.05) genotype or age*genotype effect in this experiment, post hoc pairwise comparation was carried out at each timepoint using Tukey-Kramer HSD test. Significant difference is highlighted with red text colour (p<0.05) in the statistical summary and with a star (★) on the graph.
A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

6.2.2: Pan-neural IIS reduction in adult female flies increased sleep fragmentation at middle age, but had no effect in males

Sleep was also measured in flies with adult-specific pan-neural IIS reduction using the inducible elavGS system (elavGS/InR^{DN}). The data were initially analysed with General Linear Modelling to determine significant effects of age, genotype and age*genotype interaction. When a significant (p<0.005) effect was found, post hoc pairwise comparison was carried out using Student's t-test.

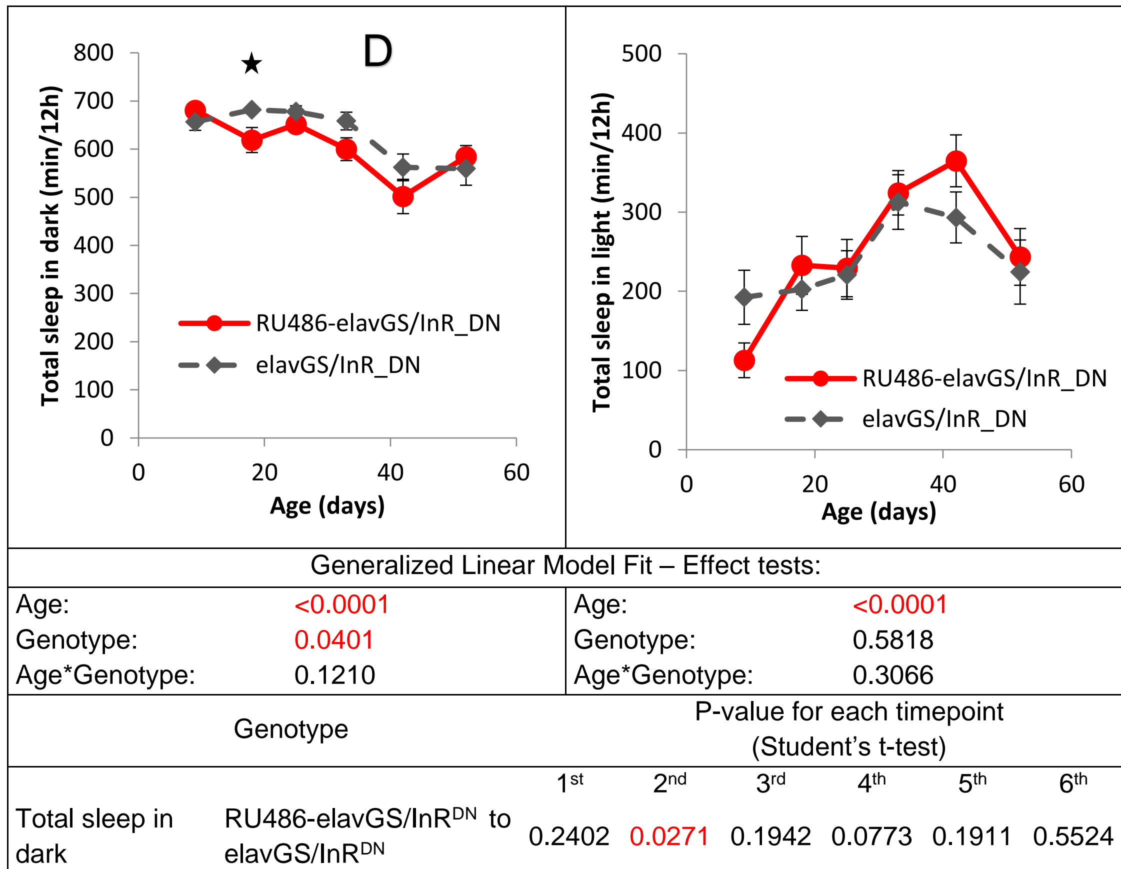
In both RU486-elavGS/InR^{DN} and control elavGS/InR^{DN} females all sleep and activity parameters showed significant changes with age indicating sleep fragmentation as shown by increased light and dark bout number and decreased light and dark bout length (**Figure 32**). Decreased IIS in adult neurons resulted in significant effects predominantly on total activity and dark sleep parameters. Although the number of minutes flies spent being active did not change in response to adult specific pan-neural IIS reduction, total activity level (number of beam crossings) was higher in RU486-elavGS/InR^{DN} females compared to controls between age 18-33 (p=0.0041, 0.0087 and 0.0365). RU486-elavGS/InR^{DN} female sleep in the light was not significantly different to controls, however their sleep in the dark became fragmented at an earlier age than controls in response to reduced IIS in their neurons throughout adulthood (**Figure 32E** and **G**), suggesting a detrimental effect. Their total sleep in dark was reduced at the age of 18 days (p=0.0271) coinciding with the early increase in number of sleep bouts (p= 0.0007) and reduction in bout length in the dark (p= 0.0224) at this age (**Figure 32**). In contrast to the effects on dark sleep, reduced IIS in adult female neurons had no effect on the normal age-related changes in light sleep.

Adult specific pan-neural IIS reduction - Females



Generalized Linear Model Fit – Effect tests:			
Age:	0.0120	Age:	0.0001
Genotype:	0.5638	Genotype:	<0.0001
Age*Genotype:	0.7731	Age*Genotype:	0.3597

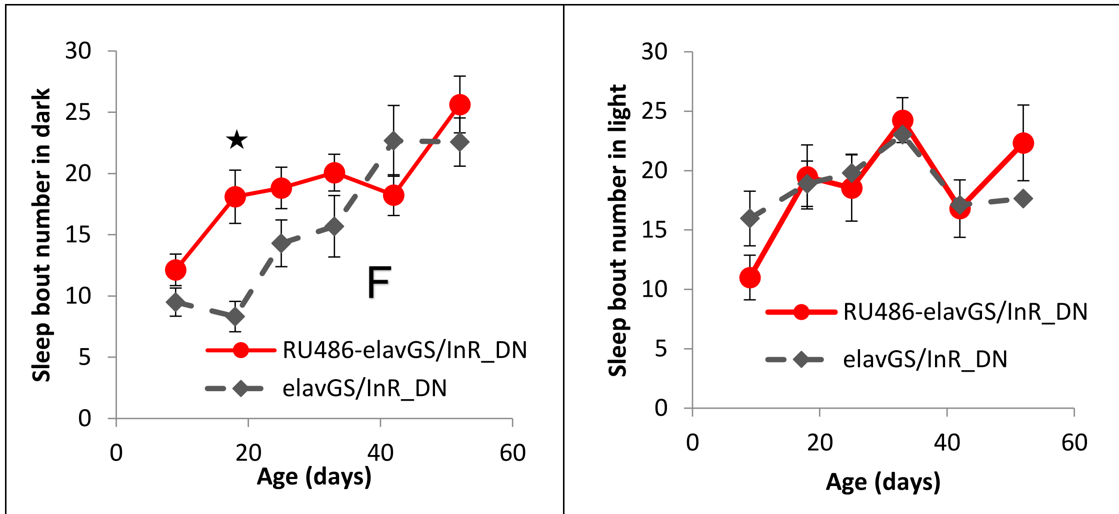
	Genotype	P-value for each timepoint (Student's t-test)					
		1st	2nd	3rd	4th	5th	6th
Total activity level	RU486-elavGS/InR^{DN} to elavGS/InR^{DN}	0.0996	0.0041	0.0087	0.0365	0.3997	0.9645



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0401	Genotype:	0.5818
Age*Genotype:	0.1210	Age*Genotype:	0.3066

	Genotype	P-value for each timepoint (Student's t-test)					
		1st	2nd	3rd	4th	5th	6th
Total sleep in dark	RU486-elavGS/InR^{DN} to elavGS/InR^{DN}	0.2402	0.0271	0.1942	0.0773	0.1911	0.5524

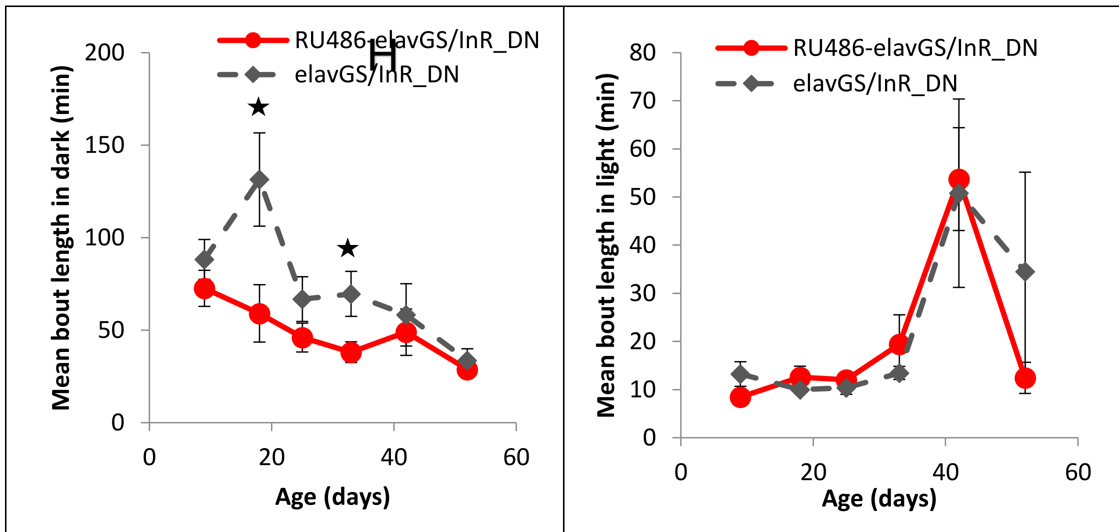
E



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.0014
Genotype:	0.0026	Genotype:	0.9955
Age*Genotype:	0.0136	Age*Genotype:	0.4497

	Genotype	P-value for each timepoint (Student's t-test)					
		1st	2nd	3rd	4th	5th	6th
Sleep bout number in dark	RU486-elavGS/InR^{DN} to elavGS/InR^{DN}	0.1382	0.0007	0.0935	0.1637	0.1942	0.3216

G



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0005	Genotype:	0.6616
Age*Genotype:	0.0917	Age*Genotype:	0.6573

	Genotype	P-value for each timepoint (Student's t-test)					
		1st	2nd	3rd	4th	5th	6th
Sleep bout length in dark	RU486-elavGS/InR^{DN} to elavGS/InR^{DN}	0.2912	0.0224	0.1700	0.0327	0.6590	0.5416

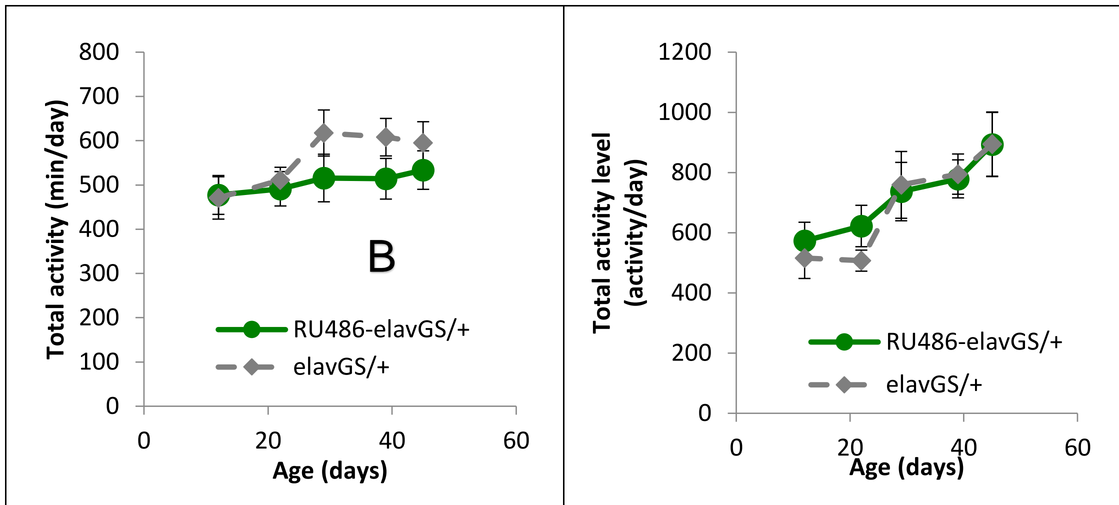
Figure 32 - Effect of inducible pan-neural IIS reduction from the age of 3 days on the sleep behaviour of female flies

The activity of the mated female flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as 'sleep'. Flies counted as 'dead' if they show less than 100 min activity per day, N=15 for each group and timepoint. RU486-elavGS/UAS-InR^{DN} group has reduced IIS induced by RU486 from the age of 3 days. The elavGS/UAS-InR^{DN} control group had no RU486 in their media at all. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. Where there was significant ($p<0.05$) genotype or age*genotype effect found by generalised linear modelling, a post hoc pairwise comparison of each timepoint was carried out using Student's t-test. Significant difference is highlighted with red text colour ($p<0.05$) in the statistical summary and with a star (★) on the graph.
A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

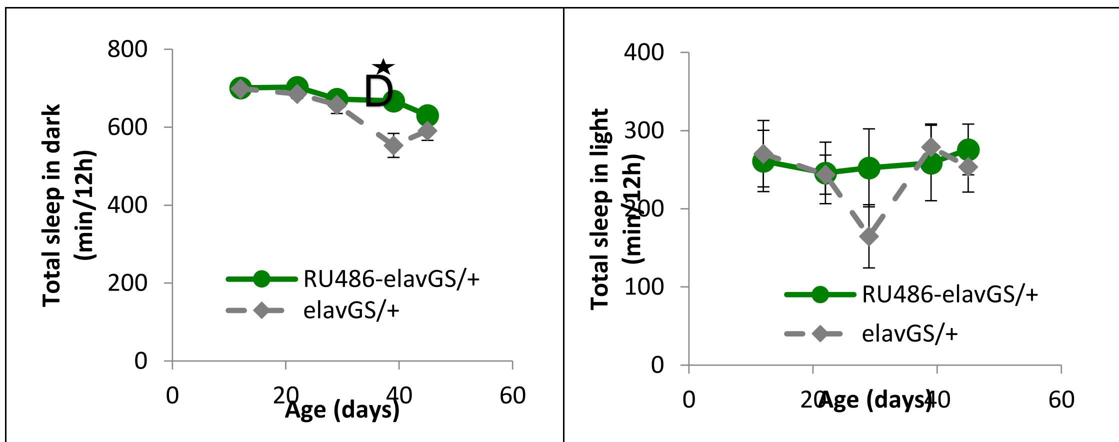
To determine if RU486 itself had any effect on sleep behaviour, an elavGS/+ experiment was run parallel to the elavGS/UAS-InR^{DN} groups. As the elavGS/+ flies did not carry the UAS-InR^{DN} transgene any effect on sleep or its senescence would be due to RU486 itself.

Total activity level and most sleep parameters showed the expected age-related changes in both RU486-elavGS/+ and elavGS/+ females (**Figure 33**). There was no significant effect of RU486 treatment on activity or sleep except for a small increase in dark sleep at age 39 days (p=0.0030) (**Figure 33C**). These data indicate that the detrimental effects on sleep fragmentation due to expression of InR^{DN} in female adult neurons was not due RU486 itself. Therefore, reduced IIS in adult female neurons resulted in detrimental effects on sleep fragmentation.

Effect of RU486 - Females

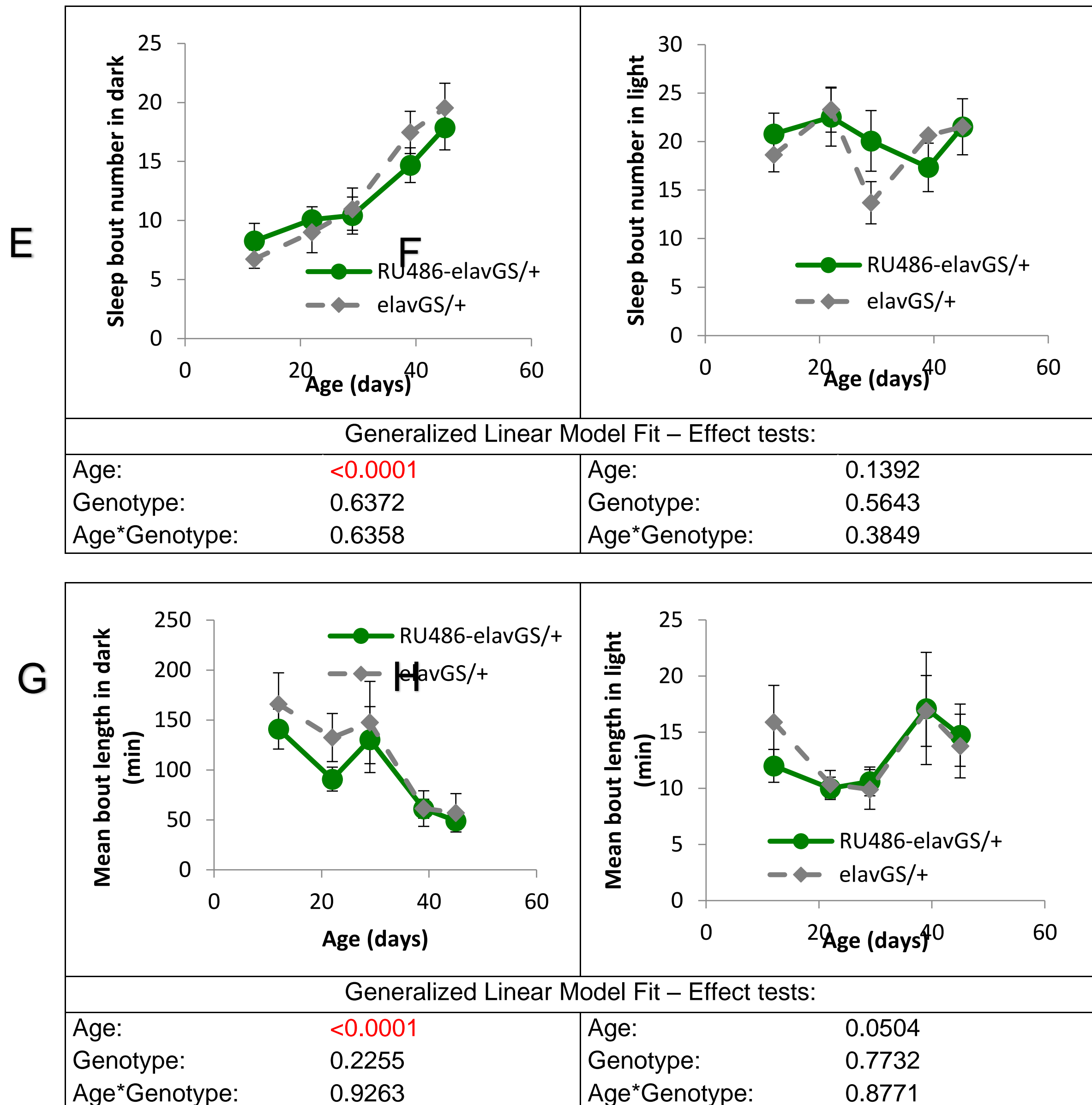


Generalized Linear Model Fit – Effect tests:			
Age:	0.1125	Age:	<0.0001
Genotype:	0.0549	Genotype:	0.6057
Age*Genotype:	0.6930	Age*Genotype:	0.9042



Generalized Linear Model Fit – Effect tests:			
Age: Genotype:	<0.0001	Age:	0.4836
Age*Genotype:	0.0007	Genotype:	0.4898
	0.0143	Age*Genotype:	0.6509

Genotype		P-value for each timepoint (Student's t-test)				
		1st	2nd	3rd	4th	5th
Total sleep in dark	RU486-elavGS/+ to elavGS/+	0.8312	0.1843	0.6257	0.0030	0.2143



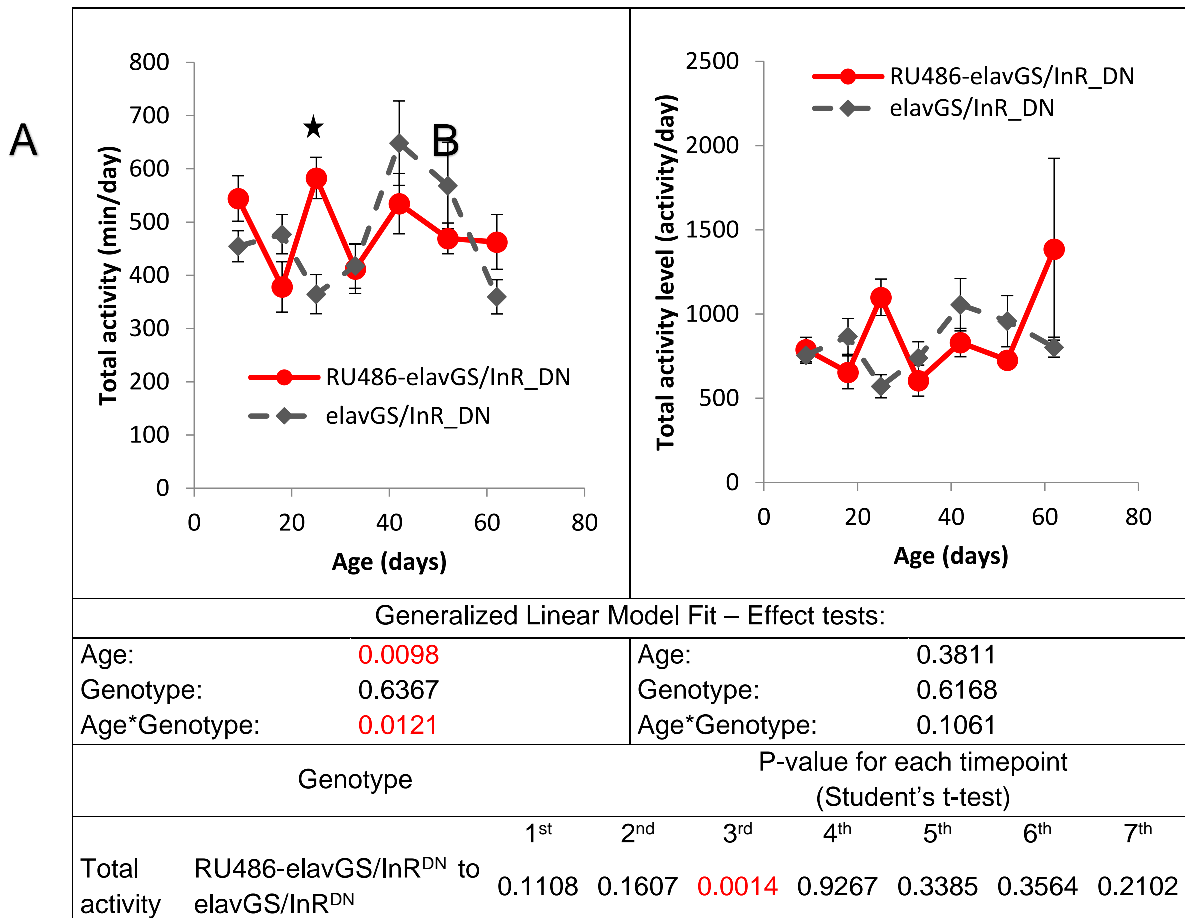
Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.1392
Genotype:	0.6372	Genotype:	0.5643
Age*Genotype:	0.6358	Age*Genotype:	0.3849

Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.0504
Genotype:	0.2255	Genotype:	0.7732
Age*Genotype:	0.9263	Age*Genotype:	0.8771

Figure 33 - Effect of RU486 from the age of 3 days on the sleep behaviour of female flies
The activity of the mated female flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as ‘sleep’. Flies counted as ‘dead’ if they show less than 100 min activity per day, N=15 for each group and timepoint. RU486-elavGS/+ group has reduced IIS induced by RU486 from the age of 3 days. The elavGS/+ control group had no RU486 in their media at all. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. Where there was significant ($p<0.05$) genotype or age*genotype effect found by generalised linear modelling, a post hoc pairwise comparison of each timepoint was carried out using Student’s t-test. Significant difference is highlighted with red text colour ($p<0.05$) in the statistical summary and with a star (★) on the graph.
A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

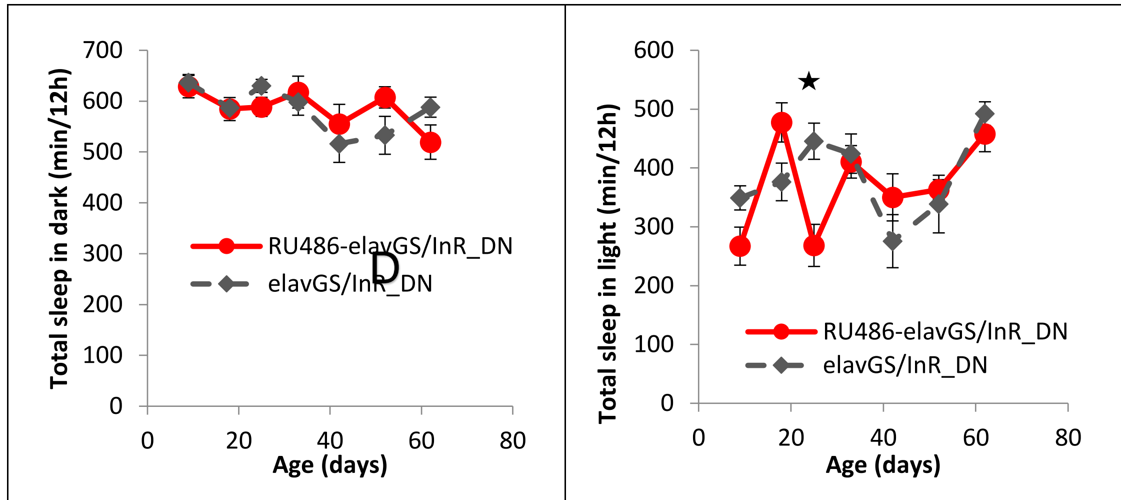
There was a significant effect of age on all parameters except mean bout length in the light and total activity level, so males show sleep fragmentation as shown by their increase in day and night sleep bout number and length with age. Unlike sleep in females which showed significant age*genotype interaction for most parameters, there was no effect of genotype on any parameter apart from increased total activity and decreased total sleep in light at age 25 days, but the data are generally very variable and do not show a clear pattern (**Figure 34**). This suggests that reduced IIS in neurons has no effect on the normal senescence (sleep fragmentation) of male sleep.

Adult specific pan-neural IIS reduction - Males



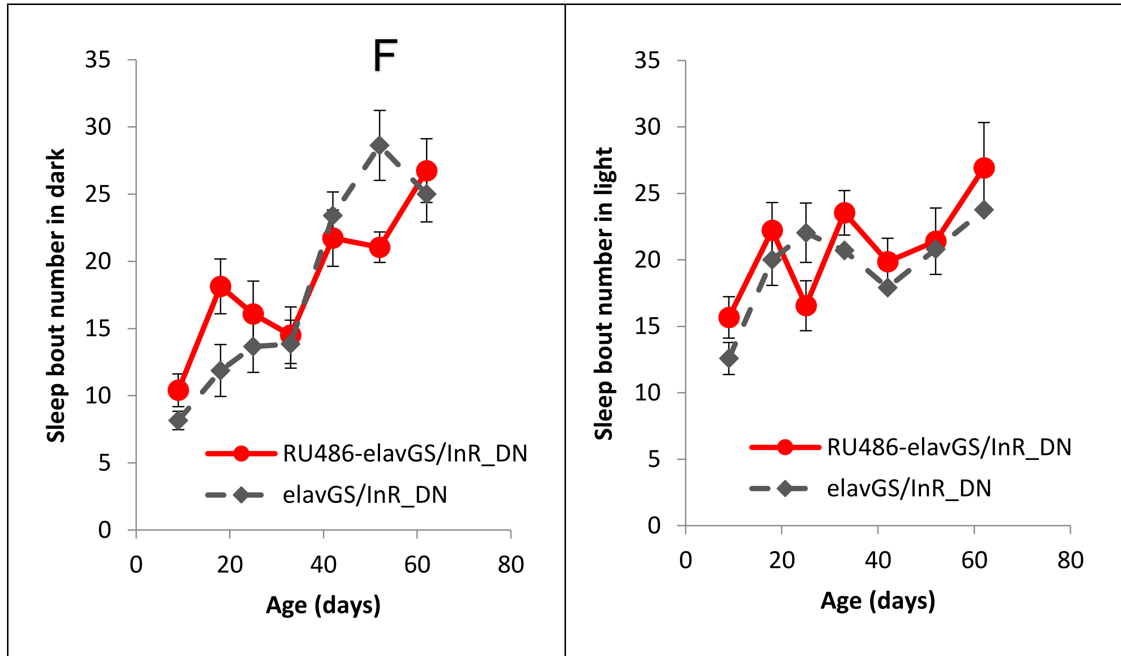
Generalized Linear Model Fit – Effect tests:			
Age:	0.0098	Age:	0.3811
Genotype:	0.6367	Genotype:	0.6168
Age*Genotype:	0.0121	Age*Genotype:	0.1061

	Genotype	P-value for each timepoint (Student's t-test)						
		1st	2nd	3rd	4th	5th	6th	7th
Total activity	RU486-elavGS/InR^{DN} to elavGS/InR^{DN}	0.1108	0.1607	0.0014	0.9267	0.3385	0.3564	0.2102

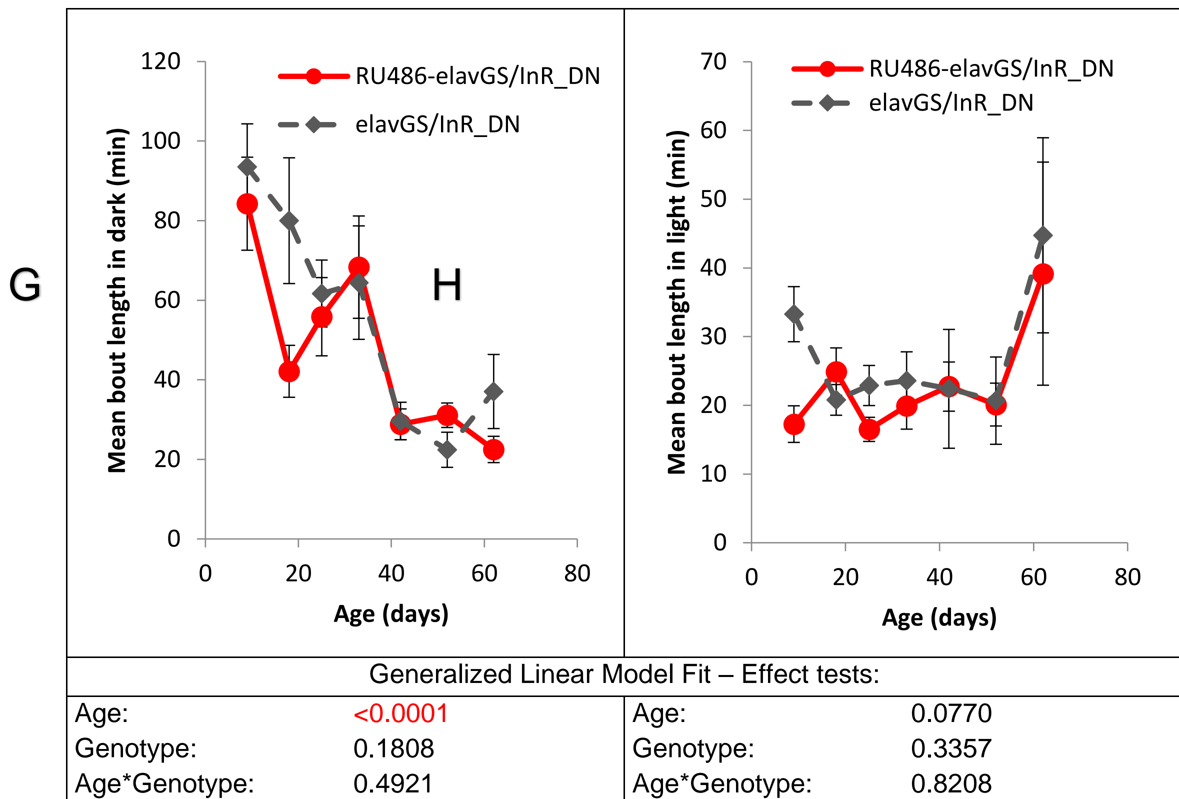


Generalized Linear Model Fit – Effect tests:			
Age:	0.0112	Age:	<0.0001
Genotype:	0.8980	Genotype:	0.4268
Age*Genotype:	0.2634	Age*Genotype:	0.0030

	Genotype	P-value for each timepoint (Student's t-test) 1st	2nd	3rd	4th	5th	6th	7th
Total sleep in light	RU486-elavGS/InR^{DN} to elavGS/InR^{DN}	0.0525	0.0688	0.0028	0.7541	0.3136	0.6997	0.4746



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.0004
Genotype:	0. 5991	Genotype:	0.3426
Age*Genotype:	0.0665	Age*Genotype:	0.5208



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.0770
Genotype:	0.1808	Genotype:	0.3357
Age*Genotype:	0.4921	Age*Genotype:	0.8208

Figure 34 - Effect of inducible pan-neural IIS reduction from the age of 3 days on the sleep behaviour of male flies

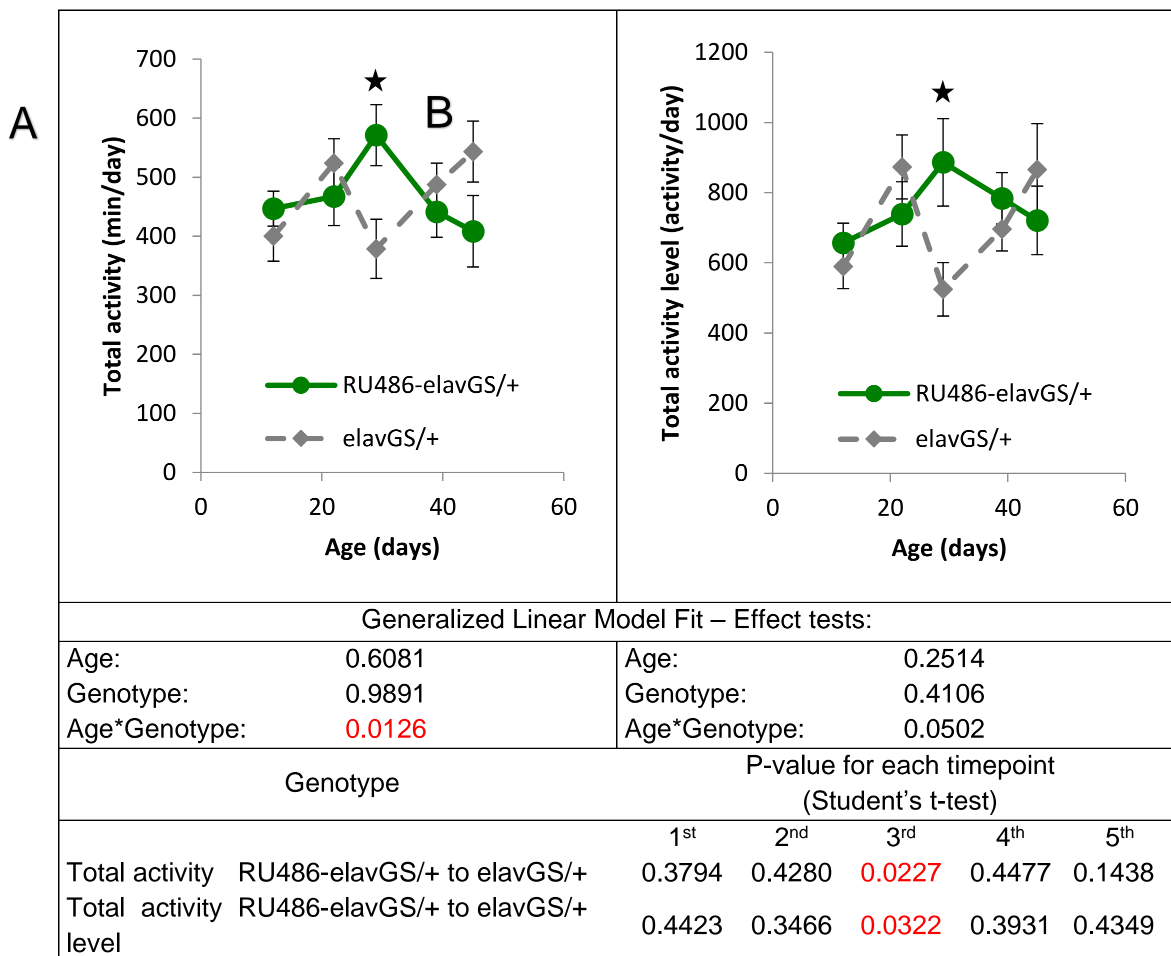
The activity of the male flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as 'sleep'. Flies counted as 'dead' if they show less than 100 min activity per day, N=15 for each group and timepoint. RU486-elavGS/UAS-InR^{DN} group has reduced IIS induced by RU486 from the age of 3 days. The elavGS/UAS-InR^{DN} control group had no RU486 in their media at all. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. Where there was significant ($p<0.05$) genotype or age*genotype effect found by generalised linear modelling, a post hoc pairwise comparison of each timepoint was carried out using Student's t-test. Significant difference is highlighted with red text colour ($p<0.05$) in the statistical summary and with a star (★) on the graph.

A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

Similarly to females, male elavGS/+ did not show significant age effects on all of the sleep parameters (Figure 35). There was no age effect on total activity, total activity level and total sleep in light in elavGS/+ male experiment. Unlike females, RU486 had detrimental effects on male sleep and activity, with increased activity of the flies at middle age and increased sleep fragmentation from young age. At age 29 days, total activity and total activity level was higher in RU486-elavGS/+ than the control

(p=0.0227 and 0.0322). At the same age, total sleep in light decreased (p=0.0112) but increased at the age of 45 (p=0.0112). Sleep in the dark was not affected significantly. Interestingly, sleep fragmentation increased in response to RU486 in males with higher number of sleep bouts in dark at age 12 and 29 days (p=0.0113 and 0.0145) and increased sleep bout number at 12 and 39 days (p=0.0054 and 0.0075). The length of the sleep bouts in the dark were lower than that of the control flies at the age of 12 (p=0.0275) and sleep bouts in the light also became shorter in response to RU486 at the age of 12 and 29 (p=0.0178 and 0.0338). Since the male sleep behaviour is affected by RU486, it masks the possible effect of adult-specific IIS reduction on sleep.

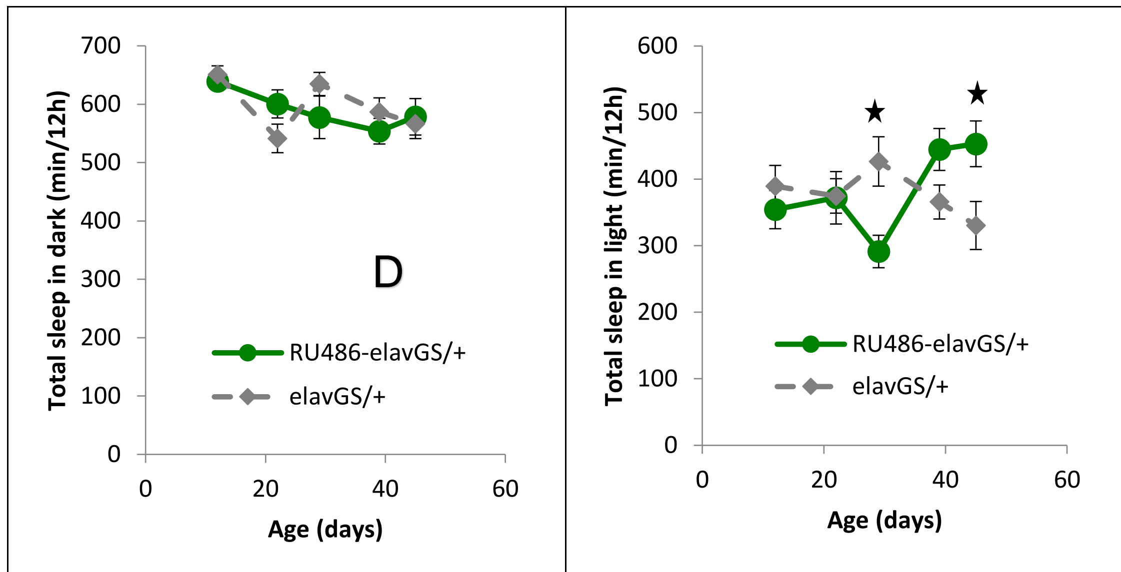
Effect of RU486 - Males



Generalized Linear Model Fit – Effect tests:			
Age:	0.6081	Age:	0.2514
Genotype:	0.9891	Genotype:	0.4106
Age*Genotype:	0.0126	Age*Genotype:	0.0502

	Genotype	P-value for each timepoint (Student's t-test)				
		1st	2nd	3rd	4th	5th
Total activity	RU486-elavGS/+ to elavGS/+	0.3794	0.4280	0.0227	0.4477	0.1438
Total activity level	RU486-elavGS/+ to elavGS/+	0.4423	0.3466	0.0322	0.3931	0.4349

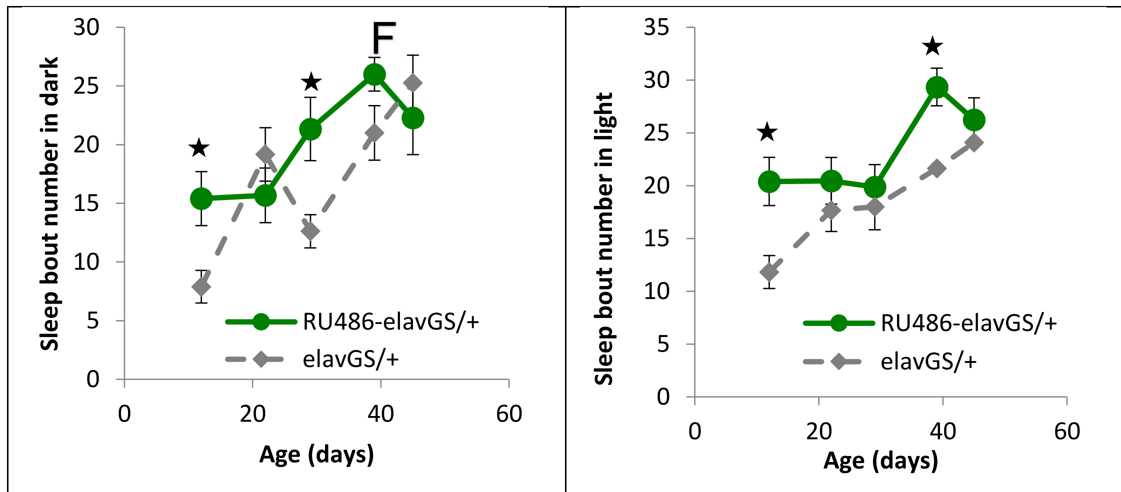
C



Generalized Linear Model Fit – Effect tests:			
Age:	0.0064	Age:	0.6559
Genotype:	0.6942	Genotype:	0.7837
Age*Genotype:	0.1765	Age*Genotype:	0.0019

Genotype	P-value for each timepoint (Student's t-test) 1st	2nd	3rd	4th	5th
Total sleep RU486-elavGS/+ to elavGS/+ in light	0.4244	0.9574	0.0112	0.0758	0.0385

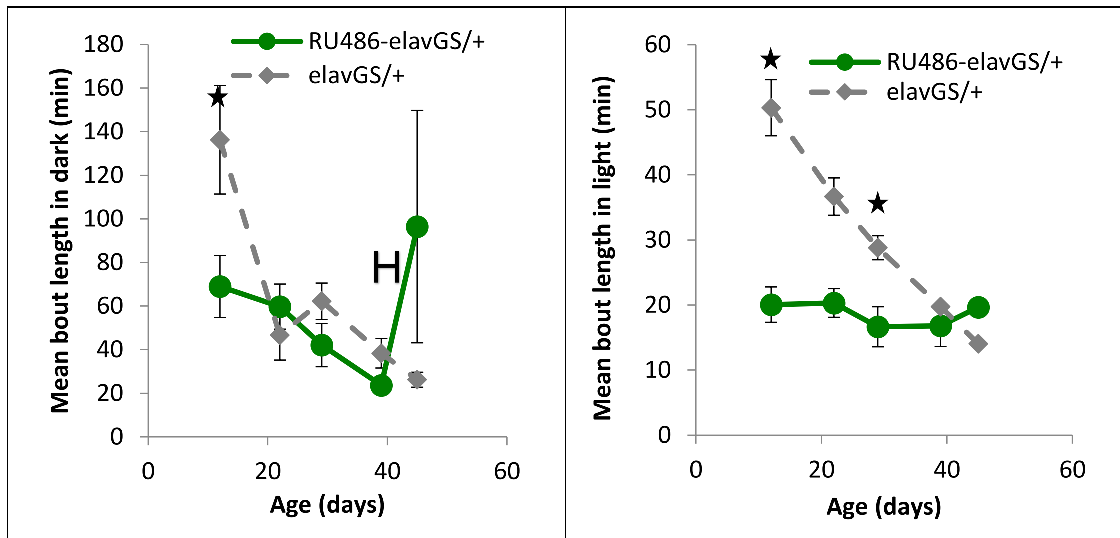
E



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0429	Genotype:	0.0006
Age*Genotype:	0.0169	Age*Genotype:	0.3044

Genotype	P-value for each timepoint (Student's t-test) 1st	2nd	3rd	4th	5th
Bout number RU486-elavGS/+ to elavGS/+ in dark	0.0113	0.3304	0.0145	0.0974	0.5086
Bout number RU486-elavGS/+ to elavGS/+ in light	0.0054	0.3945	0.5793	0.0075	0.5290

G



Generalized Linear Model Fit – Effect tests:			
Age:	0.0090	Age:	0.0071
Genotype:	0.7708	Genotype:	0.0022
Age*Genotype:	0.0207	Age*Genotype:	0.0236

Genotype		P-value for each timepoint (Student's t-test)				
		1st	2nd	3rd	4th	5th
Bout length dark	RU486-elavGS/+ to elavGS/+	0.0275	0.4415	0.1686	0.0720	0.2520
Bout length light	RU486-elavGS/+ to elavGS/+	0.0178	0.1348	0.0338	0.5128	0.1850

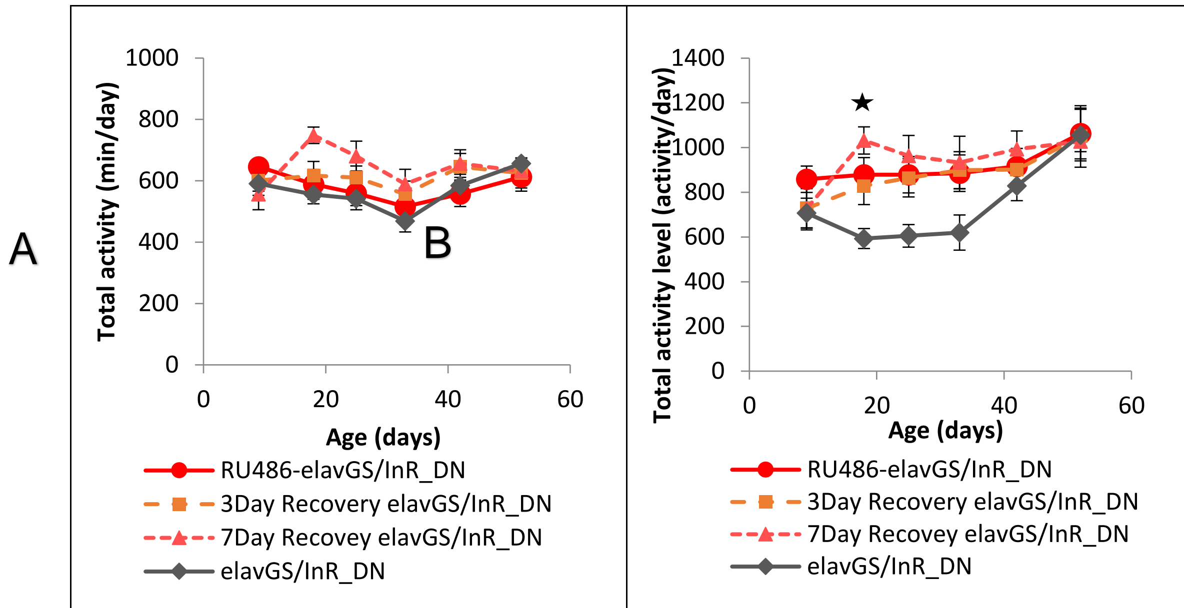
Figure 35 - Effect of RU486 from the age of 3 days on the sleep behaviour of male flies
The activity of the male flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as 'sleep'. Flies counted as 'dead' if they show less than 100 min activity per day, N=15 for each group and timepoint. RU486-elavGS/+ group has reduced IIS induced by RU486 from the age of 3 days. The elavGS/+ control group had no RU486 in their media at all. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. Where there was significant ($p<0.05$) genotype or age*genotype effect found by generalised linear modelling, a post hoc pairwise comparison of each timepoint was carried out using Student's t-test. Significant difference is highlighted with red text colour ($p<0.05$) in the statistical summary and with a star (★) on the graph.
A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

6.2.4: There is no consistent recovery from the effects of adult specific pan-neural IIS reduction

To determine if any of the observed effects of reduced adult specific pan-neural IIS on sleep and activity could be reversed, a 3 day and a 7 day recovery time was introduced prior to behavioural testing in this experiment. The experiment setup and conditions were the same as previously, but this time there were four groups: RU486-elavGS/InR^{DN} were fed and maintained on RU486 food from 3 days old such that they had reduced neuronal IIS throughout the rest of their life. The 3 day and 7 day recovery groups were fed RU486 food from the age of 3 days until 3 or 7 days before the sleep experiment timepoint in hope for IIS recovery in neurons to the normal level. The control group was the elavGS/InR^{DN} group with no RU486 at all, so they had normal levels of IIS. The data was first analysed using General Linear Modelling to determine the effect of age, genotype and age*genotype interaction. When significant ($p<0.005$) effect was found for Genotype or Age*Genotype, post hoc pairwise comparison was carried out using Dunnett's Method, were the RU486-elavGS/InR^{DN}, 7 day and 3 day recovery groups were compared to the elavGS/InR^{DN} control at each timepoint.

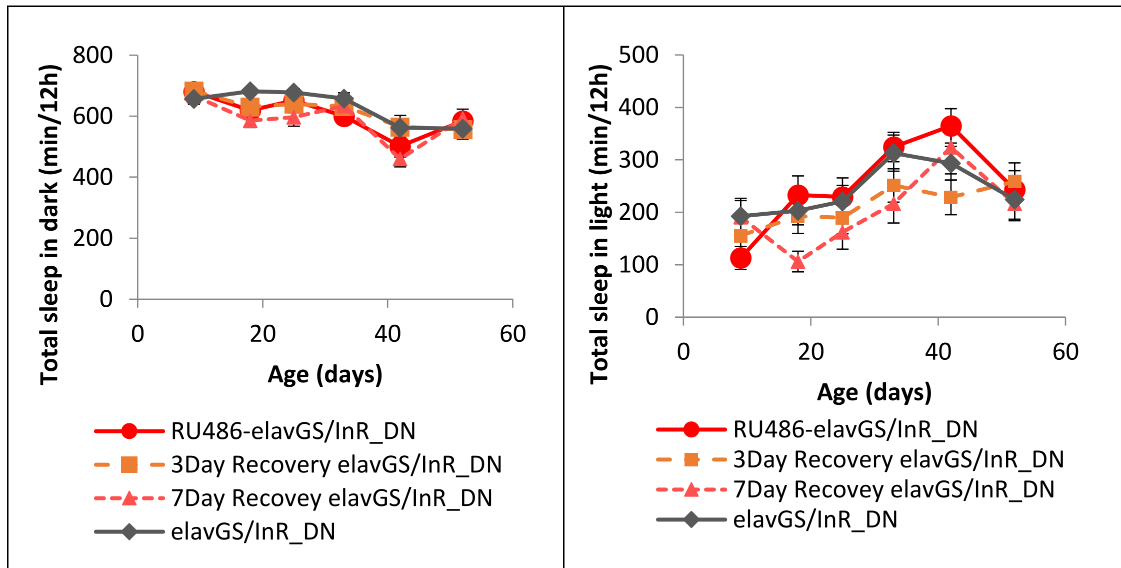
In females, age was a significant effect in all activity and sleep parameters in all groups, indicating that sleep and activity showed the expected age-related changes. Overall, the females did not show consistent recovery from the increased sleep fragmentation caused by reduced adult-specific pan-neural IIS reduction (**Figure 36**). The activity level (i.e. the number of times the flies crossed the infrared beam per day) is significantly increased in response to adult specific IIS reduction at the age of 18 days compared to the control with no RU486 ($p=0.0146$) and show no recovery, as the 7 day recovery group ($p=0.0001$) is also significantly higher than the control ($p=0.0001$), and the 3 day recovery is close to a significance ($p=0.0527$) (**Figure 36B**). There was also no recovery of the increased sleep bout number at the age of 28 days, as all 3 groups are significantly higher than the control (p values: 7 day=0.0002, 3 day=0.0004, RU486=0.0015) (**Figure 36E**).The sleep bout length in dark could not recover at the age of 18 days as all 3 groups were lower than the elavGS/InR^{DN} control (p values: 7 day=0.0004, 3 day=0.0011, RU486=0.0059), while at the age of 33 days the 7 day group showed recovery (**Figure 36G**).

Adult specific pan-neural IIS reduction with recovery in females



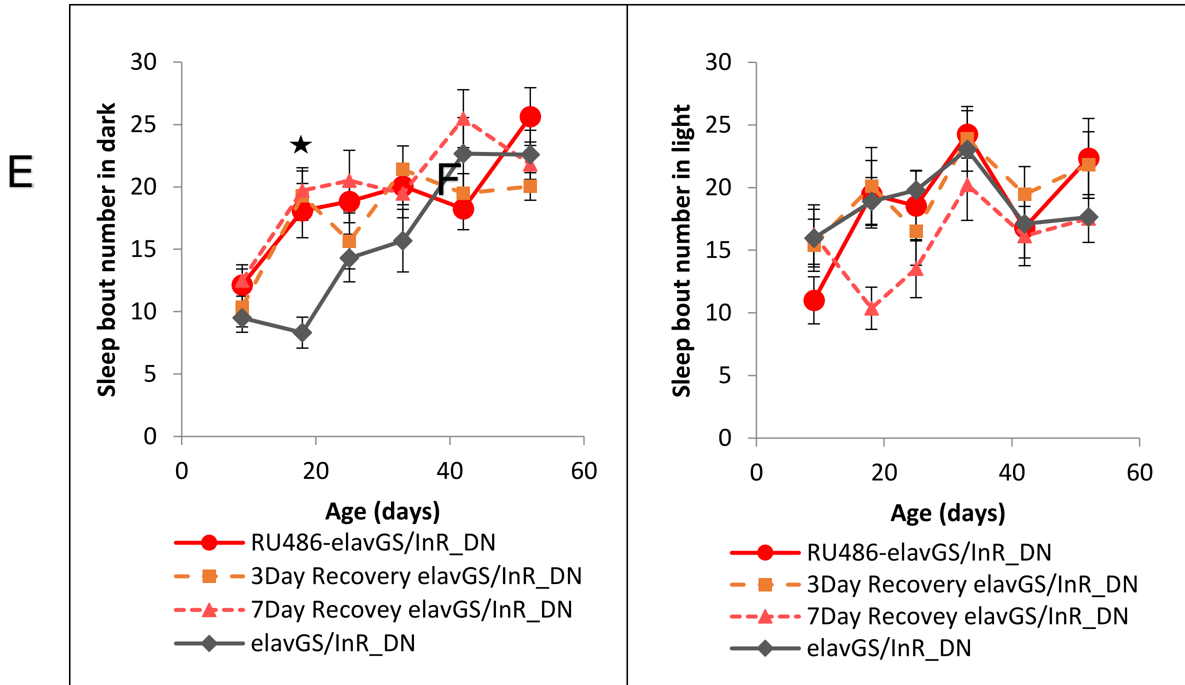
Generalized Linear Model Fit – Effect tests:			
Age:	0.0153	Age:	<0.0001
Genotype:	0.0066	Genotype:	<0.0001
Age*Genotype:	0.2426	Age*Genotype:	0.4213

Dunnett's Method using elavGS/InR^{DN} as control		P-value for each timepoint 1st	2nd	3rd	4th	5th	6th
Total activity	To 7 day Recovery elavGS/InR^{DN}	0.8615	0.0035	0.0515	0.1296	0.4796	0.9725
	To 3 day Recovery elavGS/InR^{DN}	0.9954	0.5648	0.4999	0.3610	0.5989	0.9517
	To RU486 elavGS/InR^{DN}	0.6184	0.8854	0.9814	0.7880	0.9357	0.8773
Total activity level	To 7 day Recovery elavGS/InR^{DN}	0.9960	0.0001	0.0073	0.0637	0.2600	0.9956
	To 3 day Recovery elavGS/InR^{DN}	0.9955	0.0527	0.0606	0.1179	0.8123	1.0000
	To RU486 elavGS/InR^{DN}	0.3942	0.0146	0.0504	0.1436	0.7221	0.9999



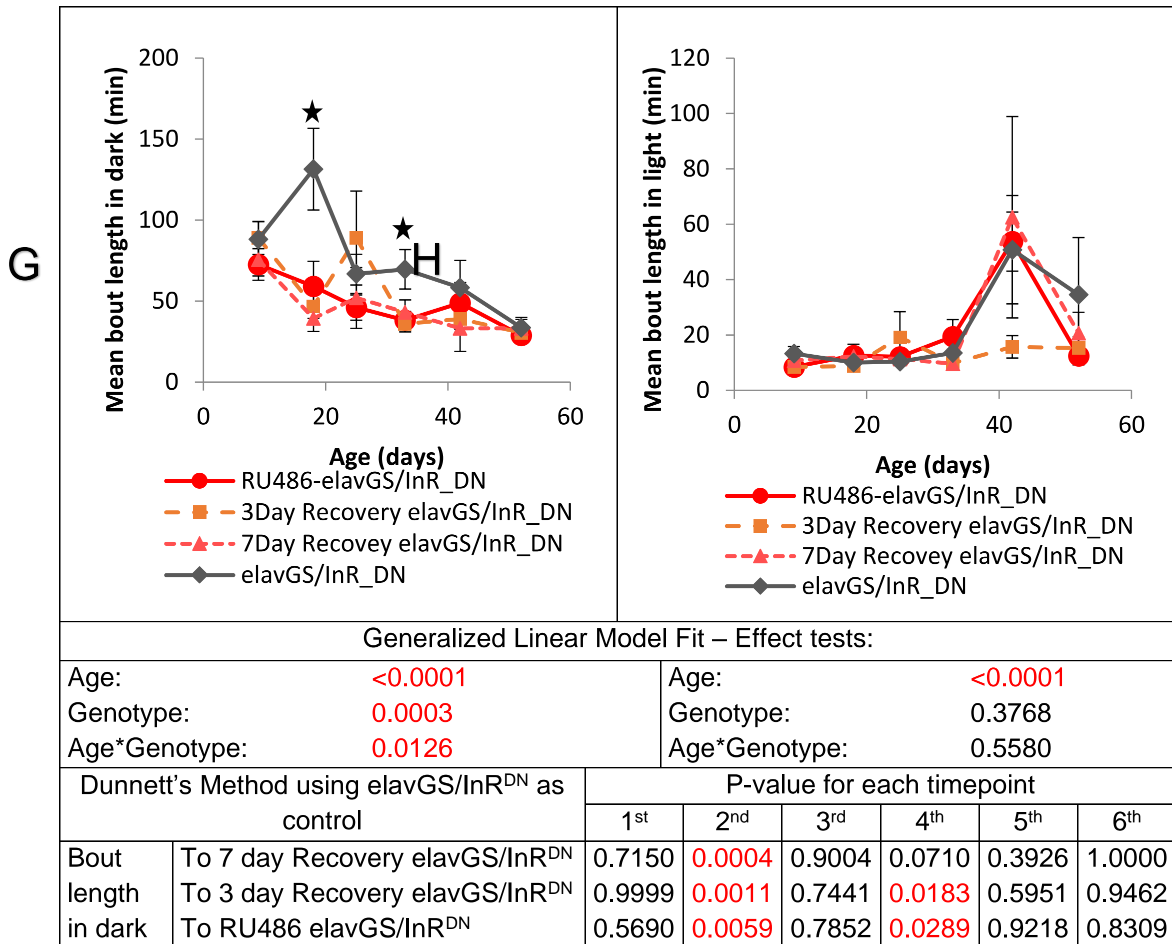
Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.0001
Genotype:	0.0139	Genotype:	0.0297
Age*Genotype:	0.0953	Age*Genotype:	0.0743

Dunnett's Method using elavGS/InR^{DN} as control		P-value for each timepoint					
		1st	2nd	3rd	4th	5th	6th
Total sleep in dark	To 7 day Recovery elavGS/InR^{DN}	0.9482	0.0067	0.0491	0.7088	0.0702	0.7729
	To 3 day Recovery elavGS/InR^{DN}	0.5450	0.2310	0.5360	0.7005	0.9996	0.9996
	To RU486 elavGS/InR^{DN}	0.6122	0.1058	0.7753	0.1216	0.4019	0.8835
Total sleep in light	To 7 day Recovery elavGS/InR^{DN}	1.0000	0.0704	0.4444	0.1289	0.8947	0.9973
	To 3 day Recovery elavGS/InR^{DN}	0.7062	0.9897	0.8388	0.4540	0.4867	0.8363
	To RU486 elavGS/InR^{DN}	0.1495	0.8200	0.9955	0.9904	0.4096	0.9643



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0004	Genotype:	0.0233
Age*Genotype:	0.0042	Age*Genotype:	0.3940

Dunnett's Method using elavGS/InR^{DN} as control		P-value for each timepoint					
		1st	2nd	3rd	4th	5th	6th
Bout number in dark	To 7 day Recovery elavGS/InR^{DN}	0.2830	0.0002	0.1032	0.4236	0.6910	0.9828
	To 3 day Recovery elavGS/InR^{DN}	0.9399	0.0004	0.9395	0.1428	0.6014	0.6490
	To RU486 elavGS/InR^{DN}	0.3704	0.0015	0.3156	0.3200	0.3481	0.5094
Bout number in light	To 7 day Recovery elavGS/InR^{DN}	0.9995	0.0453	0.1672	0.7573	0.9831	1.0000
	To 3 day Recovery elavGS/InR^{DN}	0.9955	0.9692	0.6442	0.9900	0.8170	0.5545
	To RU486 elavGS/InR^{DN}	0.2565	0.9967	0.9675	0.9728	0.9995	0.4609



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0003	Genotype:	0.3768
Age*Genotype:	0.0126	Age*Genotype:	0.5580

Dunnett's Method using elavGS/InR^{DN} as control		P-value for each timepoint					
		1st	2nd	3rd	4th	5th	6th
Bout length in dark	To 7 day Recovery elavGS/InR^{DN}	0.7150	0.0004	0.9004	0.0710	0.3926	1.0000
	To 3 day Recovery elavGS/InR^{DN}	0.9999	0.0011	0.7441	0.0183	0.5951	0.9462
	To RU486 elavGS/InR^{DN}	0.5690	0.0059	0.7852	0.0289	0.9218	0.8309

Figure 36 - Effect of inducible IIS reduction from the age of 3 days on the sleep behaviour of female flies with 7 day and 3 day recovery groups

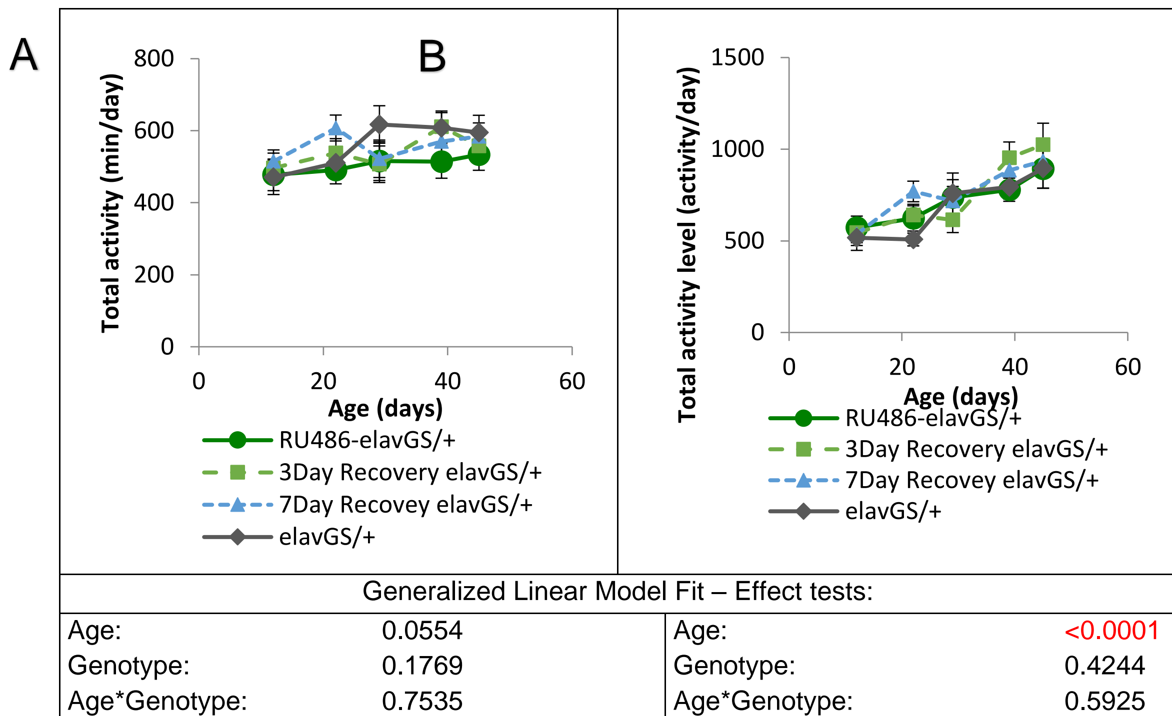
The activity of the mated female flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as 'sleep'. Flies counted as 'dead' if they show less than 100 min activity per day, N=15 for each group and timepoint. RU486-elavGS/UAS-InR^{DN} group had reduced IIS induced by RU486 from the age of 3 days. The 3 and 7 day recovery flies were removed from the RU486 food 3 or 7 days before the experiment and kept on standard food. The elavGS/UAS-InR^{DN} control group had no RU486 in their media at all. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype had a significant effect, the elavGS/InR^{DN} control group was compared to the other 3 groups using Dunnett's Method at each timepoint. Significant difference is highlighted with red text colour ($p<0.05$) in the statistical summary and with a star (★) on the graph.

A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

The recovery experiment was repeated with the elavGS/+ flies, investigating if it is possible to recover from the detrimental effects of RU486 on the sleep behaviour. It was done the same way as the recovery experiment with elavGS/InR^{DN} flies, but this time the elavGS flies were crossed with wild type w^{Dah} flies, so they did not have the UAS-InR^{DN} transgene. General Linear Modelling was used to check the effect of age, genotype and age*genotype interaction. When significant ($p<0.005$) effect was found for Genotype or Age*Genotype, post hoc pairwise comparison was carried out using Dunnett's Method, were the RU486-elavGS/+, 7 day and 3 day recovery groups were compared to the elavGS/+control at each timepoint.

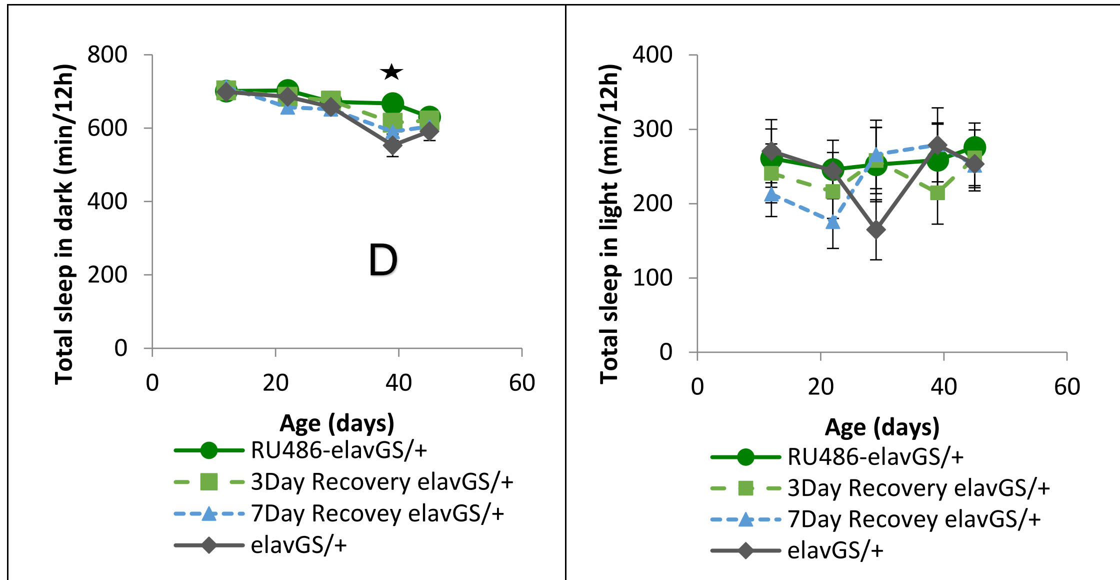
Female flies did not show significant age effect in their total activity, total sleep in light and sleep bout number in light (**Figure 37**). The only significant difference at each timepoint is more sleep in dark at the age of 39 days (p= 0.0052), which recovered with both 3- and 7-day recovery time (**Figure 37C**).

Effect of RU486 on elavGS female recovery



Generalized Linear Model Fit – Effect tests:			
Age:	0.0554	Age:	<0.0001
Genotype:	0.1769	Genotype:	0.4244
Age*Genotype:	0.7535	Age*Genotype:	0.5925

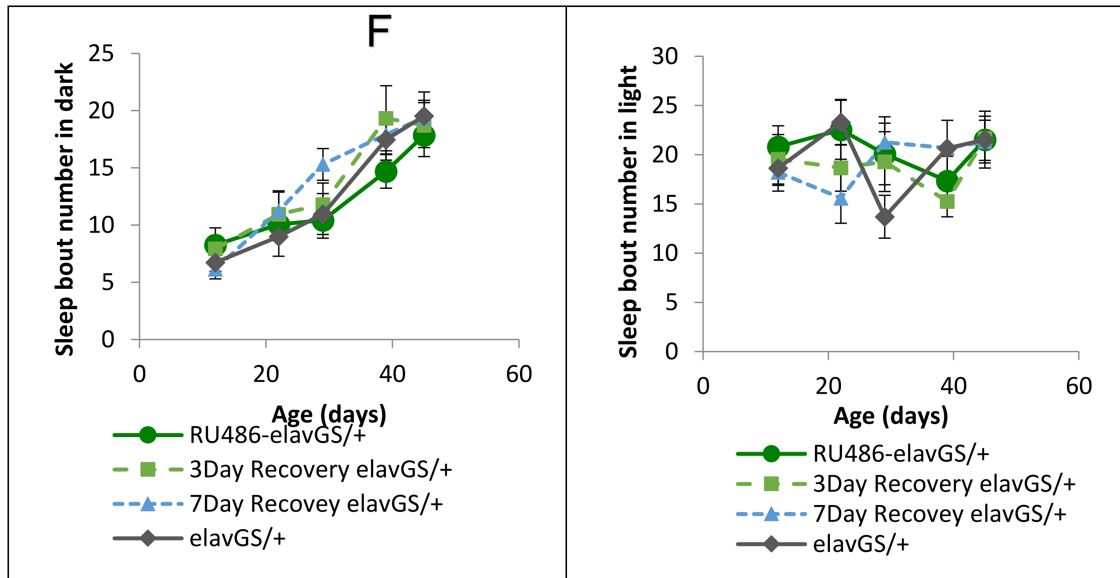
C



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.5572
Genotype:	0.0015	Genotype:	0.8035
Age*Genotype:	0.1360	Age*Genotype:	0.6913

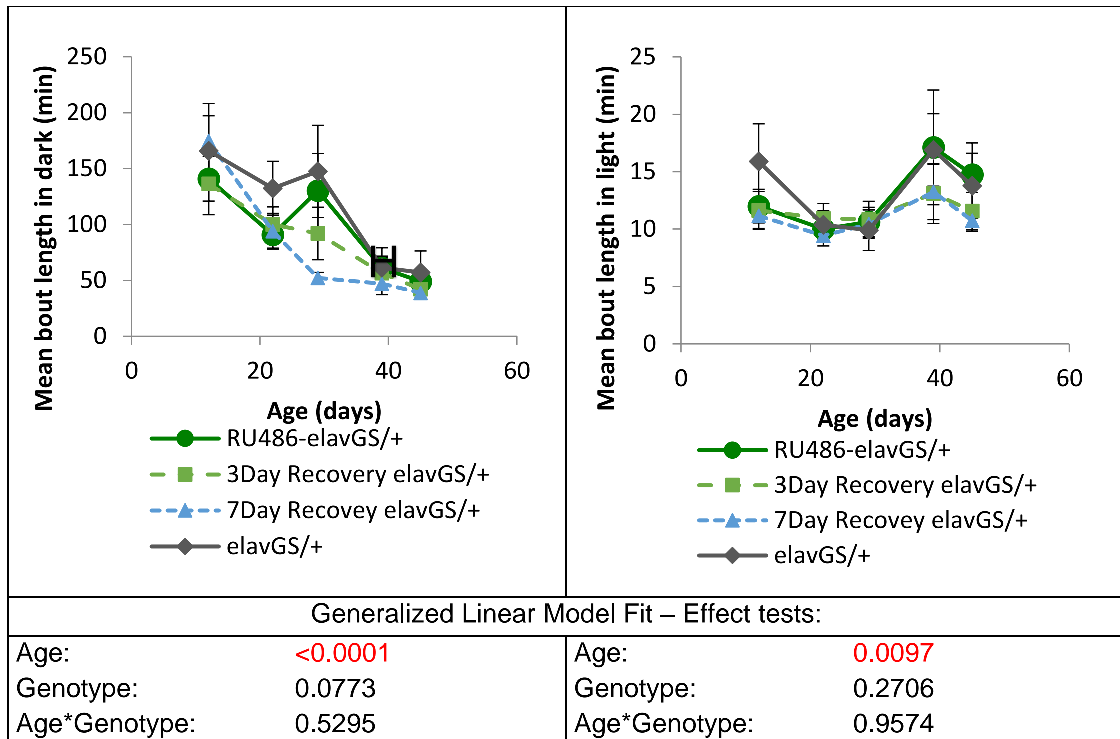
Dunnett's Method using elavGS/+ as control		P-value for each timepoint				
		1st	2nd	3rd	4th	5th
Total sleep in dark	To 7 day Recovery elavGS/+	0.4858	0.1929	0.9877	0.5477	0.9338
	To 3 day Recovery elavGS/+	0.9683	1.0000	0.8039	0.1756	0.5789
	To RU486 elavGS/+	0.9908	0.5845	0.8902	0.0052	0.3582

E



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.3652
Genotype:	0.3130	Genotype:	0.7837
Age*Genotype:	0.7746	Age*Genotype:	0.2117

G



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.0097
Genotype:	0.0773	Genotype:	0.2706
Age*Genotype:	0.5295	Age*Genotype:	0.9574

Figure 37 - Effect of RU486 from the age of 3 days on the sleep behaviour of female flies with 3 and 7 day recovery

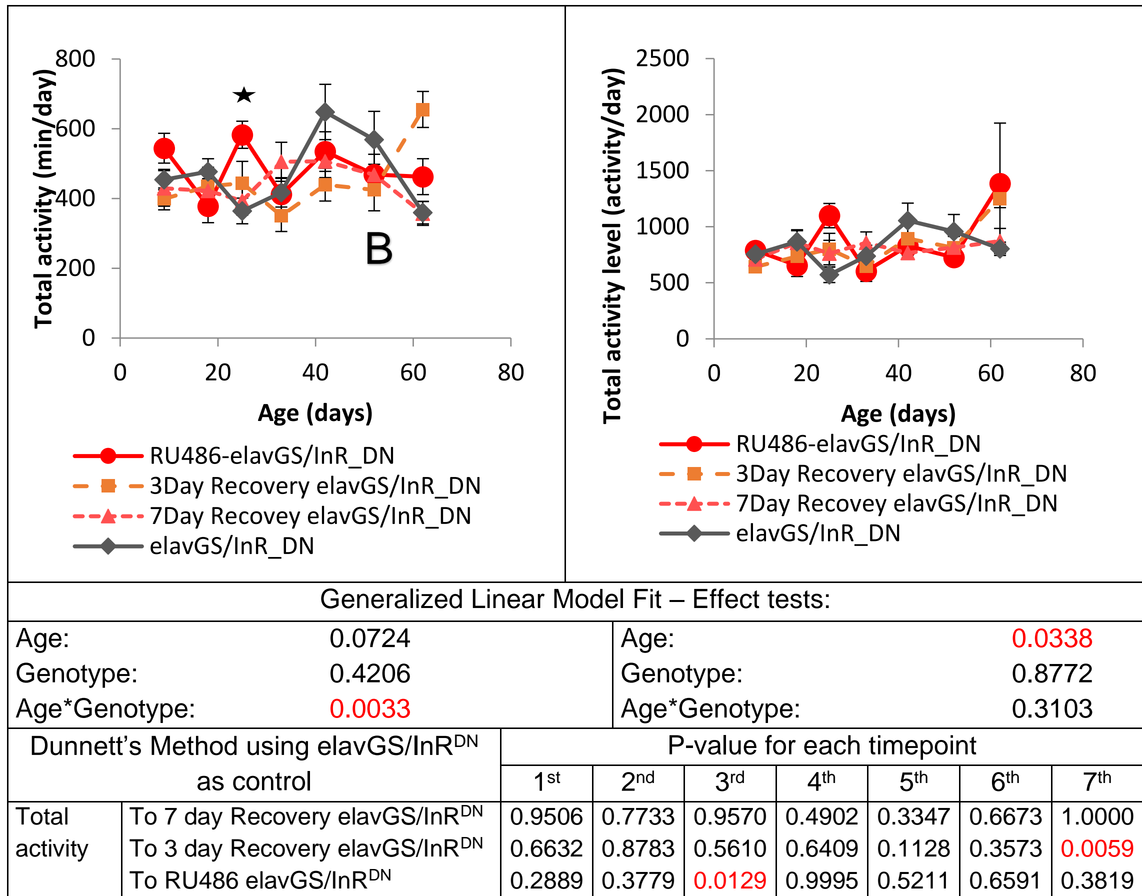
The activity of the mated female flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as 'sleep'. Flies counted as 'dead' if they show less than 100 min activity per day, N=15 for each group and timepoint. RU486-elavGS/+ group had reduced IIS induced by RU486 from the age of 3 days. The 3 and 7 day recovery flies were removed from the RU486 food 3 or 7 days before the experiment and kept on standard food. The elavGS/+ control group had no RU486 in their media at all. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype had a significant effect, the elavGS/+ control group was compared to the other 3 groups using Dunnett's Method at each timepoint. Significant difference is highlighted with red text colour ($p<0.05$) in the statistical summary and with a star (★) on the graph.

A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

When looking at the male recovery data, the only parameter that did not show any significant age effect by Generalised Linear Modelling is the total activity. When comparing the elavGS/InR^{DN} group to the other three groups at each timepoint, only two parameters showed any significant difference (**Figure 38**). At the age of 25 days, the RU486 group showed higher total activity (p 0.0129) and less sleep in light ($p=0.0183$) compared to the control, which recovered with 3 or 7 days off the RU486 food (**Figure 38A** and **D**).

Adult specific pan-neural IIS reduction with recovery in males

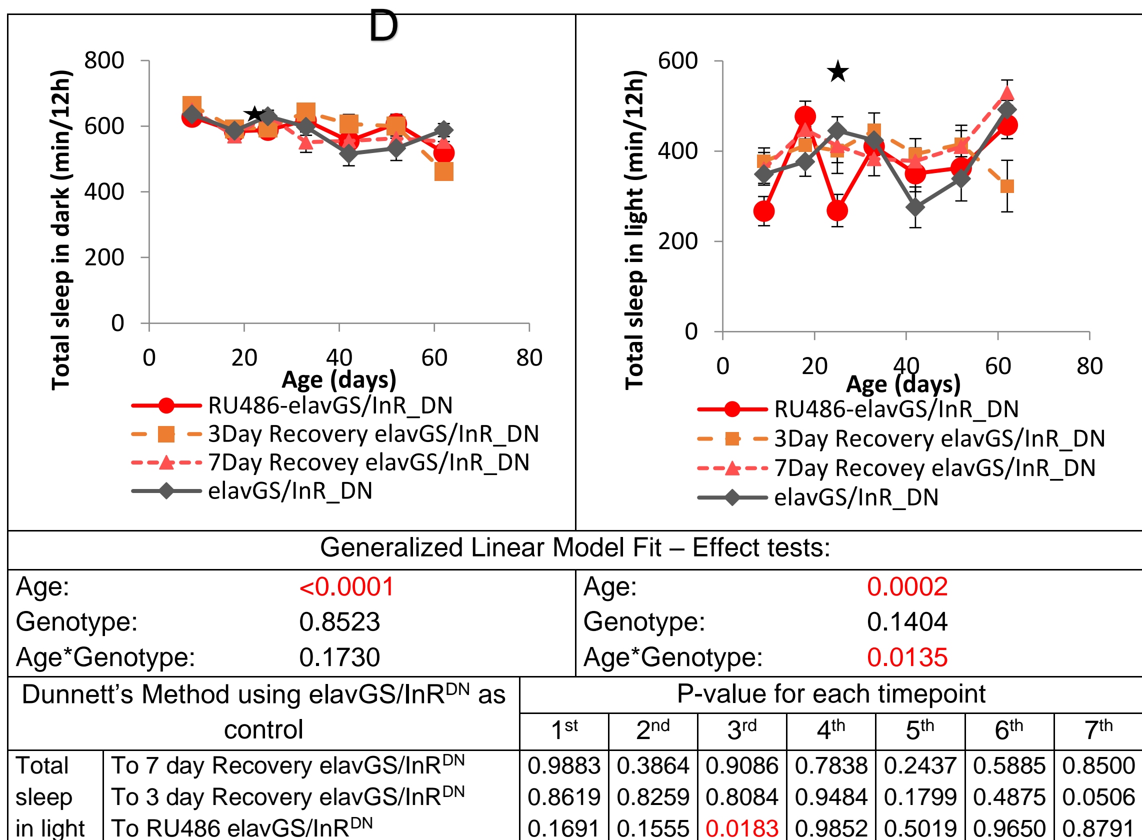
A



Generalized Linear Model Fit – Effect tests:			
Age:	0.0724	Age:	0.0338
Genotype:	0.4206	Genotype:	0.8772
Age*Genotype:	0.0033	Age*Genotype:	0.3103

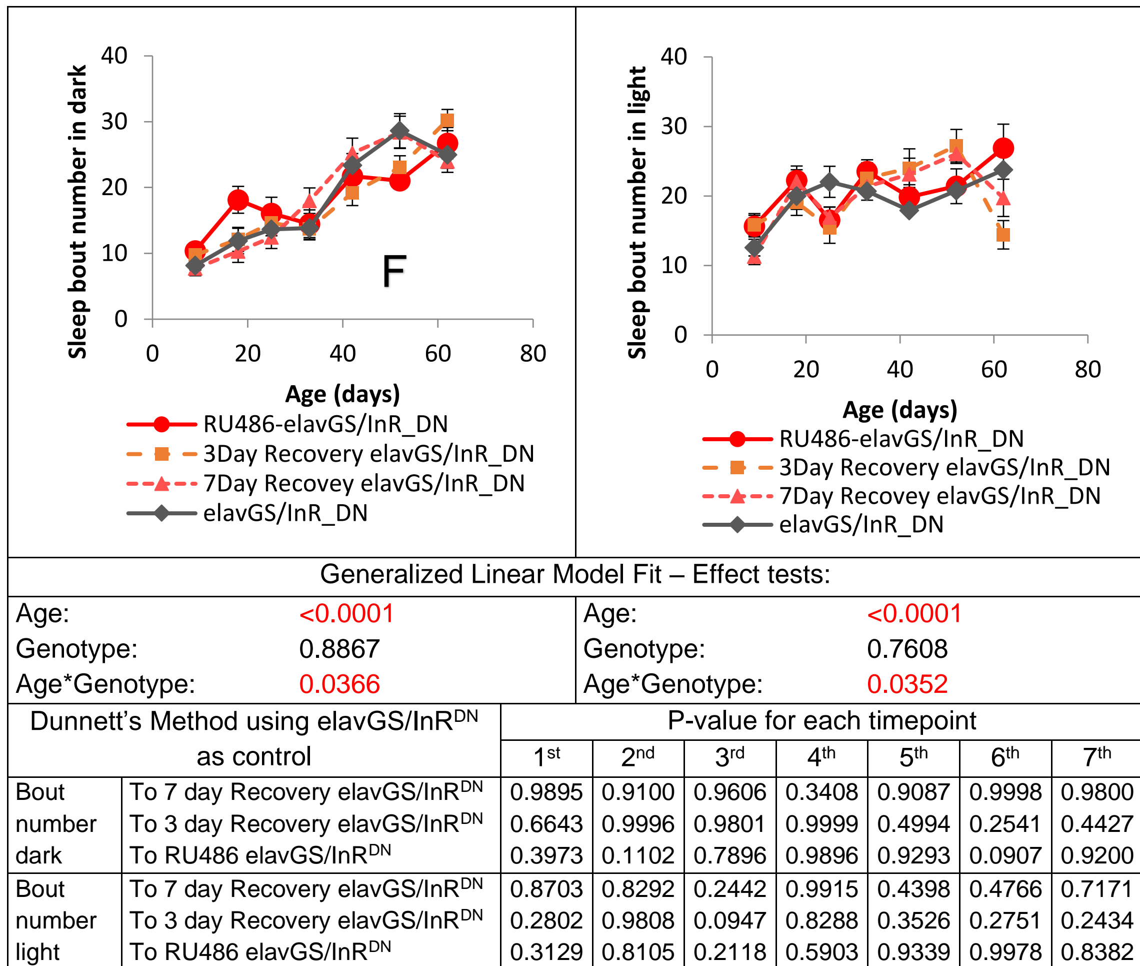
Dunnett's Method using elavGS/InR^{DN} as control		P-value for each timepoint						
		1st	2nd	3rd	4th	5th	6th	7th
Total activity	To 7 day Recovery elavGS/InR^{DN}	0.9506	0.7733	0.9570	0.4902	0.3347	0.6673	1.0000
	To 3 day Recovery elavGS/InR^{DN}	0.6632	0.8783	0.5610	0.6409	0.1128	0.3573	0.0059
	To RU486 elavGS/InR^{DN}	0.2889	0.3779	0.0129	0.9995	0.5211	0.6591	0.3819

C



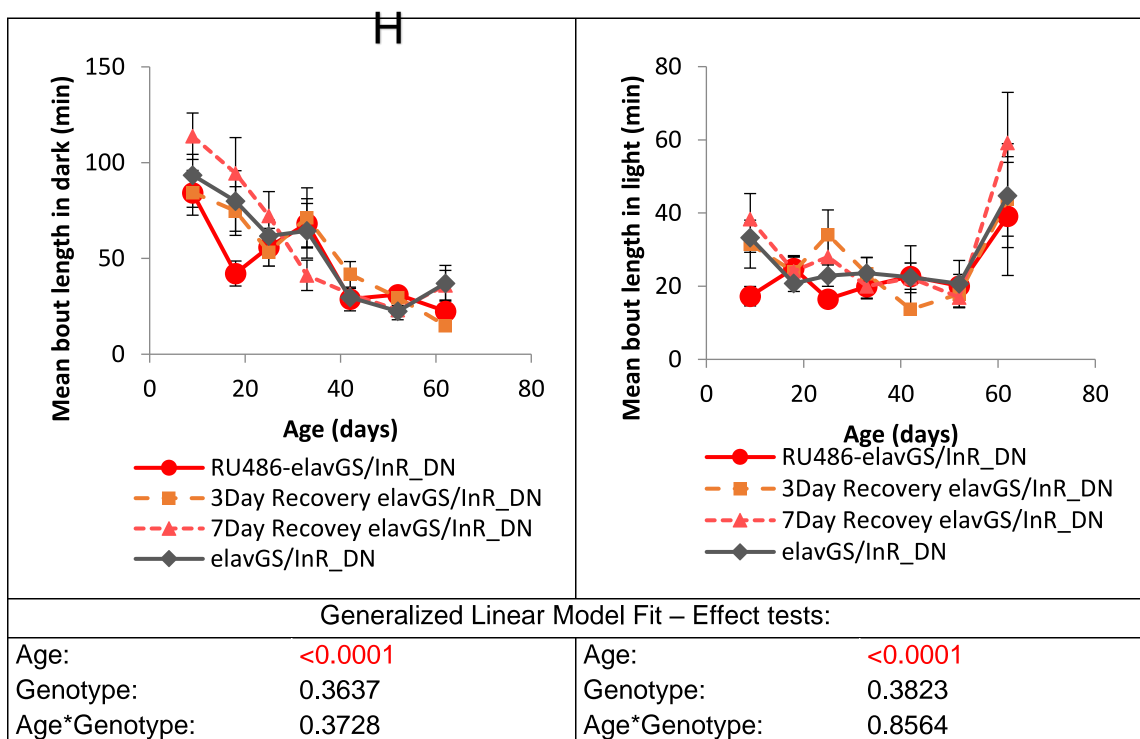
Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.0002
Genotype:	0.8523	Genotype:	0.1404
Age*Genotype:	0.1730	Age*Genotype:	0.0135

Dunnett's Method using elavGS/InR^{DN} as control		P-value for each timepoint						
		1st	2nd	3rd	4th	5th	6th	7th
Total sleep in light	To 7 day Recovery elavGS/InR^{DN}	0.9883	0.3864	0.9086	0.7838	0.2437	0.5885	0.8500
	To 3 day Recovery elavGS/InR^{DN}	0.8619	0.8259	0.8084	0.9484	0.1799	0.4875	0.0506
	To RU486 elavGS/InR^{DN}	0.1691	0.1555	0.0183	0.9852	0.5019	0.9650	0.8791



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.8867	Genotype:	0.7608
Age*Genotype:	0.0366	Age*Genotype:	0.0352

Dunnett's Method using elavGS/InR^{DN} as control		P-value for each timepoint						
		1st	2nd	3rd	4th	5th	6th	7th
Bout number dark	To 7 day Recovery elavGS/InR^{DN}	0.9895	0.9100	0.9606	0.3408	0.9087	0.9998	0.9800
	To 3 day Recovery elavGS/InR^{DN}	0.6643	0.9996	0.9801	0.9999	0.4994	0.2541	0.4427
	To RU486 elavGS/InR^{DN}	0.3973	0.1102	0.7896	0.9896	0.9293	0.0907	0.9200
Bout number light	To 7 day Recovery elavGS/InR^{DN}	0.8703	0.8292	0.2442	0.9915	0.4398	0.4766	0.7171
	To 3 day Recovery elavGS/InR^{DN}	0.2802	0.9808	0.0947	0.8288	0.3526	0.2751	0.2434
	To RU486 elavGS/InR^{DN}	0.3129	0.8105	0.2118	0.5903	0.9339	0.9978	0.8382



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.3637	Genotype:	0.3823
Age*Genotype:	0.3728	Age*Genotype:	0.8564

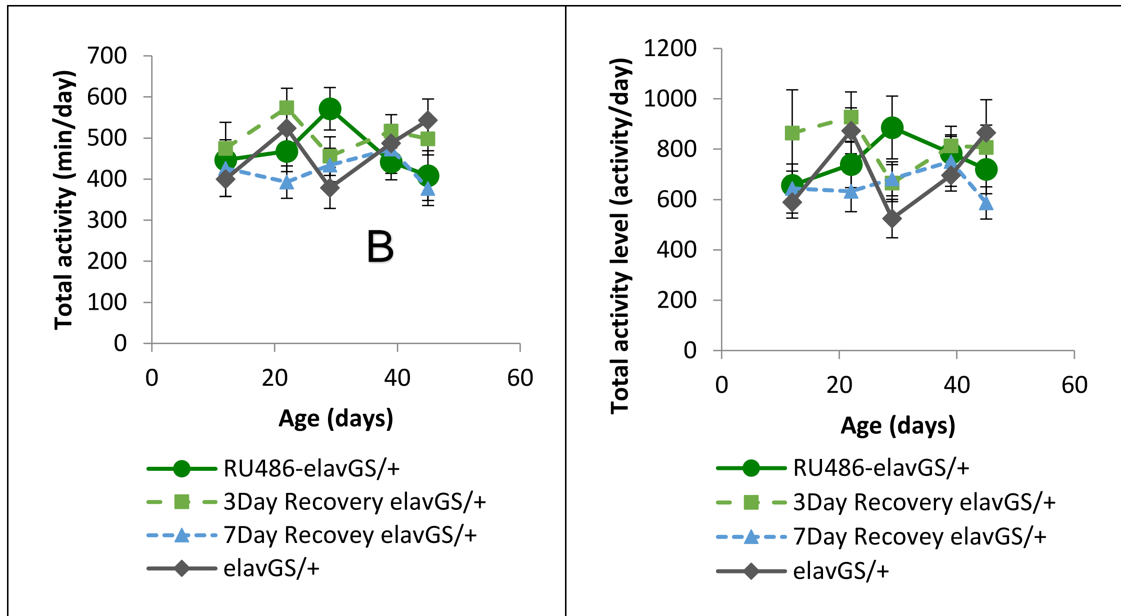
<u>Figure 38</u> - Effect of inducible IIS reduction from the age of 3 days on the sleep behaviour of male flies with 7 day and 3 day recovery groups

The activity of the male flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as

‘sleep’. Flies counted as ‘dead’ if they show less than 100 min activity per day, N=15 for each group and timepoint. RU486-elavGS/UAS-InR^{DN} group had reduced IIS induced by RU486 from the age of 3 days. The 3 and 7 day recovery flies were removed from the RU486 food 3 or 7 days before the experiment and kept on standard food. The elavGS/UAS-InR^{DN} control group had no RU486 in their media at all. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype had a significant effect, the elavGS/InR^{DN} control group was compared to the other 3 groups using Dunnett’s Method at each timepoint. Significant difference is highlighted with red text colour (p<0.05) in the statistical summary and with a star (★) on the graph.
A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

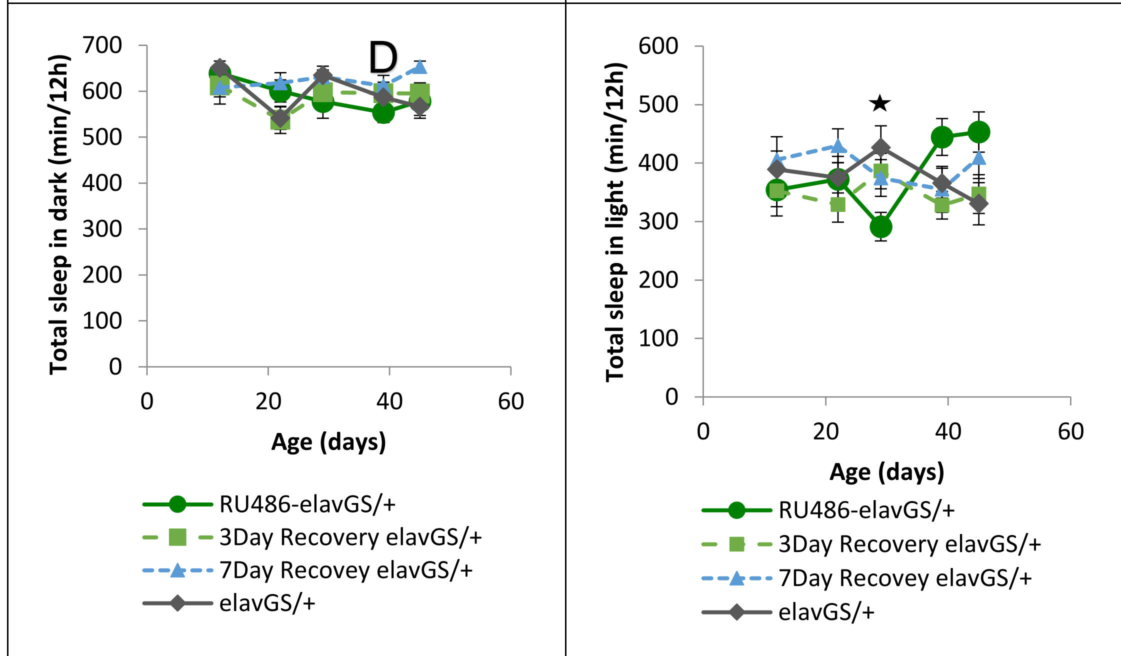
In contrast to females, RU486 treatment of males increased the sleep fragmentation which could be reversed by recovery time off the drug (**Figure 39**). Total sleep in light was reduced by RU486 (p=0.0205) at the age of 29 days and it recovered with 3 or 7 days off the RU486 food (**Figure 39D**). The number of sleep bouts in the dark were increased by RU486 at the age of 12 (p=0.0143) and 29 days (p=0.0241) and the number of sleep bouts in the light was higher at age 12 (p=0.0037) and 39 days (p=0.0084). The two recovery groups in both sleep bout parameters did not show significant difference from the elavGS/+ control, so they recovered (**Figure 39E** and **F**). The sleep bout length in the dark was reduced by RU486 at the age of 12 (p=0.0494) and it could not be reversed by 3 day recovery (p=0.0408), however, after 7 days of recovery the significant difference from elavGS/+ disappeared. The sleep bout length in the dark was shortened by RU486 at the age of 12 (p=0.0189 and 29 days (p=0.0477) and the 3 day recovery at the age of 12 days was close to being significantly different from elvGS/+ (p=0.0539) (**Figure 39G** and **H**). Thus, 3 and 7 day recovery time could reverse the negative effects of RU486. Since RU486 affects the sleep behaviour of the male flies, it is hard to interpret the effect of adult-specific pan-neural IIS reduction on males.

The effect of RU486 on elavGS/+ males with recovery



Generalized Linear Model Fit – Effect tests:

Age:	0.5868	Age:	0.4515
Genotype:	0.0765	Genotype:	0.0691
Age*Genotype:	0.1667	Age*Genotype:	0.2376

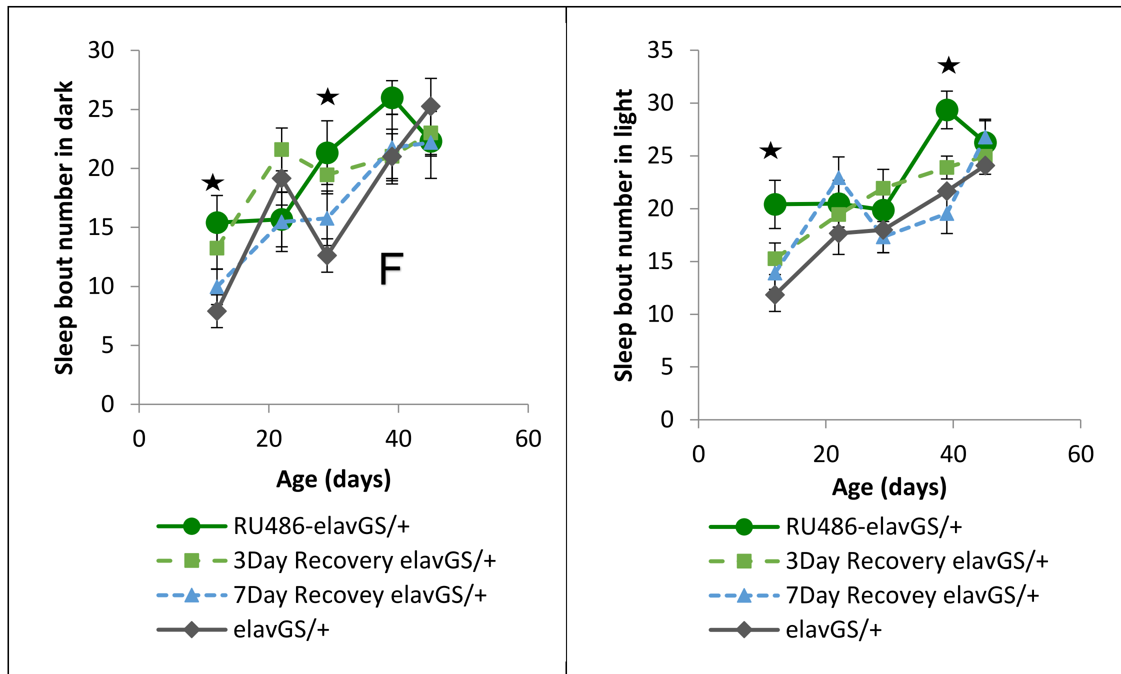


Generalized Linear Model Fit – Effect tests:

Age:	0.0265	Age:	0.9814
Genotype:	0.0857	Genotype:	0.1705
Age*Genotype:	0.1789	Age*Genotype:	0.0317

Dunnett's Method using elavGS/+ as control		P-value for each timepoint 1st	2nd	3rd	4th	5th
Total sleep in light	To 7 day Recovery elavGS/+	0.9692	0.5554	0.5632	0.9911	0.3715
	To 3 day Recovery elavGS/+	0.8264	0.6547	0.7322	0.7334	0.9816
	To RU486 elavGS/+	0.8377	0.9999	0.0205	0.2094	0.0800

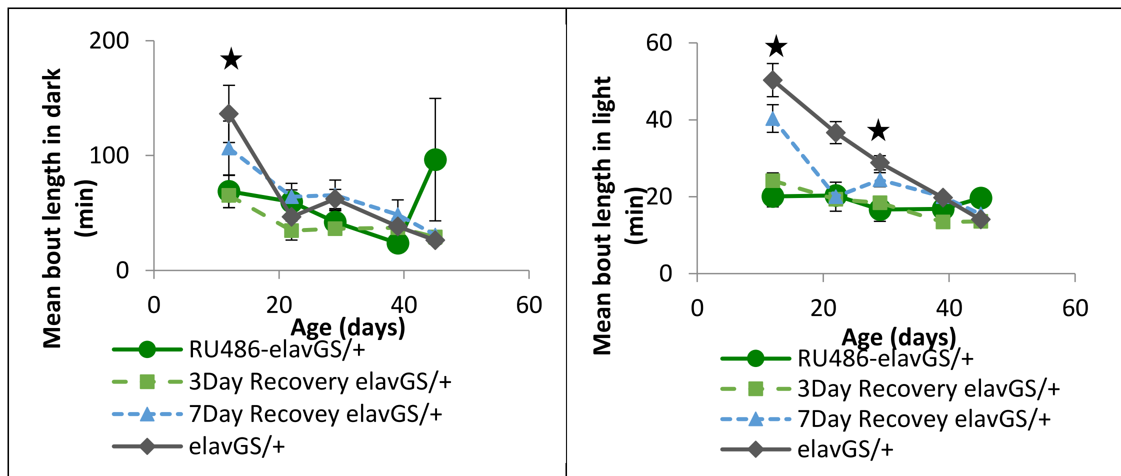
E



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0401	Genotype:	0.0018
Age*Genotype:	0.1073	Age*Genotype:	0.1204

Dunnett's Method using elavGS/+ as control		P-value for each timepoint 1st	2nd	3rd	4th	5th
Bout number in dark	To 7 day Recovery elavGS/+	0.7581	0.5867	0.6242	0.9909	0.7394
	To 3 day Recovery elavGS/+	0.1143	0.8100	0.0860	1.0000	0.8658
	To RU486 elavGS/+	0.0143	0.6040	0.0241	0.2980	0.7476
Bout number in light	To 7 day Recovery elavGS/+	0.7471	0.2505	0.9906	0.7317	0.7268
	To 3 day Recovery elavGS/+	0.4069	0.6894	0.3981	0.6801	0.9842
	To RU486 elavGS/+	0.0037	0.6894	0.8629	0.0084	0.8223

G H



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.1212	Genotype:	0.0003
Age*Genotype:	0.0357	Age*Genotype:	0.0714

Dunnett's Method using elavGS/+ as control	P-value for each timepoint 1st	2nd	3rd	4th	5th

Bout length in dark	To 7 day Recovery elavGS/+	0.5746	0.5868	0.9900	0.7440	0.9991
	To 3 day Recovery elavGS/+	0.0408	0.7881	0.1887	0.9993	0.9998
	To RU486 elavGS/+	0.0494	0.7476	0.3870	0.4948	0.2523
Bout length in light	To 7 day Recovery elavGS/+	0.6760	0.1265	0.6965	0.9999	0.9591
	To 3 day Recovery elavGS/+	0.0539	0.0916	0.0942	0.3059	0.9983
	To RU486 elavGS/+	0.0189	0.1177	0.0477	0.8317	0.2576

Figure 39 - Effect of RU486 from the age of 3 days on the sleep behaviour of male flies with 3 and 7 day recovery

The activity of the male flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as 'sleep'. Flies counted as 'dead' if they show less than 100 min activity per day, N=15 for each group and timepoint. RU486-elavGS/+ group had reduced IIS induced by RU486 from the age of 3 days. The 3 and 7 day recovery flies were removed from the RU486 food 3 or 7 days before the experiment and kept on standard food. The elavGS/+ control group had no RU486 in their media at all. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype had a significant effect, the elavGS/+ control group was compared to the other 3 groups using Dunnett's Method at each timepoint. Significant difference is highlighted with red text colour ($p<0.05$) in the statistical summary and with a star (★) on the graph.

A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

6.3: Discussion

The aim of the sleep experiments presented here was to investigate the effects of reduced IIS in *Drosophila* neurons on age-related sleep fragmentation in flies. Metaxakis et al. (2014) had previously shown using the *dilp2-3,5* mutant and daGAL4/InR^{DN} fly models that sleep fragmentation increases with age in flies and systemic reduction of IIS can ameliorate age-related sleep fragmentation in *Drosophila*. The effect of pan-neural IIS reduction on age-related sleep fragmentation, however, was not known. Based on previous work in our lab (Ismail et al. 2015) showing neutral or detrimental effects of reduced IIS on the senescence of other behavioural functions, we hypothesised that pan-neural IIS reduction would not be beneficial to the neural circuitry regulating sleep behaviour.

Overall, the age-related sleep behavioural changes were not as characteristic as they are in the exploratory walking or negative geotaxis experiments, and often age did

not have a significant effect. When age did have an effect on sleep behaviour in our study, it showed similar patterns to those seen in Metaxakis et al. (2014) such as increased number of sleep bouts and shorter length of sleep bouts with age indicating age-related sleep fragmentation.

Constitutive pan-neural IIS reduction did not alter the sleep behaviour of the flies (**Figure 30** and **Figure 31**). When IIS was reduced from adulthood only using the elavGS system, the female sleep fragmentation was increased at middle age, therefore it had detrimental effects (**Figure 32**). While sleep in the light did not change in response to adult specific pan-neural IIS reduction, at the age of 18 days, their sleep bout numbers in dark doubled and the length of the sleep bouts in the dark was reduced by 45%. The total sleep in dark was also reduced by 10% at the age of 18 days and while the total time the flies spent active did not change, the amount they moved during their active time increased by about 65% between the age of 18-33 days. RU486 only affected the total dark sleep parameter (**Figure 33**), therefore the increased sleep fragmentation in females was caused by the reduced IIS in their neurons.

Males did not show any clear change in response to reduced IIS (**Figure 34**), however their sleep fragmentation was increased by RU486 itself (**Figure 35**). Since the chemical used as the inducer of IIS reduction had detrimental effects on the behaviour, it is impossible to tell from this data how the sleep behaviour of the males responds to IIS reduction in the neurons, as the detrimental effect of RU486 can mask any positive or negative effect of reduced IIS in adult fly neurons.

The recovery experiment tested if it is possible to reverse the detrimental effects of reduced IIS in the adult neurons. There was no consistent recovery of the sleep fragmentation in females, as the detrimental effect of adult specific IIS reduction in the neurons, namely increased total activity level, shorter sleep bouts in the dark and higher number of sleep bouts in the dark, did not improve after 3 or 7 days recovery time off RU486 (**Figure 36** and **Figure 37**).

It is hard to draw conclusions from the male data, as the chemical RU486 affects their behaviour. The recovery experiment with the elavGS/+ males showed that the negative effect of RU486 can be reversed by 3 or 7 day recovery time off the drug (**Figure 38** and **Figure 39**).

Similarly to the negative geotaxis and exploratory walking behaviours, while systemic IIS reduction improves some of the behavioural functions, in this case the sleep fragmentation with age, pan-neural IIS signalling reduction has no or detrimental

effect on sleep behaviour. In our experiments, many of the detrimental effects happened at middle age (in around 18-30 days old flies), suggesting that pan-neural IIS reduction affects the sleep fragmentation itself, not its age-related increase. Based on the findings of Erion, et. al (2012), IIS does not seem to play a role in sleep regulation at young age and it is not known IIS in neurons effects age-related sleep fragmentation. This could be caused by reduced function of the neurons due to reduced IIS. The detrimental effects of reduced pan-neural IIS could be caused by reduced function of the neurons. All neuronal subtypes play a role in regulating sleep (reviewed by Ly, et al. 2018, further discussed in Chapter 10), therefore disrupted function of any of the neuronal subtypes caused by reduced IIS in the neurons could alter sleep behaviour.

Metaxakis, et al. (2014) showed that different mechanisms regulate day and night sleep. The increased daytime activity due to systemic IIS reduction is mediated through dFOXO, furthermore, AKH and octopaminergic signalling also regulate daytime activity. On the other hand, night sleep is mediated by TOR and S6K signalling (Metaxakis, et al. 2014). In our experiment, adult-specific pan-neural IIS reduction affected the total activity level of the female flies, but not their total activity, therefore they moved more when they were active, but did not spend more time being active. The daytime sleep of the females was normal, but adult specific pan-neural IIS reduction increased sleep fragmentation at night, therefore the detrimental effects of reduced pan-neural IIS could possibly be caused by changes in TOR or S6K. Metaxakis, et al. (2014) used virgin female flies for their experiments, while our experiments used mated females and males. Previous studies have shown that mating affects sleep in females (Garbe, et al. 2016 and Dove et al. 2017), therefore mating could affect the sleep behaviour of the female flies in out experiments.

To sum up, sleep behaviour in our experiments shows less characteristic change with age compared to other behaviours measured before, therefore less suitable for measuring ageing. Similarly to other behaviours, while systemic IIS reduction shows improvement in the behaviour, pan-neural IIS reduction either causes no or detrimental effects. It is also worth mentioning that similarly to the exploratory walking experiment, males show behavioural changes in response to the chemical RU486, therefore it should be used cautiously for fly behavioural studies.

Chapter 7: Endocrine and peripheral effects of reduced IIS in neurons

7.1: Introduction

As discussed in the previous chapters, numerous studies, including those presented here, have shown a disconnection between lifespan and health span such that it is possible to extend lifespan with no improvement or even detrimental effects on behavioural function (Tomioka, et al. 2006, Bhandari, et al. 2007, Ismail, et al. 2015). For locomotor behavioural function, Ismail et al. (2015) found that constitutive pan-neural IIS reduction using the elavGAL4 driver and UAS-InR^{DN} transgene extended the lifespan of female flies, but it did not improve the age-related decline in negative geotaxis and had detrimental effects on exploratory walking senescence. In Chapters 4 and 5 of this study, down-regulation of IIS in neurons only during adulthood had a similar effect indicating that the detrimental effects on locomotor behavioural senescence in Ismail et al. (2015) were not due to developmental effects.

In Chapter 6, the investigation was extended further to determine the role of reduced neuronal IIS on another behaviour - age-related sleep fragmentation. Interestingly, the age-related sleep fragmentation of flies with constitutive neuronal IIS reduction was unaffected, but the sleep of females with adult specific neuronal IIS reduction showed sleep fragmentation at earlier ages than controls. Together, these data indicate that in general reduced neuronal IIS extends lifespan but with detrimental effects on behavioural senescence. However, different behavioural functions do not respond in the same way to reduced IIS during development and adulthood; and males and females do not always respond in the same way. The primary aim of the experiments presented in this chapter is to investigate additional phenotypes that will give insight into how reduced IIS in neurons influences lifespan and neuronal function in males and females.

Firstly, we measured the effects of pan-neural IIS reduction (both constitutive and adult-specific) on the expression of *Drosophila* insulin like peptides (DILPs) from fly heads and bodies. *Drosophila* have a single Insulin Receptor (dInR) and multiple ligands that are able to bind to the receptor (Mathew, et al. 2017). Seven of these ligands, the *Drosophila* insulin-like peptides (DILPs1-7), were identified by their similarity to human insulin (Brogiolo et al., 2001), while DILP8 was discovered more

recently (Colombani et al, 2012). Our study measured the expression of *dilp2-7*. Each DILP has a unique cell and developmental stage specific expression pattern, which is nicely summarised in the review article of Nässel, Liu and Lu, (2015). In the larval brain, DILP1,2, 3 and 5 are expressed in the Insulin Producing Cells (IPCs), but DILP1 expression is transient in adults. DILP2, 3 and 5 are expressed in a set of 14 median neurosecretory cells (MNCs) in the brain, the Insulin producing cells (IPCs). Furthermore, DILP3 is also expressed in muscle cells of the adult midgut and DILP5 in the follicle cells of ovary and principal cells in renal tubules (Nässel, Liu and Luo, 2015). DILP 4 is expressed in the anterior midgut in larvae, but its expression in the adult is scarce and has not been fully analysed yet (Brogiolo et al., 2001, Nässel, Liu and Luo, 2015). DILP6 is expressed in adipose cells (fat body), salivary glands, heart and the glial cells of the CNS in larvae and only in the fat body in adult flies (Nässel, Liu and Luo, 2015). There are about 20 neurons responsible for producing DILP7 in the abdominal ganglia in both larvae and adult flies, and some of these have axons terminating in the hindgut. They also project to the sub-esophageal ganglion in the brain and to the female internal reproductive tract (Yang, et al. 2008). Lastly, DILP8 was found in larval imaginal discs (Colombani et al., 2012; Garelli et al., 2012) and the FlyAtlas gene expression data base also shows expression in adult ovaries. **Figure 40** shows the location of the expression of various *dilp*s in the adult fly body.

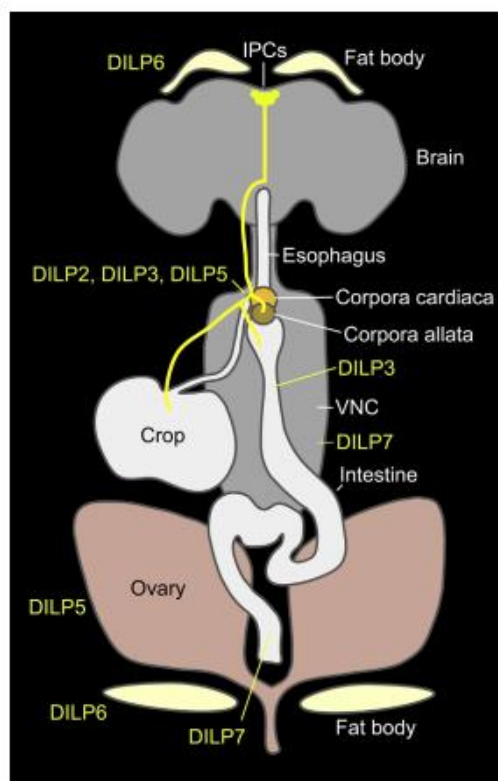


Figure 40 - Summary of DILP production and release sites in the adult Drosophila
The Insulin Producing Cells (IPCs) are shown in yellow, where DILP2,3 and 5 are produced. They are released from the axon terminations in the corpora cardiaca (pair of neuroglandular bodies behind the brain and on both sides of the aorta) and the corpora allata (paired glandular bodies at either side of the foregut), crop and anterior intestine. DILP5 is also produced in the ovaries and renal tubules. DILP6 is produced in the fat body in the fly head and body. DILP7 is produced in the abdominal neuromeres and released into the posterior intestine, female internal reproductive tract and CNS as well. The illustration is from the review article of Nässel, Liu and Luo (2015).

Previous studies have investigated the individual functions and effects of the *dilps* and revealed some functional redundancy and coordination of expression between the IPC and fat body *dilps*. For instance, Grönke, et al. (2010) measured the egg-to-adult survival of *dilp* mutants (except *dilp8*, which was discovered in 2012) and found that all single *dilp* knock out mutants, *dilp2-3* and *dilp1-4* mutants were homozygous viable. *Dilp2–3,5* homozygous females are viable, but only 50-60% of the males survive, similarly to *dilp7;2–3,5* mutant flies. Survival is reduced in *dilp1–4,5* and *dilp7;1–4,5* mutants. Although the latter group lacks all *dilp*s except *dilp6* (and dilp8), it still develops into adulthood. However, when *dilp2, 3, 5 and 6* were knocked out (*dilp7* and *8* remaining), complete lethality resulted in males and females, suggesting that DILP6 acts redundantly to the *dilp*s expressed in the IPCs (Grönke, et al., 2010). Grönke, et al. (2010) also measured the transcript levels of the 4E-BP, which is a direct target of the dFOXO transcription factor when it is in the nucleus following reduction of IIS. No significant upregulation of 4E-BP was found in any of the single *dilp* mutants, except for *dilp3* in the heads (Grönke et al. 2010). The transcript levels of 4E-BP were also elevated in *dilp2–3,5* mutants. Grönke et al. (2010) suggest that this could mean that DILPs function redundantly in a negative feedback system and the loss of one DILP can be compensated for by the upregulation of other DILPs. Grönke, et al. (2010) also found that *dilp5* was upregulated in *dilp2* and *dilp2-3* mutants and *dilp3* was up-regulated in *dilp2* and *dilp5* mutants, showing that there is a compensatory transcriptional regulation of *dilp*s expressed in the IPCs. The expression of *dilp*4 and 7 did not change significantly with the loss of *dilp2– 3,5* but *dilp6* (expressed in the fat body) was highly upregulated, suggesting that there could be a negative feedback system coordinating the expression of *dilp*s between the IPCs in the brain and in the peripheral tissues, such as the fat body (Grönke et. al, 2010).

In terms of the roles of the individual DILPs, there is evidence that although there is some functional specificity, there is much functional redundancy. Ikeya, et al. (2002) showed that all DILPs can promote growth, with DILP2 having the strongest effect. Later studies identified the role of the DILPs in lifespan, fecundity, stress resistance and metabolism (Broughton, et al. 2005 and 2008, Grönke, et al. 2010, Bai, et al. 2012). The role of the DILP2,3 5 producing IPCs in lifespan was first identified by Broughton et al (2005) and it was later shown that reduction of DILP2 alone via RNAi was sufficient to modulate trehalose storage but not lifespan (Broughton et al, 2008). RNAi knockdown of *dilp*2 resulted in a compensatory increase in *dilp*3 and 5 via autocrine insulin signalling in the IPCs. To determine the roles of all the individual DILPs Grönke, et al. (2010) measured the lifespan of *dilp null* mutant flies and found no extension of

lifespan in the single *dilp1,3,4,5,6,7* mutant. In contrast to the *dilp2* RNAi hypomorphs which showed no lifespan extension (Broughton, et al. 2008), the *dilp2* knock out flies had significantly longer lifespan than the controls in both males and females. *Dilp2–3* mutants also had extended lifespan, but they didn't live longer than *dilp2* mutants. Heterozygous *dilp2-3, 5* mutants were slightly long-lived, but the homozygous *dilp2–3,5* mutants and the *dilp1–4* mutants had normal lifespan. This was contradictory to the results of Broughton, et al. (2005), were flies with IPC ablation and therefore reduced *dilp2,3* and *5* had extended lifespan. Those flies, however contained the intracellular symbiont bacteria *Wolbachia pipientis*, which has previously shown to affect IIS in flies (Ikeya, et al. 2009). The experiments of Grönke, et al. (2010) used *Wolbachia* negative flies in the w^{1118} background. When they repeated the experiments using *Wolbachia* positive *dilp2,3* and *5* null mutant flies in the w^{Dah} background, the female maximum lifespan was increased by 22% on standard diet and by 27% on high yeast diet while *Wolbachia* had no effect on the lifespan of the wild type w^{Dah} flies (Grönke, et al. 2010). The lifespan and other phenotypic effects of dilp null mutant flies are summarised in **Table 6**. Bai et al. (2012) have found that overexpressing dilp6 in the adult fat body lengthen the lifespan and represses *dilp2* and *dilp5* expression in the brain and DILP2 release into the haemolymph. Recent study of Post, et al. (2018) shows that *dilp1* can promote longevity. They found that *dilp2 null* mutation extends lifespan, *Akh* mRNA and protein levels and also increase *dilp1* mRNA expression 14-fold. *Dilp1 null* mutant flies and *dilp1-2 null* mutants have normal lifespan, but transgenic expression of *dilp1* in *dilp1-2* double mutants extend lifespan. Post, et al. (2018) suggests that the reduction or loss of dilp2 extends lifespan because its depletion promotes *Akh* and *dilp1* expression, which functions as a pro-longevity factor.

As DILPs function redundantly in flies and their expression is regulated by autocrine IIS in the IPCs and by DILP6 from the fat body, it is possible that altering IIS in neurons may affect IIS or *dilp* expression in the IPCs or elsewhere in the body, which in turn could affect the lifespan of the flies. Ismail et al. (2015) measured *dilp* expression in elavGAL4/UAS-InR^{DN} male and female fly heads and bodies with constitutive pan-neural IIS reduction and found no change in *dilp* expression. We repeated this experiment using the elavGAL4/UAS-InR^{DN} flies and additionally measured *dilp* expression in heads and bodies of RU486-elavGS/UAS-InR^{DN} flies with reduced pan-neuronal IIS only during the adult period.

<u>Table 6</u> - Summary of the effect of *dilp* mutants

Based on the results of Grönke et al. (2010), also containing the results of Post, et al. (2018). ND: not determined, NC: not changed. W+/-: *Wolbachia* positive/negative. Most females are *Wolbachia* negative, unless otherwise stated.

Background: orange: reduction, yellow: not changed, green: increase

Mutant	Viability	Egg to adult development time	Body weight	Median lifespan	Lifetime fecundity	Paraquat resistance	Starvation resistance	Lipid	Glycogen	Trehalose
dilp1	100%	NC	m: -7% f:-7%	NC	NC	NC	Reduced (Post, et.al 2018)	NC	Reduced (Post, et.al 2018)	ND
dilp2	100%	m: +8h f: +17h	m: -5% f: -11%	m:+9% f:+8-13%	-25%	NC	NC	NC	NC	+64%
dilp3	100%	NC	NC	NC	-22%	NC	NC	NC	NC	NC
dilp4	100%	NC	NC	NC	NC	NC	NC	NC	NC	ND
dilp5	100%	NC	NC	NC	-18% (p<0.07)	NC	NC	NC	NC	NC
$dilp6^{41}$	100%	m:+4h	m:-10% f:-20%	NC	-46%	NC	NC	+21%	NC	NC
$dilp6^{68}$	100%	m:+4h	m:-13% f:-20%	ND	ND	NC	NC	ND	ND	ND
dilp7	100%	NC	NC	NC	NC	NC	NC	NC	NC	ND
dilp1-2	ND	ND	NC	NC	ND	ND	Reduced (Post, et.al 2018)	ND	Reduced (Post, et.al 2018)	ND
dilp2-3	100%	ND	ND	f:+12%	-27%	NC	NC	ND	ND	ND
dilp1-4	100%	f:+25h	m:-13% f:-11%	NC	-14%	+21%	+18%	ND	ND	ND
dilp2,3,5 (W-)	f:100% m:60%	m:+10-17d f:+8-17d	f:-42%	NC	-69%	+25%	NC	+19%	+72%	ND
dilp2,3,5 (W+)	ND	ND	less reduction than W-	f:29%	Same as W-	NC	NC	ND	ND	ND
dilp1-4,5	<100%	m:+10-17d f:+8-17d	f:-53%	ND	ND		ND	ND	ND	ND
dilp2,3,5,6	0%	-	-	-	-	-	-	-	-	-
dilp1-4,5,6	0%	-	-	-	-	-	-	-	-	-
dilp2,3,5,7	f:100% m:60%	m:+10-17d f:+8-17d	f:-41%	ND	ND	ND	ND	ND	ND	ND
dilp1-4,5,7	<100%	m:+10-17d f:+8-17d	f:-52%	ND	ND	ND	ND	ND	ND	ND

To further investigate the possible endocrine effects of pan-neural IIS reduction, we also measured the effects of reduced IIS on haemolymph glucose content, fecundity, starvation resistance and oxidative stress resistance phenotypes which are often found to be altered in long-lived IIS mutants (Clancy, et al. 2001, Broughton, et al. 2005, Hwangbo et al., 2004; Giannakou et al. 2004, 2007).

Reduced fecundity and enhanced resistance to various stresses are often linked to lifespan extension. Broughton et al. (2005) found that long lived flies with ablated median neurosecretory cells have reduced female fecundity and they show resistance to oxidative stress (using paraquat) and starvation. In contrast, these flies were more sensitive to heat shock and showed slower recovery from cold shock.

Lastly, we attempted to measure apoptosis levels in the brain in response to reduced pan-neural IIS. Reducing IIS promotes the transcription factor FOXO localisation in the nucleus, which can then promote apoptosis (Zhang, et al. 2011). One of our hypotheses for the disconnection between lifespan and health span in response to pan-neural IIS reduction is that reducing IIS in neurons damages or reduces the function of the neurons. FOXO induced apoptosis in neurons in response to reduced IIS could be one explanation for the detrimental effects of reduced pan-neural IIS.

7.1.1:Aims

To measure the expression of *dilp2-6* in male and female fly heads and *dilp4-7* in male and female fly bodies in response to constitutive and adult specific pan-neural IIS reduction using the elavGAL4 and elavGS drivers.

To investigate if constitutive and adult specific pan-neural IIS reduction using the elavGAL4 and elavGS drivers has any effect on the haemolymph glucose content.

To measure the fecundity of female flies with constitutive and adult specific pan-neural IIS reduction using the elavGAL4 and elavGS drivers.

To measure how long flies with constitutive and adult specific pan-neural IIS reduction using the elavGAL4 and elavGS drivers can survive on agar media containing no sugar or yeast.

To study the oxidative stress resistance of flies with constitutive and adult specific pan-neural IIS reduction using the elavGAL4 and elavGS drivers by measuring the lifespan of flies fed on food containing H_2O_2.

To investigate if constitutive and adult specific pan-neural IIS reduction using the elavGAL4 and elavGS drivers causes apoptosis in neurons by tagging apoptotic cells in dissected fly brains and visualising them under fluorescent microscopy.

7.1.2: Research design

Similarly to Chapters 4-6, constitutive pan-neural IIS reduction was achieved using the elavGAL4 driver to express the UAS-InR^{DN} transgene, where elavGAL4/UAS-

InR^{DN} is the experimental group with constitutive IIS reduction in neurons and the two control groups are the elavGAL4/+ and UAS-InR^{DN}/+. The inducible elavGS driver was used for adult specific pan-neural IIS reduction where the experimental group is RU486-elavGS/UAS-InR^{DN} and the control group is elavGS/UAS-InR^{DN}. The effect of RU486 itself was measured using the RU486-elavGS/+ and elavGS/+ groups.

The expression of *dilp2, 3, 4, 5, 6* was measured in fly heads and the expression of *dilp4, 5, 6, 7* was measured in the bodies of both male and female flies at the age of 10 days for elavGAL4 flies and at the age of 12 days for elavGS flies using RT-PCR (qPCR), as described in Chapter 2.17-2.19. β-actin, Tubulin and Rlp32 were used as reference genes for all qPCR experiments.

Haemolymph was extracted from elavGAL4 and elavGS flies using centrifugation and the glucose concentration of the haemolymph was measured using Thermo Scientific Infinity^{TM} Glucose affinity reagent, as described in Chapter 2.15.

One female fecundity experiment was carried out measuring individual fecundity, were mated females were kept on standard food as 1 fly per vials and their eggs were counted after 24 hours (N=30) as described in Chapter 2.10. In the second experiment, mated female flies were kept as 10 fly per vial (N=10 vials). Both fecundity experiments were performed using elavGAL4 flies at the age of 10 days and elavGS flies at the age of 12 days.

Starvation resistance was measured by keeping elavGAL4 and elavGS flies on a 1% agar media containing no sugar or yeast (N=100). ElavGAL4 flies were sorted onto the starvation food at the age of 10 days, while elavGS at the age of 12 days, the number of dead flies were counted daily.

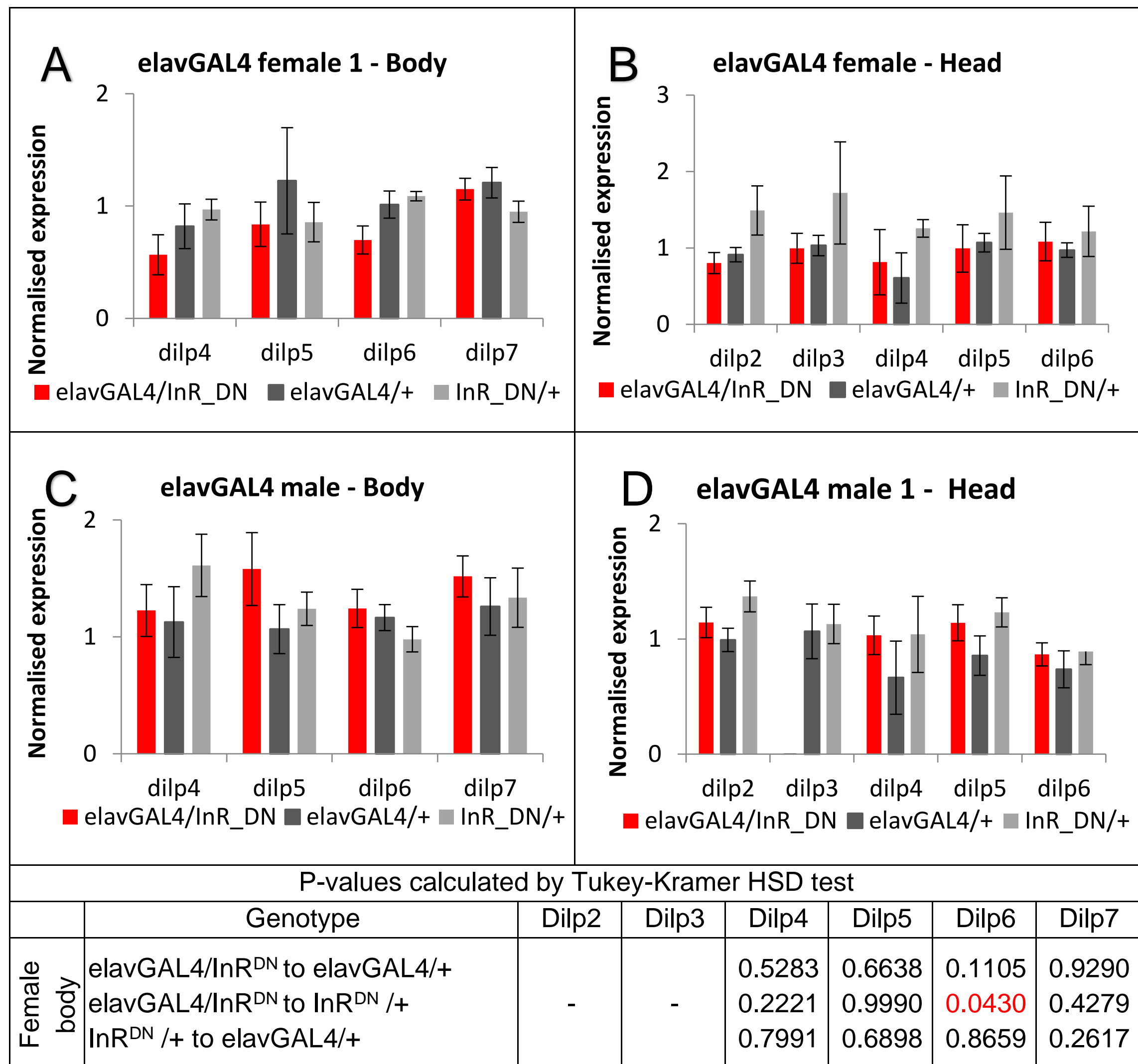
Oxidative stress resistance was measured by keeping elavGAL4 and elavGS flies on a media containing 5% H_2O_2, and 5% sugar (N=100). ElavGAL4 flies were sorted onto the starvation food at the age of 10 days, while elavGS at the age of 12 days, the number of dead flies were counted twice a day.

For the apoptosis assay the brains of 10 days and 35 days old female elavGAL4 and elavGS flies were dissected and the apoptotic cells were tagged using Millipore ApopTag® Red in situ apoptosis detection kit and visualised under fluorescent microscopy as described in Chapter 2.16.

7.2: Results

7.2.1: Constitutive pan-neural IIS reduction has no effect on *dilp* expression

Transcript levels of *dilp*s were measured in 10 day old fly heads and bodies using Trizol RNA extraction and qPCR. *Dilps 2-6* were measured in heads and *dilps 4-7* in bodies. The female and male *dilp* expression data presented in **Figure 41** show that there was no significant effect on *dilp* expression due to expression of UAS-InR^{DN} driven by elavGAL4 in neurons throughout development and adulthood. This result is in agreement with Ismail et al. (2015) suggesting that the lifespan extension of elavGAL4/UAS-InR^{DN} was not due to an endocrine regulation of *dilp* expression from the IPCs or elsewhere.



P-values calculated by Tukey-Kramer HSD test

	Genotype	Dilp2	Dilp3	Dilp4	Dilp5	Dilp6	Dilp7
Female body	elavGAL4/InR^{DN} to elavGAL4/+	-	-	0.5283	0.6638	0.1105	0.9290
	elavGAL4/InR^{DN} to InR^{DN} /+			0.2221	0.9990	0.0430	0.4279
	InR^{DN} /+ to elavGAL4/+			0.7991	0.6898	0.8659	0.2617

Female head	elavGAL4/InR^{DN} to elavGAL4/+	0.9272	0.9978	0.8913	0.9862	0.9448	-
	elavGAL4/InR^{DN} to InR^{DN} /+	0.1029	0.4549	0.6048	0.6048	0.9221	
	InR^{DN} /+ to elavGAL4/+	0.1794	0.4885	0.3609	0.6980	0.7658	
Male body	elavGAL4/InR^{DN} to elavGAL4/+	-	-	0.9630	0.3842	0.9049	0.7083
	elavGAL4/InR^{DN} to InR^{DN} /+			0.5690	0.5639	0.3450	0.8399
	InR^{DN} /+ to elavGAL4/+			0.4183	0.8569	0.5818	0.9704
Male head	elavGAL4/InR^{DN} to elavGAL4/+	0.6694	0.8842	0.6400	0.4105	0.7582	-
	elavGAL4/InR^{DN} to InR^{DN} /+	0.4237	0.7559	0.9998	0.9085	0.9890	
	InR^{DN} /+ to elavGAL4/+	0.1183	0.9672	0.6284	0.2307	0.6751	

Figure 41 - The effect of constitutive IIS reduction on *dilp* expression in fly heads and bodies

Male and female flies were snap frozen in liquid nitrogen at the age of 10 days and kept at -80ºC until RNA extraction. For each qPCR experiment, 20 heads and 10 bodies were used to generate about 500 ng RNA from the heads and 1000 ng RNA from the bodies. The experimental group is the elavGAL4/UAS-InR^{DN} with constitutive IIS reduction in their neurons, while the two control groups are elavGAL4/+ and UAS-InR^{DN}/+. Data shown as mean relative expression level +/- SEM, the means were compared using Tukey-Kramer HSD test. Significant difference is highlighted with red text colour (p<0.05) in the statistical summary and with a star (★) on the graph.

A) The effect of constitutive pan-neural IIS reduction on the *dilp4-7* expression in female bodies. N=6 for *dilp4-6*, N=5 for *dilp7*.

B) The effect of constitutive pan-neural IIS reduction on the *dilp2-6* expression in female heads. N=4 for *dilp2-6*.

C) The effect of constitutive pan-neural IIS reduction on the *dilp4-7* expression in male bodies. N=7 for *dilp4-7*.

D) The effect of constitutive pan-neural IIS reduction on the *dilp2-6* expression in male heads. N=5 for *dilp2-3* and *dilp5-6*, N=4 for *dilp4*

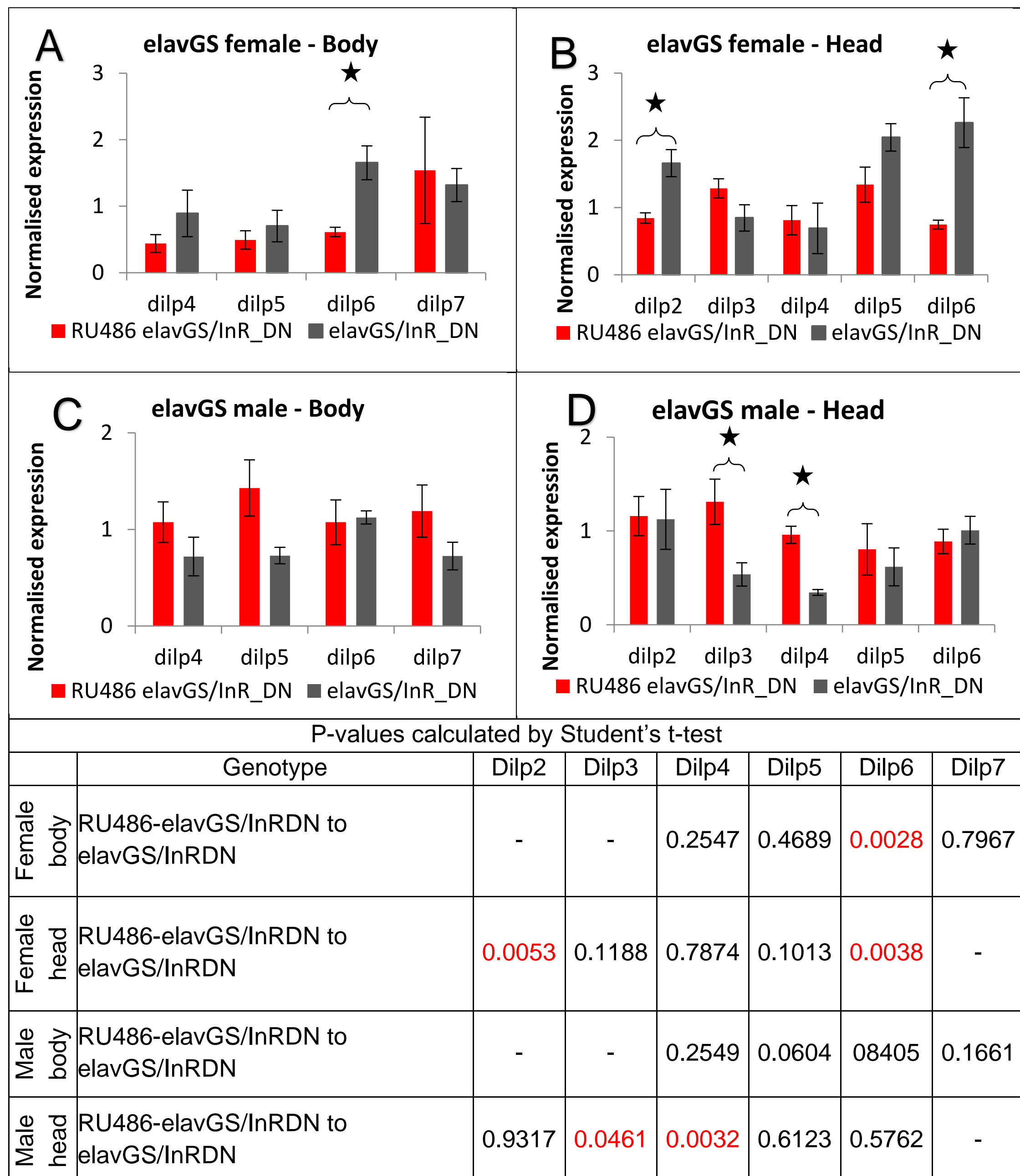
7.2.2: Adult-specific pan-neural IIS reduction lowers *dilp6* and *dilp2* expression in females, and increases *dilp3* and *dilp4* in male heads

To study how adult-specific IIS reduction in neurons affects the *dilp* expression, the transcript levels of *dilp*s were similarly measured in 12 day old fly heads and bodies of elavGS/UAS-InR^{DN} flies with and without RU486.

The data in **Figure 42** show that *dilp6* was significantly lower than control in RU486-elavGS/UAS-InR^{DN} female heads (p=0.0028) and bodies (p=0.0038) and *dilp2* was lower than control in RU486-elavGS/UAS-InR^{DN} female heads (p=0.0053). There were no significant *dilp* expression changes in male bodies, but *dilp3* and *dilp4* were higher than control in RU486-elavGS/UAS-InR^{DN} male heads (p=0.0461 and p=0.0032).

To determine if RU486 itself had any effect *dilp* expression, the transcript levels of *dilp*s were measured in 12 days old fly elavGS/+ heads and bodies using Trizol RNA

extraction and qPCR. *Dilp2-6* was measured in heads and *dilp4-7* in bodies. RU486-elavGS/+ flies were fed on RU486 food from the age of 3 days and the elavGS/+ control was kept on standard food. The results in **Figure 43** show only one significant change in *dilp* expression due to RU486, that is increased levels of dilp6 in female bodies (p=0.0161).



P-values calculated by Student's t-test

	Genotype	Dilp2	Dilp3	Dilp4	Dilp5	Dilp6	Dilp7
Female body	RU486-elavGS/InRDN to elavGS/InRDN	-	-	0.2547	0.4689	0.0028	0.7967
Female head	RU486-elavGS/InRDN to elavGS/InRDN	0.0053	0.1188	0.7874	0.1013	0.0038	-
Male body	RU486-elavGS/InRDN to elavGS/InRDN	-	-	0.2549	0.0604	08405	0.1661
Male head	RU486-elavGS/InRDN to elavGS/InRDN	0.9317	0.0461	0.0032	0.6123	0.5762	-

Figure 42 - The effect of constitutive IIS reduction on *dilp* expression in fly heads and bodies

Male and female flies were snap frozen in liquid nitrogen at the age of 12 days and kept at -80ºC until RNA extraction. For each qPCR experiment, 20 heads and 10 bodies were used to generate about 500 ng RNA from the heads and 1000 ng RNA from the bodies. The experimental group is the RU486-elavGS/UAS-InR^{DN} with reduced pan-neural IIS from the age

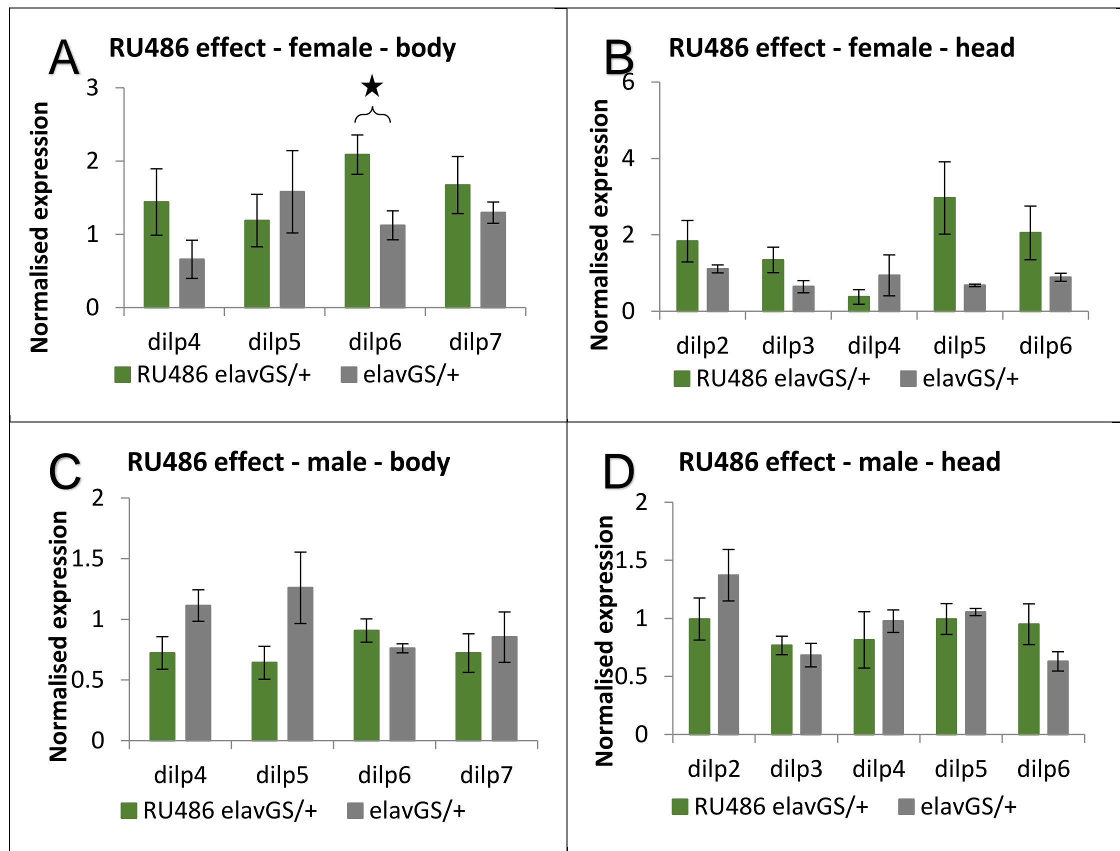
of 3 days, while the control group is elavGS/ UAS-InR^{DN} with no RU486 in their food. Data shown as mean relative expression level +/- SEM, the means were compared using Student's t-test. Significant difference is highlighted with red text colour ($p<0.05$) in the statistical summary and with a star (★) on the graph.

A) The effect of adult-specific pan-neural IIS reduction on the *dilp4-7* expression in female bodies. N=6 for *dilp4,5 and 7*, N=5 for *dilp5*.

B) The effect of adult-specific pan-neural IIS reduction on the *dilp2-6* expression in female heads. N=5 for *dilp2, 4 and 6,* N=4 for *dilp3* and N=3 for *dilp5.*

C) The effect of adult-specific pan-neural IIS reduction on the *dilp4-7* expression in male bodies. N=5 for *dilp4,6 and 7,* and N=4 for *dilp5*.

D) The effect of adult-specific pan-neural IIS reduction on the *dilp2-6* expression in male heads. N=3 for *dilp2-6.*

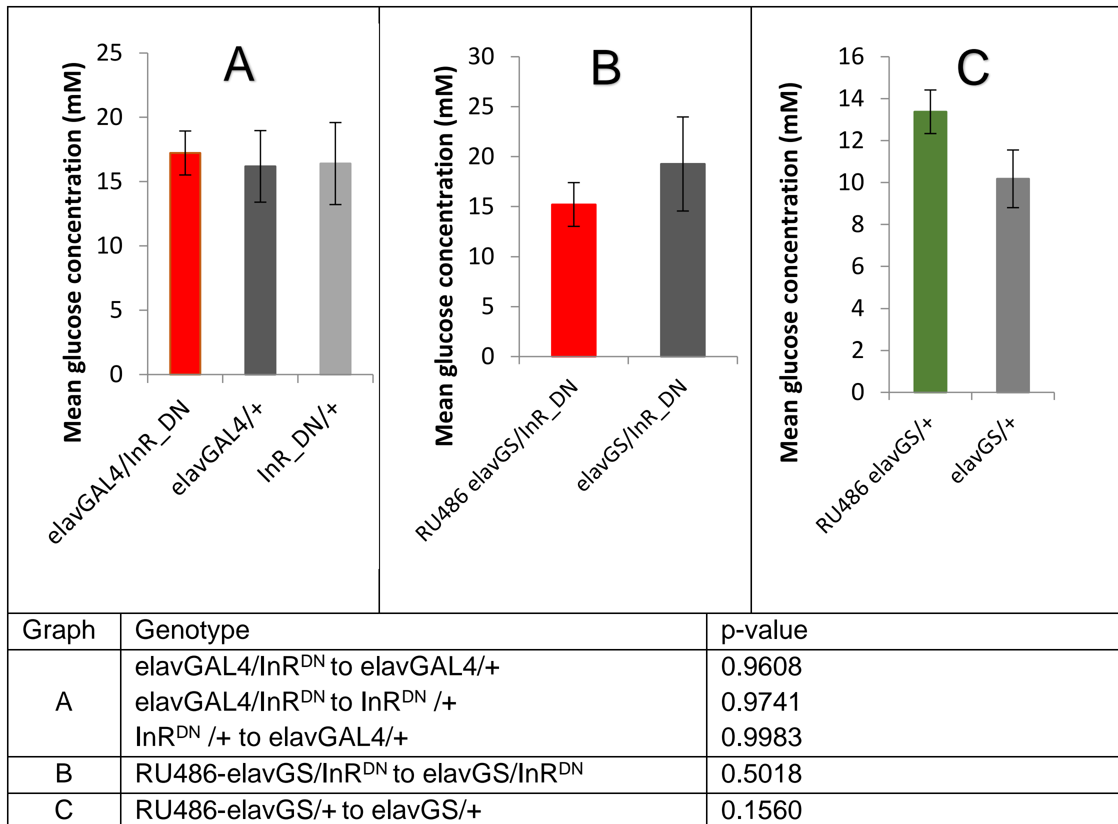


P-values calculated by Student's t-test							
	Genotype	Dilp2	Dilp3	Dilp4	Dilp5	Dilp6	Dilp7
Female body	RU486-elavGS/+ to elavGS/+	-	-	0.1654	0.5719	0.0161	0.4276
Female head	RU486-elavGS/+ to elavGS/+	0.2263	0.1070	0.3500	0.0732	0.1401	-
Male body	RU486-elavGS/+ to elavGS/+	-	-	0.0899	0.1169	0.2441	0.6450
Male head	RU486-elavGS/+ to elavGS/+	0.2579	0.5518	0.5714	0.6829	0.1744	-

Figure 43 - The effect of RU486 on *dilp* expression in fly heads and bodies
Male and female flies were snap frozen in liquid nitrogen at the age of 12 days and kept at -80ºC until RNA extraction. For each qPCR experiment, 20 heads and 10 bodies were used to generate about 500 ng RNA from the heads and 1000 ng RNA from the bodies. The two groups are the RU486-elavGS/+ which was fed on RU486 food from the age of 3 days and the other is elavGS/+ kept on standard food. Data shown as mean relative expression level +/- SEM, the means were compared using Student's t-test. Significant difference is highlighted with red text colour ($p<0.05$) in the statistical summary and with a star (★) on the graph.
A) The effect of RU486 on the *dilp4-7* expression in female bodies. N=6 for *dilp4,5 and 7*, N=5 for *dilp5*.
B) The effect of RU486 on the *dilp2-6* expression in female heads. N=5 for *dilp2, 4 and 6,* N=4 for *dilp3* and N=3 for *dilp5*.
C) The effect of RU486 on the *dilp4-7* expression in male bodies. N=5 for *dilp4,6 and 7,* and N=4 for *dilp5*.
D) The effect of RU486 on the *dilp2-6* expression in male heads. N=3 for *dilp2-6.*

7.2.3: Pan-neural IIS reduction did not affect the haemolymph glucose concentration in our experiment

To investigate if pan neural IIS reduction had any effect on fly haemolymph glucose concentration, haemolymph was extracted from 4 days old female elavGAL4 and 6 days old female elavGS flies and the haemolymph glucose was measured using Thermo Scientific Infinity™ Glucose affinity reagent. The data in **Figure 44A** and **B** show no effect of constitutive or adult-specific IIS neuronal reduction on haemolymph glucose content. There was also no effect of RU486 itself on haemolymph glucose (**Figure 44C**). However, due to difficulties with extracting sufficient volume of pure haemolymph, the variability of the haemolymph glucose concentration between samples is large. We were unable to compensate with increasing the number of samples (N=12 or 10 for each experiment), therefore the results are preliminary and need to be repeated with a more efficient method of haemolymph extraction.



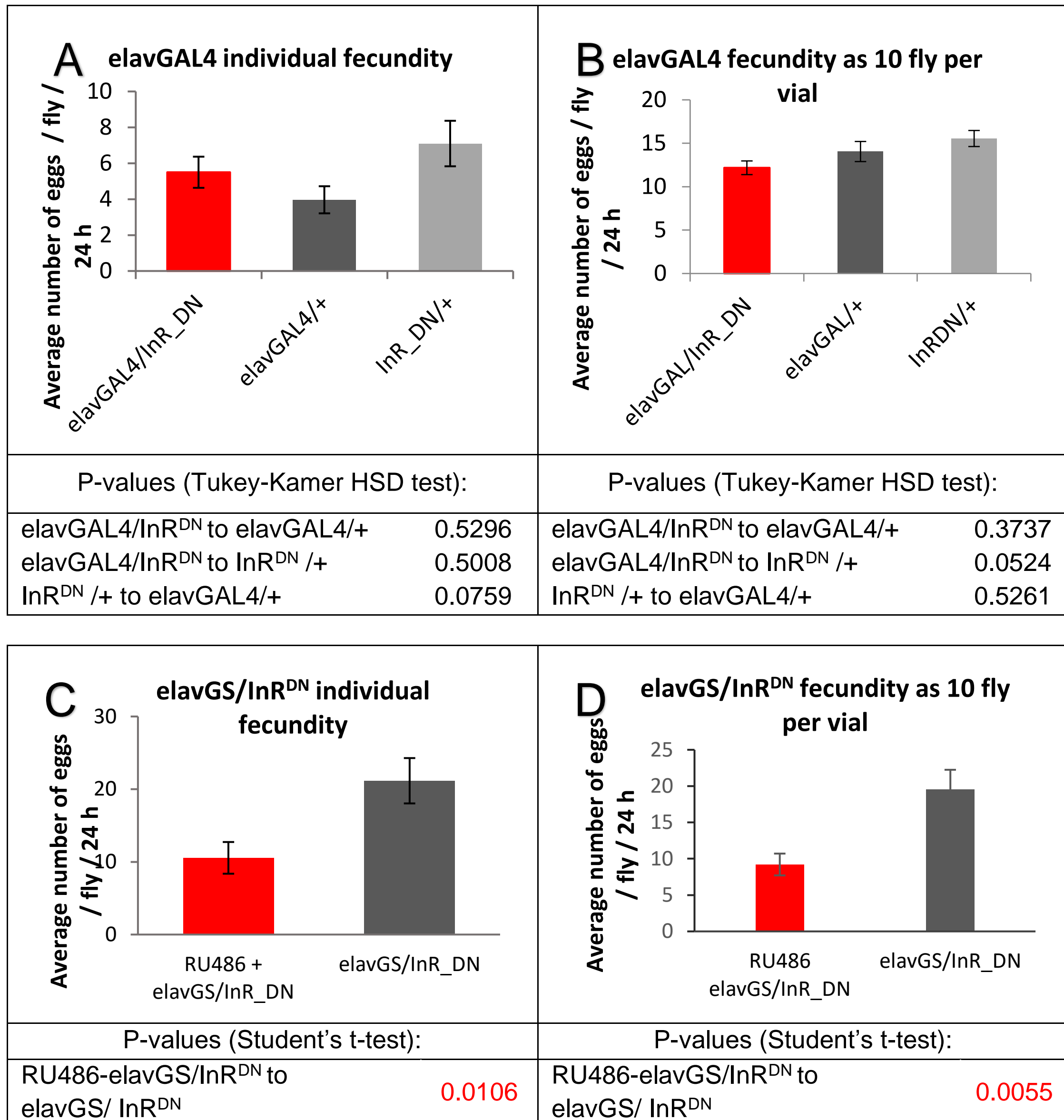
Graph	Genotype	p-value
A	elavGAL4/InR^{DN} to elavGAL4/+ elavGAL4/InR^{DN} to InR^{DN} /+ InR^{DN} /+ to elavGAL4/+	0.9608 0.9741 0.9983
B	RU486-elavGS/InR^{DN} to elavGS/InR^{DN}	0.5018
C	RU486-elavGS/+ to elavGS/+	0.1560

Figure 44 - Haemolymph glucose content of female flies in response to pan-neural IIS reduction
Haemolymph was extracted from 4 days old elavGAL4 and 6 days old female elavGS flies and the haemolymph glucose was measured using Thermo Scientific Infinity^{TM} Glucose affinity reagent. Error bars represent SEM.
A) The effect of constitutive IIS reduction in neurons on female haemolymph glucose content. The experimental group is the elavGAL4/UAS-InR^{DN} with constitutive IIS reduction in their neurons, while the two control groups are elavGAL4/+ and UAS-InR^{DN}/+, N=12. The means were compared using Tukey-Kramer HSD test.
B) The effect of adult specific IIS reduction in neurons on female haemolymph glucose content. The experimental group is the RU486-elavGS/UAS-InR^{DN} where IIS was reduced from the age of 3 days and the control group is the elavGS/UAS-InR^{DN} without RU486, N=10. The means were compared using Student's t-test.
C) The effect of RU486 on female haemolymph glucose content. The two groups are the RU486-elavGS/+ which was fed on RU486 food from the age of 3 days and the other is elavGS/+ kept on standard food, N=10. The means were compared using Student's t-test.

7.2.4: Pan-neural IIS reduction does not affect female fecundity, but RU486 does

The fecundity of mated females with constitutive and adult specific IIS reduction in their neurons was measured by counting eggs laid over a 24 hour period. The elavGAL4 flies were 10 days old and the elavGS were 12 days. Two experiments were carried out, in one of them, flies were kept individually in separate vials, while in the second the flies were kept as 10 females per vial. The data in **Figure 45A** and **B** show

no significant effect of constitutive IIS reduction in neurons on female fecundity. Although females with adult-specific pan-neural IIS reduction (RU486-elavGS/UAS-InR^{DN}) laid significantly fewer eggs than the control group (**Figure 45C** and **D**), this is likely due to RU486 itself, as the RU486-elavGS/+ flies also had reduced fecundity compared to elavGS/+ (**Figure 45E** and **F**).



P-values (Tukey-Kamer HSD test):		P-values (Tukey-Kamer HSD test):	
elavGAL4/InR^{DN} to elavGAL4/+	0.5296	elavGAL4/InR^{DN} to elavGAL4/+	0.3737
elavGAL4/InR^{DN} to InR^{DN} /+	0.5008	elavGAL4/InR^{DN} to InR^{DN} /+	0.0524
InR^{DN} /+ to elavGAL4/+	0.0759	InR^{DN} /+ to elavGAL4/+	0.5261

P-values (Student's t-test):		P-values (Student's t-test):	
RU486-elavGS/InR^{DN} to elavGS/ InR^{DN}	0.0106	RU486-elavGS/InR^{DN} to elavGS/ InR^{DN}	0.0055

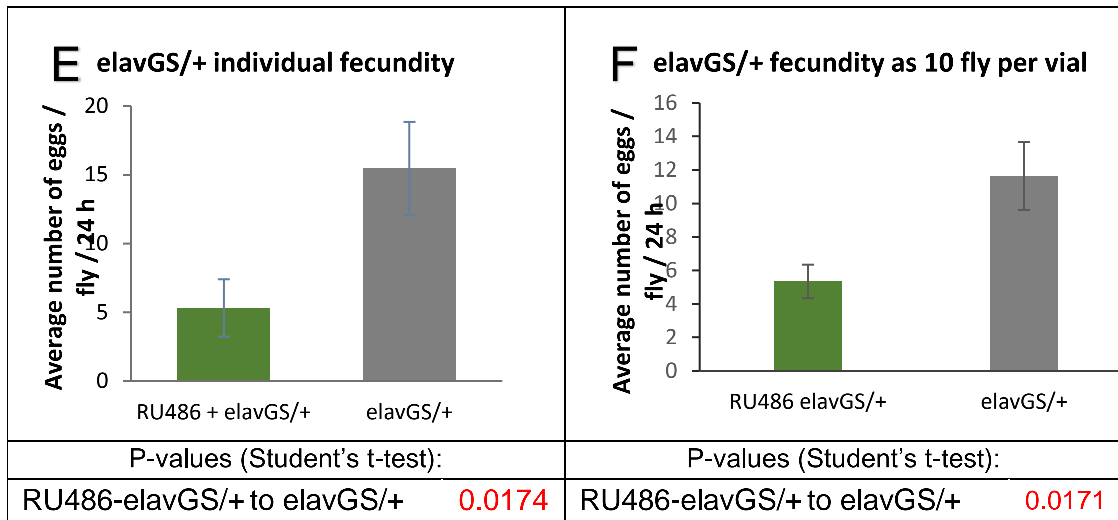


Figure 45 - Female fecundity in response to pan-neural IIS reduction

Eggs laid over a 24 hour period was counted using 10 days old elavGAL4 and 12 days old elavGS mated female flies. Females were kept individually in vials for the individual fecundity experiment (N=30) and as 10 fly/vial for the second experiment (N=10 vials). Error bars represent +/- SEM. Significant difference is highlighted with red text colour ($p<0.05$) in the statistical summary. Individual fecundity experiment was carried out by Alise Eihmane and fecundity as 10 flies per vial was done by Tommy Shaw undergraduate project students.

The effect of constitutive IIS reduction in neurons on female individual fecundity (**A**) and fecundity measured as 10 fly/vial (**B**). The experimental group is the elavGAL4/UAS-InR^{DN} with constitutive IIS reduction in their neurons, while the two control groups are elavGAL4/+ and UAS-InR^{DN}/+. The means were compared using Tukey-Kramer HSD test.

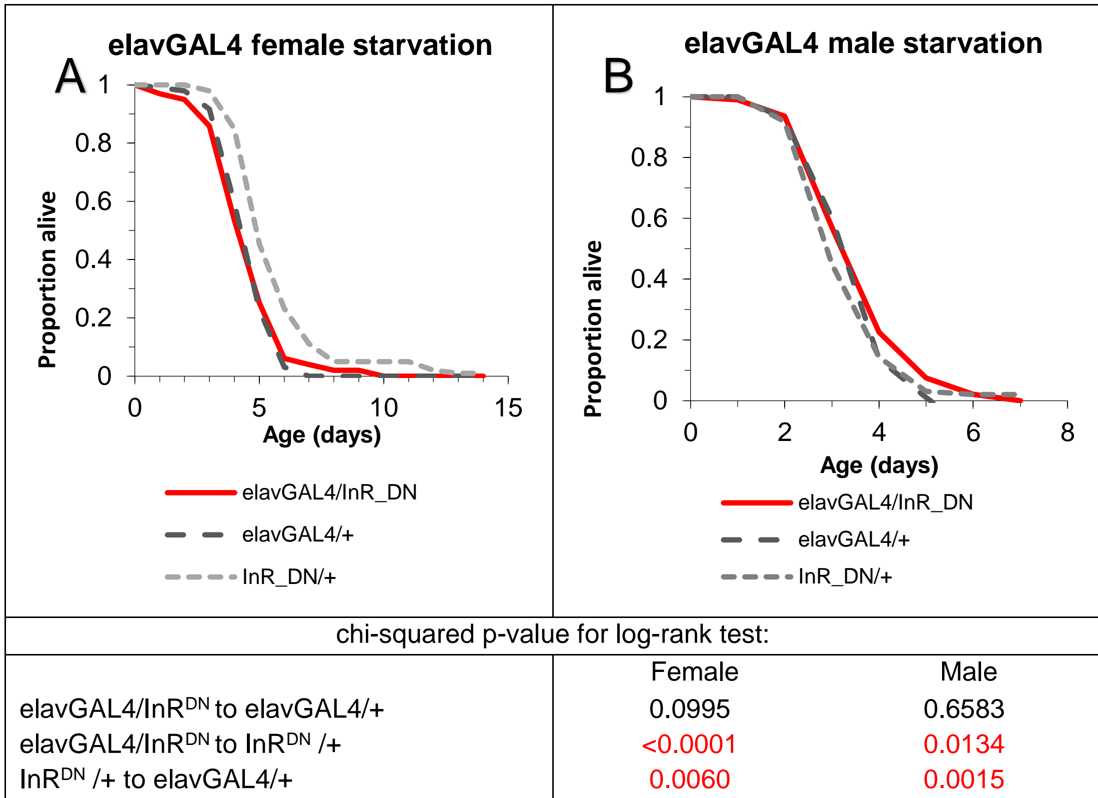
The effect of adult specific IIS reduction in neurons on female individual fecundity (**C**) and fecundity measured as 10 fly/vial (**D**). The experimental group is the RU486-elavGS/UAS-InR^{DN} where IIS was reduced from the age of 3 days and the control group is the elavGS/UAS-InR^{DN} without RU486. The means were compared using Student's t-test.

The effect of RU486 on female individual fecundity (**E**) and fecundity measured as 10 fly/vial (**F**). The two groups are the RU486-elavGS/+ which was fed on RU486 food from the age of 3 days and the other is elavGS/+ kept on standard food, The means were compared using Student's t-test.

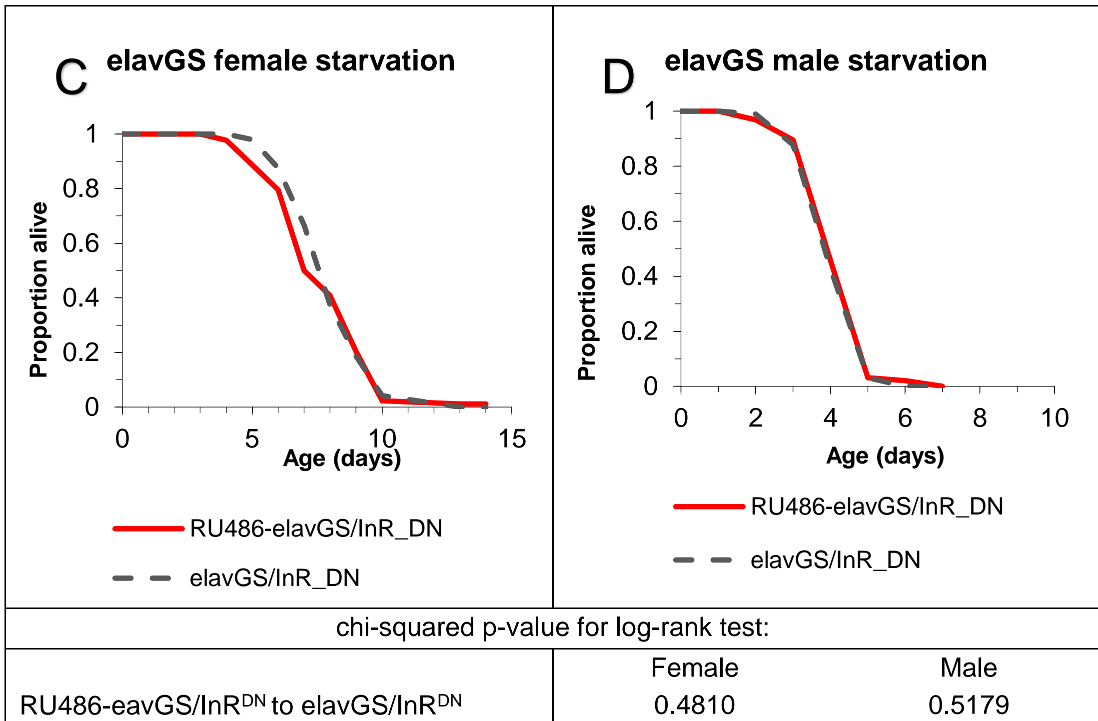
7.2.5: Pan-neural IIS reduction does not affect starvation resistance, but RU486 does

To measure the starvation resistance of flies with constitutive or adult specific IIS reduction, 10 days old elavGAL4 and 12 days old elavGS flies were transferred onto agar media without any sugar or yeast and their survival was measured every day.

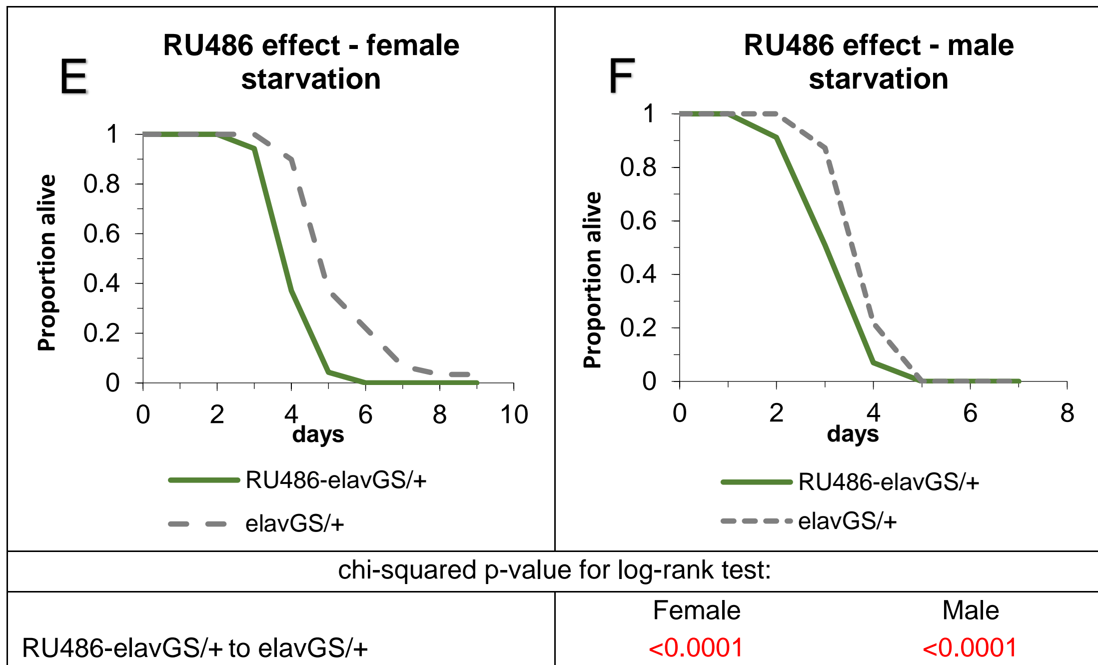
The results show no effect of constitutive pan-neural IIS reduction on male or female starvation resistance. The UAS-InR^{DN}/+ group is significantly different from elavGAL4/+ and elavGAL4/UAS-InR^{DN} in both males and females, but the experimental group is not different from elavGAL4/+. The experiments with the inducible elavGS driver showed no effect on female or male starvation resistance, RU486 however significantly shortened the lifespan of both males and females ($p<0.0001$) (**Figure 46**).



chi-squared p-value for log-rank test:		
	Female	Male
elavGAL4/InR^{DN} to elavGAL4/+	0.0995	0.6583
elavGAL4/InR^{DN} to InR^{DN} /+	<0.0001	0.0134
InR^{DN} /+ to elavGAL4/+	0.0060	0.0015



chi-squared p-value for log-rank test:		
	Female	Male
RU486-eavGS/InR^{DN} to elavGS/InR^{DN}	0.4810	0.5179



chi-squared p-value for log-rank test:		
	Female	Male
RU486-elavGS/+ to elavGS/+	<0.0001	<0.0001

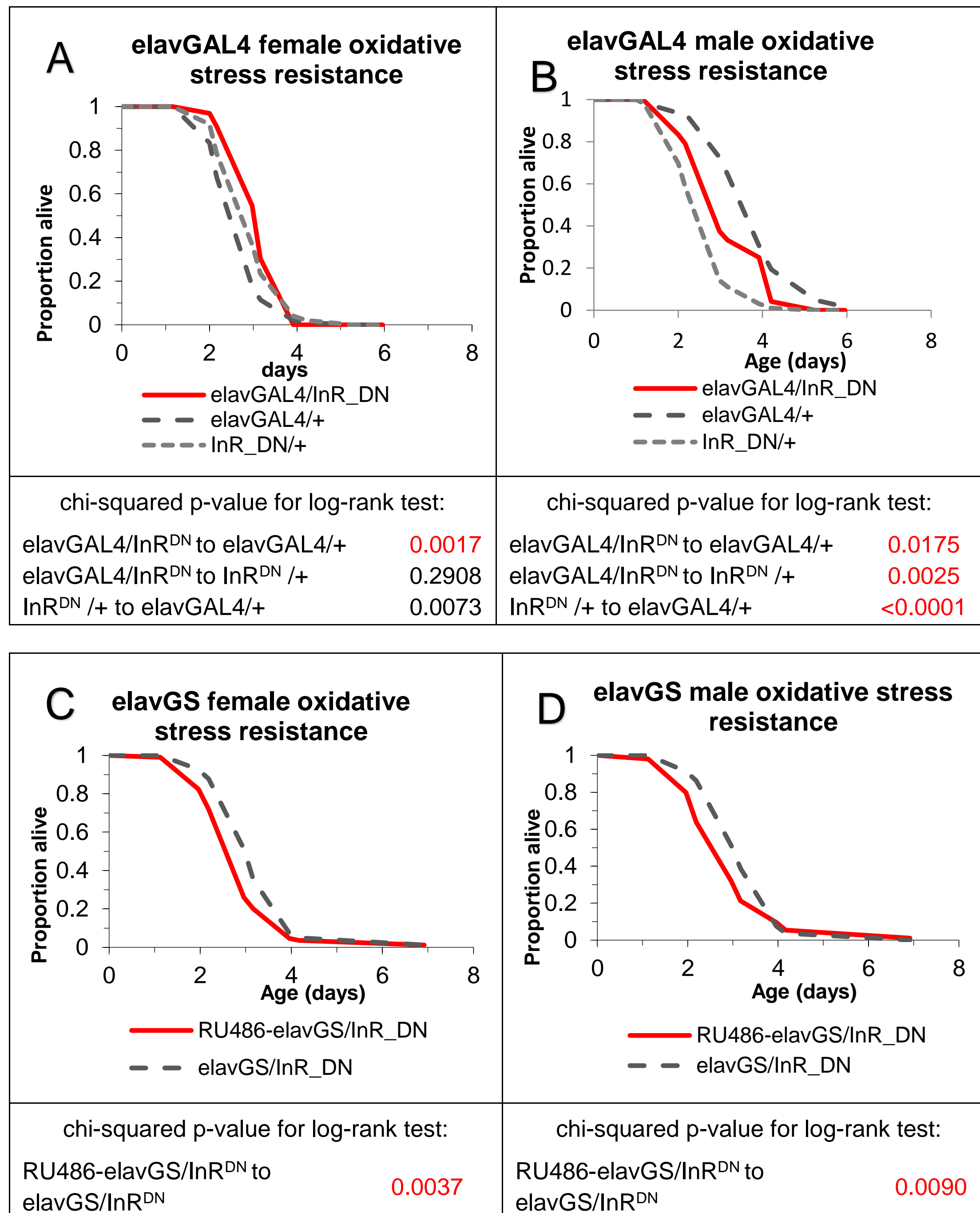
<u>Figure 46</u> - Starvation resistance in response to pan-neural IIS reduction
10 days old elavGAL4 and 12 days old elavGS flies were transferred onto agar media without any sugar and yeast and their survival was measured daily (N=100). Survival curves were compared using nonparametric log rank tests and p values are shown under the graphs, with significant differences highlighted in red ($p<0.05$). The experiments were carried out by Alison Tse undergraduate project student.
The effect of constitutive IIS reduction in neurons on female (**A**) and male (**B**) starvation resistance. The experimental group is the elavGAL4/UAS-InR^{DN} with constitutive IIS reduction in their neurons, while the two control groups are elavGAL4/+ and UAS-InR^{DN}/+.
The effect of adult specific IIS reduction in neurons on female (**C**) and male (**D**) starvation resistance. The experimental group is the RU486-elavGS/UAS-InR^{DN} where IIS was reduced from the age of 3 days and the control group is the elavGS/UAS-InR^{DN} without RU486.
The effect of RU486 on female (**E**) and male (**F**) starvation resistance. The two groups are the RU486-elavGS/+ which was fed on RU486 food from the age of 3 days and the other is elavGS/+ kept on standard food.

7.2.6: Adult-specific pan-neural IIS reduction reduces resistance to oxidative stress

Oxidative stress resistance in flies with constitutive or adult specific IIS reduction was measured by transferring 10 days old elavGAL4 and 12 days old elavGS flies onto media containing 5% H_2O_2 and 5% sugar and their survival was measured twice a day.

The results with constitutive pan-neural IIS reduction did not show any consistent effect on oxidative stress resistance. The elavGAL4/InR^{DN} females were not significantly different from both control groups and in males, all three groups were significantly different from each other and the experimental group was doing better than the UAS-InR^{DN}/+ control, but worse than the elavGAL4/+ control group. When the inducible elavGS driver was used to reduce IIS in adult fly neurons, both males and

females were significantly more sensitive to oxidative stress than the control (p=0.0037 for females and p=0.0090 for males). RU486 had no effect on oxidative stress resistance (**Figure 47**).



A – chi-squared p-value for log-rank test:

Comparison	p-value
elavGAL4/InR^{DN} to elavGAL4/+	0.0017
elavGAL4/InR^{DN} to InR^{DN} /+	0.2908
InR^{DN} /+ to elavGAL4/+	0.0073

B – chi-squared p-value for log-rank test:

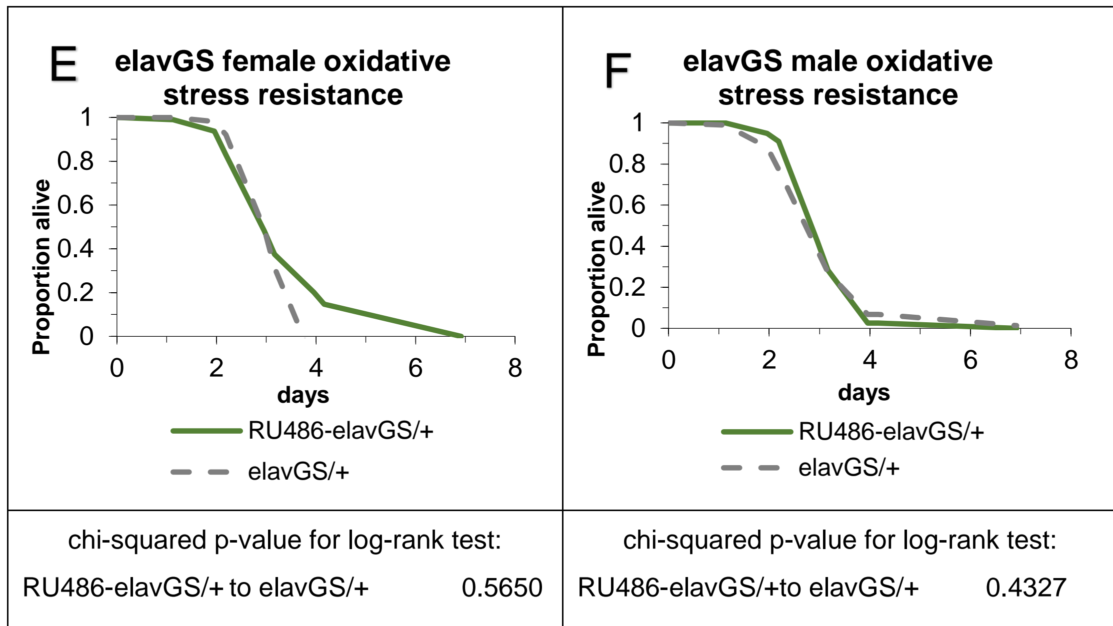
Comparison	p-value
elavGAL4/InR^{DN} to elavGAL4/+	0.0175
elavGAL4/InR^{DN} to InR^{DN} /+	0.0025
InR^{DN} /+ to elavGAL4/+	<0.0001

C – chi-squared p-value for log-rank test:

Comparison	p-value
RU486-elavGS/InR^{DN} to elavGS/InR^{DN}	0.0037

D – chi-squared p-value for log-rank test:

Comparison	p-value
RU486-elavGS/InR^{DN} to elavGS/InR^{DN}	0.0090



chi-squared p-value for log-rank test:		chi-squared p-value for log-rank test:	
RU486-elavGS/+ to elavGS/+	0.5650	RU486-elavGS/+to elavGS/+	0.4327

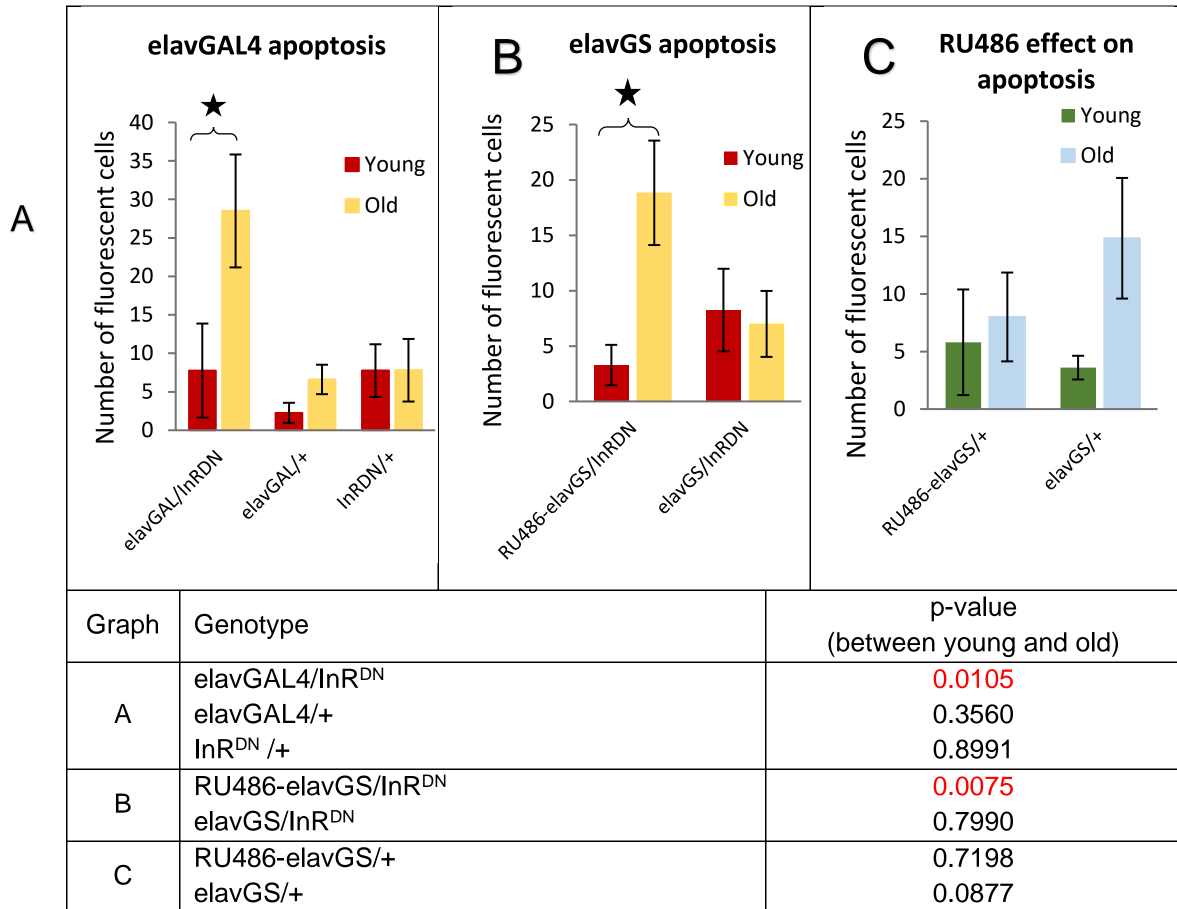
Figure 47 - Oxidative stress resistance in response to pan-neural IIS reduction
10 days old elavGAL4 and 12 days old elavGS flies were transferred onto agar media with 5% H_2O_2 and 5% sugar (N=100). Survival curves were compared using nonparametric log rank tests and p values are shown under the graphs, with significant differences highlighted in red ($p<0.05$). The experiments were carried out by Alison Tse undergraduate project student.
The effect of constitutive IIS reduction in neurons on female (**A**) and male (**B**) oxidative stress resistance. The experimental group is the elavGAL4/UAS-InR^{DN} with constitutive IIS reduction in their neurons, while the two control groups are elavGAL4/+ and UAS-InR^{DN}/+.
The effect of adult specific IIS reduction in neurons on female (**C**) and male (**D**) oxidative stress resistance. The experimental group is the RU486-elavGS/UAS-InR^{DN} where IIS was reduced from the age of 3 days and the control group is the elavGS/UAS-InR^{DN} without RU486.
The effect of RU486 on female (**E**) and male (**F**) oxidative stress resistance. The two groups are the RU486-elavGS/+ which was fed on RU486 food from the age of 3 days and the other is elavGS/+ kept on standard food.

7.2.7: Reduced pan-neural IIS may induce apoptosis in the fly brain at older ages

For the apoptosis assay the brains of 10 days and 35 days old female elavGAL4 and elavGS flies were dissected and the apoptotic cells were tagged using Millipore ApopTag® Red in situ apoptosis detection kit and visualised under fluorescent microscopy.

The results show that there was no increase in the number of apoptotic cells at young age (10 days old), but at older age (35 days old) the number of apoptotic cells significantly increased in the experimental groups with constitutive and with adult-specific IIS reduction in the neurons. There was no significant difference between the old and young age of any of the control groups and RU486 itself had no significant effect on apoptosis in the brain (**Figure 48**).

It is worth to mention that the apoptosis experiment was only carried out using female flies, and due to limitation of time and resources, it has not been yet repeated, therefore, it only shows preliminary data. This is further discussed in Chapter 11.5 at the Limitations and future directions section.



Graph	Genotype	p-value (between young and old)
A	elavGAL4/InR^{DN}	0.0105
	elavGAL4/+	0.3560
	InR^{DN} /+	0.8991
B	RU486-elavGS/InR^{DN}	0.0075
	elavGS/InR^{DN}	0.7990
C	RU486-elavGS/+	0.7198
	elavGS/+	0.0877

Figure 48 - Number of apoptotic cells in young and old female flies in response to pan-neural IIS reduction

The brains of 10 days (young) and 35 days (old) old female elavGAL4 and elavGS flies were dissected and the apoptotic cells were tagged using Millipore ApopTag® Red in situ apoptosis detection kit and visualised under fluorescent microscopy. Six brains were visualised for each genotype. Z-stack images were analysed by ImageJ (NIH, Bethesda, MD, USA) and the number of apoptotic cells was determined using the analyse particle function. The statistical analysis was done using t-test in oneway ANOVA. Error bars represent +/- SEM. The experiments were carried out by Tommy Shaw undergraduate project student.

A) The number of apoptotic cells in young and old female flies in response to constitutive IIS reduction in neurons. The experimental group is the elavGAL4/UAS-InR^{DN} with constitutive IIS reduction in their neurons, while the two control groups are elavGAL4/+ and UAS-InR^{DN}/+.

B) The number of apoptotic cells in young and old female flies in response to adult-specific IIS reduction in neurons. The experimental group is the RU486-elavGS/UAS-InR^{DN} where IIS was reduced from the age of 3 days and the control group is the elavGS/UAS-InR^{DN} without RU486.

C) The number of apoptotic cells in young and old female flies in response to RU486. The two groups are the RU486-elavGS/+ which was fed on RU486 food from the age of 3 days and the other is elavGS/+ kept on standard food.

7.3: Discussion

In this chapter we studied some of the possible endocrine and peripheral effects of constitutive and adult specific pan-neural IIS reduction in *Drosophila* by measuring *dilp* expression in heads and bodies, haemolymph glucose content, female fecundity, starvation resistance and oxidative stress resistance.

Constitutive IIS reduction had no effect on *dilp* expression levels, similarly to the findings of Ismail et al. (2015), suggesting that lifespan extension was not due to a neuroendocrine regulation of *dilp* transcripts, although we did not measure effects on DILP proteins. Adult specific IIS reduction in neurons however did result in changes in *dilp* expression suggesting such endocrine regulation could be involved in the phenotypes of the RU486-elavGS/UAS-InR^{DN} flies. The reduced expression of *dilp6* in both heads and bodies of RU486-elavGS/UAS-InR^{DN} female flies was somewhat surprising given the results of Bai et al. (2012), which found that overexpression of dilp6 extended lifespan via repression of *dilp2* and *dilp5* expression and DILP2 release. However, the effects we observed on *dilp* transcripts were due to a reduction in IIS in neurons, and not due to direct manipulation of the *dilp*s from either the fat body or the IPCs. Moreover, our data show that IIS in neurons can influence the expression of *dilp*s in the IPCs and the Fat Body via an unknown endocrine mechanism.

However, although we have shown effects on *dilp* expression due to reduced IIS in neurons, the question remains as to whether or not such changes are causal in the lifespan extension of RU486-elavGS/UAS-InR^{DN} females. A complete knock out of *dilp*2 has been shown to be sufficient for lifespan extension (Grönke et al. 2009) but a partial knock down of *dilp2* expression is not (Broughton et al. 2008). Together with the fact that the partial reduction in *dilp*2 transcript in our studies was only observed in RU486-elavGS/UAS-InR^{DN} females but not in long-lived elavGAL/UAS-InR^{DN} females, it is unlikely that reduced *dilp*2 expression in the head is the main cause of the extended lifespan. That said, we did not measure effects of reduced neuronal IIS on levels of DILP protein production and secretion. Such measurements, which are planned for the future, would further our understanding of how reduced neuronal IIS influences lifespan. It would also be interesting to measure *dilp* expression and protein levels in older flies around the age of 30-35 days, which is the age when the detrimental effects on pan-neural IIS reduction were first observed.

Our experiments studying the effect of reduced pan-neural IIS on haemolymph glucose content and fecundity are showing preliminary results and need to be confirmed by using more robust experimental designs. Broughton et al. (2005) reported 2-fold increase in haemolymph glucose concentration in response to the ablation of the IPCs using the same experimental design and repeat number. Therefore, if there was a big change in haemolymph glucose concentration in response to reduced pan-neural IIS, our experiments could have spotted it. The circulation of most insects contains two types of sugars: glucose and trehalose. While glucose is obtained from the diet, the role of trehalose is carbohydrate distribution to peripheral tissues and it originates from the fat body (Klowden, 2002). Broughton et al. (2005) showed that haemolymph trehalose concentration was reduced by 15% in response to IPC ablation. We did not measure trehalose concentration in our experiments, but it would be interesting to do so in the future. Semaniuk et al. (2018) showed that *dilp3* and *dilp7* can influence haemolymph glucose in a diet dependent manner and DILP3 plays a role in regulating haemolymph trehalose on low-protein and high-carbohydrate diet.

Or fecundity experiments did not show any significant effect of reduced pan-neural IIS. However, it showed that RU4486 has negative effect on female fecundity. As our experiment was carried out sampling from the flies kept for lifespan measurements, we wanted to keep them in the same environment and diet. Our original plan was to measure fecundity over the lifetime of the flies, as done by Broughton et al (2005) showing that IPC ablation reduces fecundity. We attempted to measure fecundity every 10 days throughout the lifespan of the flies, however their fecundity dropped almost to zero after the second timepoint. Therefore, we only presented the first timepoint in this chapter. Repeating the fecundity experiment did not fit into our timescale unfortunately, but in the future, it needs to be repeated in order to gain more reliable results. Recording egg number every 5 days, as in Broughton, et al. (2005) would probably give a better picture of the fecundity decline. Alternatively, we could measure cumulative egg number laid by female flies over the first 3-4 weeks of their life, as done by Grönke, et al. (2009) and we could possibly boost their egg laying by adding live yeast paste into their vial (Clancy et al. 2001).

In contrast to the lack of effect on starvation resistance, RU486-elavGS/UAS-InR^{DN} flies were more sensitive to oxidative stress than controls. Previous studies (Clancy et al. 2001, Broughton et al. 2005) have found that extension of lifespan due to reduced IIS is often associated with enhanced oxidative stress resistance. Our finding of oxidative stress sensitivity further suggests that reduced neuronal IIS does not influence peripheral IIS. The effects on survival under oxidative conditions could

instead be due to cell autonomous effects on neuronal survival. The excitatory neurotransmitter, glutamate increases mitochondrial respiration, therefore it may elevate the levels of reactive oxygen species in the post-synaptic cell, which in high levels can promote cell death. Moreover, glutamate inactivates AKT, counteracting the neuroprotective role of IIS (Garcia-Galloway, et al. 2003, Yang, et al. 2011).

In order to determine if reduced IIS in neurons led to increased neuronal cell death, we attempted to assess levels of apoptosis, although time constraints resulted in the data being preliminary in nature. However, we did observe increased numbers of apoptotic cells due to both constitutive and adult-specific neuronal reduction in females at old age, whereas control flies did not show this increase. Studies of Zheng, et al. (2005) showed that apoptosis in flies shows gradual increase with age in muscle cells and induced in fat cells at old age. However, the fly nervous system does not show signs of apoptosis with age. The relationship between IIS and neuronal cell death has not yet been studied in flies, however mouse IGF-1 shows anti-apoptotic actions in cultured neurons (Baker et al., 1999). Furthermore, the overexpression of IGF-1 in the CNS of transgenic mice attenuates cerebellar apoptosis, *in vivo* supporting its anti-apoptotic role (Chrysis, Calikoglu, Ye and D'Ercole, 2001). Future experiments are planned to further investigate neuronal cell death under normal and oxidative stress conditions.

In conclusion, long-lived flies with reduced pan-neural IIS do not show increased oxidative stress or starvation resistance, that is common in response to systemic IIS signalling reduction. Furthermore, our preliminary results on fecundity and haemolymph glucose concentration did not show any significant reduction in response to reduced pan-neural IIS, these experiments however need to be repeated in the future. Reducing IIS in neurons during adulthood changes some of the *dilp* expression levels in both males and females, but these changes were not present in response to constitutive IIS reduction, therefore unlikely to be the reason for the lifespan extension. Lastly, the detrimental effects of reduced pan-neural IIS on behaviour could possibly be explained by increased programmed cell death at older age, however, further apoptosis studies are required to confirm these results.

Chapter 8: The effects of neuronal subtype-specific IIS reduction on lifespan

8.1: Introduction

As discussed previously, the lifespan extending effect of IIS reduction has been widely studied in various model organisms and it is well supported (Kenyon, et al. 1993, McElwee et al. 2003, 2004, Tatar et al. 2001, Clancy et al. 2001, Broughton, et al. 2005, Bluher et al. 2003, Holzenberger et al. 2003.) and it has been shown that tissue or time specific IIS reduction can still promote longevity (Mathew, et al. 2017). However, the effects of reduced IIS on the brain is controversial, since numerous studies show that reduction of IIS in the CNS leads to detrimental effects on memory and behaviour despite extending lifespan (Broughton and Partridge, 2009) - further discussed in Chapter 1.10. As presented in Chapters 4 and 5, reduced IIS throughout the development of the flies was not necessary for lifespan extension or the detrimental effects on behavioural decline. The fact that reduced IIS in adult neurons can influence lifespan and behavioural senescence raises a number of questions regarding how it occurs and which neuronal subtypes are responsible for the effects on lifespan and behaviour. In this chapter, studies on the role of different neuronal subtypes in the response to reduced IIS are presented.

Descending neurons have their cells bodies in the brain and their axons carry information to the body ganglia, controlling the behaviour of the organism. Hsu and Bhandawat (2016) estimated ~1100 descending neurons in the *Drosophila* brain distributed in 6 clusters and measured the distribution of neuronal subtypes based on their neurotransmitter. They found that none of the clusters expresses exclusively a single neurotransmitter, rather the clusters contain a mixture of neuronal subtypes. The two major neurotransmitters are the excitatory acetylcholine and the inhibitory GABA. Approximately 38% of the descending neurons are cholinergic, and 37% are GABAergic and the equal amount of inhibitory and excitatory neuronal types stands in contrast to the descending neuron distribution in vertebrates, as it is predominantly excitatory (Hsu and Bhandawat, 2016). Their study localised four minor neurotransmitters in the fly brain: 6% of the descending neurons are glutamatergic, about 3% is serotoninergic, 1% is octopaminergic and only 0.2% is dopaminergic. The remaining 15% of the descending neurons are like likely to express neurotransmitters

that were not examined in the study of Hsu and Bhandawat (2016), such as histamine, tyramine or peptidergic neurotransmitters. The localisation of the neuronal subtypes is shown in . In their recent study, Deng, et al. (2019) visualised the location of small-molecule neurotransmitters, including glycine and histamine and while no glycine receptor was found in the fly brain, they confirmed the presence of histidine decarboxylase, thus histamine is also used as a neurotransmitter in flies.

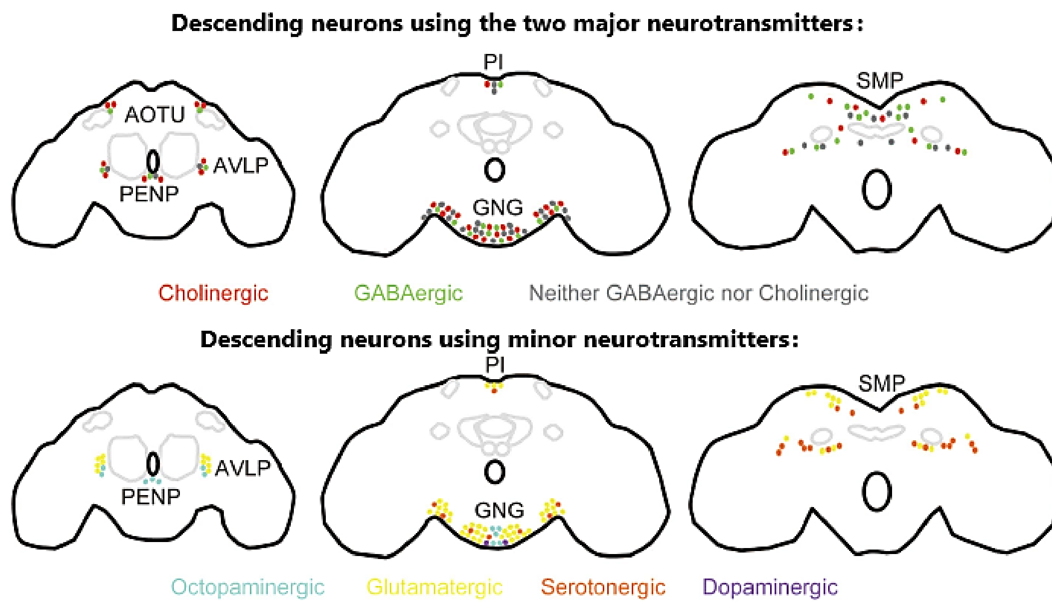


Figure 49 - Descending neurons expressing major or minor neurotransmitters
Localisation was done by and the picture is adapted from Hsu and Bhandawat (2016). The dots for the major neurotransmitters represent the proportion of the given type of neuron, while the dots for the minor neurotransmitters represent a single descending neuron.

While it was believed that each neuron expresses a single neurotransmitter (known as 'Dale's principle' (Dale, 1935 and Eccles et al., 1954)), recent studies have witnessed the co-expression of more than one type of neurotransmitter in neurons. As an example, Granger et al., (2017) showed the co-release of acetylcholine – glutamate and dopamine – glutamate in the mammalian midbrain. Deng et al. (2019) examined the possible co-expression of neurotransmitters using dopaminergic neurons as an example, showing that dopaminergic neurons also express enzymes related to glutamate, acetylcholine and GABA transport or synthesis. However, excitatory and inhibitory neurotransmitters were not found to be co-released from the same neuron. Contrary, in mammals the co-expression of excitatory and inhibitory neurotransmitters has been shown (Kao et al., 2004, Ottem et al., 2004, Boulland et al., 2009, Zander et al., 2010, Granger et al., 2017).

The role of the neuronal subtypes has been widely investigated in a variety of behaviours usually one or a few neurotransmitters at a time, but there is no systematic overview of the role of each neurotransmitter in all aspects of fly behaviour. The role of dopamine has been widely studied and it was shown to modulate aggression (Alekseyenko, et al. 2013), arousal (Andretic, et al. 2005, Sitaraman, 2015), locomotion (Draper, et al. 2007), courtship (Keleman, et al. 2012) and foraging (Landayan, et. a. 2018). Octopamine was also found to play an important role in regulating aggression (Zhou, et al. 2008, Andrews, et al. 2014), it is necessary for adaptation to endurance exercise (Sujkowski, et al. 2017), and it also regulates odour-based decision making (Claßen and Scholz, 2018). Li, et al. (2016) showed that flies lacking octopamine are more resistant to starvation, have increased body fat deposit, reduced physical activity and reduced metabolic rate compared to the control flies. These octopamine deficient flies have a shorter lifespan and increased rate of insulin release. Serotonin promotes aggression in flies (Dierick and Greenspan, 2007, Alekseyenko, et. a. 2014), regulates attraction to ethanol (Xu, et al. 2016), sleep (Qian, et al. 2017) and regulates memory formation (Lee, et al. 2011, Haynes, et al. 2015, Scheunemann, et al. 2018). Acetylcholine also modulates sleep behaviour (Muraro and Ceriani, 2015) and promotes aggression (Alekseyenko, et. a. 2019). GABA on the other hand decreases aggression (Alekseyenko, et. a. 2019), and it also modulates sleep (Hamasaka, et al. 2005), mediates the behaviour-impairing effect of ethanol (Dzitoyeva, et. a. 2003), promote food consumption (Cheung and Scott, 2017) and appetitive long-term memory formation (Pavlowsky, et al. 2018). Glutamate promotes wakefulness in flies (Zimmerman, et al. 2017) and regulates odour responses (Liu and Wilson, 2013). Lastly, histamine was suggested to be a major mechanosensory transmitter in flies (Buchner, et al. 1993), it promotes wakefulness (Oh, et al. 2013) and modulates temperature preference and sensitivity (Yusein, et al. 2010).

Various components of the CNS may have individual sensitivities to IIS (Broughton and Partridge, 2009). Therefore, one of our hypotheses to explain the disconnection between extended lifespan and decreased behavioural health is that individual neuronal subtypes may respond to reduced IIS differently, and what we see during pan-neural IIS reduction is the sum of positive, negative and/or neutral effects of reduced IIS in each neuronal subtype. To determine how different neuronal types respond to reduced IIS, we obtained GAL4 drivers for 4 of the 7 neuronal subtypes from the Bloomington *Drosophila* Stock Centre; dopaminergic, cholinergic, glutamatergic and GABAergic. This chapter presents how reduction of IIS in specific

neuronal subtypes by the GAL4 drivers and the UAS-InR^{DN} transgene affects lifespan.

8.1.1: Aims

To investigate the effect of neuronal subtype specific IIS reduction on lifespan using dopaminergic, cholinergic, GABA-ergic and glutamatergic neuron specific drivers.

8.1.2: Research design

Dopaminergic (ThGAL4), glutamatergic (VglutGAL4), cholinergic (ChAT-GAL4) and GABAergic (Gad1-GAL4) *Drosophila* lines were ordered from Bloomington *Drosophila* Stock Centre and backcrossed into our w^{Dah} background for 5 generations. The expression pattern of each GAL4 was validated following backcrossing (Chapter 3). Each line was crossed with the UAS-InR^{DN} transgenic fly and their lifespans were measured.

To reduce IIS selectively in dopaminergic neurons, the UAS-InR^{DN} transgene was crossed to the ThGAL4 (BDSC 8848) driver. ThGAL4 has its GAL4 driver fused to the *pale* gene encoding for Tyrosine 3-monooxygenase, which is a tyrosine hydroxylase which is the first a rate limiting step during dopamine synthesis and expressed in dopaminergic neurons (Friggi-Grelin, et al. 2003).

The VglutGAL4 (BDSC 26160) *Drosophila* line was used to reduce IIS in the glutamatergic neurons. It works by the OK371 enhancer trap element being inserted close to the *CG9887* gene encoding for vesicular glutamate transporter (Vglut), which is essential for the uptake of the neurotransmitter glutamate into synaptic vesicles (Mahr and Aberle, 2006).

The Gad1-GAL4 driver (BDSC 51630) was used to reduce IIS selectively in GABAergic neurons. The expression of GAL4 is driven by a sequence immediately upstream of Gad1 translation site. Glutamic acid decarboxylase 1 (Gad1) encodes for a glutamic acid decarboxylase, which is an enzyme required for gamma-Aminobutyric acid (GABA) synthesis (Ng et al., 2002).

The GAL4 driver chosen to reduce IIS in cholinergic neurons was the ChAT-GAL4 (BDSC 6798). The expression of GAL4 is regulated from the “cholinergic” locus

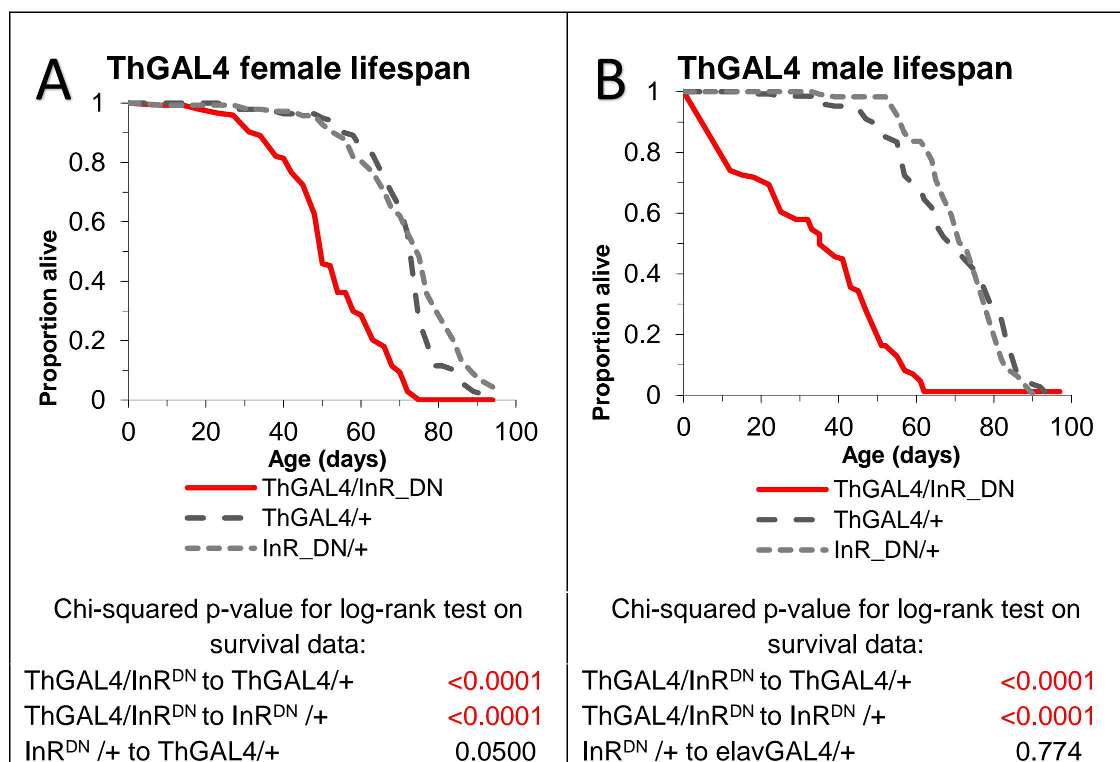
encoding for both ChAT and VAChT. Choline acetyltransferase (ChAT) encodes for the catalysator of acetylcholine biosynthesis, while Vesicular acetylcholine transporter (VAChT) is a transport protein responsible for packaging acetylcholine into synaptic vesicles (Salvaterra and Kitamoto, 2001).

8.2: Results

8.2.1: Reducing IIS in dopaminergic neurons shortens the lifespan of female and male fruit flies

The experimental group with reduced IIS in the dopaminergic neurons was the ThGAL4/UAS-InR^{DN}. The two control groups were ThGAL4/+ and UAS-InR^{DN}/+ both containing the driver or the transgene alone, crossed to the wild type w^{Dah} background. All groups were kept on standard food throughout their life and their lifespan was measured.

As shown in **Figure 50**, constitutive reduction of IIS in dopaminergic neurons significantly reduced the lifespan of both male and female flies (p<0.001). There was about 30% reduction in female median lifespan and around 50% in males.



Chi-squared p-value for log-rank test on survival data:

ThGAL4/InR^{DN} to ThGAL4/+	<0.0001
ThGAL4/InR^{DN} to InR^{DN} /+	<0.0001
InR^{DN} /+ to ThGAL4/+	0.0500

Chi-squared p-value for log-rank test on survival data:

ThGAL4/InR^{DN} to ThGAL4/+	<0.0001
ThGAL4/InR^{DN} to InR^{DN} /+	<0.0001
InR^{DN} /+ to elavGAL4/+	0.774

Figure 50 - Lifespan of male and female flies with constitutive IIS reduction in their dopaminergic neurons

The experimental group with reduced IIS is the ThGAL4/InR^{DN} cross, while ThGAL4/+ and InR^{DN} /+ are the control groups of the driver and the transgene crossed with wild type flies (N=150). Survival curves were compared using nonparametric log rank tests and p values are shown under the graphs, with significant differences highlighted in red ($p<0.05$).

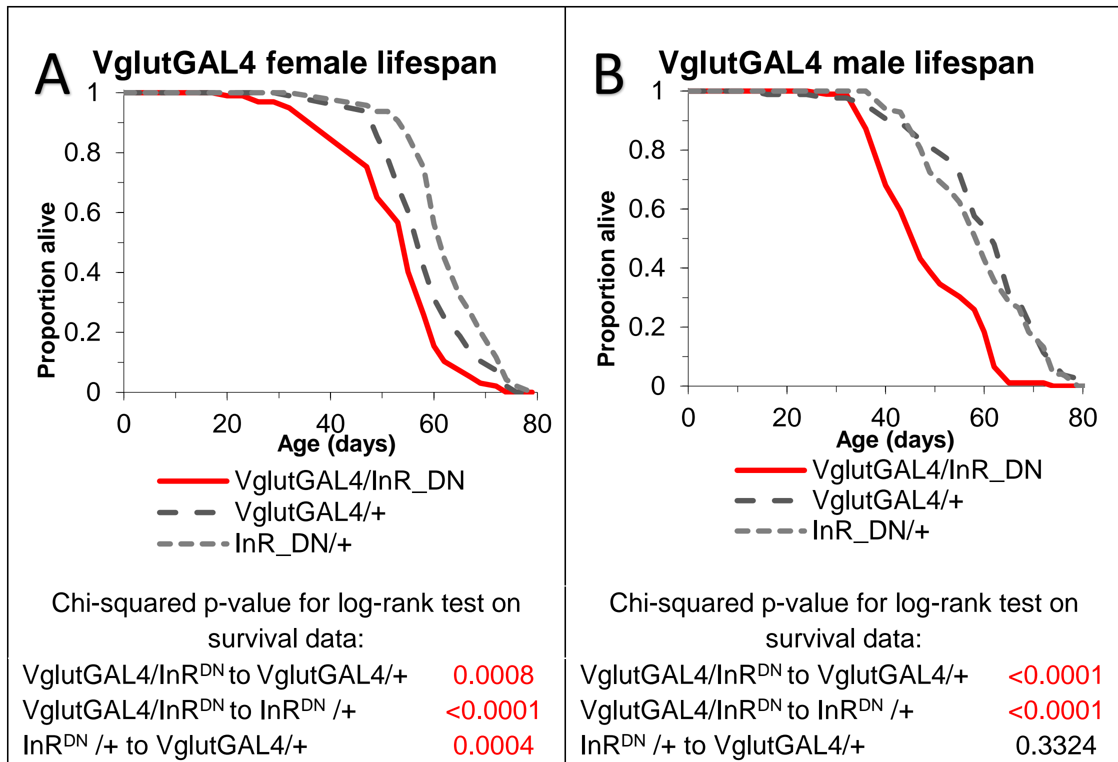
A) Female lifespan. Median lifespan: ThGAL4/InR^{DN}: 49 days, ThGAL4/+: 73.5 days, InR^{DN} /+: 73.5 days,

B) Male lifespan. Median lifespan: ThGAL4/InR^{DN}: 35 days, ThGAL4/+: 68 days, InR^{DN} /+: 72 days.

8.2.2: Reducing IIS in glutamatergic neurons slightly shortens the lifespan of female and male fruit flies

The experimental group is the VglutGAL4/UAS-InR^{DN} expressing the dominant negative insulin receptor in the glutamatergic neurons. The control groups for the driver and the transgene are VglutGAL4/+ and UAS-InR^{DN}/+, both crossed with wild type w^{Dah} background. All three groups were kept on standard food throughout their life and their lifespan was measured.

The results of glutamatergic neuron specific IIS reduction on lifespan are presented in **Figure 51**, showing significant reduction in lifespan for both females ($p=0.0008$ to VglutGAL4/+ and $p<0.0001$ to InR^{DN}/+) and males ($p<0.0001$). The reduction in median lifespan was, around 10% in females and 25% in males. The VglutGAL4 driver itself had some negative effect on the female lifespan as the VglutGAL4/+ control group had significantly shorter lifespan compared to the InR^{DN}/+ group.



Chi-squared p-value for log-rank test on survival data:

Comparison	p-value
VglutGAL4/InR^{DN} to VglutGAL4/+	0.0008
VglutGAL4/InR^{DN} to InR^{DN} /+	<0.0001
InR^{DN} /+ to VglutGAL4/+	0.0004

Chi-squared p-value for log-rank test on survival data:

Comparison	p-value
VglutGAL4/InR^{DN} to VglutGAL4/+	<0.0001
VglutGAL4/InR^{DN} to InR^{DN} /+	<0.0001
InR^{DN} /+ to VglutGAL4/+	0.3324

Figure 51 - Lifespan of male and female flies with constitutive IIS reduction in their glutamatergic neurons

The experimental group with reduced IIS is the VglutGAL4/InR^{DN} cross, while VglutGAL4/+ and InR^{DN} /+ are the control groups of the driver and the transgene crossed with wild type flies (N=100). Survival curves were compared using nonparametric log rank tests and p values are shown under the graphs, with significant differences highlighted in red ($p<0.05$). The experiments were carried out by Emma Zhang, taught master's student.

A) Female lifespan. Median lifespan: VglutGAL4/InR^{DN}: 54 days, VglutGAL4/+: 56.5 days, InR^{DN}/+: 61 days,

B) Male lifespan. Median lifespan: VglutGAL4/InR^{DN}: 45 days, VglutGAL4/+: 61 days, InR^{DN} /+: 59 days.

8.2.3: Reducing IIS in GABAergic neurons does not affect lifespan

The experimental group is the Gad1GAL4/UAS-InR^{DN} expressing the dominant negative insulin receptor in the GABAergic neurons. The control groups for the driver and the transgene are Gad1GAL4/+ and UAS-InR^{DN}/+, both crossed with wild type w^{Dah} background. All three groups were kept on standard food throughout their life and their lifespan was measured. The results are presented in **Figure 52**, showing no significant lifespan increase or reduction in either males or females.

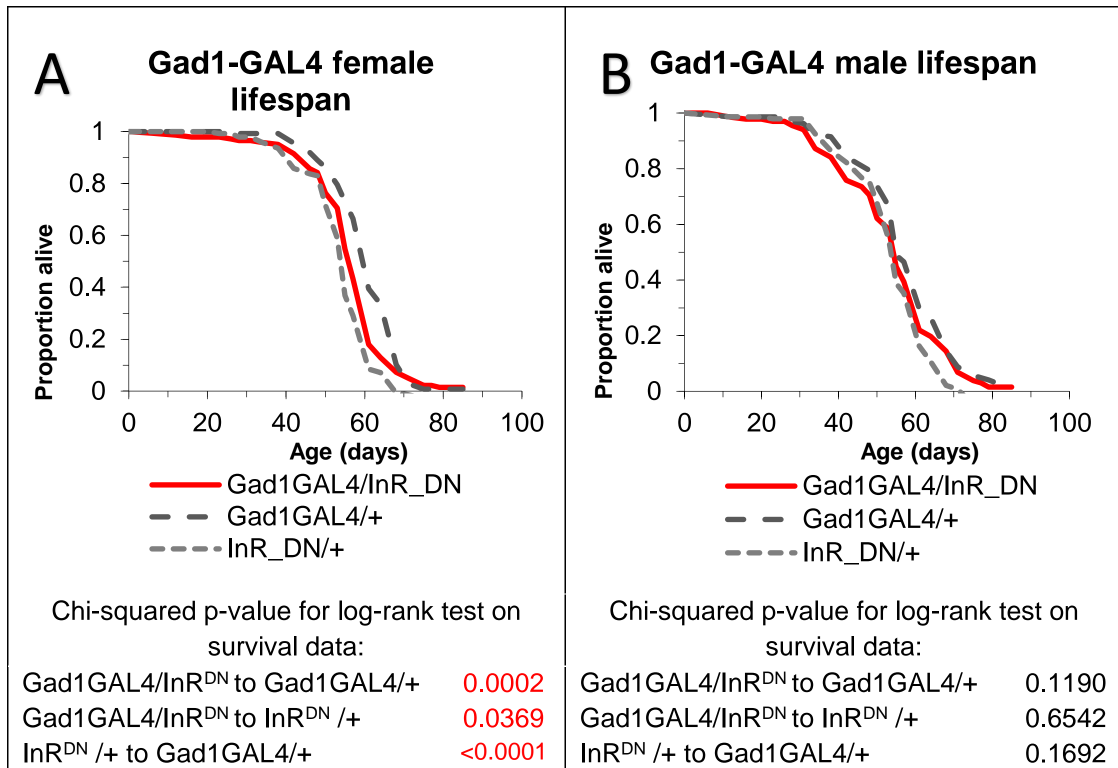


Figure 52 - Lifespan of male and female flies with constitutive IIS reduction in their GABAergic neurons

The experimental group with reduced IIS is the Gad1GAL4/InR^{DN} cross, while Gad1GAL4/+ and InR^{DN} /+ are the control groups of the driver and the transgene crossed with wild type flies (N=150). Survival curves were compared using nonparametric log rank tests and p values are shown under the graphs, with significant differences highlighted in red ($p<0.05$).

A) Female lifespan. Median lifespan: Gad1GAL4/InR^{DN}: 56 days, Gad1GAL4/+: 59 days, InR^{DN}/+: 54 days,

B) Male lifespan. Median lifespan: Gad1GAL4/InR^{DN}: 54 days, Gad1GAL4/+: 54 days, InR^{DN} /+: 54 days.

8.2.4: Reducing IIS in cholinergic neurons shortens male lifespan and may also shorten female lifespan.

The experimental group (ChAT-GAL4/UAS-InR^{DN}) expressed the dominant negative insulin receptor selectively in cholinergic neurons. The control groups for the driver and the transgene were ChAT-GAL4/+ and UAS-InR^{DN}/+, both crossed with wild type *w^{Dah}* background. All three groups were kept on standard food throughout their life and their lifespan was measured.

The results presented in **Figure 53** show that the ChAT-GAL4 driver itself significantly shortened the lifespan of both male and female flies. It is thus difficult to separate the effect of the GAL4 driver on lifespan from that of IIS reduction. However, although the lifespan of ChAT-GAL4/UAS-InR^{DN} females was not significantly different

from that of the ChAT-GAL4/+ control group, ChAT-GAL4/UAS-InR^{DN} male were significantly shorter lived than both controls suggesting a negative effect on at least male lifespan of reduced IIS in cholinergic neurons.

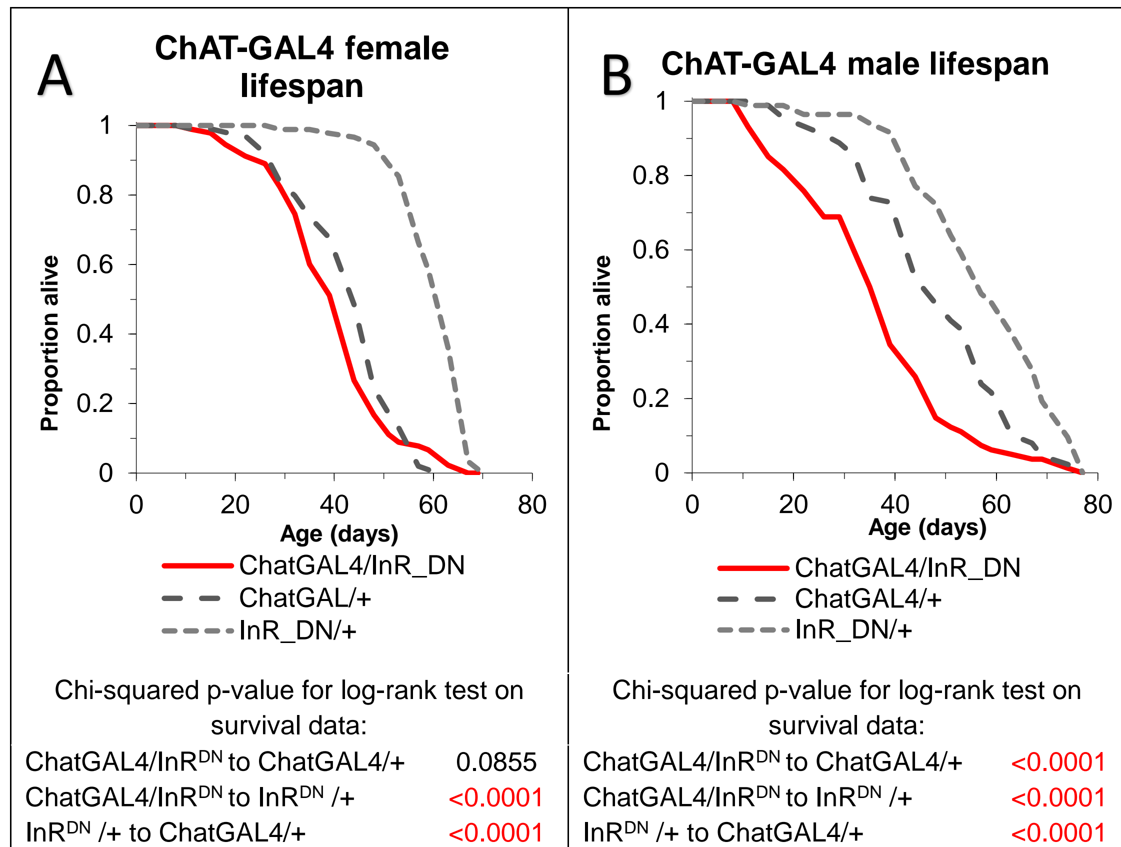


Figure 53 - Lifespan of male and female flies with constitutive IIS reduction in their cholinergic neurons

The experimental group with reduced IIS is the ChatGAL4/InR^{DN} cross, while ChatGAL4/+ and InR^{DN} /+ are the control groups of the driver and the transgene crossed with wild type flies (N=100). Survival curves were compared using nonparametric log rank tests and p values are shown under the graphs, with significant differences highlighted in red ($p<0.05$).

A) Female lifespan. Median lifespan: ChatGAL4/InR^{DN}: 41.5 days, ChatGAL4/+: 41.5 days, InR^{DN}/+: 61 days,

B) Male lifespan. Median lifespan: ChatGAL4/InR^{DN}: 33.5 days, ChatGAL4/+: 46 days, InR^{DN} /+: 55 days. The p-values from the log-rank tests are shown under the graphs and the significant ones are highlighted in red font colour ($p<0.05$).

8.3: Discussion

In this chapter we investigated the effects of neuronal subtype specific IIS reduction on the lifespan of flies. Due to time constraints, we tested four of the seven neuronal subtypes. We found that UAS-InR^{DN} expression in each neuronal subtype resulted in different effects on lifespan, however there was no positive effect seen for any of the neuronal types, only negative or neutral. Reduction of IIS in GABAergic neurons had no effect on the lifespan of the flies, whereas reducing IIS in dopaminergic neurons led to a massive decrease in lifespan and reducing IIS in glutamatergic neurons also shortened lifespan but to a lesser extent. The results using the ChAT-GAL4 cholinergic driver are difficult to interpret because the driver itself shortened lifespan. Males showed further lifespan shortening when they expressed UAS-InR^{DN} through the ChAT-GAL4 driver, but females did not. Since the driver itself made the flies short-lived, it is difficult to separate the effect of the driver from the effect of the IIS reduction and draw conclusions from this lifespan experiment.

Together the data presented here show that reducing IIS in the specific neuronal subtypes tested had neutral or detrimental effects on fly lifespan. Unlike reducing IIS in all neurons, neuronal subtype specific IIS reduction did not extend lifespan. Currently the mechanism of lifespan extension by pan-neural IIS reduction is not known, so we can only speculate why reduced IIS in neuronal subtypes could not increase lifespan. Since we did not have the time in this project to test all the neuronal subtypes, it is possible that we did not modulate IIS in the neuronal subtype that is promoting longevity. Li, et al. (2016) showed that flies lacking octopamine have a shorter lifespan and increased rate of insulin release, therefore octopaminergic neurons are an interesting target for IIS reduction to promote longevity. It is equally possible that reduced IIS in all neurons is required to promote longevity, especially if reduced IIS in neurons slows down the ageing of the neurons and this is limiting for lifespan. In that case, reducing IIS in only a subset of neurons may not be sufficient to affect the lifespan of the whole organism. Reducing IIS in a subset of neurons can also lead to dysregulation of the health and function of the brain, shortening lifespan.

It is also possible, that the UAS-InR^{DN} transgene is expressed in different levels by the various neuronal subtype specific drivers. In this case, the different effects on the lifespan of the flies are at least partially due to the level of InR^{DN} expression in the specific neuronal subtypes. To test the expression of the InR^{DN} transgene using qPCR,

we would need to collect specific neuronal subtypes for RNA extraction, which unfortunately did not fit into the timescale or budget of the project.

We hypothesised that the disconnection between lifespan and health-span can be due to reduced IIS having detrimental effects on the function of the neurons but slows down the ageing of the neurons, thus extends lifespan. In Chapter 5 we showed that pan-neural IIS reduction has reversible detrimental effects on the exploratory walking behaviour of the flies, therefore reduced IIS does reduce the function of the neurons and does not accelerate the ageing of the neurons. However, we still do not have evidence of reduced IIS slowing down neuronal ageing. The results of this chapter fit into this hypothesis, as the neuronal subtypes expressing minor neurotransmitters (dopaminergic and glutamatergic) shortened the lifespan, therefore it is possible that the positive effect on the ageing of those neurons could not compensate the negative effect of reduced IIS on the function of the neurons, as they are fewer in number. GABA is the major inhibitory neurotransmitter in flies and reduced IIS in GABAergic neurons did not affect the lifespan of the flies, which supports this hypothesis. However, reduced IIS in cholinergic neurons - which are equally as abundant as GABAergic neurons (Hsu and Bhandawat, 2016) - shortened the lifespan of the flies. The results of the cholinergic flies are unfortunately unreliable as the GAL4 driver affected the health and survival of the flies.

In the future, the experiment in cholinergic neurons needs to be repeated with a different driver that does not affect the health of the flies. It would also be interesting to see how serotoninergic, octopaminergic and histaminergic neurons respond to reduced IIS, however these experiments did not fit into the limited time of this project. It would be also necessary to measure the expression of InR^{DN} in each specific neuronal subtype to exclude that the differences in lifespan are due to different expression levels of the transgene by the GAL4 drivers.

Chapter 9: The effect of neuronal subtype specific IIS reduction on exploratory walking and negative geotaxis senescence

9.1: Introduction

The experiments presented in previous chapters using pan-neural IIS reduction showed a disconnection between lifespan and health span. Despite female flies being long lived, they either showed no improvement in their behaviour or an earlier decline. A possible explanation for this is that the beneficial effects of reduced IIS on peripheral organs outweighs the detrimental effects of IIS reduction on the CNS (Ismail et. al, 2015). This could explain why only the senescence of negative geotaxis and exploratory walking parameters that require muscle health were ameliorated in response to reduced IIS. However, the decision-making parameters of exploratory walking did not. The recovery experiments presented in Chapter 5 revealed that the detrimental effects on exploratory walking due to reduced neuronal IIS were due to effects on neuronal function are not due to an acceleration of neuronal ageing. However, it is still unclear as to whether or not neuronal ageing is slowed by reduced IIS, similarly to the effect of reduced IIS on the periphery.

In this Chapter we address our second hypothesis that individual neuronal subtypes show different sensitivities to IIS such that the effect of pan-neural IIS reduction on behavioural senescence is the sum of the positive, negative and neutral effects of reduced IIS on individual neuronal subtypes. In the previous chapter we have found that neuronal subtype specific IIS reduction of 4 neuronal subtypes (dopaminergic, cholinergic, glutamatergic and GABAergic) affected lifespan differently, with no lifespan extension observed with any of the subtypes. In this chapter, the effects of neuronal subtype specific IIS reduction on negative geotaxis and exploratory walking are investigated using the same dopaminergic, glutamatergic, GABAergic and cholinergic GAL4 drivers as for the lifespan experiments.

Various components of the CNS may have individual sensitivities to IIS (Broughton and Partridge, 2009), therefore one our hypotheses to explain the disconnection between extended lifespan and decreased health suggests that

individual neuronal subtypes may respond to reduced IIS differently, and what we see during pan-neural IIS reduction is the sum of positive, negative and neutral effects of reduced IIS in each neuronal subtype. IIS reduction in dopaminergic, glutamatergic and cholinergic neurons shortened the lifespan of flies, while it had no effect on lifespan in GABAergic neurons.

9.1.1: Aims

To investigate the effect of neuronal subtype specific IIS reduction on negative geotaxis and exploratory walking behavioural senescence of fruit flies using dopaminergic, cholinergic, GABA-ergic and glutamatergic neuron specific drivers.

9.1.2: Research design

Dopaminergic (ThGAL4), glutamatergic (VglutGAL4), cholinergic (ChAT-GAL4) and GABAergic (Gad1-GAL4) *Drosophila* lines (described in Chapter 8.1.2) were ordered from Bloomington *Drosophila* Stock Centre and backcrossed into our w^{Dah} background for 5 generations. For the experimental groups, each line was crossed with the UAS-InR^{DN} line to reduce the IIS selectively in the specific neuronal subtype. The control groups were the GAL4 driver and the UAS-InR^{DN} transgene crossed with wild type w^{Dah} flies.

Flies were sorted onto standard food vials as 10 per vial at the age of 3 days, separated by gender. They were stored at standard conditions at 25ºC, 70% humidity 12 h dark/light cycle throughout their lifespan. Experiments were also carried out at the same conditions. Flies were sampled from the population about every 10 days for negative geotaxis and exploratory walking experiment throughout their life. Experiments were done at the same time of the day to avoid the effects of their varying daily activity as described in Chapter 2.

The negative geotaxis data was initially analysed by Generalised Linear Modelling (GLM) to see if Age, Genotype or Age*Genotype has any significant effect on the groups. If there was a significant Genotype or Age*Genotype effect, post hoc Tukey-Kramer pairwise comparison was carried out at each timepoint.

The exploratory walking videos were analysed using EthoVision XT video tracking software (Nodus) and the data was first analysed by GLM to see if Age, Genotype or Age*Genotype has any significant effect on the groups. Where there was a significant genotype or age*genotype effect, post hoc pairwise comparation using Tukey-Kramer HSD test was carried out for each timepoint.

9.2: Results

9.2.1: Constitutive reduction of IIS in dopaminergic neurons had no effect on negative geotaxis senescence and exploratory walking behaviour

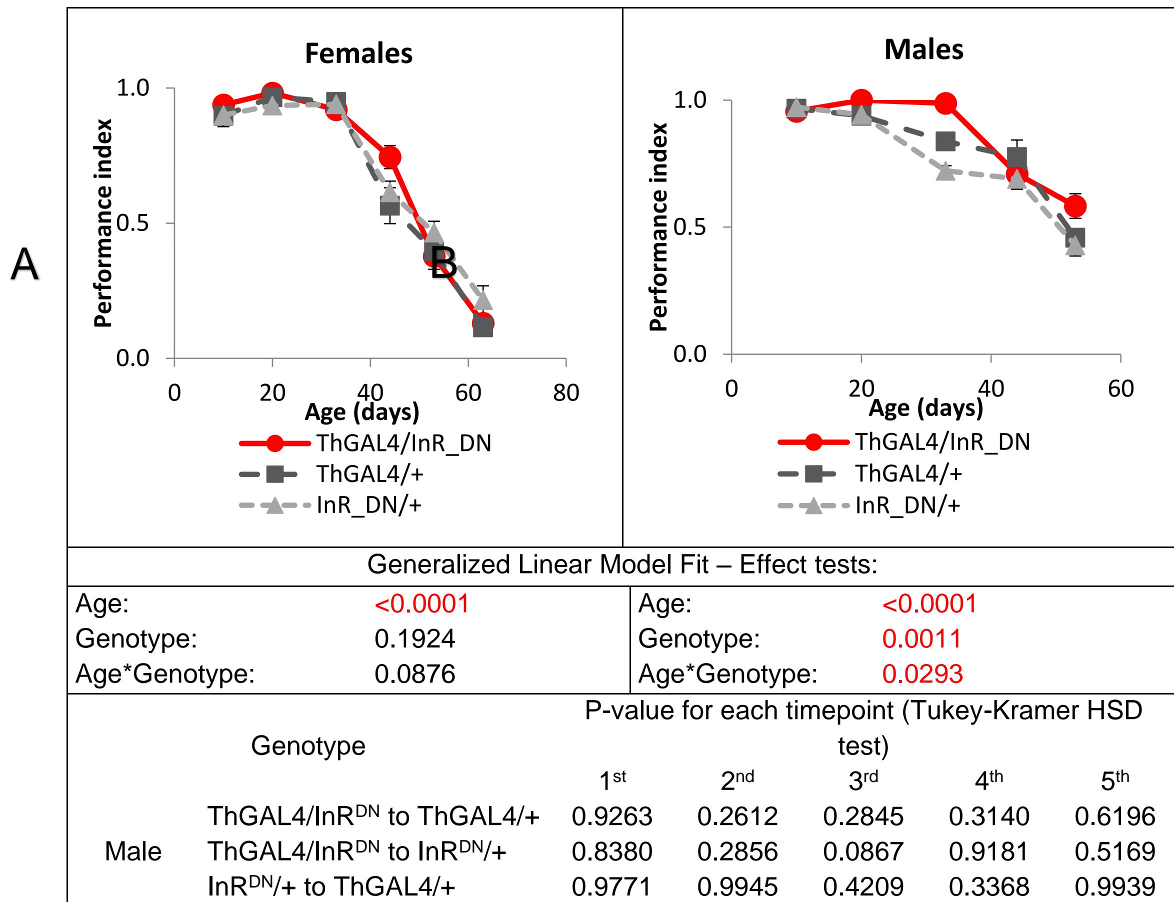
The experimental group with reduced IIS in the dopaminergic neurons was the ThGAL4/UAS-InR^{DN}. The two control groups were ThGAL4/+ and UAS-InR^{DN}/+ both containing the driver or the transgene alone, crossed to the wild type w^{Dah} background.

Figure 54 shows that negative geotaxis declined with age similarly in all genotypes. For females, there was no significant effect of genotypes on negative geotaxis senescence. Generalised Linear Modelling (GLM) showed significant genotype effect on the male flies (p=0.0011), however host hoc pairwise comparisons of each timepoint using Tukey-Kramer HSD test did not find any significant differences between the ThGAL4/UAS-InR^{DN} and both controls. Thus, constitutive IIS reduction in dopaminergic neurons has no effect on negative geotaxis behaviour.

In parallel with the negative geotaxis experiment, an exploratory walking experiment was carried out sampling from the same population of flies. **Figure 55** shows that age had a significant effect on all the male and female exploratory walking parameters, but genotype had no effect on the normal age-related decline of all walking parameters. Therefore, constitutive IIS reduction in dopaminergic neurons does not affect exploratory walking behaviour.

Dopaminergic neurons – Negative geotaxis

A



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.1924	Genotype:	0.0011
Age*Genotype:	0.0876	Age*Genotype:	0.0293

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test)				
		1st	2nd	3rd	4th	5th
Male	ThGAL4/InR^{DN} to ThGAL4/+	0.9263	0.2612	0.2845	0.3140	0.6196
	ThGAL4/InR^{DN} to InR^{DN}/+	0.8380	0.2856	0.0867	0.9181	0.5169
	InR^{DN}/+ to ThGAL4/+	0.9771	0.9945	0.4209	0.3368	0.9939

Figure 54 - Effect of constitutive IIS reduction in dopaminergic neurons on negative geotaxis senescence

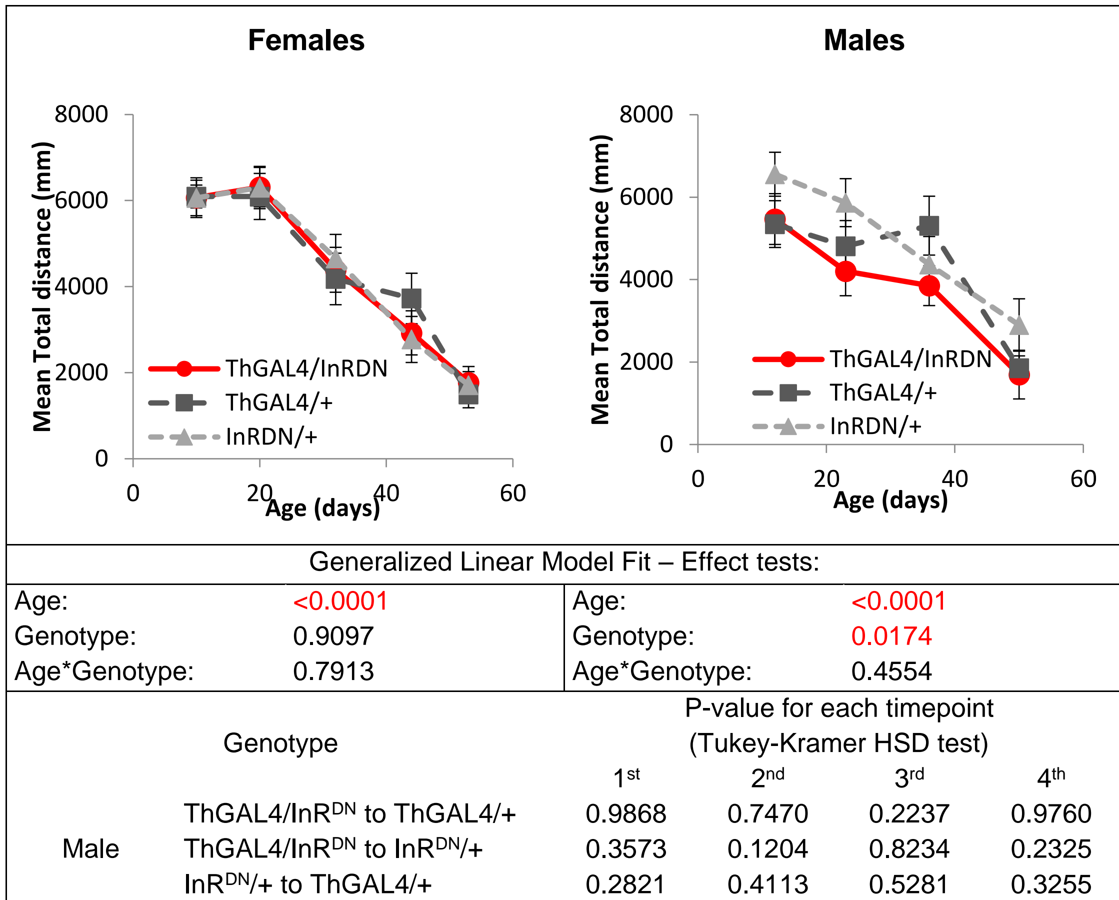
Negative geotaxis performance index of flies over the lifespan. N=3 (group of 10 flies) for each measurement. Error bars represent +/- SEM. Data was analysed by JMP statistical software. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype has a significant effect, post hoc pairwise comparison was carried out using Tukey-Kramer HSD test at each timepoint. Significant difference is highlighted with red text colour ($p<0.05$)

A) ThGAL4/UAS-InR^{DN} female flies compared to ThGAL4/+ and UAS-InR^{DN}/+ control groups.

B) ThGAL4/UAS-InR^{DN} male flies compared to ThGAL4/+ and UAS-InR^{DN}/+ control groups.

Dopaminergic neurons – Exploratory Walking

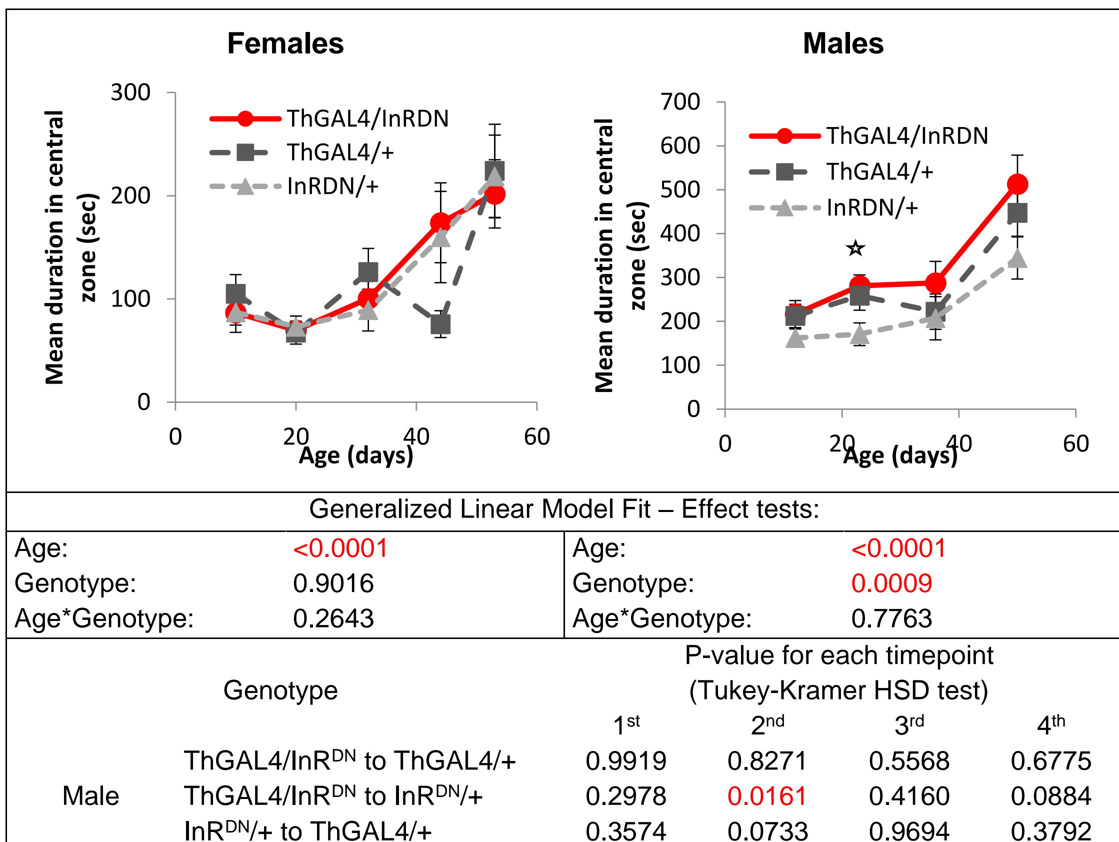
A



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.9097	Genotype:	0.0174
Age*Genotype:	0.7913	Age*Genotype:	0.4554

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test) 1st	2nd	3rd	4th
Male	ThGAL4/InR^{DN} to ThGAL4/+	0.9868	0.7470	0.2237	0.9760
	ThGAL4/InR^{DN} to InR^{DN}/+	0.3573	0.1204	0.8234	0.2325
	InR^{DN}/+ to ThGAL4/+	0.2821	0.4113	0.5281	0.3255

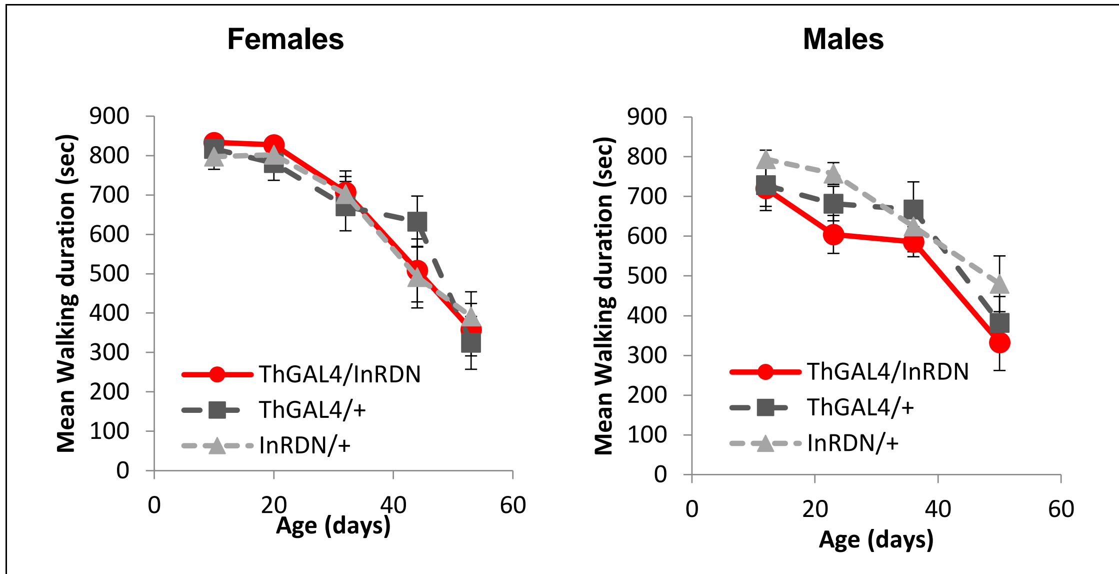
B



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.9016	Genotype:	0.0009
Age*Genotype:	0.2643	Age*Genotype:	0.7763

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test) 1st	2nd	3rd	4th
Male	ThGAL4/InR^{DN} to ThGAL4/+	0.9919	0.8271	0.5568	0.6775
	ThGAL4/InR^{DN} to InR^{DN}/+	0.2978	0.0161	0.4160	0.0884
	InR^{DN}/+ to ThGAL4/+	0.3574	0.0733	0.9694	0.3792

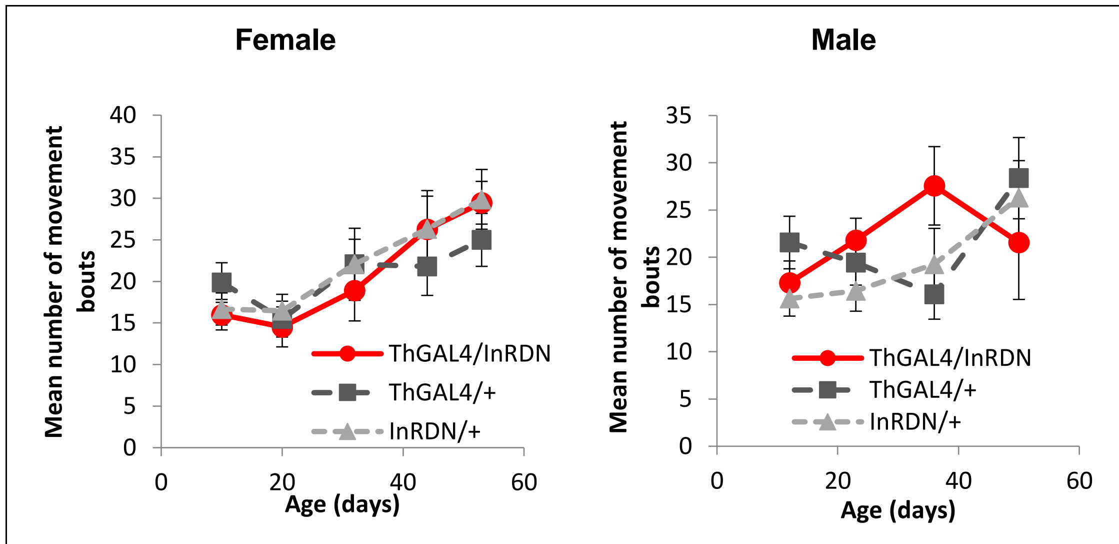
C



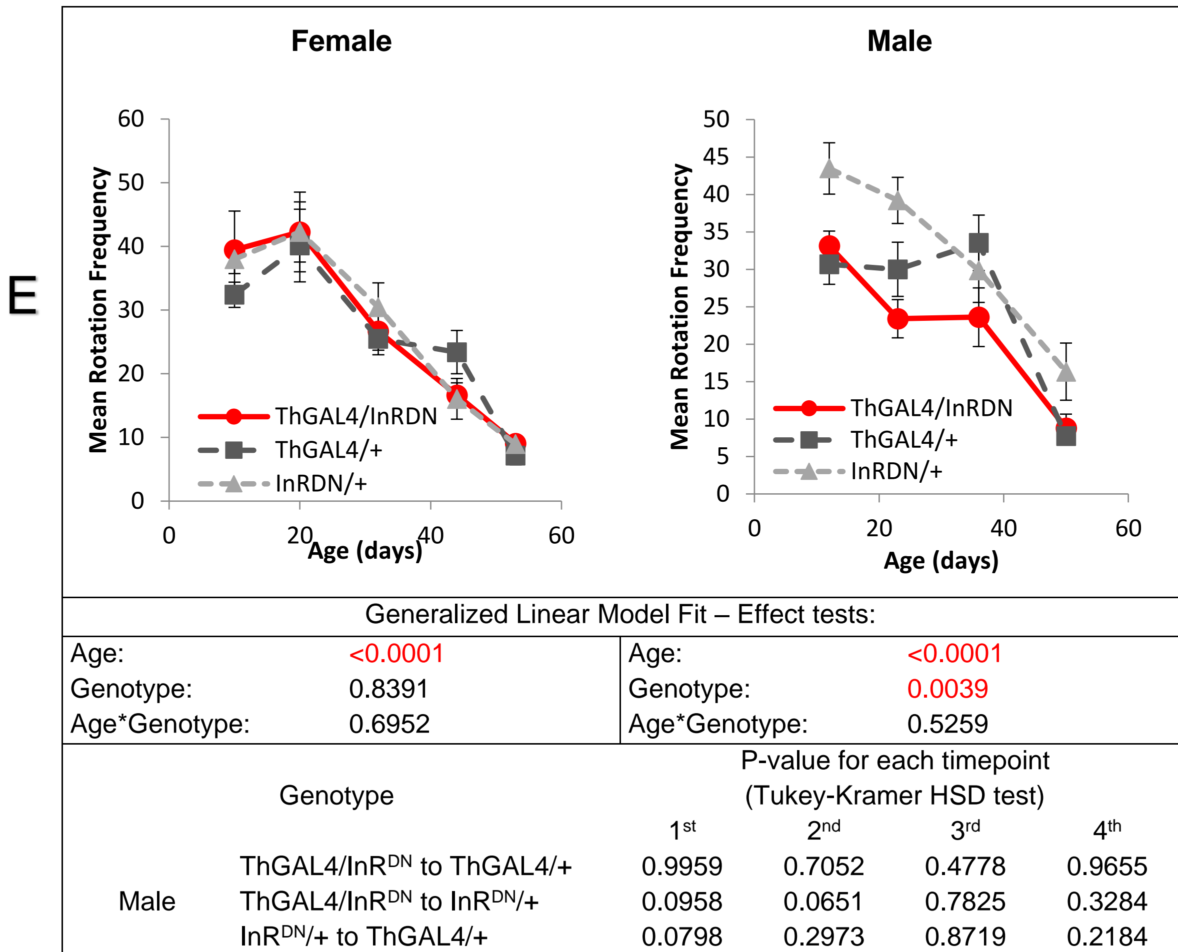
Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.9109	Genotype:	0.0197
Age*Genotype:	0.6788	Age*Genotype:	0.7901

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test)			
		1st	2nd	3rd	4th
Male	ThGAL4/InR^{DN} to ThGAL4/+	0.9910	0.3604	0.5679	0.8702
	ThGAL4/InR^{DN} to InR^{DN}/+	0.4790	0.0219	0.8818	0.2978
	InR^{DN}/+ to ThGAL4/+	0.5570	0.3785	0.8510	0.5567

D

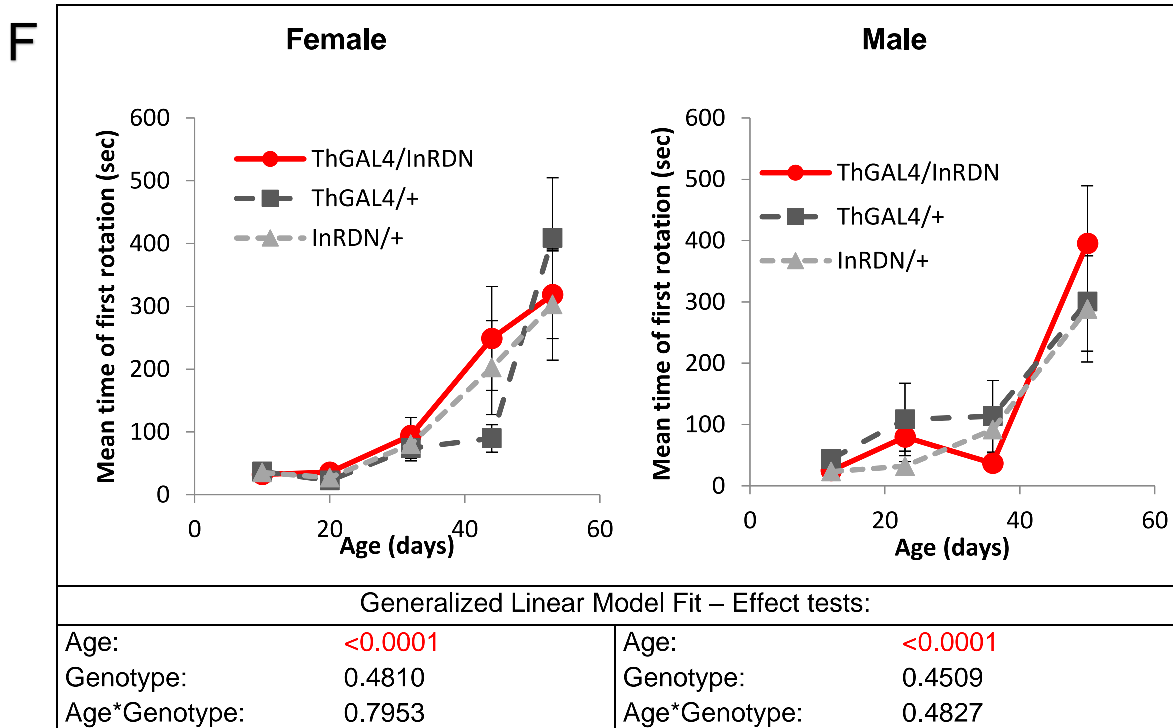


Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.0332
Genotype:	0.7774	Genotype:	0.4809
Age*Genotype:	0.9132	Age*Genotype:	0.0995

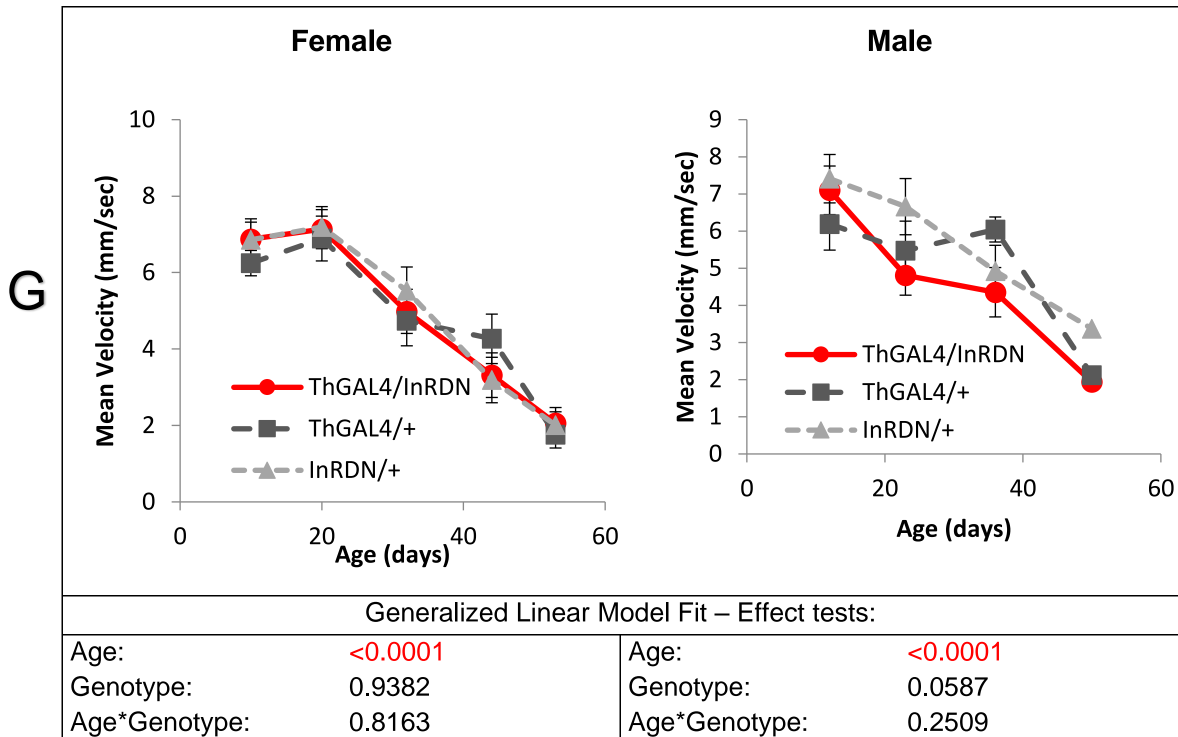


Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.8391	Genotype:	0.0039
Age*Genotype:	0.6952	Age*Genotype:	0.5259

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test)			
		1st	2nd	3rd	4th
Male	ThGAL4/InR^{DN} to ThGAL4/+	0.9959	0.7052	0.4778	0.9655
	ThGAL4/InR^{DN} to InR^{DN}/+	0.0958	0.0651	0.7825	0.3284
	InR^{DN}/+ to ThGAL4/+	0.0798	0.2973	0.8719	0.2184



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.4810	Genotype:	0.4509
Age*Genotype:	0.7953	Age*Genotype:	0.4827



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.9382	Genotype:	0.0587
Age*Genotype:	0.8163	Age*Genotype:	0.2509

Figure 55 - The exploratory walking senescence of female and male flies with reduced IIS in their dopaminergic neuronal subtypes

Exploratory walking of ThGAL4/UAS-InR^{DN} flies compared to ThGAL4/+ and UAS-InR^{DN}/+ control groups. Female data: left column, male data: right column. N=16 for each genotype. Error bars represent +/- SEM. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype has a significant effect, post hoc pairwise comparison was carried out using Tukey-Kramer HSD test. Significant difference is highlighted with red text colour ($p<0.05$) in the statistical summary and with a star (★) on the graph.

A) Total distance walked over the lifespan **B)** Duration in central zone over the lifespan **C)** Walking duration over the lifespan **D)** Number of movement bouts over the lifespan **E)** Rotation frequency over the lifespan **F)** First rotation time over the lifespan **G)** Velocity over the lifespan

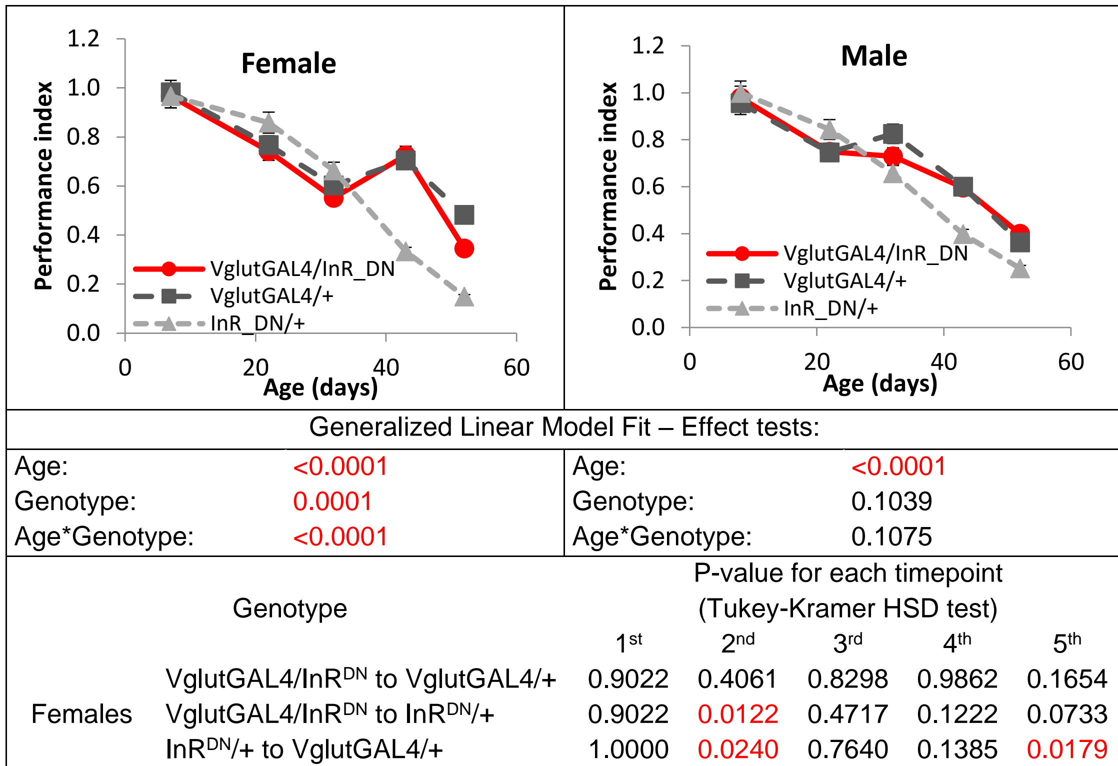
9.2.2: The effect of constitutive reduction of IIS in glutamatergic neurons

The experimental group is the VglutGAL4/UAS-InR^{DN} expressing the dominant negative insulin receptor in the glutamatergic neurons. The control groups for the driver and the transgene are VglutGAL4/+ and UAS-InR^{DN}/+, both crossed with wild type w^{Dah} background. **Figure 56** is showing the performance index over the lifespan of male and female flies with reduced IIS in their glutamatergic neurons.

As shown in **Figure 56**, all groups showed a significant decline in their negative geotaxis behaviour with age. In females, there was a significant effect of genotype and genotype*age interaction (p=0.0001 and p<0.0001) but post hoc pairwise comparisons at each timepoint showed that VglutGAL4/InR^{DN} females were not significantly different to both controls at individual time points. The InR^{DN}/+ group was significantly different from VglutGAL4/InR^{DN} (p=0.0122) and VglutGAL4/+ (p=0.0240) at age 22 days and from VglutGAL4/+ only (p=0.0179) at the age of 52 days. Therefore, there was no significant effect of reduced IIS in glutamatergic neurons on negative geotaxis senescence in males and females.

The exploratory walking experiment was carried out parallel to the negative geotaxis experiment, sampling from the same population of VglutGAL4 flies. In **Figure 57** it can be seen that age had a significant effect on all male and female parameters of exploratory walking in all genotypes. For most parameters in males and females the experimental VglutGAL4/InR^{DN} group was not significantly different to both controls at each time point suggesting that reduced IIS in glutamatergic neurons has little effect on the normal senescence of exploratory walking. However, there was one exception to this. Female VglutGAL4/InR^{DN} had significantly fewer movement bouts than both controls (VglutGAL4/+: p=0.0201 and InR^{DN}/+: p=0.0040) at age 43 days. In the male experiment the VglutGAL4/InR^{DN} group was not significant from both controls for any of the parameters. The InR^{DN}/+ control showed a reoccurring significant increase at age 32 and 43 days in the male experiment in the total distance walked, walking duration, rotation frequency and velocity parameters. This could suggest that the VglutGAL4 driver itself has an effect on the male exploratory walking behaviour.

Glutamategic neurons – Negative geotaxis



Generalized Linear Model Fit – Effect tests:

	Female		Male
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0001	Genotype:	0.1039
Age*Genotype:	<0.0001	Age*Genotype:	0.1075

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test) 1st	2nd	3rd	4th	5th
Females	VglutGAL4/InR^{DN} to VglutGAL4/+	0.9022	0.4061	0.8298	0.9862	0.1654
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.9022	0.0122	0.4717	0.1222	0.0733
	InR^{DN}/+ to VglutGAL4/+	1.0000	0.0240	0.7640	0.1385	0.0179

Figure 56 - Effect of constitutive IIS reduction in glutamatergic neurons on negative geotaxis senescence

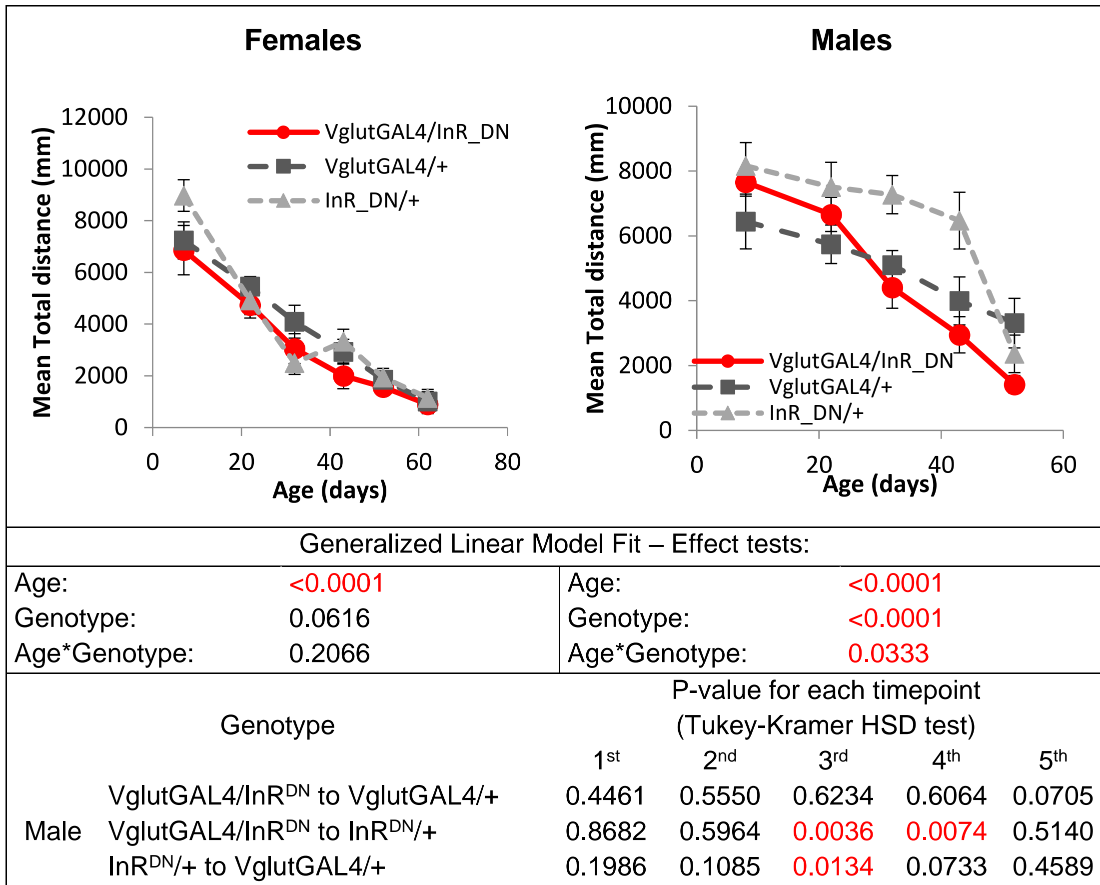
Negative geotaxis performance index of flies over the lifespan. N=3 (group of 10 flies) for each measurement. Error bars represent +/- SEM. Data was analysed by JMP statistical software. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype has a significant effect, post hoc pairwise comparison was carried out using Tukey-Kramer HSD test at each timepoint. Significant difference is highlighted with red text colour ($p<0.05$)

A) VglutGAL4/UAS-InR^{DN} female flies compared to VglutGAL4/+ and UAS-InR^{DN}/+ control groups.

B) VglutGAL4/UAS-InR^{DN} male flies compared to VglutGAL4/+ and UAS-InR^{DN}/+ control groups.

Glutamatergic neurons - Exploratory Walking

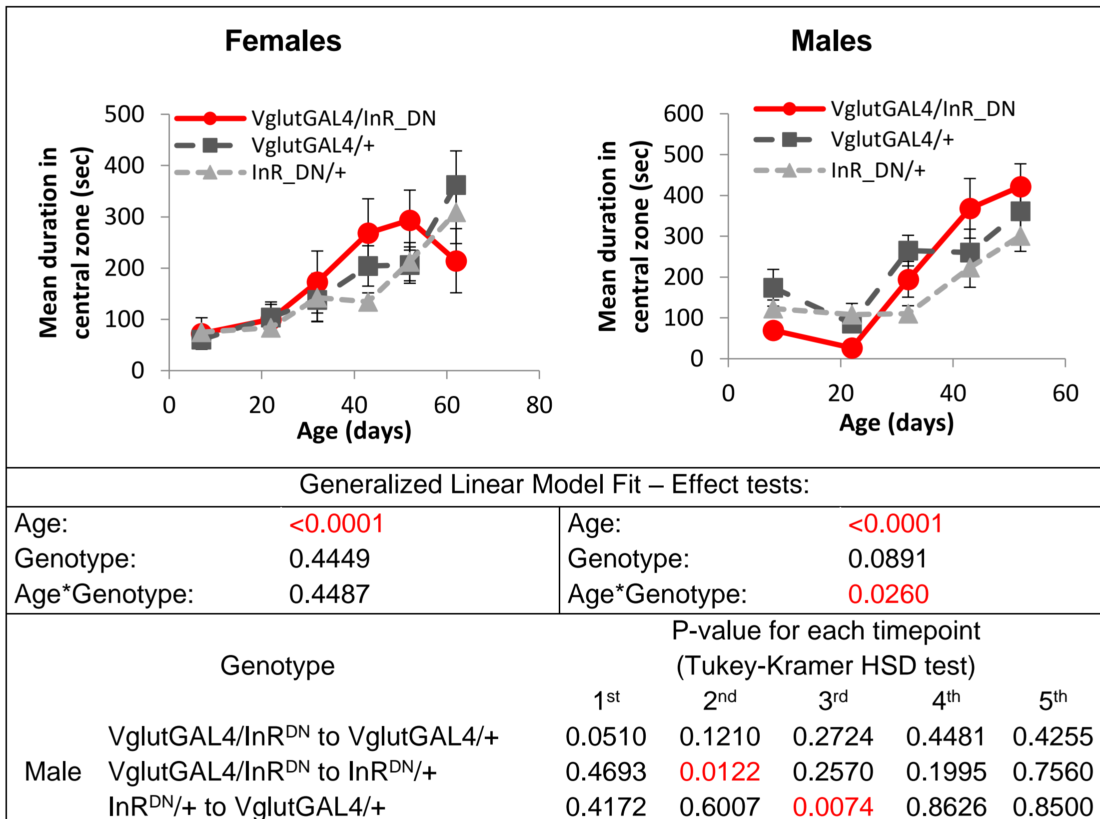
A



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0616	Genotype:	<0.0001
Age*Genotype:	0.2066	Age*Genotype:	0.0333

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test)				
		1st	2nd	3rd	4th	5th
Male	VglutGAL4/InR^{DN} to VglutGAL4/+	0.4461	0.5550	0.6234	0.6064	0.0705
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.8682	0.5964	0.0036	0.0074	0.5140
	InR^{DN}/+ to VglutGAL4/+	0.1986	0.1085	0.0134	0.0733	0.4589

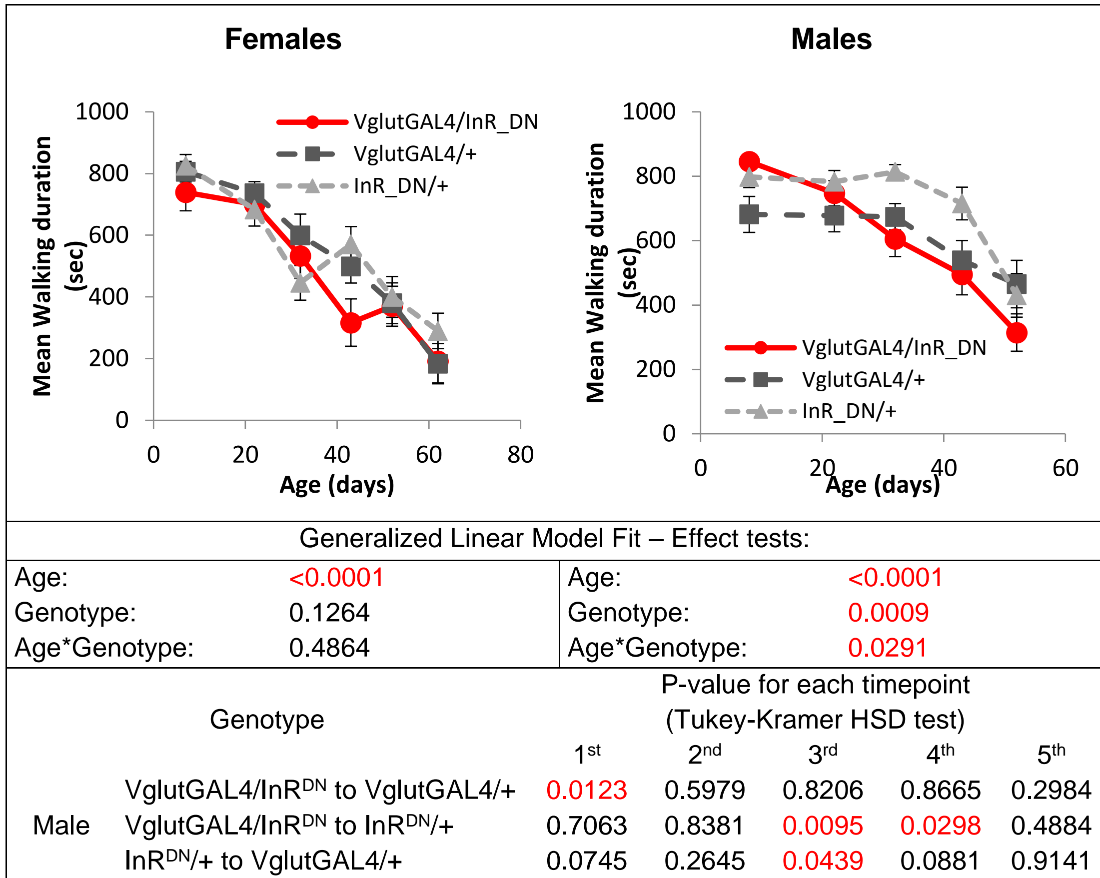
B



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.4449	Genotype:	0.0891
Age*Genotype:	0.4487	Age*Genotype:	0.0260

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test)				
		1st	2nd	3rd	4th	5th
Male	VglutGAL4/InR^{DN} to VglutGAL4/+	0.0510	0.1210	0.2724	0.4481	0.4255
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.4693	0.0122	0.2570	0.1995	0.7560
	InR^{DN}/+ to VglutGAL4/+	0.4172	0.6007	0.0074	0.8626	0.8500

C

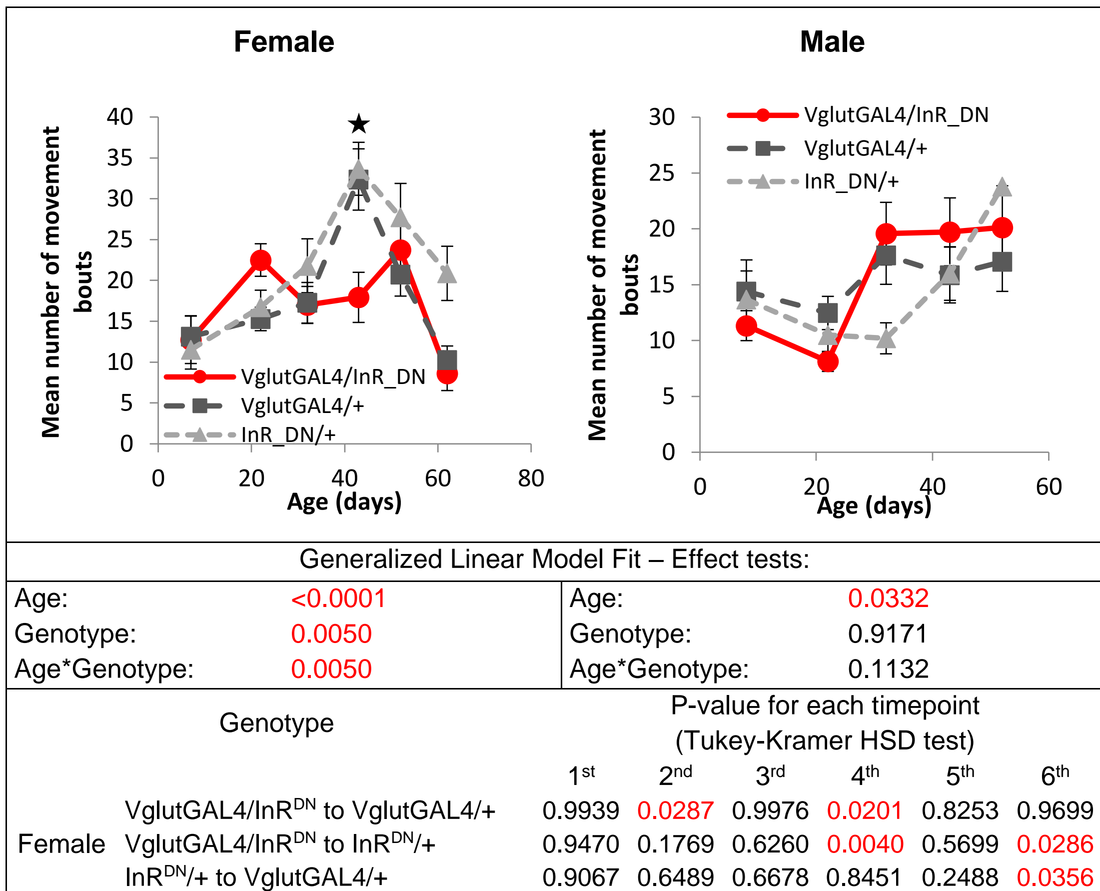


Generalized Linear Model Fit – Effect tests:

Age:	<0.0001	Age:	<0.0001
Genotype:	0.1264	Genotype:	0.0009
Age*Genotype:	0.4864	Age*Genotype:	0.0291

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test) 1st	2nd	3rd	4th	5th
Male	VglutGAL4/InR^{DN} to VglutGAL4/+	0.0123	0.5979	0.8206	0.8665	0.2984
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.7063	0.8381	0.0095	0.0298	0.4884
	InR^{DN}/+ to VglutGAL4/+	0.0745	0.2645	0.0439	0.0881	0.9141

D

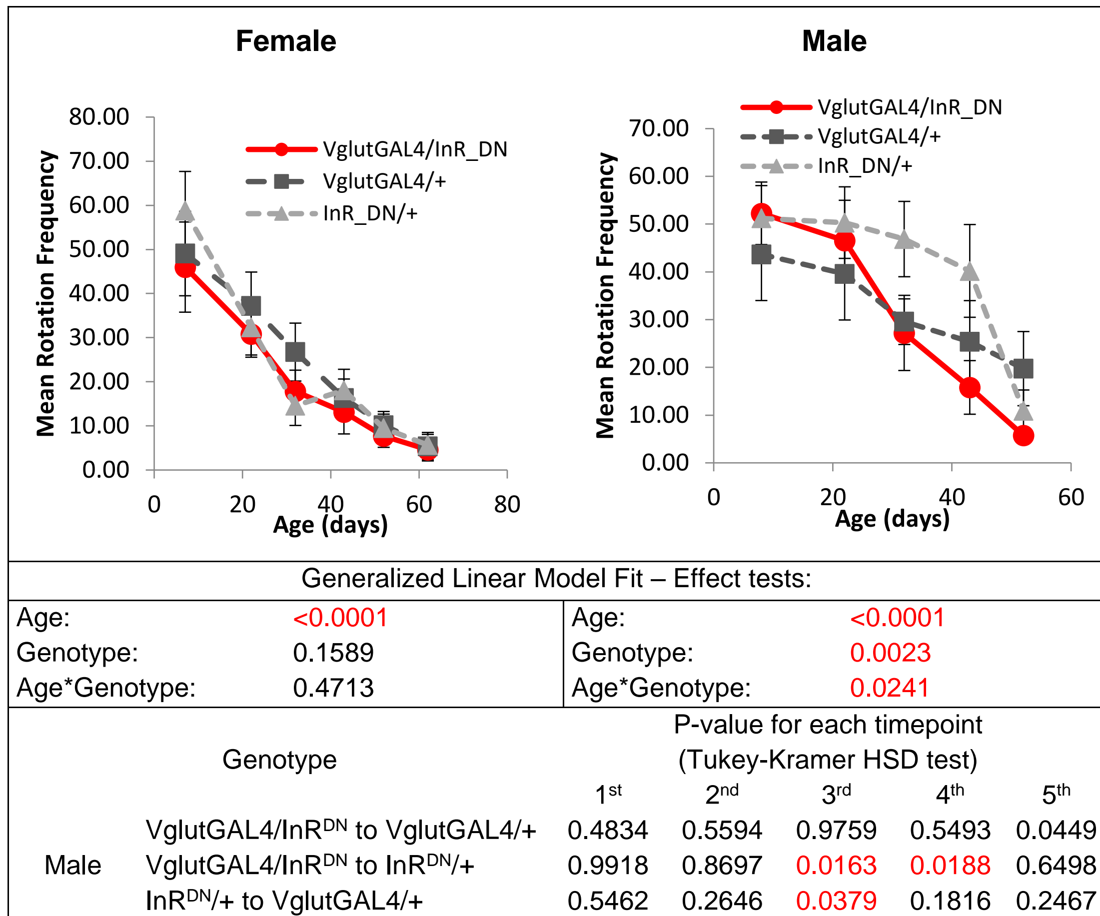


Generalized Linear Model Fit – Effect tests:

Age:	<0.0001	Age:	0.0332
Genotype:	0.0050	Genotype:	0.9171
Age*Genotype:	0.0050	Age*Genotype:	0.1132

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test) 1st	2nd	3rd	4th	5th	6th
Female	VglutGAL4/InR^{DN} to VglutGAL4/+	0.9939	0.0287	0.9976	0.0201	0.8253	0.9699
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.9470	0.1769	0.6260	0.0040	0.5699	0.0286
	InR^{DN}/+ to VglutGAL4/+	0.9067	0.6489	0.6678	0.8451	0.2488	0.0356

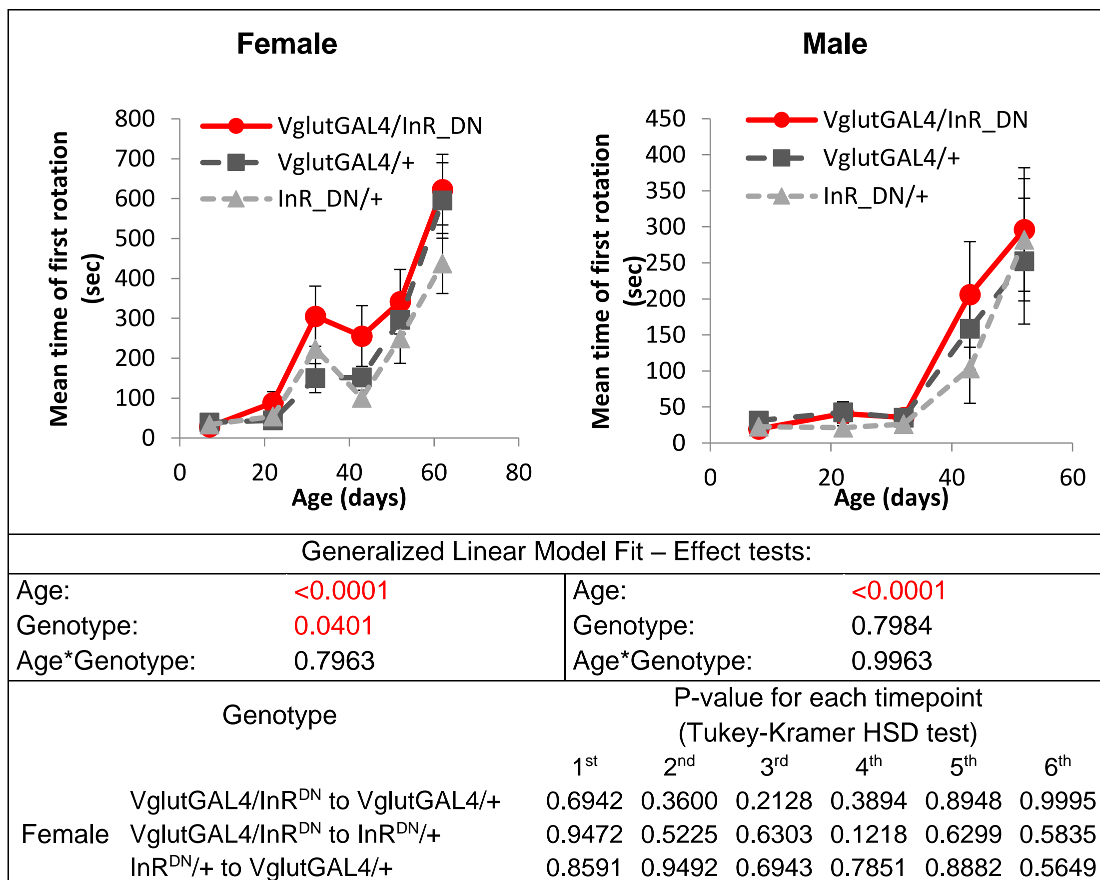
E



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.1589	Genotype:	0.0023
Age*Genotype:	0.4713	Age*Genotype:	0.0241

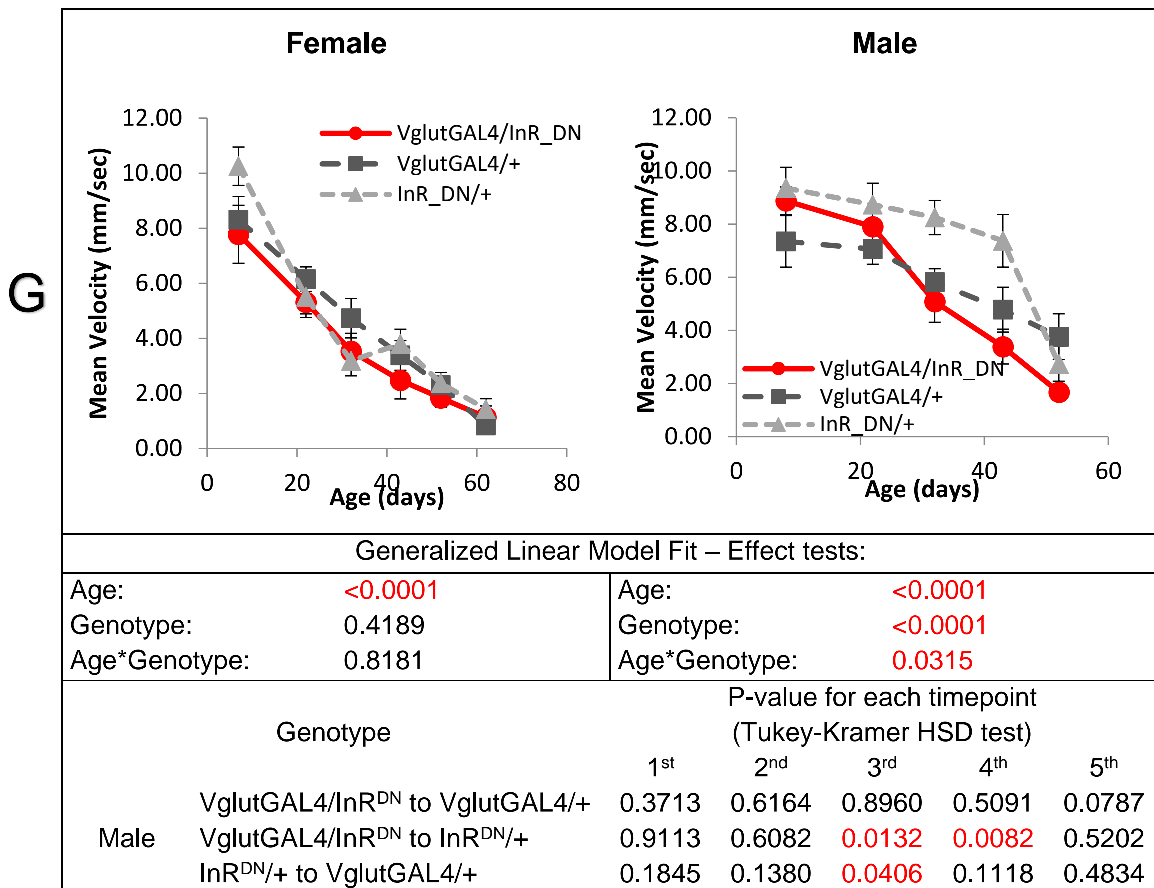
	Genotype	P-value for each timepoint (Tukey-Kramer HSD test) 1st	2nd	3rd	4th	5th
Male	VglutGAL4/InR^{DN} to VglutGAL4/+	0.4834	0.5594	0.9759	0.5493	0.0449
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.9918	0.8697	0.0163	0.0188	0.6498
	InR^{DN}/+ to VglutGAL4/+	0.5462	0.2646	0.0379	0.1816	0.2467

F



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0401	Genotype:	0.7984
Age*Genotype:	0.7963	Age*Genotype:	0.9963

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test) 1st	2nd	3rd	4th	5th	6th
Female	VglutGAL4/InR^{DN} to VglutGAL4/+	0.6942	0.3600	0.2128	0.3894	0.8948	0.9995
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.9472	0.5225	0.6303	0.1218	0.6299	0.5835
	InR^{DN}/+ to VglutGAL4/+	0.8591	0.9492	0.6943	0.7851	0.8882	0.5649



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.4189	Genotype:	<0.0001
Age*Genotype:	0.8181	Age*Genotype:	0.0315

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test)				
		1st	2nd	3rd	4th	5th
Male	VglutGAL4/InR^{DN} to VglutGAL4/+	0.3713	0.6164	0.8960	0.5091	0.0787
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.9113	0.6082	0.0132	0.0082	0.5202
	InR^{DN}/+ to VglutGAL4/+	0.1845	0.1380	0.0406	0.1118	0.4834

Figure 57 - The exploratory walking senescence of female and male flies with reduced IIS in their glutamatergic neuronal subtypes

Exploratory walking of VglutGAL4/UAS-InR^{DN} flies compared to VglutGAL4/+ and UAS-InR^{DN}/+ control groups. Female data: left column, male data: right column. N=16 for each genotype. Error bars represent +/- SEM. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype has a significant effect, post hoc pairwise comparison was carried out using Tukey-Kramer HSD test. Significant difference is highlighted with red text colour ($p<0.05$) in the statistical summary and with a star (★) on the graph.

A) Total distance walked over the lifespan **B)** Duration in central zone over the lifespan **C)** Walking duration over the lifespan **D)** Number of movement bouts over the lifespan **E)** Rotation frequency over the lifespan **F)** First rotation time over the lifespan **G)** Velocity over the lifespan

9.2.3: Constitutive reduction of IIS in GABAergic neurons does not affect negative geotaxis senescence and exploratory walking behaviour

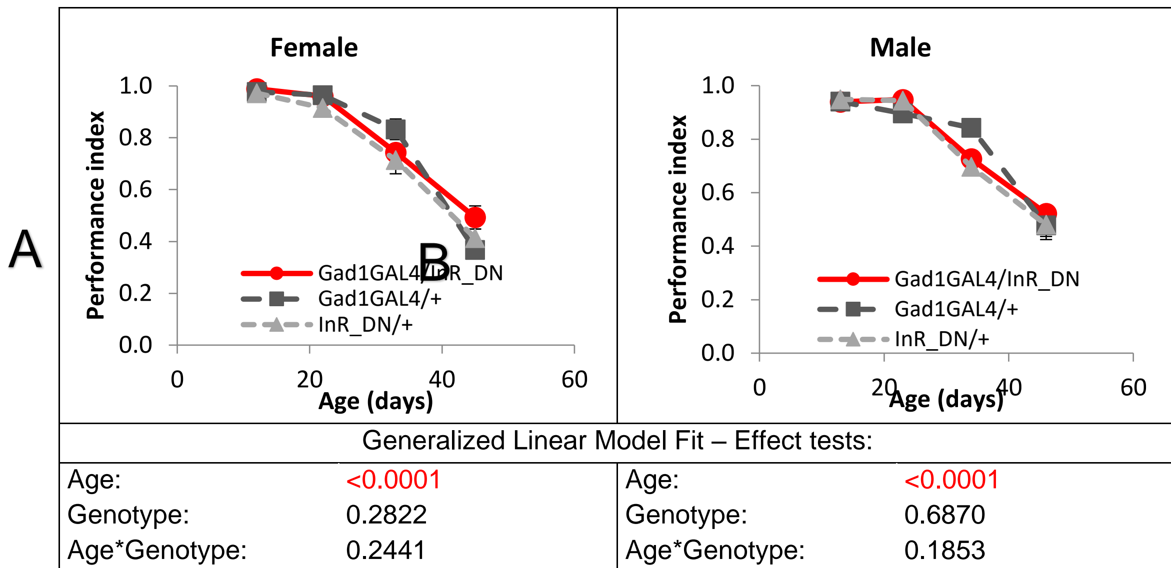
The experimental group is the Gad1GAL4/UAS-InR^{DN} expressing the dominant negative insulin receptor in the GABAergic neurons. The control groups for the driver and the transgene are Gad1GAL4/+ and UAS-InR^{DN}/+, both crossed with wild type w^{Dah} background.

Figure 58 shows the performance index over the lifespan of male and female flies with reduced IIS in their GABAergic neurons. In both males and females, age had significant effect on the performance index over the lifespan of the flies, but reduced IIS in the GABAergic neurons had no effect on the age-related decline of negative geotaxis.

The exploratory walking experiment was performed in parallel with the negative geotaxis experiment sampling from the same population of Gad1-GAL4 flies. Age had a significant effect on all the male and female exploratory walking parameters according to GLM and it found significant genotype and/or age*genotype effect on both female and male negative geotaxis, however pairwise comparation of each timepoint using Tukey-Kramer HSD test never showed that the experimental Gad1-GAL4/InR^{DN} group was significantly different from both two control groups (**Figure 59**). Therefore, constitutive IIS reduction in GABAergic neurons does not affect exploratory walking behaviour.

GABAergic neurons – Negative geotaxis

A



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.2822	Genotype:	0.6870
Age*Genotype:	0.2441	Age*Genotype:	0.1853

Figure 58 - Effect of constitutive IIS reduction in GABAergic neurons on negative geotaxis senescence

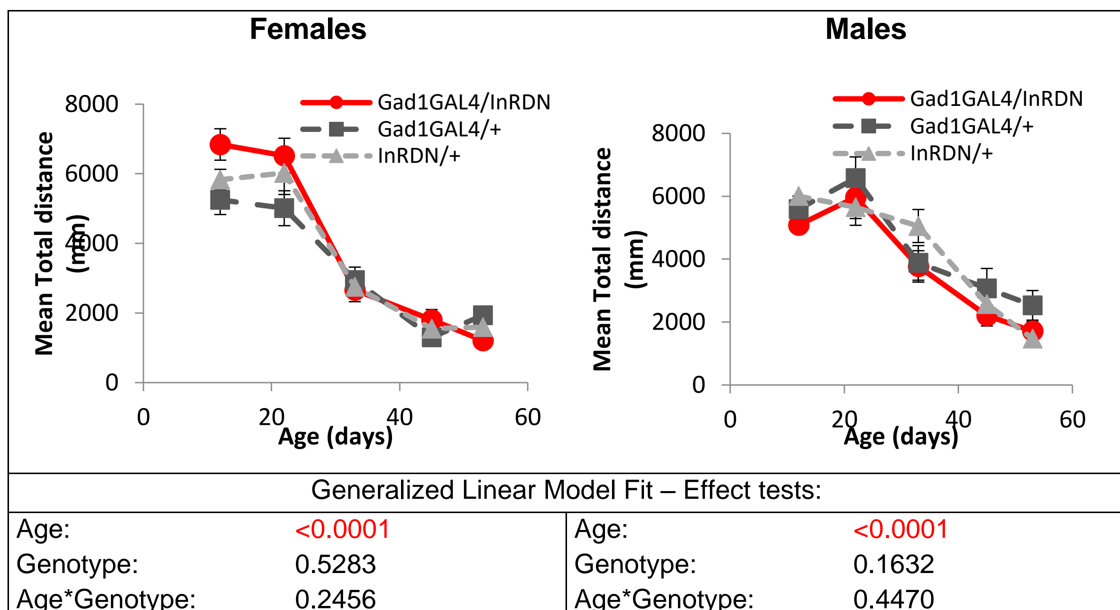
Negative geotaxis performance index of flies over the lifespan. N=3 (group of 10 flies) for each measurement. Error bars represent +/- SEM. Data was analysed by JMP statistical software. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype has a significant effect, post hoc pairwise comparison was carried out using Tukey-Kramer HSD test at each timepoint. Significant difference is highlighted with red text colour ($p<0.05$)

A) Gad1-GAL4/UAS-InR^{DN} female flies compared to Gad1-GAL4/+ and UAS-InR^{DN}/+ control groups.

B) Gad1-GAL4/UAS-InR^{DN} male flies compared to Gad1-GAL4/+ and UAS-InR^{DN}/+ control groups.

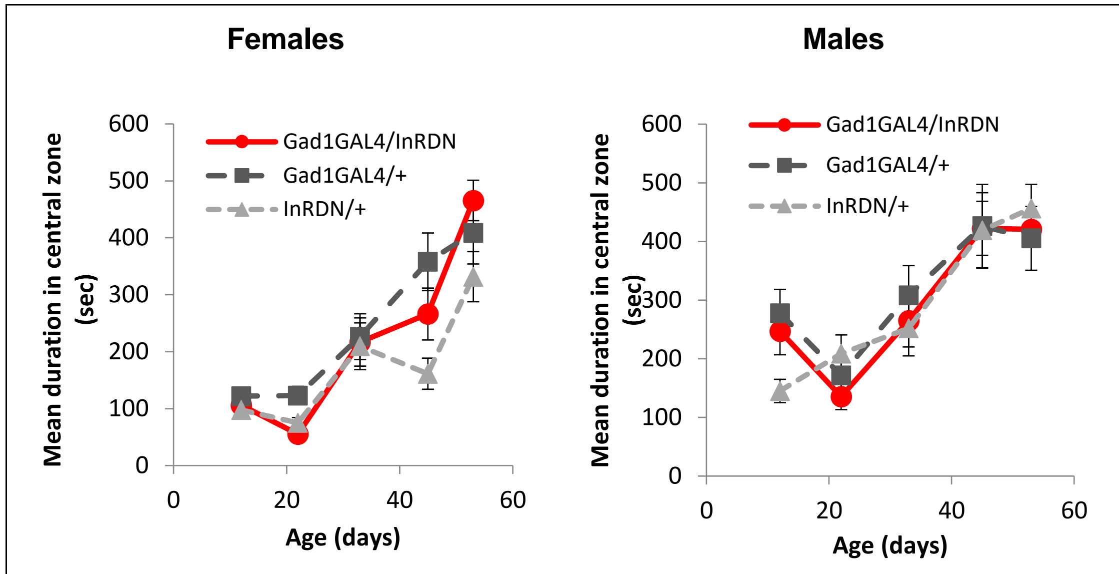
A

GABAergic neurons – Exploratory Walking



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.5283	Genotype:	0.1632
Age*Genotype:	0.2456	Age*Genotype:	0.4470

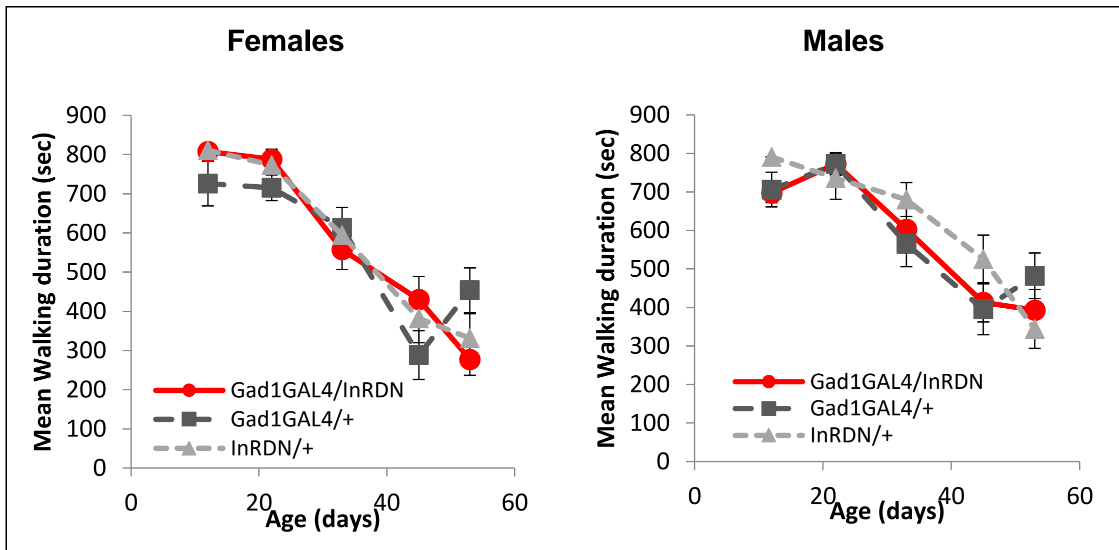
B



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0027	Genotype:	0.6831
Age*Genotype:	0.0514	Age*Genotype:	0.4871

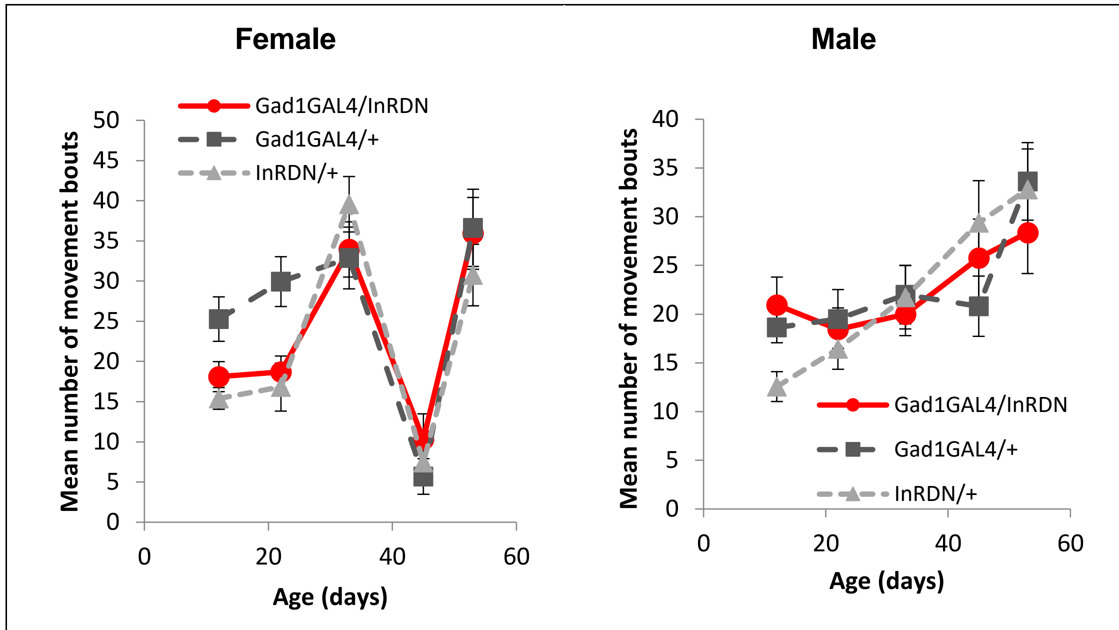
	Genotype	P-value for each timepoint (Tukey-Kramer HSD test)				
		1st	2nd	3rd	4th	5th
Female	Gad1GAL4/InR^{DN} to Gad1GAL4/+	0.2849	0.0012	0.9859	0.3048	0.6516
	Gad1GAL4/InR^{DN} to InR^{DN}/+	0.1282	0.5106	0.9909	0.2050	0.1121
	InR^{DN}/+ to Gad1GAL4/+	0.8952	0.0267	0.9550	0.0063	0.4719

C



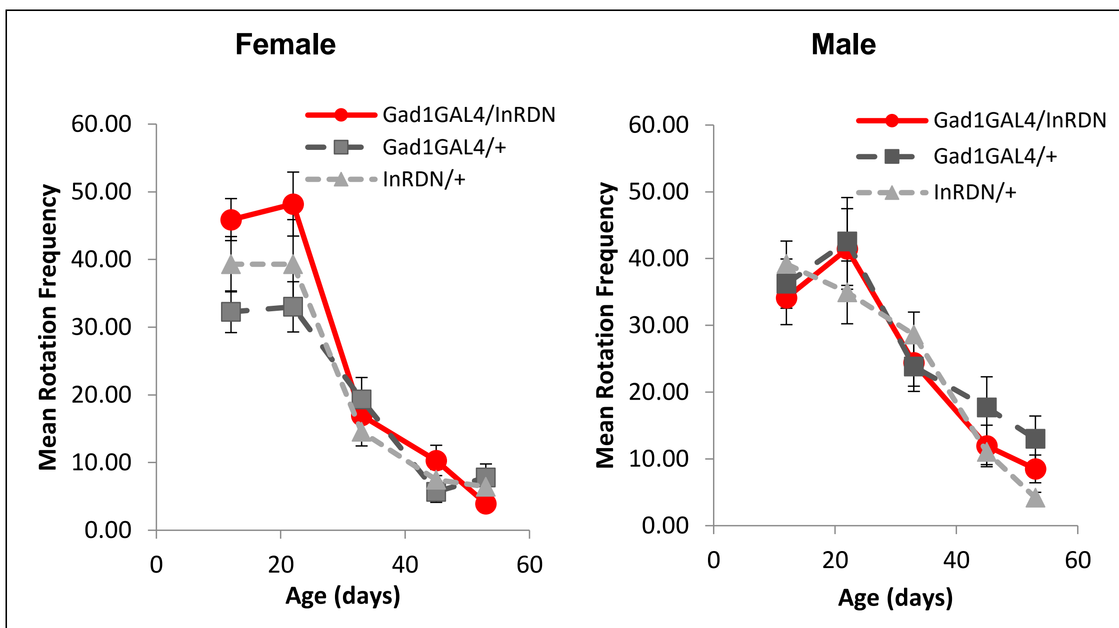
Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.7102	Genotype:	0.3913
Age*Genotype:	0.0938	Age*Genotype:	0.1407

D

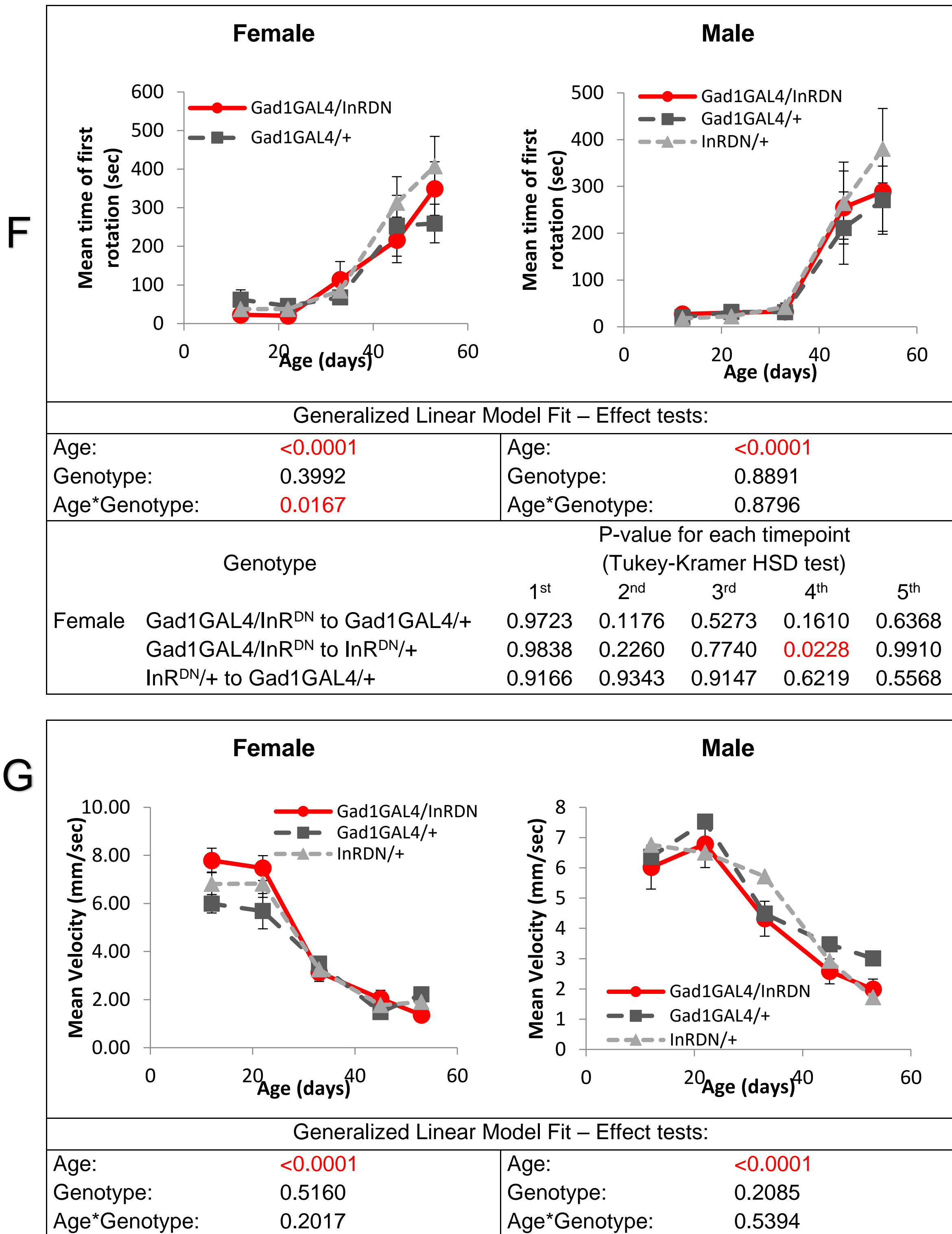


Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.3341	Genotype:	0.9854
Age*Genotype:	0.2282	Age*Genotype:	0.2562

E



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.2474	Genotype:	0.3875
Age*Genotype:	0.1469	Age*Genotype:	0.5684



F

Generalized Linear Model Fit – Effect tests:

Female		Male	
Age:	<0.0001	Age:	<0.0001
Genotype:	0.3992	Genotype:	0.8891
Age*Genotype:	0.0167	Age*Genotype:	0.8796

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test) 1st	2nd	3rd	4th	5th
Female	Gad1GAL4/InR^{DN} to Gad1GAL4/+	0.9723	0.1176	0.5273	0.1610	0.6368
	Gad1GAL4/InR^{DN} to InR^{DN}/+	0.9838	0.2260	0.7740	0.0228	0.9910
	InR^{DN}/+ to Gad1GAL4/+	0.9166	0.9343	0.9147	0.6219	0.5568

G

Generalized Linear Model Fit – Effect tests:

Female		Male	
Age:	<0.0001	Age:	<0.0001
Genotype:	0.5160	Genotype:	0.2085
Age*Genotype:	0.2017	Age*Genotype:	0.5394

Figure 59 - The exploratory walking senescence of female and male flies with reduced IIS in their GABAergic neuronal subtypes

Exploratory walking of Gad1-GAL4/UAS-InR^{DN} flies compared to Gad1-GAL4/+ and UAS-InR^{DN}/+ control groups. Female data: left column, male data: right column. N=16 for each genotype. Error bars represent +/- SEM. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype has a significant effect, post hoc pairwise comparison was carried out using Tukey-Kramer HSD test. Significant difference is highlighted with red text colour ($p<0.05$) in the statistical summary and with a star (★) on the graph.

A) Total distance walked over the lifespan **B)** Duration in central zone over the lifespan **C)** Walking duration over the lifespan **D)** Number of movement bouts over the lifespan **E)** Rotation frequency over the lifespan **F)** First rotation time over the lifespan **G)** Velocity over the lifespan

9.2.4 The effect of constitutive IIS reduction in cholinergic neurons on negative geotaxis senescence and exploratory walking

The experimental group is the ChAT-GAL4/UAS-InR^{DN} expressing the dominant negative insulin receptor selectively in cholinergic neurons. The control groups for the driver and the transgene are ChAT-GAL4/+ and UAS-InR^{DN}/+, both crossed with wild type w^{Dah} background.

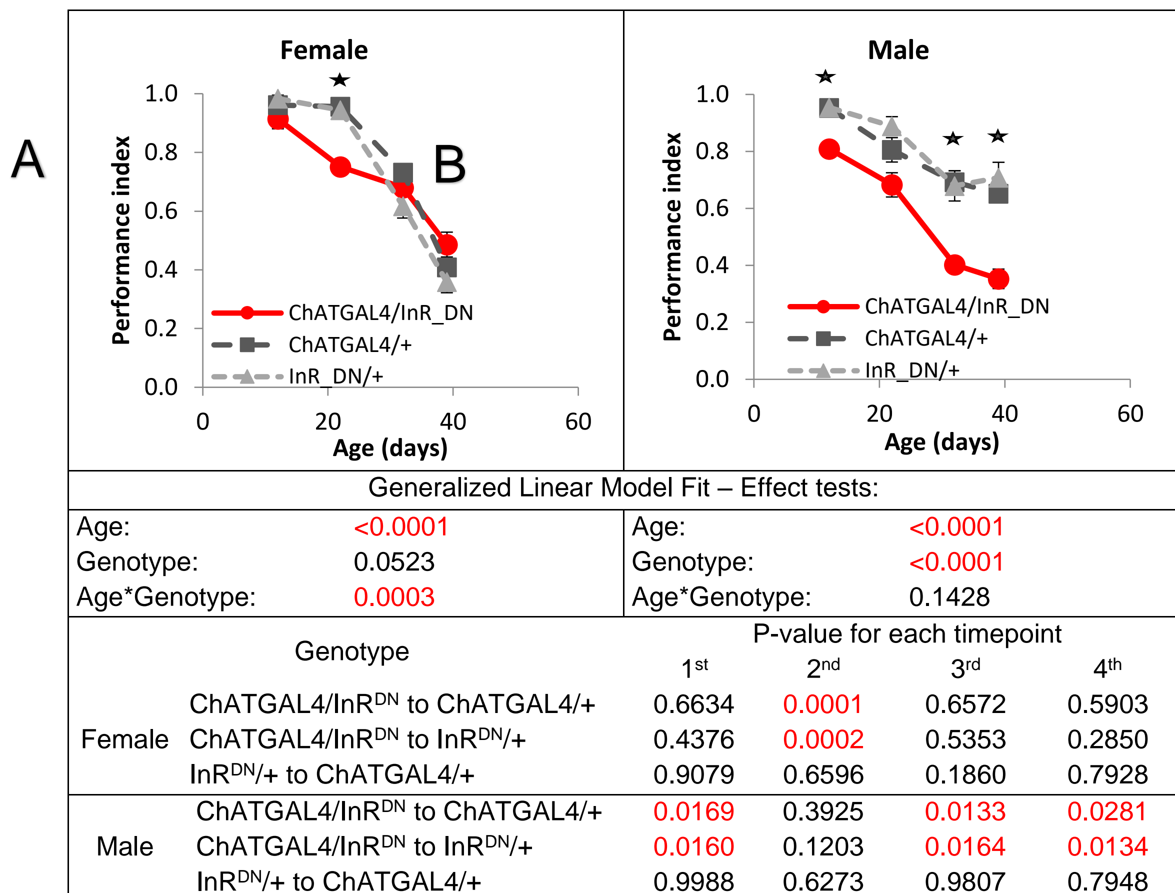
Figure 60 shows the performance index over the lifespan of male and female flies with reduced IIS in their cholinergic neurons. Genotype had significant effect on performance index in both male and female experiments (**Figure 60**). At the age of 22 days, the ChAT-GAL4/UAS-InR^{DN} experimental group showed faster negative geotaxis decline (p=0.0001 to ChAT-GAL4/+ and p=0.0002 to InR^{DN}/+). Males had reduced negative geotaxis compared to the controls at the age of 12 (p=0.0169 to ChAT-GAL4/+ and p=0.0160 to InR^{DN}/+), 32 (p=0.0133 to ChAT-GAL4/+ and p=0.0164 to InR^{DN}/+) and 39 days (p=0.0281 to ChAT-GAL4/+ and p=0.0134 to InR^{DN}/+). This means that reducing IIS in cholinergic neurons had detrimental effects on negative geotaxis in both genders.

The exploratory walking experiment was performed in parallel with the negative geotaxis experiment sampling from the same population of ChAT-GAL4 flies. Age had a significant effect on all male and female exploratory walking parameters except for duration in central zone and first rotation time in males. Significant effects of genotype and/or age*genotype interaction was found for female exploratory walking, however pairwise comparisons of each timepoint using Tukey-Kramer HSD test did not show significant differences between the ChAT-GAL4/InR^{DN} group and both control groups. These data indicate that reduced IIS in female cholinergic neurons had no effect on the normal senescence of exploratory walking (**Figure 61**).

In contrast, young males showed reduced function in response to lower IIS in the cholinergic neurons at the age of 13 days in numerous parameters (**Figure 61**). The experimental ChAT-GAL4/InR^{DN} group was significantly different to controls in the following parameters: Total distance walked reduced at age 13 days (p=0.0036 to ChAT-GAL4/+ and p=0.0001 to InR^{DN}/+), walking duration reduced at the age of 13

(p=0.0011 to ChAT-GAL4/+ and p=0.0001 to InR^{DN}/+), number of movement bouts increased at the age of 33 days (p=0.0405 to ChAT-GAL4/+ and p=0.0405 to InR^{DN}/+), rotation frequency reduced (p=0.0193 to ChAT-GAL4/+ , p<0.0164 to InR^{DN}/+ and p=0.0019 between InRDN/+ and ChAT- GAL4/+), increased first rotation time at age of 13 is close to significance (p=0.0519 to ChAT-GAL4/+ and p=0.0031 to InR^{DN}/+) and finally velocity was reduced at the age of 13 days (p=0.0045 to ChAT-GAL4/+ and p<0.0001 to InR^{DN}/+). These data show that reducing IIS in cholinergic neurons in male flies has detrimental effects on their walking behaviour at young age.

Cholinergic neurons – Negative Geotaxis



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0523	Genotype:	<0.0001
Age*Genotype:	0.0003	Age*Genotype:	0.1428

	Genotype	P-value for each timepoint 1st	2nd	3rd	4th
Female	ChATGAL4/InR^{DN} to ChATGAL4/+	0.6634	0.0001	0.6572	0.5903
	ChATGAL4/InR^{DN} to InR^{DN}/+	0.4376	0.0002	0.5353	0.2850
	InR^{DN}/+ to ChATGAL4/+	0.9079	0.6596	0.1860	0.7928
Male	ChATGAL4/InR^{DN} to ChATGAL4/+	0.0169	0.3925	0.0133	0.0281
	ChATGAL4/InR^{DN} to InR^{DN}/+	0.0160	0.1203	0.0164	0.0134
	InR^{DN}/+ to ChATGAL4/+	0.9988	0.6273	0.9807	0.7948

Figure 60 - Effect of constitutive IIS reduction in cholinergic neurons on negative geotaxis senescence

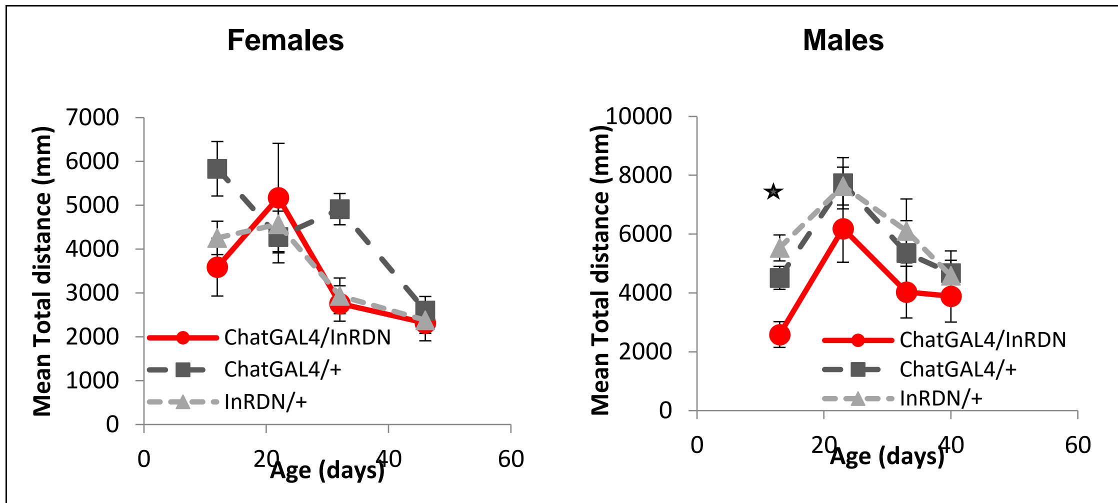
Negative geotaxis performance index of flies over the lifespan. N=3 (group of 10 flies) for each measurement. Error bars represent +/- SEM. Data was analysed by JMP statistical software. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype has a significant effect, post hoc pairwise comparison was carried out using Tukey-Kramer HSD test at each timepoint. Significant difference is highlighted with red text colour (p<0.05)

A) ChATGAL4/UAS-InR^{DN} female flies compared to ChATGAL4/+ and UAS-InR^{DN}/+ control groups.

B) ChATGAL4/UAS-InR^{DN} male flies compared to ChATGAL4/+ and UAS-InR^{DN}/+ control groups.

Cholinergic neurons - Exploratory Walking

A

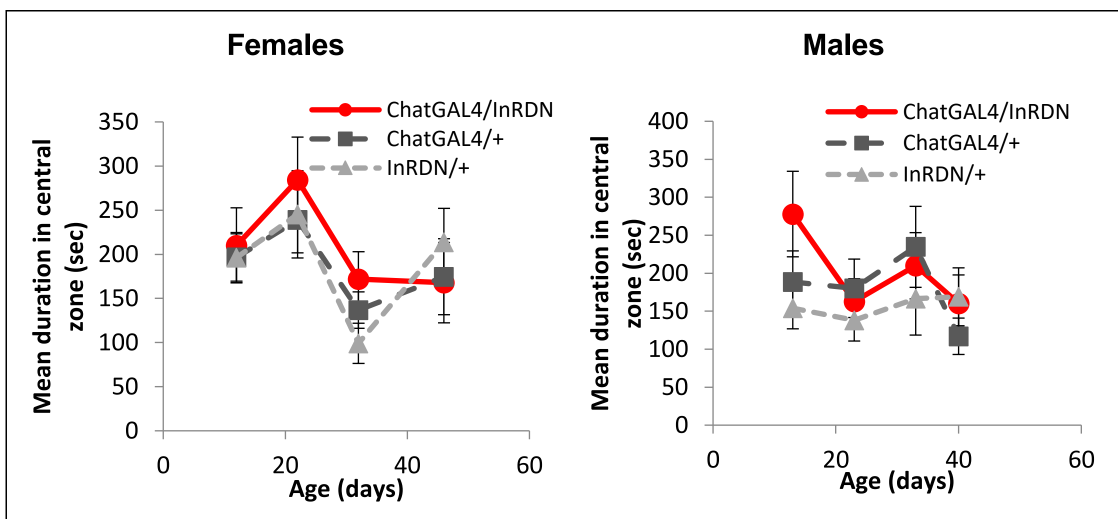


Generalized Linear Model Fit – Effect tests:

Age:	<0.0001	Age:	<0.0001
Genotype:	0.0272	Genotype:	0.0037
Age*Genotype:	0.0575	Age*Genotype:	0.7167

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test) 1st	2nd	3rd	4th
Female	ChATGAL4/InR^{DN} to ChATGAL4/+	0.0109	0.7495	0.0009	0.8295
	ChATGAL4/InR^{DN} to InR^{DN}/+	0.5297	0.8764	0.9477	0.9912
	InR^{DN}/+ to ChATGAL4/+	0.1362	0.9698	0.0027	0.8916
Male	ChATGAL4/InR^{DN} to ChATGAL4/+	0.0036	0.4634	0.6333	0.7350
	ChATGAL4/InR^{DN} to InR^{DN}/+	<0.0001	0.6989	0.3325	0.7840
	InR^{DN}/+ to ChATGAL4/+	0.1164	0.9086	0.8630	0.9961

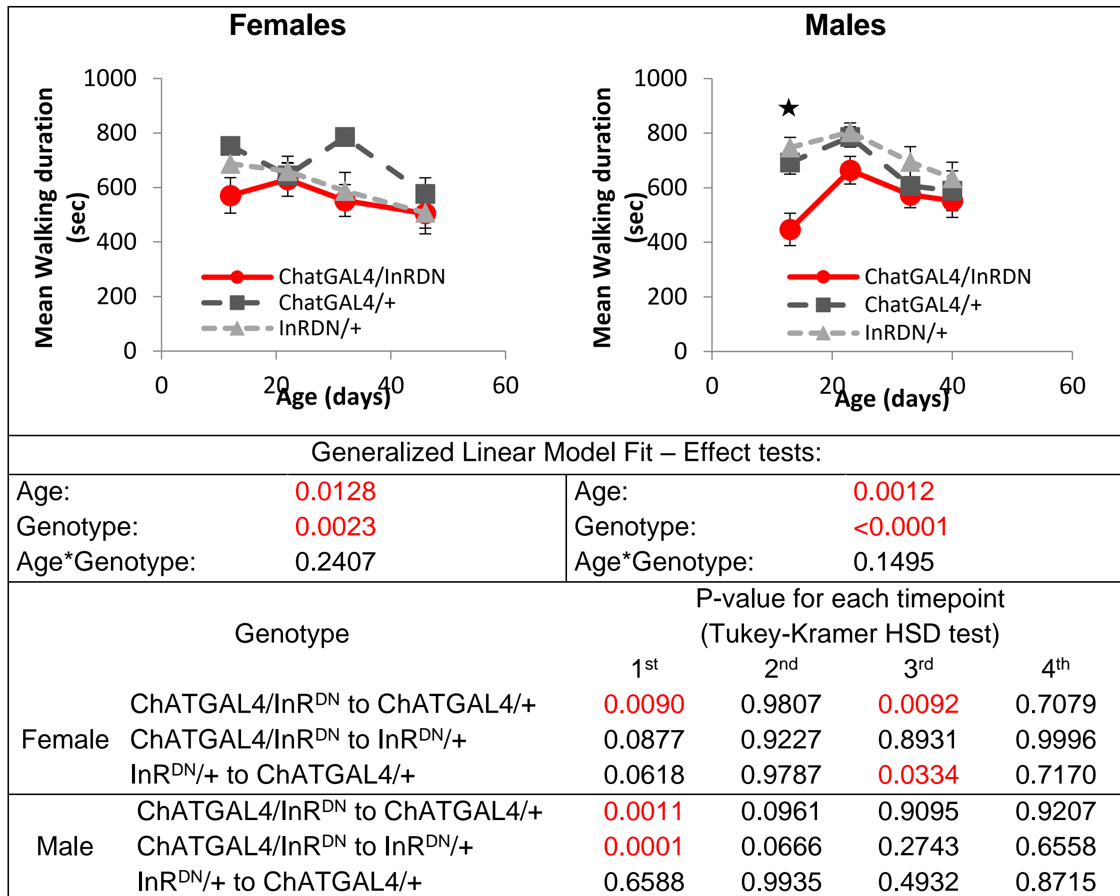
B



Generalized Linear Model Fit – Effect tests:

Age:	0.0012	Age:	0.1950
Genotype:	0.7185	Genotype:	0.2513
Age*Genotype:	0.7997	Age*Genotype:	0.4293

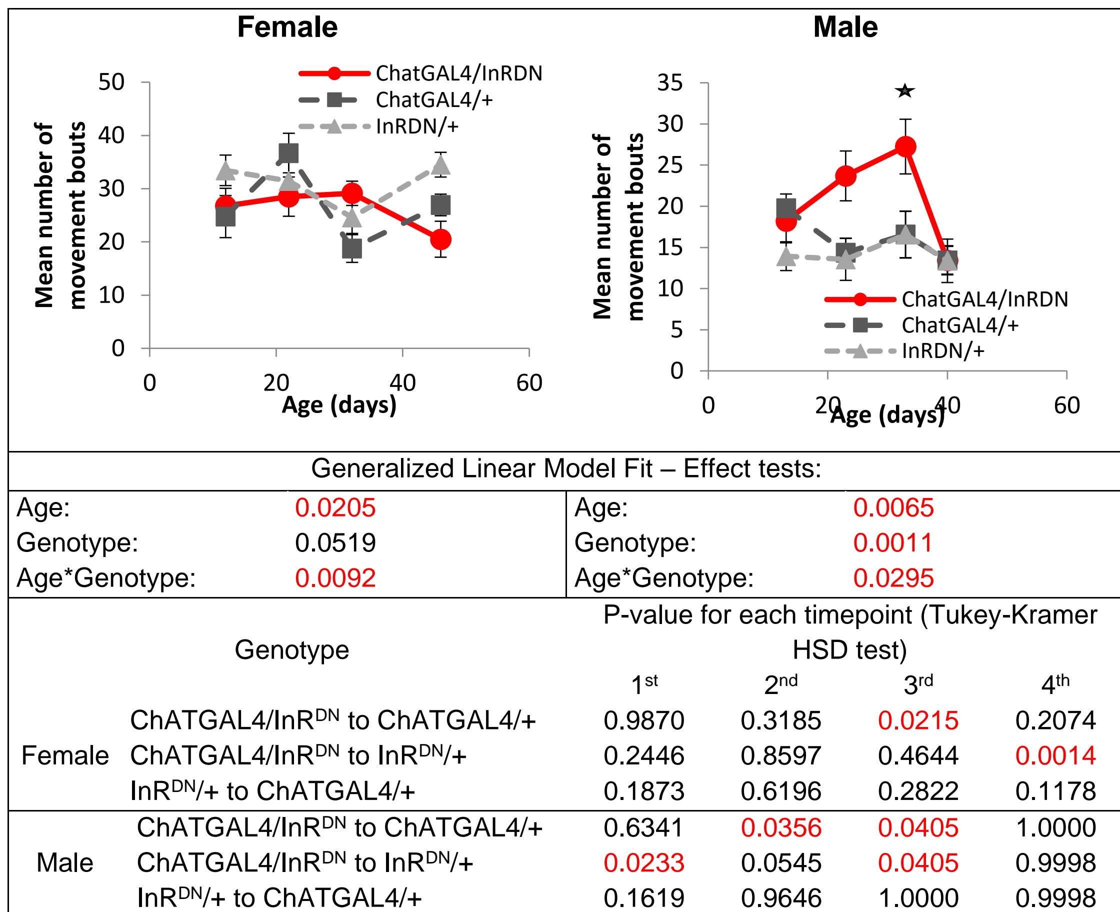
C



Generalized Linear Model Fit – Effect tests:			
Age:	0.0128	Age:	0.0012
Genotype:	0.0023	Genotype:	<0.0001
Age*Genotype:	0.2407	Age*Genotype:	0.1495

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test)			
		1st	2nd	3rd	4th
Female	ChATGAL4/InR^{DN} to ChATGAL4/+	0.0090	0.9807	0.0092	0.7079
	ChATGAL4/InR^{DN} to InR^{DN}/+	0.0877	0.9227	0.8931	0.9996
	InR^{DN}/+ to ChATGAL4/+	0.0618	0.9787	0.0334	0.7170
Male	ChATGAL4/InR^{DN} to ChATGAL4/+	0.0011	0.0961	0.9095	0.9207
	ChATGAL4/InR^{DN} to InR^{DN}/+	0.0001	0.0666	0.2743	0.6558
	InR^{DN}/+ to ChATGAL4/+	0.6588	0.9935	0.4932	0.8715

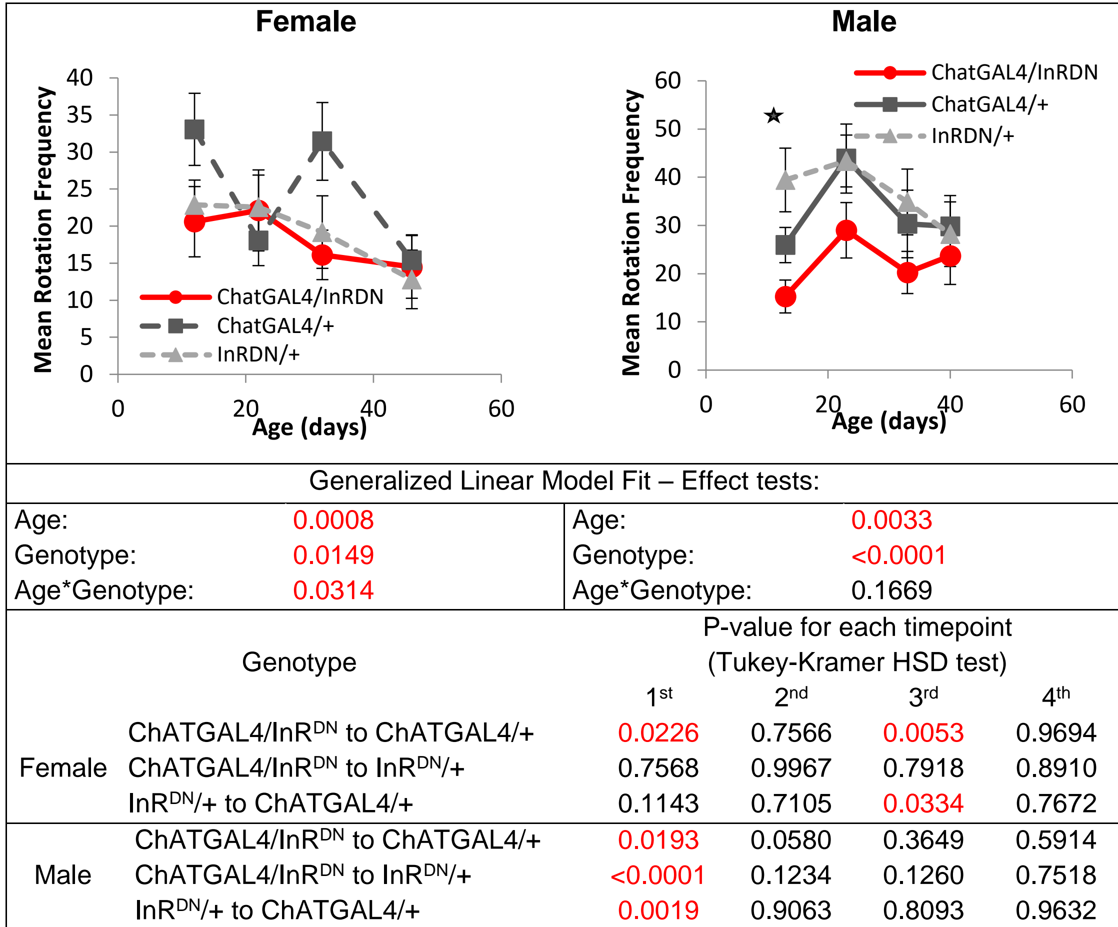
D



Generalized Linear Model Fit – Effect tests:			
Age:	0.0205	Age:	0.0065
Genotype:	0.0519	Genotype:	0.0011
Age*Genotype:	0.0092	Age*Genotype:	0.0295

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test)			
		1st	2nd	3rd	4th
Female	ChATGAL4/InR^{DN} to ChATGAL4/+	0.9870	0.3185	0.0215	0.2074
	ChATGAL4/InR^{DN} to InR^{DN}/+	0.2446	0.8597	0.4644	0.0014
	InR^{DN}/+ to ChATGAL4/+	0.1873	0.6196	0.2822	0.1178
Male	ChATGAL4/InR^{DN} to ChATGAL4/+	0.6341	0.0356	0.0405	1.0000
	ChATGAL4/InR^{DN} to InR^{DN}/+	0.0233	0.0545	0.0405	0.9998
	InR^{DN}/+ to ChATGAL4/+	0.1619	0.9646	1.0000	0.9998

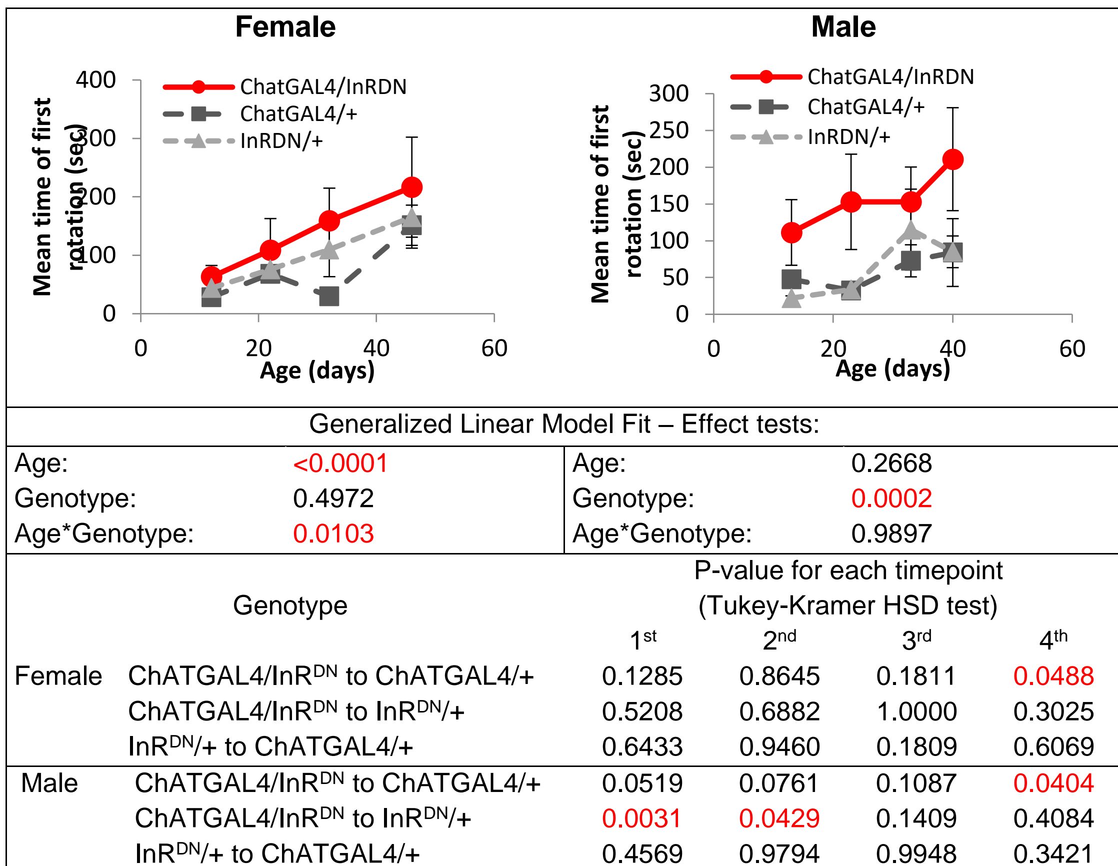
E



Generalized Linear Model Fit – Effect tests:			
Age:	0.0008	Age:	0.0033
Genotype:	0.0149	Genotype:	<0.0001
Age*Genotype:	0.0314	Age*Genotype:	0.1669

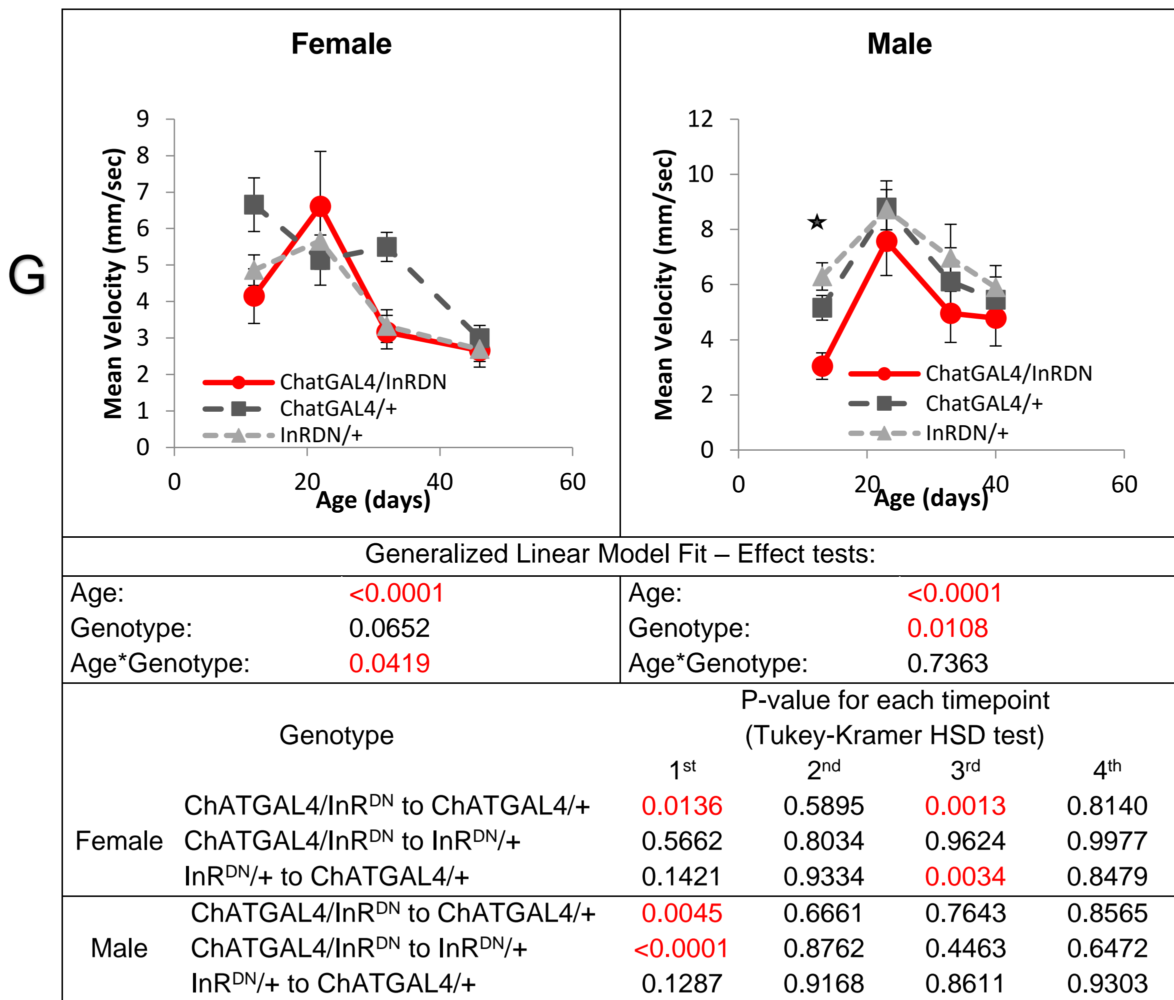
	Genotype	P-value for each timepoint (Tukey-Kramer HSD test) 1st	2nd	3rd	4th
Female	ChATGAL4/InR^{DN} to ChATGAL4/+	0.0226	0.7566	0.0053	0.9694
	ChATGAL4/InR^{DN} to InR^{DN}/+	0.7568	0.9967	0.7918	0.8910
	InR^{DN}/+ to ChATGAL4/+	0.1143	0.7105	0.0334	0.7672
Male	ChATGAL4/InR^{DN} to ChATGAL4/+	0.0193	0.0580	0.3649	0.5914
	ChATGAL4/InR^{DN} to InR^{DN}/+	<0.0001	0.1234	0.1260	0.7518
	InR^{DN}/+ to ChATGAL4/+	0.0019	0.9063	0.8093	0.9632

F



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.2668
Genotype:	0.4972	Genotype:	0.0002
Age*Genotype:	0.0103	Age*Genotype:	0.9897

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test) 1st	2nd	3rd	4th
Female	ChATGAL4/InR^{DN} to ChATGAL4/+	0.1285	0.8645	0.1811	0.0488
	ChATGAL4/InR^{DN} to InR^{DN}/+	0.5208	0.6882	1.0000	0.3025
	InR^{DN}/+ to ChATGAL4/+	0.6433	0.9460	0.1809	0.6069
Male	ChATGAL4/InR^{DN} to ChATGAL4/+	0.0519	0.0761	0.1087	0.0404
	ChATGAL4/InR^{DN} to InR^{DN}/+	0.0031	0.0429	0.1409	0.4084
	InR^{DN}/+ to ChATGAL4/+	0.4569	0.9794	0.9948	0.3421



Generalized Linear Model Fit – Effect tests:

Female		Male	
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0652	Genotype:	0.0108
Age*Genotype:	0.0419	Age*Genotype:	0.7363

	Genotype	P-value for each timepoint (Tukey-Kramer HSD test) 1st	2nd	3rd	4th
Female	ChATGAL4/InR^{DN} to ChATGAL4/+	0.0136	0.5895	0.0013	0.8140
	ChATGAL4/InR^{DN} to InR^{DN}/+	0.5662	0.8034	0.9624	0.9977
	InR^{DN}/+ to ChATGAL4/+	0.1421	0.9334	0.0034	0.8479
Male	ChATGAL4/InR^{DN} to ChATGAL4/+	0.0045	0.6661	0.7643	0.8565
	ChATGAL4/InR^{DN} to InR^{DN}/+	<0.0001	0.8762	0.4463	0.6472
	InR^{DN}/+ to ChATGAL4/+	0.1287	0.9168	0.8611	0.9303

Figure 61 - The exploratory walking senescence of female and male flies with reduced IIS in their cholinergic neuronal subtypes

Exploratory walking of ChAT-GAL4/UAS-InR^{DN} flies compared to ChAT-GAL4/+ and UAS-InR^{DN}/+ control groups. Female data: left column, male data: right column. N=16 for each genotype. Error bars represent +/- SEM. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. When genotype or age*genotype has a significant effect, post hoc pairwise comparison was carried out using Tukey-Kramer HSD test. Significant difference is highlighted with red text colour ($p<0.05$) in the statistical summary and with a star (★) on the graph.

A) Total distance walked over the lifespan **B)** Duration in central zone over the lifespan **C)** Walking duration over the lifespan **D)** Number of movement bouts over the lifespan **E)** Rotation frequency over the lifespan **F)** First rotation time over the lifespan **G)** Velocity over the lifespan
.

9.3: Discussion

Reducing IIS in neurons of *Drosophila* using the elavGAL4 or elavGS driver extended the lifespan but had either no or detrimental effects on walking and negative geotaxis behaviour, thus showing a disconnection between lifespan and healthspan. In order to understand more about how different neuronal subtypes respond to IIS reduction, the previous chapter investigated the effect of reduced IIS in neuronal subtypes on lifespan and the aims of this chapter were to determine if negative geotaxis and exploratory walking senescence changes when IIS is reduced in specific neuronal subtypes. Out of the seven neuronal subtypes, we tested the effect of reduced IIS in four because of time constraints. It will be interesting to determine the effects of reduced IIS in octopaminergic, serotoninergic and histaminergic neurons but unfortunately these experiments did not fit in the time and resources of this project.

Overall, we found that negative geotaxis was not affected by reduced IIS in dopaminergic, glutamatergic and GABAergic neurons, but IIS reduction in cholinergic neurons negatively affected both male and female negative geotaxis. There was no consistent effect of reduced IIS in dopaminergic, glutamatergic and GABAergic neurons on any of the exploratory walking parameters, except that males with reduced IIS in cholinergic neurons showed significant reduction of behavioural function at young age. In the glutamatergic experiment, the UAS-InR^{DN}/+ control groups were found to be significantly different from the other two groups, VglutGAL4/InR^{DN} and VglutGAL4/+, raising the possibility that the VglutGAL4 driver itself affected the behaviour of the flies negatively.

Since flies with reduced IIS in dopaminergic and glutamatergic neurons were short lived, it is interesting that their negative geotaxis and exploratory walking declined similarly to control groups with normal lifespan. Therefore, shorter lifespan does not necessarily lead to faster functional decline, again showing that effects of reduced IIS on lifespan and behavioural healthspan are independent of each other. Reduction of IIS in GABAergic neurons had no effect on lifespan, negative geotaxis and exploratory walking. Not much is known about the role of IIS in GABAergic neurons. In female mice, the knockout of insulin receptor on GABAergic neurons disrupts the energy homeostasis but does not affect reproductive maturation or fertility (Evans, et al. 2014). The insulin producing cells in flies express the GABA$_B$ receptor but not the GABA$_A$. Disruption of the GABA$_B$ receptor in the IPCs by RNA interference shortens the lifespan, reduces metabolic stress resistance, alters metabolism under stress and

increase DILP production. Therefore, $GABA_B$ receptor plays a role in the inhibitory control of DILP production and release in the IPCs under metabolic stress (Enell et al. 2010).

The ChAT-GAL4 cholinergic driver without the UAS-InR^{DN} transgene shortened the lifespan of both male and female flies, and the lifespan of males was further reduced when the UAS-InR^{DN} transgene was expressed. Therefore, reduced IIS in cholinergic neurons had a detrimental effect on male lifespan but did not shorten female lifespan more than the driver on its own. The ChAT-GAL4 driver did not affect the walking or negative geotaxis significantly in both males and females and ChAT-GAL4/InR^{DN} experimental female flies did not show exploratory walking behavioural change compared to the controls. The negative geotaxis of females with cholinergic IIS reduction showed a lower function at the age of 22 days, and the negative geotaxis of males with IIS reduction in cholinergic neurons was reduced throughout their lifespan. ChAT-GAL4/InR^{DN} experimental males also showed reduced function at the age of 13 days in total distance walked, walking, rotation frequency, first rotation time and velocity, and their number of movement bouts increased at the age of 33 days, showing that reducing IIS in the cholinergic neurons in male flies had detrimental effects on their walking behaviour at young age.

In summary, reducing IIS in dopaminergic, glutamatergic and GABAergic neurons had no effect on negative geotaxis and exploratory walking senescence. Reduced IIS in cholinergic neurons had detrimental effects on female and male negative geotaxis and male exploratory walking. Future experiments are planned to fully characterise the role of individual neuronal subtypes including a repeat of the experiment with cholinergic neurons with a different driver that does not affect the health of the flies and an analysis of flies with reduced IIS in serotoninergic, octopaminergic and histaminergic neurons.

Chapter 10: The effect of neuronal subtype specific IIS reduction on sleep and activity

10.1: Introduction

The studies of Metaxakis et al. (2014) showed that sleep fragmentation increases with age in *Drosophila* showing similar characteristics to human sleep at old age, with increased day sleep, reduced night sleep, increased number of sleep bouts in day and night and decreased night sleep bout duration. The study also showed that systemically reduced IIS rescued age-related sleep fragmentation using *dilp2-3,5* mutant and daGAL4/UAS-InR^{DN} flies (further described in Chapter 6.1).

In Chapter 6, sleep behaviour in response to pan-neural IIS reduction was studied using the constitutive elavGAL4 and the inducible elavGS driver with the UAS-InR^{DN} transgene. Our results did not always show characteristic age-related sleep behavioural changes as described previously, but when age had a significant effect on sleep behavioural parameters, it followed similar patterns to the studies of Metaxakis et al. (2014). Constitutive pan-neural IIS reduction did not alter sleep behaviour, whereas IIS reduction from adulthood using the elavGS system increased female sleep fragmentation at middle age. Therefore, reduced neuronal IIS during adulthood had detrimental effects on sleep fragmentation. The chemical RU486 used to induce IIS reduction with the elavGS driver had negative effects on sleep fragmentation in males, therefore it is difficult to determine the effect of adult specific pan-neural IIS reduction in males.

In this chapter we tested how reduction of IIS in specific neuronal subtypes affected daily activity and sleep fragmentation changes over the lifespan of flies.

10.1.1: Aims

To investigate the effect of neuronal subtype specific IIS reduction on sleep behavioural changes of fruit flies using dopaminergic, cholinergic, GABA-ergic and glutamatergic neuron specific drivers.

10.1.2: Research design

To measure the daily activity of flies with reduced IIS in specific neuronal subtypes, we used Drosophila Activity Monitors (DAMs) as shown on **Figure 12**. About every 10 days, flies were sampled from the population and individual flies were transferred into DAM tubes (N=15) and were kept under standard conditions for 4 days. The data were recorded in 1 minute bins and 'sleep' was defined as a minimum of 5 minutes with no activity (Shaw, et al. 2000). If a fly shows less than 100 min activity per day, it is considered dead. Only the data from day 2 and 3 was used during the analysis, as on the first day the flies could still be affected by CO_2 and on the 4th day the food in the DAM tubes may start to dry out, affecting the behaviour of the flies. The data was initially analysed with General Linear Modelling (GLM) seeking for an effect of age, genotype and age*genotype interaction. When significant ($p<0.005$) effect was found for Genotype or Age*Genotype, post hoc pairwise comparison was carried out using Tukey-Kramer HSD test.

This study uses 8 parameters to measure sleep behaviour. Total activity is the number of active minutes throughout a 24 h period, while Total activity level shows how many times the fly crossed the infrared beam in that 24 hour period. Total sleep in dark or in light shows the total number of minutes the flies spent asleep (being inactive for 5 or minutes) in a 12 h period with or without light. The number of sleep bouts in dark or light shows the number of uninterrupted sleep sections in a 12 h dark or light period. More sleep bouts mean that the sleep of the flies is more fragmented. Mean sleep bout length in the dark or light shows the average length of the sleep bouts, where shorter sleep bouts suggest more fragmented sleep.

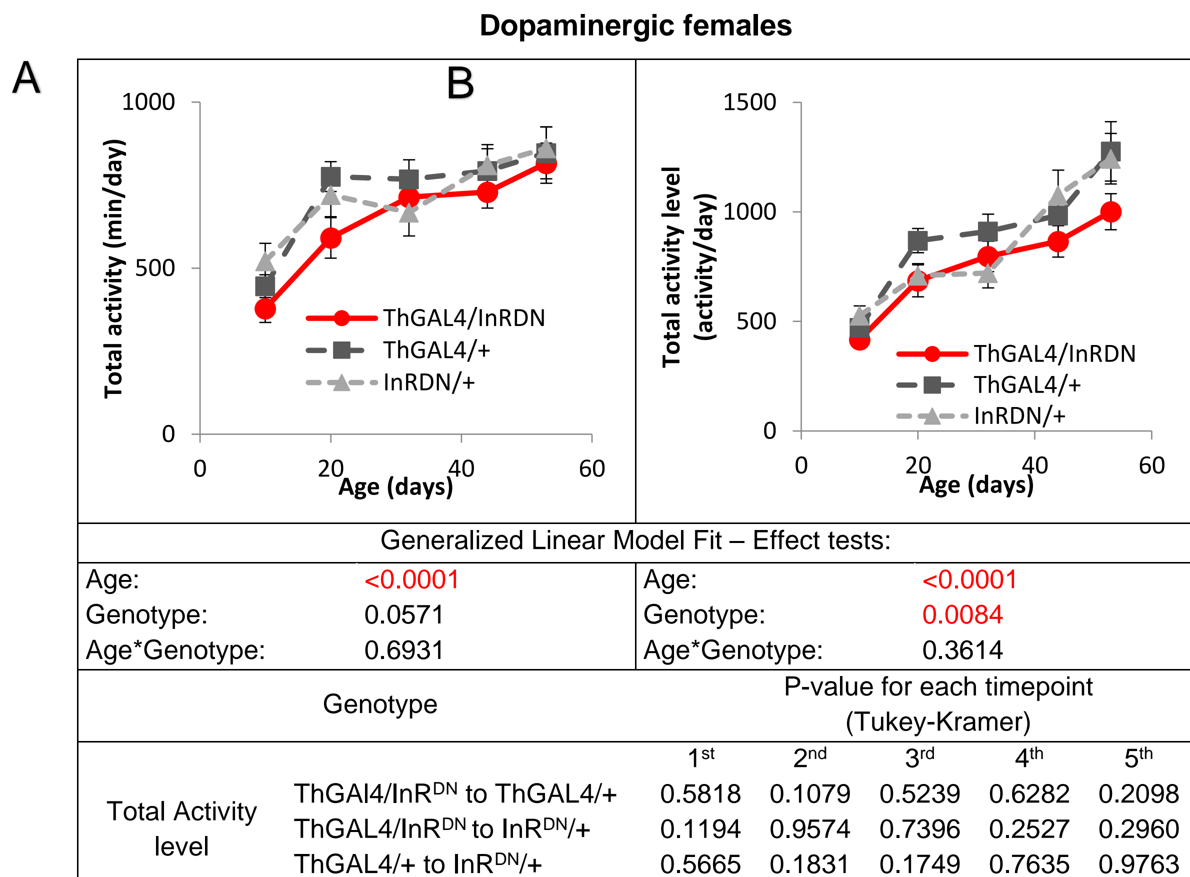
Sleep was analysed in flies with the UAS-InR^{DN} transgene driven by dopaminergic (ThGAL4), glutamatergic (VglutGAL4), cholinergic (ChAT-GAL4) and GABAergic (Gad1-GAL4) *Drosophila* GAL4 lines (described in Chapter 8.1.2), compared to the GAL4/+ and UAS-InR^{DN}/+ control genotypes.

10.2: Results

10.2.1: Constitutive reduction of IIS in dopaminergic neurons does not affect sleep behaviour

The experimental group with reduced IIS in the dopaminergic neurons was the ThGAL4/UAS-InR^{DN}. The two control groups were ThGAL4/+ and UAS-InR^{DN}/+ both containing the driver or the transgene alone, crossed to the wild type w^{Dah} background.

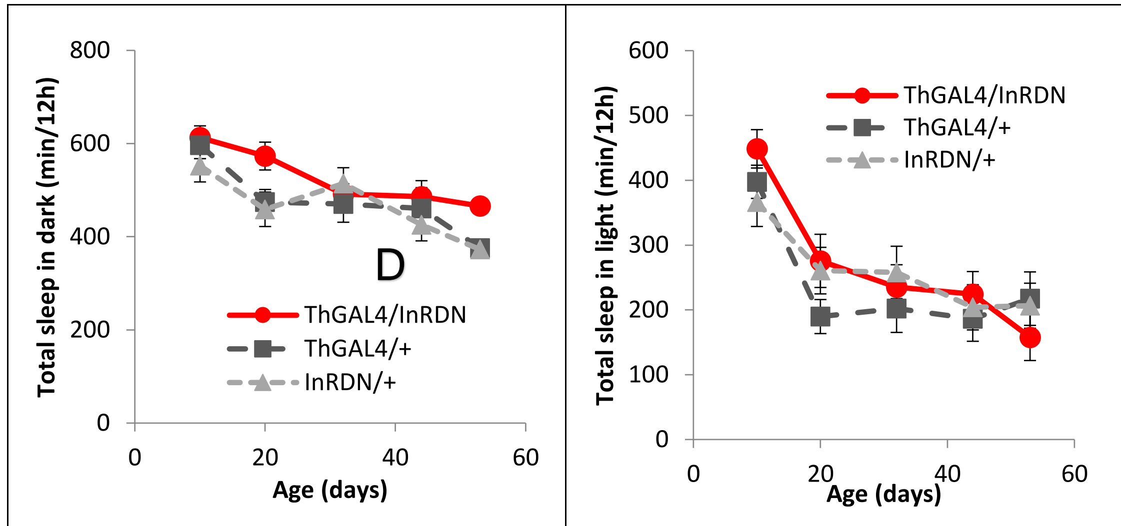
Most sleep and activity parameters in females showed significant age-related changes except for number of bouts in dark (**Figure 62**), and the sleep of ThGAL4/InR^{DN} females was not significantly different to controls. Fewer sleep and activity parameters in male flies changed with age with total activity, activity level per day and on total sleep in dark not changing with age. Similarly to the females, ThGAL4/InR^{DN} males were not significantly different to controls (**Figure 63**). Therefore, reduction of IIS in dopaminergic neurons does not affect total activity or the normal age-related sleep fragmentation of male and female flies.



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0571	Genotype:	0.0084
Age*Genotype:	0.6931	Age*Genotype:	0.3614

	Genotype	P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Total Activity level	ThGAl4/InR^{DN} to ThGAL4/+	0.5818	0.1079	0.5239	0.6282	0.2098
	ThGAL4/InR^{DN} to InR^{DN}/+	0.1194	0.9574	0.7396	0.2527	0.2960
	ThGAL4/+ to InR^{DN}/+	0.5665	0.1831	0.1749	0.7635	0.9763

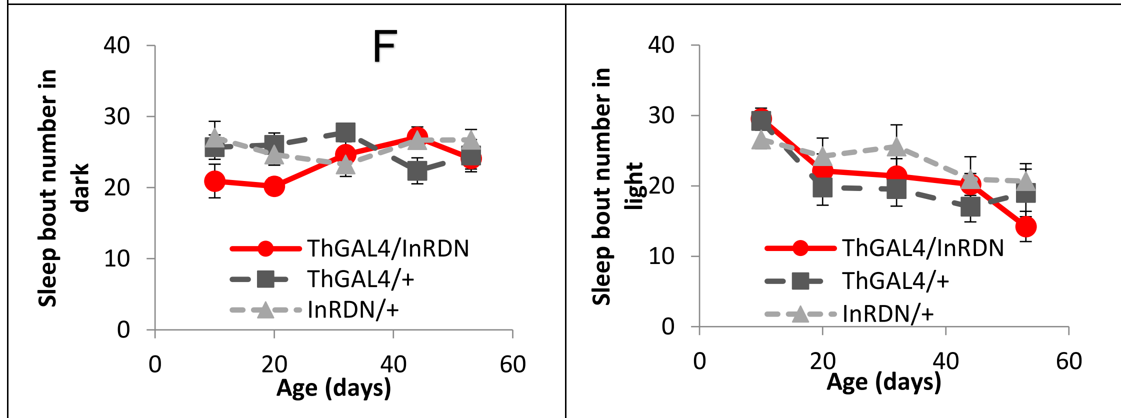
C



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0037	Genotype:	0.3744
Age*Genotype:	0.4361	Age*Genotype:	0.4220

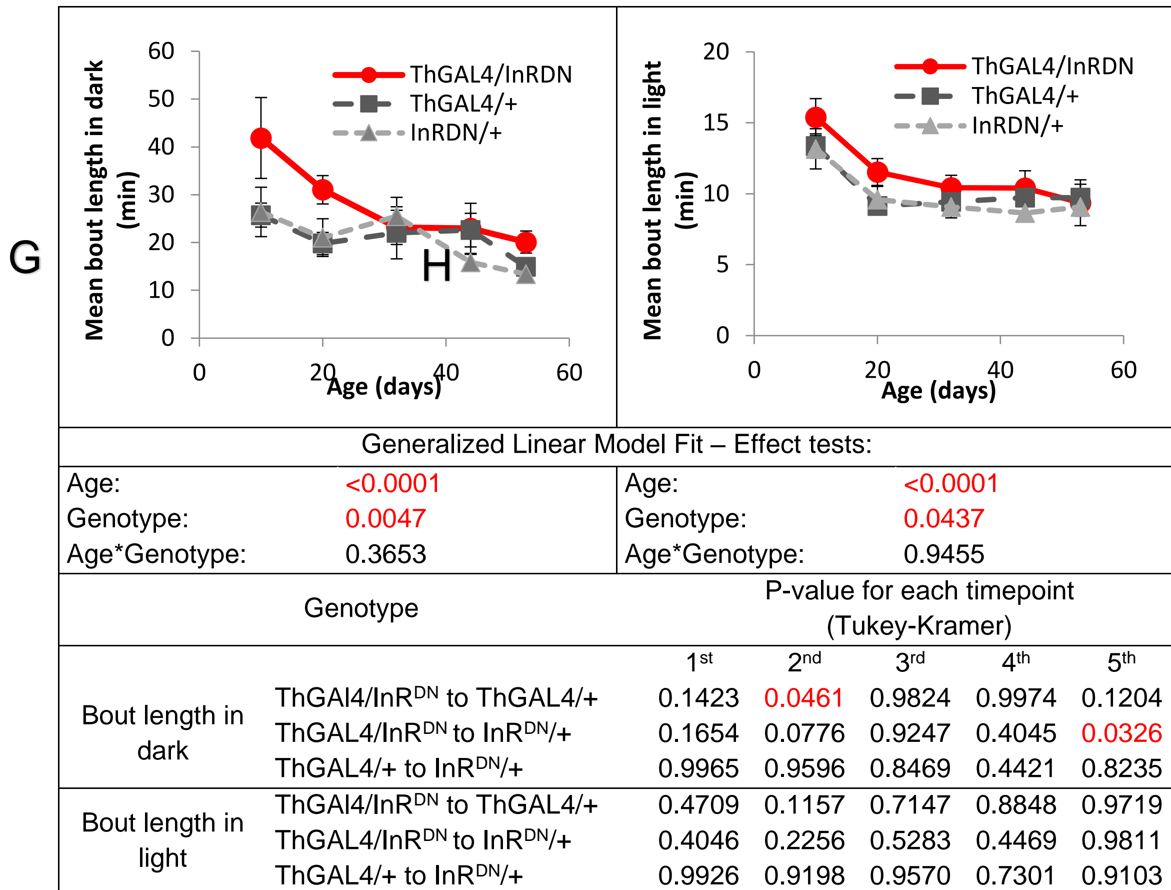
Genotype		P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Total Sleep in Dark	ThGAl4/InR^{DN} to ThGAL4/+	0.8641	0.0690	0.8906	0.8469	0.2277
	ThGAL4/InR^{DN} to InR^{DN}/+	0.1446	0.0267	0.8631	0.4146	0.2069
	ThGAL4/+ to InR^{DN}/+	0.3460	0.9297	0.6010	0.7398	0.9983

E



Generalized Linear Model Fit – Effect tests:			
Age:	0.7024	Age:	<0.0001
Genotype:	0.0765	Genotype:	0.1570
Age*Genotype:	0.0211	Age*Genotype:	0.3942

Genotype		P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Bout number in dark	ThGAl4/InR^{DN} to ThGAL4/+	0.2696	0.0224	0.3890	0.0864	0.9886
	ThGAL4/InR^{DN} to InR^{DN}/+	0.1224	0.0914	0.8139	0.9829	0.5814
	ThGAL4/+ to InR^{DN}/+	0.8994	0.7919	0.1436	0.1334	0.6698



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.0047	Genotype:	0.0437
Age*Genotype:	0.3653	Age*Genotype:	0.9455

Genotype		P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Bout length in dark	ThGAl4/InR^{DN} to ThGAL4/+	0.1423	0.0461	0.9824	0.9974	0.1204
	ThGAL4/InR^{DN} to InR^{DN}/+	0.1654	0.0776	0.9247	0.4045	0.0326
	ThGAL4/+ to InR^{DN}/+	0.9965	0.9596	0.8469	0.4421	0.8235
Bout length in light	ThGAl4/InR^{DN} to ThGAL4/+	0.4709	0.1157	0.7147	0.8848	0.9719
	ThGAL4/InR^{DN} to InR^{DN}/+	0.4046	0.2256	0.5283	0.4469	0.9811
	ThGAL4/+ to InR^{DN}/+	0.9926	0.9198	0.9570	0.7301	0.9103

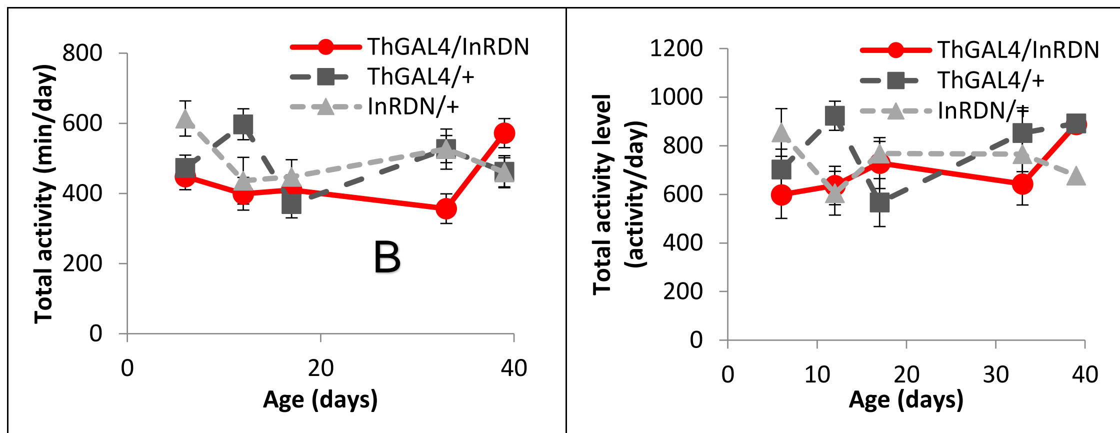
Figure 62 - Effect of constitutive IIS reduction in dopaminergic neurons on the sleep behaviour of female flies

The activity of the mated female flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as ‘sleep’. Flies counted as ‘dead’ if they show less than 100 min activity per day, N=15 for each group and timepoint. The experimental ThGAL4/UAS-InR^{DN} group with constitutive pan-neural IIS reduction was compared to ThGAL4/+ and UAS-InR^{DN}/+ control groups. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. As there was no significant ($p<0.05$) genotype or age*genotype effect in this experiment, there was no need for a post hoc test pairwise comparation.

A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

Dopaminergic males

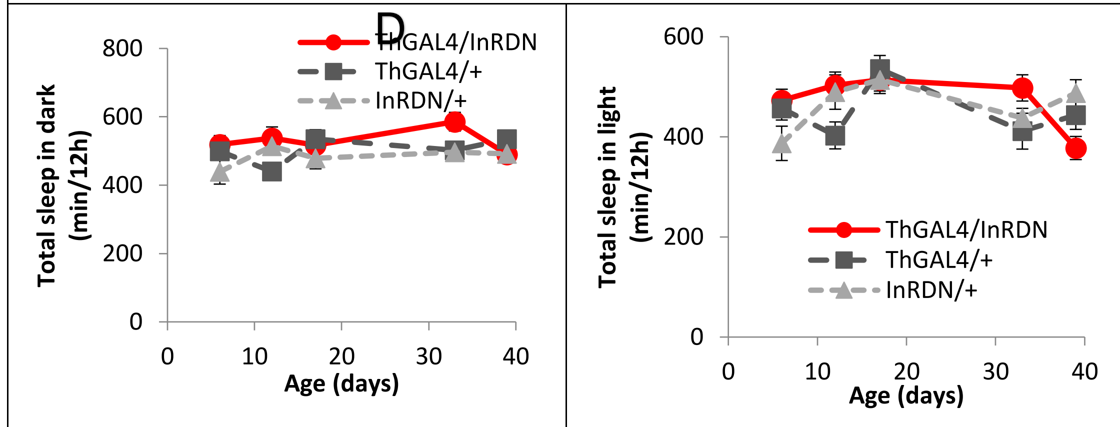
A



Generalized Linear Model Fit – Effect tests:			
Age:	0.0580	Age:	0.3288
Genotype:	0.1102	Genotype:	0.2266
Age*Genotype:	0.0009	Age*Genotype:	0.0054

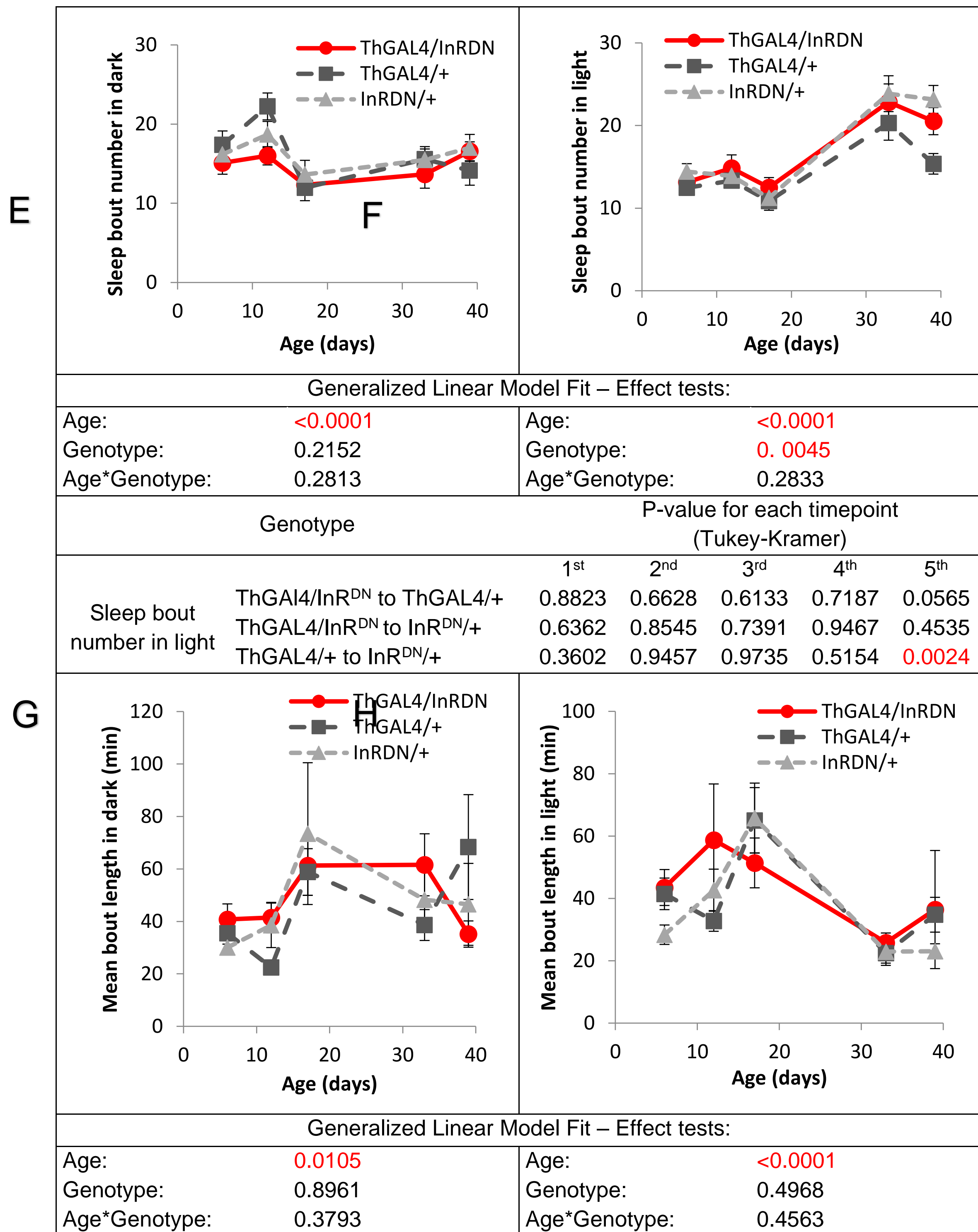
	Genotype	P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Total activity day	ThGAl4/InR^{DN} to ThGAL4/+	0.9149	0.0306	0.8143	0.0545	0.1782
	ThGAL4/InR^{DN} to InR^{DN}/+	0.0242	0.8776	0.8254	0.0939	0.1689
	ThGAL4/+ to InR^{DN}/+	0.0675	0.1005	0.4518	0.9520	0.9995
Total activity level day	ThGAl4/InR^{DN} to ThGAL4/+	0.4707	0.0880	0.3360	0.2640	0.9995
	ThGAL4/InR^{DN} to InR^{DN}/+	0.0170	0.9697	0.9348	0.6143	0.2243
	ThGAL4/+ to InR^{DN}/+	0.2109	0.0573	0.1814	0.7830	0.2134

C



Generalized Linear Model Fit – Effect tests:			
Age:	0.3858	Age:	0.0006
Genotype:	0.0235	Genotype:	0.3990
Age*Genotype:	0. 0460	Age*Genotype:	0.0014

	Genotype	P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Sleep in dark	ThGAl4/InR^{DN} to ThGAL4/+	0.8269	0.0644	0.8949	0.1254	0.4360
	ThGAL4/InR^{DN} to InR^{DN}/+	0.0552	0.8492	0.5984	0.0889	0.9984
	ThGAL4/+ to InR^{DN}/+	0.1793	0.2056	0.3356	0.9901	0.4671
Sleep in light	ThGAl4/InR^{DN} to ThGAL4/+	0.9144	0.0512	0.8346	0.1145	0.1903
	ThGAL4/InR^{DN} to InR^{DN}/+	0.0840	0.9430	0.9999	0.3243	0.0131
	ThGAL4/+ to InR^{DN}/+	0.1826	0.1126	0.8354	0.8026	0.4584



Generalized Linear Model Fit – Effect tests:

E		F	
Age:	<0.0001	Age:	<0.0001
Genotype:	0.2152	Genotype:	0. 0045
Age*Genotype:	0.2813	Age*Genotype:	0.2833

	Genotype	P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Sleep bout number in light	ThGAl4/InR^{DN} to ThGAL4/+	0.8823	0.6628	0.6133	0.7187	0.0565
	ThGAL4/InR^{DN} to InR^{DN}/+	0.6362	0.8545	0.7391	0.9467	0.4535
	ThGAL4/+ to InR^{DN}/+	0.3602	0.9457	0.9735	0.5154	0.0024

Generalized Linear Model Fit – Effect tests:

G		H	
Age:	0.0105	Age:	<0.0001
Genotype:	0.8961	Genotype:	0.4968
Age*Genotype:	0.3793	Age*Genotype:	0.4563

Figure 63 - Effect of constitutive IIS reduction in dopaminergic neurons on the sleep behaviour of male flies

The activity of the male flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as 'sleep'. Flies counted as 'dead' if they show less than 100 min activity per day, N=15 for each group and timepoint. The experimental ThGAL4/UAS-InR^{DN} group with constitutive pan-neural IIS reduction was compared to ThGAL4/+ and UAS-InR^{DN}/+ control groups. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype

interaction. As there was no significant ($p<0.05$) genotype or age*genotype effect in this experiment, there was no need for a post hoc test pairwise comparation.

A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

10.2.2: Constitutive reduction of IIS in glutamatergic neurons increases total activity and reduces daytime sleep in males

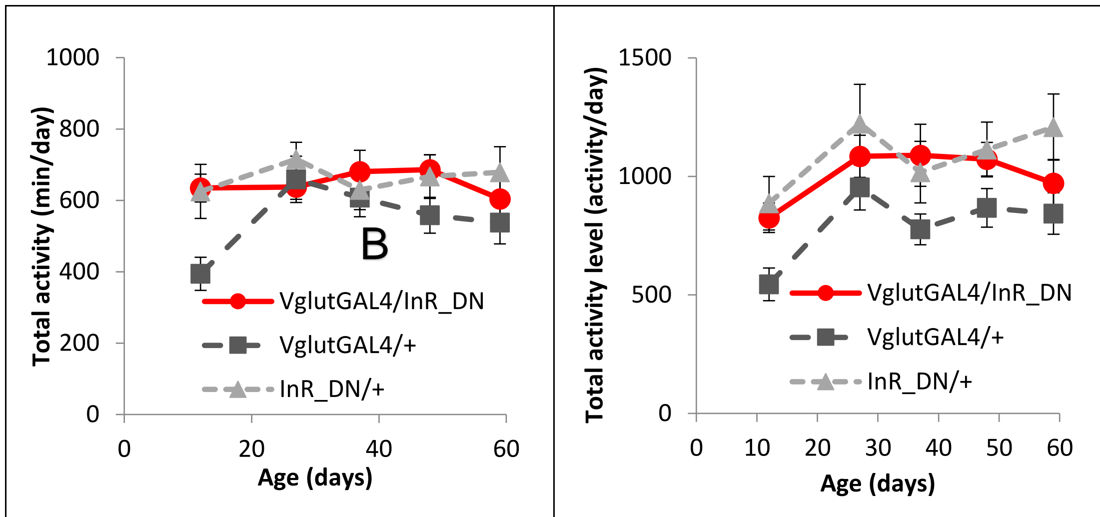
The experimental group is the VglutGAL4/UAS-InR^{DN} expressing the dominant negative insulin receptor in the glutamatergic neurons. The control groups for the driver and the transgene are VglutGAL4/+ and UAS-InR^{DN}/+, both crossed with wild type w^{Dah} background.

Age had significant effect on some of the sleep parameters, but not on all, with female total activity and sleep bout number and length in dark not showing significant change with age. The sleep and activity behaviour of female VglutGAL4/UAS-InR^{DN} flies was not significantly different to controls (**Figure 65**). Reducing IIS in glutamatergic neurons in male flies however changed the sleep behaviour (**Figure 65**). Age had a significant effect on most of the parameters except for the number of sleep bouts in light in all genotypes. The VglutGAL4/UAS-InR^{DN} males showed significantly more total activity than both controls at age 27 days ($p=0.0015$ compared to VglutGAL4/+ and $p=0.0252$ compared to UAS-InR^{DN}/+) and at the age of 12 days, the experimental group was significantly different from the VglutGAL4/+ control ($p=0.0148$) and close to significance with UAS-InR^{DN}/+ ($p=0.0577$). Surprisingly, the total activity level was unaffected, therefore the flies spent more time active, but did not cross the infrared beam more times than the control groups. Sleep in the dark was unaffected, but the total sleep during daytime was reduced significantly in VglutGAL4/UAS-InR^{DN} males at the age of 12 days ($p=0.0182$ compared to VglutGAL4/+ and $p=0.0097$ compared to UAS-InR^{DN}/+) and 27 days ($p=0.0138$ compared to VglutGAL4/+ and $p=0.0103$ compared to UAS-InR^{DN}/+). The number of sleep bouts did not change and the length of the sleep bouts in light was only significantly different from the UAS-InR^{DN}/+ control at the age of 12, 27 and 37, but not different from the VglutGAL4/+ control.

Therefore, these data show that reducing IIS in glutamatergic neurons does not affect female behaviour, but it increases the total activity of the males, predominantly at young ages, by reducing the amount they sleep during daytime.

Glutamatergic females

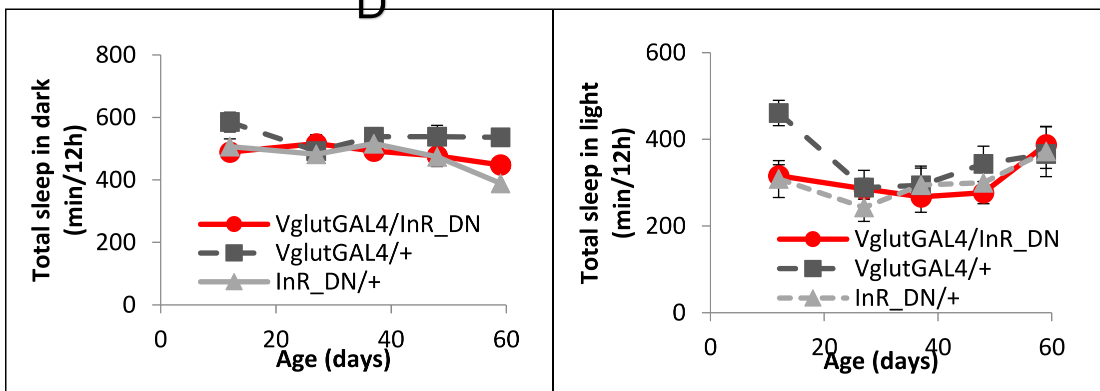
A



Generalized Linear Model Fit – Effect tests:			
Age:	0.0878	Age:	0.0014
Genotype:	0.0032	Genotype:	<0.0001
Age*Genotype:	0.4026	Age*Genotype:	0.9519

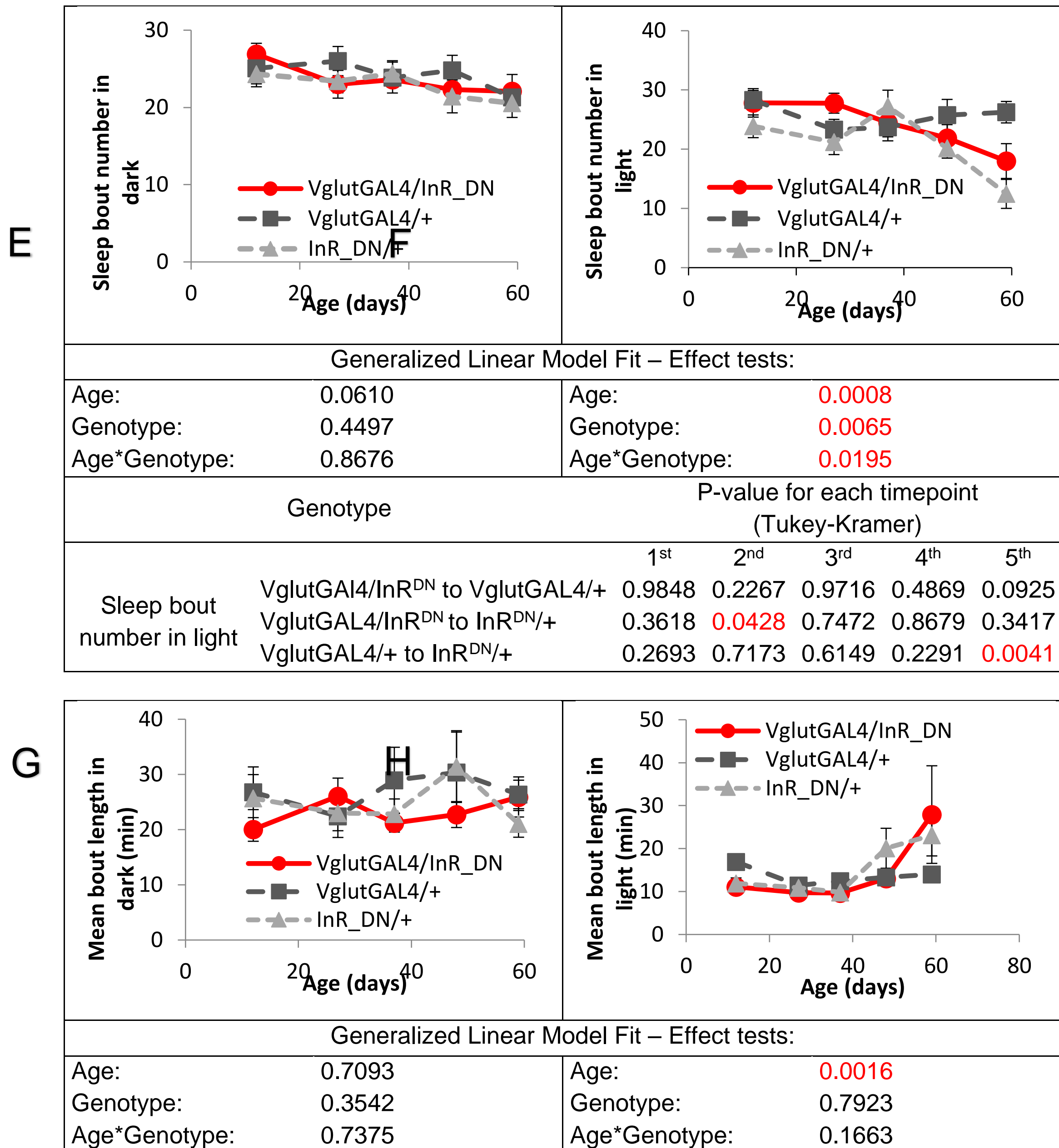
	Genotype	P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Total activity day	VglutGAl4/InR^{DN} to VglutGAL4/+	0.0138	0.9533	0.6395	0.2331	0.7738
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.9927	0.5229	0.8179	0.9662	0.7388
	VglutGAL4/+ to InR^{DN}/+	0.0162	0.7168	0.9665	0.4373	0.3586
Total activity level day	VglutGAl4/InR^{DN} to VglutGAL4/+	0.0669	0.7361	0.1297	0.1788	0.7340
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.8709	0.7041	0.9054	0.2969	0.3745
	VglutGAL4/+ to InR^{DN}/+	0.0182	0.2848	0.3283	0.9525	0.1153

C



Generalized Linear Model Fit – Effect tests:			
Age:	0.0354	Age:	0.0016
Genotype:	0.0004	Genotype:	0.0840
Age*Genotype:	0.2172	Age*Genotype:	0.3511

	Genotype	P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Sleep in dark	VglutGAl4/InR^{DN} to VglutGAL4/+	0.0475	0.8032	0.4034	0.2519	0.1805
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.9026	0.6284	0.7978	0.9961	0.4666
	VglutGAL4/+ to InR^{DN}/+	0.1114	0.9591	0.8294	0.2183	0.0180



Generalized Linear Model Fit – Effect tests:

	E		F
Age:	0.0610	Age:	0.0008
Genotype:	0.4497	Genotype:	0.0065
Age*Genotype:	0.8676	Age*Genotype:	0.0195

Genotype		P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Sleep bout number in light	VglutGAl4/InR^{DN} to VglutGAL4/+	0.9848	0.2267	0.9716	0.4869	0.0925
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.3618	0.0428	0.7472	0.8679	0.3417
	VglutGAL4/+ to InR^{DN}/+	0.2693	0.7173	0.6149	0.2291	0.0041

Generalized Linear Model Fit – Effect tests:

	G		H
Age:	0.7093	Age:	0.0016
Genotype:	0.3542	Genotype:	0.7923
Age*Genotype:	0.7375	Age*Genotype:	0.1663

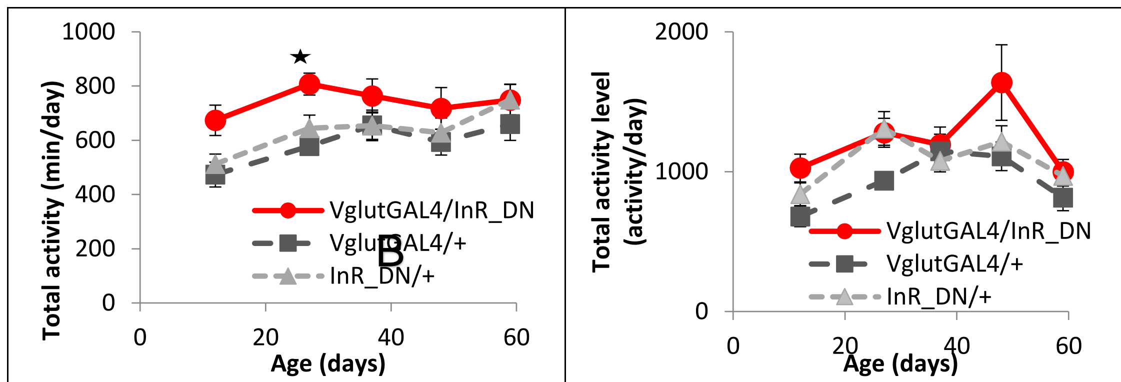
Figure 64 - Effect of constitutive IIS reduction in glutamatergic neurons on the sleep behaviour of female flies

The activity of the mated female flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as 'sleep'. Flies counted as 'dead' if they show less than 100 min activity per day, N=15 for each group and timepoint. The experimental VglutGAL4/UAS-InR^{DN} group with constitutive pan-neural IIS reduction was compared to VglutGAL4/+ and UAS-InR^{DN}/+ control groups. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. As there was no significant ($p<0.05$) genotype or age*genotype effect in this experiment, there was no need for a post hoc test pairwise comparation.

A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

Glutamatergic males

A B

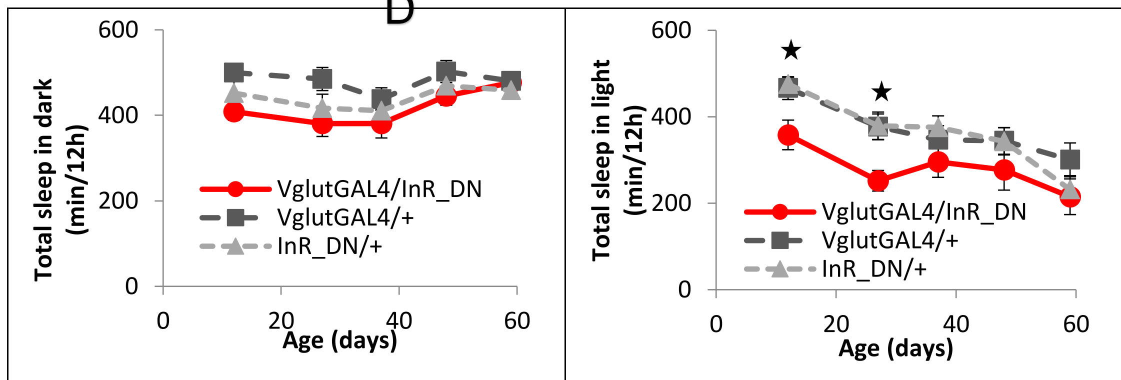


Generalized Linear Model Fit – Effect tests:

Age:	0.0015	Age:	<0.0001
Genotype:	<0.0001	Genotype:	0.0003
Age*Genotype:	0.7968	Age*Genotype:	0.3581

	Genotype	P-value for each timepoint (Tukey-Kramer) 1st	2nd	3rd	4th	5th
Total Activity	VglutGAl4/InR^{DN} to VglutGAL4/+	0.0148	0.0015	0.3892	0.4258	0.5339
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.0577	0.0252	0.3817	0.6279	0.9998
	VglutGAL4/+ to InR^{DN}/+	0.8271	0.5192	0.9999	0.9181	0.5224
Total Activity level	VglutGAl4/InR^{DN} to VglutGAL4/+	0.0215	0.0604	0.9538	0.1472	0.2774
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.2963	0.9742	0.7447	0.2737	0.9653
	VglutGAL4/+ to InR^{DN}/+	0.3895	0.0334	0.9017	0.9003	0.4035

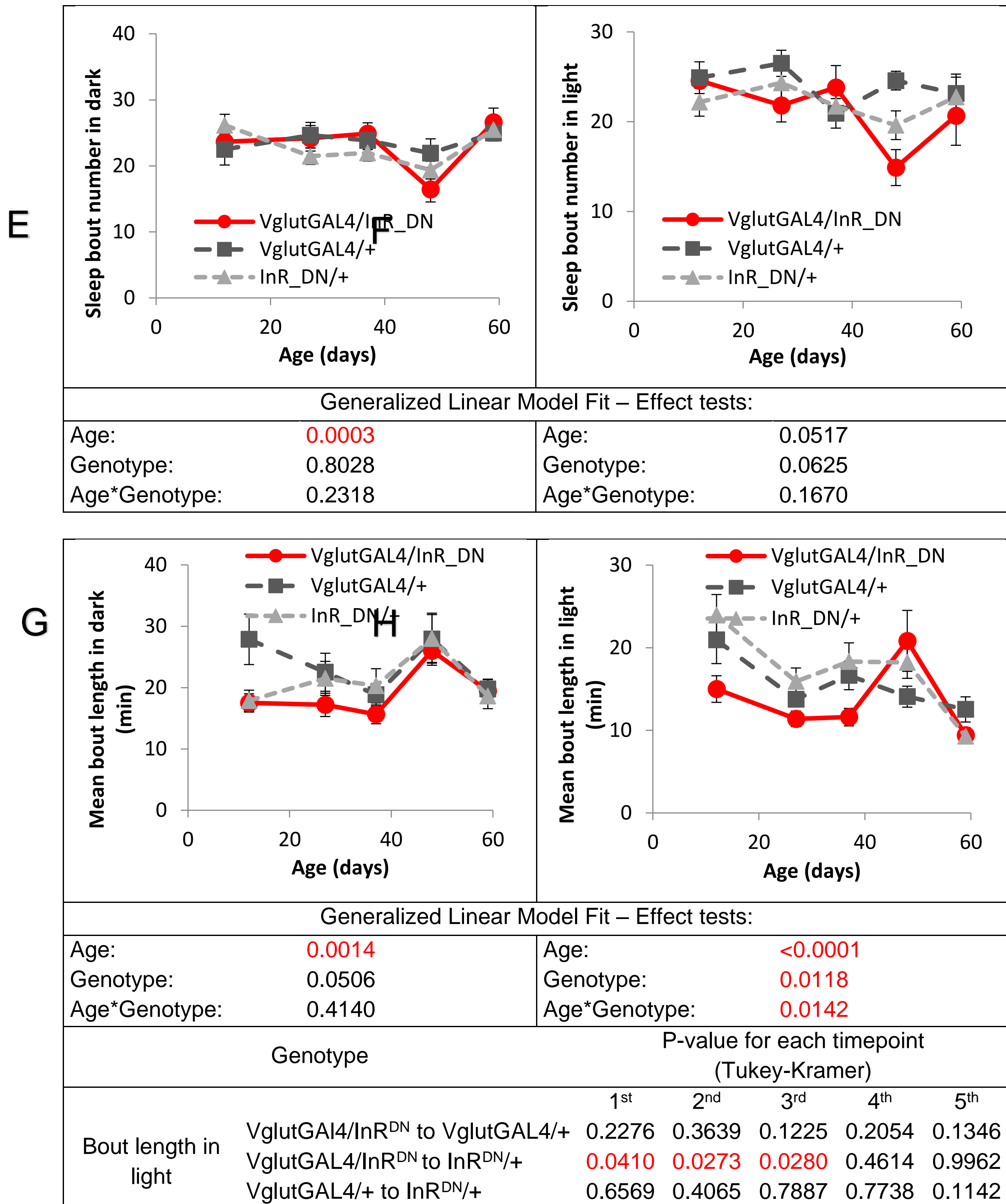
C D



Generalized Linear Model Fit – Effect tests:

Age:	0.0175	Age:	<0.0001
Genotype:	0.0016	Genotype:	<0.0001
Age*Genotype:	0.7993	Age*Genotype:	0.7004

	Genotype	P-value for each timepoint (Tukey-Kramer) 1st	2nd	3rd	4th	5th
Total Sleep in Dark	VglutGAl4/InR^{DN} to VglutGAL4/+	0.0643	0.0107	0.3897	0.4795	0.9955
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.5130	0.5371	0.7680	0.8774	0.8798
	VglutGAL4/+ to InR^{DN}/+	0.4280	0.1153	0.8100	0.7208	0.8352
Total Sleep in Light	VglutGAl4/InR^{DN} to VglutGAL4/+	0.0182	0.0138	0.5145	0.5047	0.2566
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.0097	0.0103	0.2084	0.5042	0.9481
	VglutGAL4/+ to InR^{DN}/+	0.9652	0.9980	0.8174	0.9998	0.4090



Generalized Linear Model Fit – Effect tests:			
Age:	0.0003	Age:	0.0517
Genotype:	0.8028	Genotype:	0.0625
Age*Genotype:	0.2318	Age*Genotype:	0.1670

Generalized Linear Model Fit – Effect tests:			
Age:	0.0014	Age:	<0.0001
Genotype:	0.0506	Genotype:	0.0118
Age*Genotype:	0.4140	Age*Genotype:	0.0142

Genotype		P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Bout length in light	VglutGAl4/InR^{DN} to VglutGAL4/+	0.2276	0.3639	0.1225	0.2054	0.1346
	VglutGAL4/InR^{DN} to InR^{DN}/+	0.0410	0.0273	0.0280	0.4614	0.9962
	VglutGAL4/+ to InR^{DN}/+	0.6569	0.4065	0.7887	0.7738	0.1142

Figure 65 - Effect of constitutive IIS reduction in glutamatergic neurons on the sleep behaviour of male flies

The activity of the male flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as 'sleep'. Flies counted as 'dead' if they show less than 100 min activity per day, N=15 for each group and timepoint. The experimental VglutGAL4/UAS-InR^{DN} group with constitutive pan-neural IIS reduction was compared to VglutGAL4/+ and UAS-InR^{DN}/+ control groups. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. As there was no significant (p<0.05) genotype or age*genotype effect in this experiment, there was no need for a post hoc test pairwise comparation.

A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

10.2.3: Constitutive reduction of IIS in GABAergic neurons does not affect sleep behaviour, however the Gad1-GAL4 driver increases total activity by reducing daytime sleep

The experimental group is the Gad1GAL4/UAS-InR^{DN} expressing the dominant negative insulin receptor in the GABAergic neurons. The control groups for the driver and the transgene are Gad1GAL4/+ and UAS-InR^{DN}/+, both crossed with wild type w^{Dah} background.

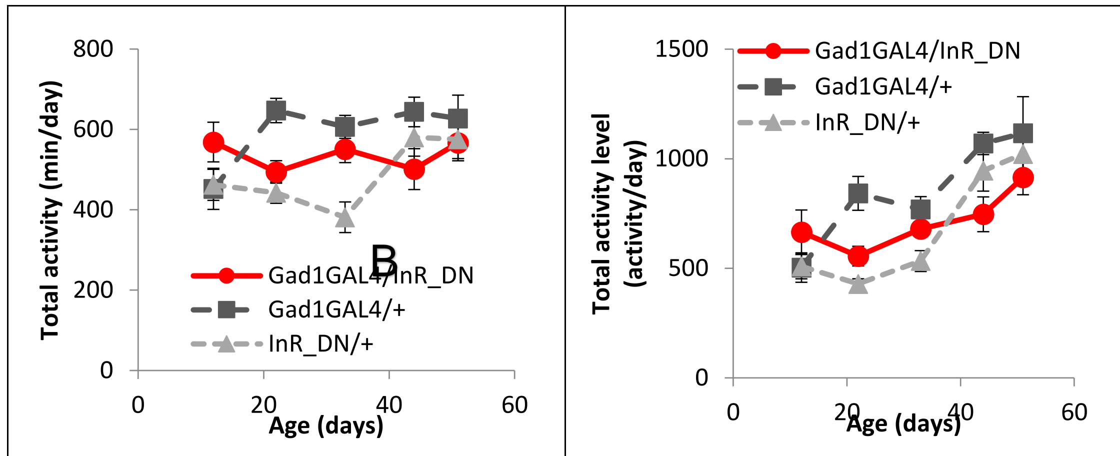
Age had a significant effect on all the female sleep behavioural parameters (**Figure 66**). The experimental group with reduced IIS in the GABAergic neurons showed no significant difference from both control groups at any time. The Gad1-GAL4/+ control for the driver however showed significantly higher total activity (at age 22 and 33 days) and total activity level (at age 22, 33 and 44 days) compared to the UAS-InR^{DN}/+ control. The Gad1-GAL4 driver did not affect sleep in dark, however the total amount of daytime sleep was reduced at age 22 and 33 days along with the number of sleep bouts in light compared to the UAS-InR^{DN}/+ control. The length of the sleep bouts in light did not change.

The male GABAergic sleep experiment did not show significant age effect according to GLM in the total activity, total sleep in light and sleep bout length in light (**Figure 67**). Even though GLM showed significant Genotype or Genotype*Age effect, Tukey-Kramer HSD test did not show that reducing IIS in GABAergic neurons affects any of the sleep parameters significantly at any timepoint.

The results show that reduction of IIS in GABAergic neurons using the Gad1-GAL4 driver did not affect the sleep behaviour significantly, however the Gad1-GAL4 driver itself increased the total activity and activity level of female flies by reducing daytime sleep or increasing daytime activity.

GABAergic females

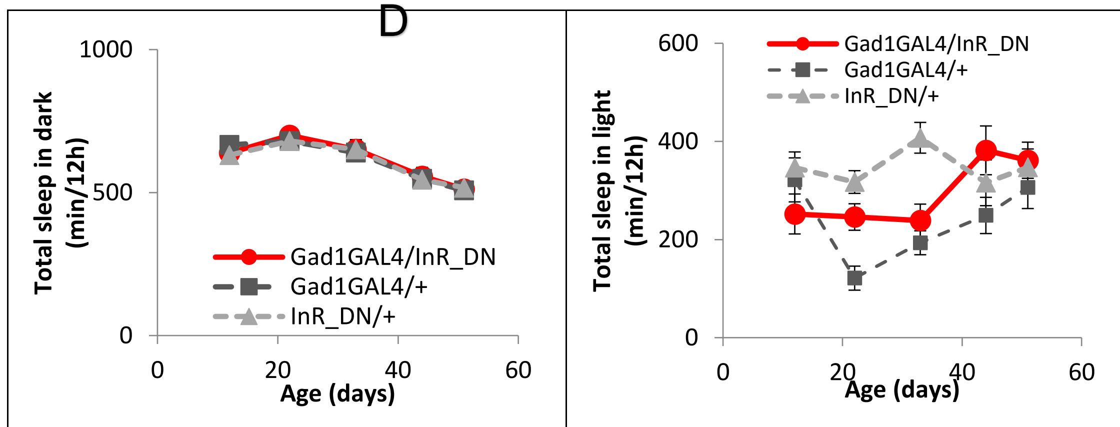
A



Generalized Linear Model Fit – Effect tests:			
Age:	0.0288	Age:	<0.0001
Genotype:	0.0004	Genotype:	0.0014
Age*Genotype:	0.0049	Age*Genotype:	0.0233

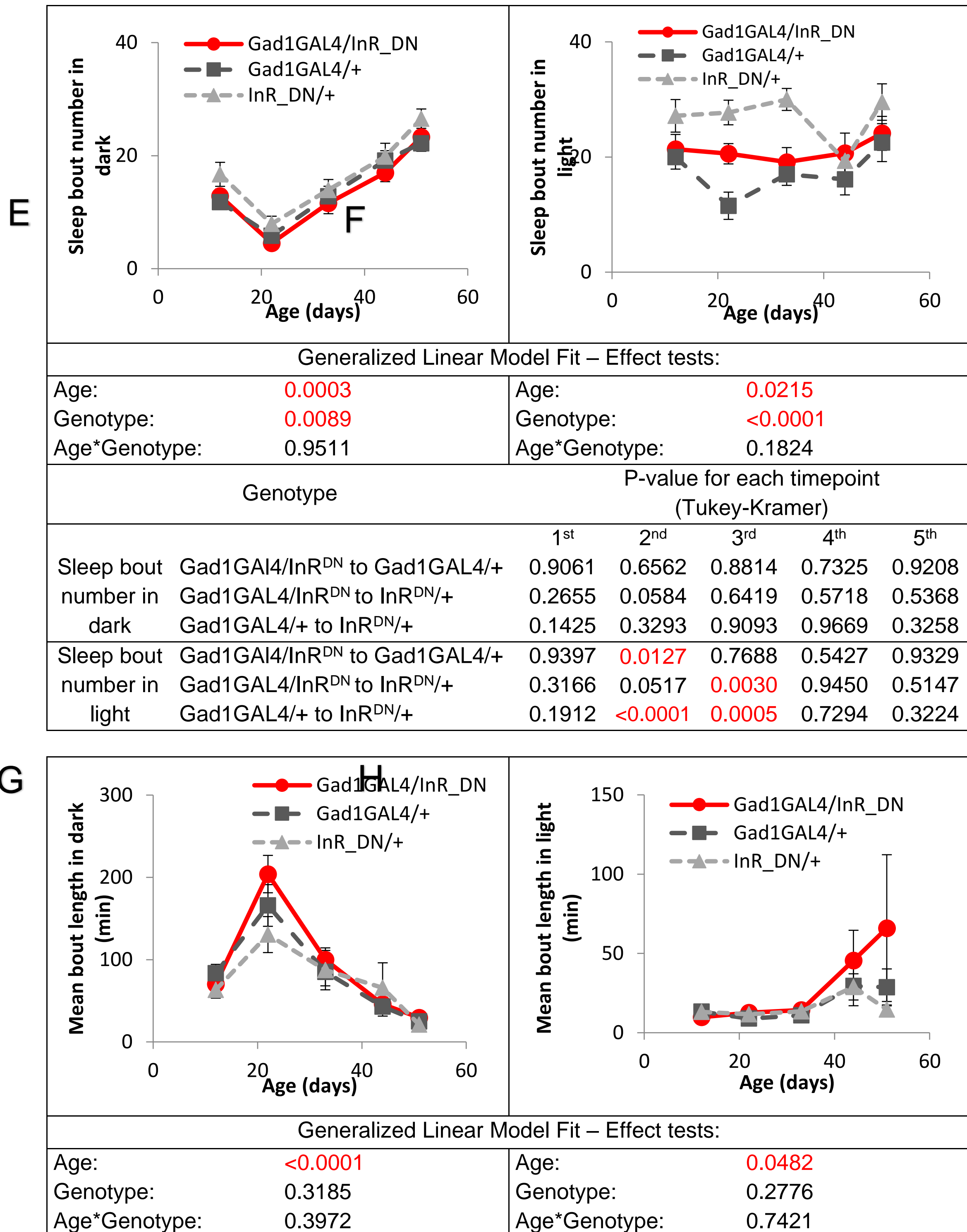
	Genotype	P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Total Activity	Gad1GAl4/InR^{DN} to Gad1GAL4/+	0.2677	0.0015	0.4794	0.0929	0.6917
	Gad1GAL4/InR^{DN} to InR^{DN}/+	0.2993	0.4102	0.0031	0.4514	0.9930
	Gad1GAL4/+ to InR^{DN}/+	0.9899	<0.0001	0.0001	0.5998	0.7906
Total Activity level	Gad1GAl4/InR^{DN} to Gad1GAL4/+	0.4046	0.0014	0.3969	0.0067	0.4840
	Gad1GAL4/InR^{DN} to InR^{DN}/+	0.3959	0.2171	0.0992	0.0903	0.8377
	Gad1GAL4/+ to InR^{DN}/+	0.9991	<0.0001	0.0042	0.2719	0.8673

C



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.0035
Genotype:	0.8793	Genotype:	<0.0001
Age*Genotype:	0.9821	Age*Genotype:	0.0067

	Genotype	P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Total Sleep in Light	Gad1GAl4/InR^{DN} to Gad1GAL4/+	0.5221	0.0043	0.5530	0.1197	0.5926
	Gad1GAL4/InR^{DN} to InR^{DN}/+	0.2776	0.1206	0.0010	0.5655	0.9709
	Gad1GAL4/+ to InR^{DN}/+	0.9141	<0.0001	<0.0001	0.5609	0.7739



Generalized Linear Model Fit – Effect tests:			
Age:	0.0003	Age:	0.0215
Genotype:	0.0089	Genotype:	<0.0001
Age*Genotype:	0.9511	Age*Genotype:	0.1824

Genotype		P-value for each timepoint (Tukey-Kramer) 1st	2nd	3rd	4th	5th
Sleep bout number in dark	Gad1GAl4/InR^{DN} to Gad1GAL4/+	0.9061	0.6562	0.8814	0.7325	0.9208
	Gad1GAL4/InR^{DN} to InR^{DN}/+	0.2655	0.0584	0.6419	0.5718	0.5368
	Gad1GAL4/+ to InR^{DN}/+	0.1425	0.3293	0.9093	0.9669	0.3258
Sleep bout number in light	Gad1GAl4/InR^{DN} to Gad1GAL4/+	0.9397	0.0127	0.7688	0.5427	0.9329
	Gad1GAL4/InR^{DN} to InR^{DN}/+	0.3166	0.0517	0.0030	0.9450	0.5147
	Gad1GAL4/+ to InR^{DN}/+	0.1912	<0.0001	0.0005	0.7294	0.3224

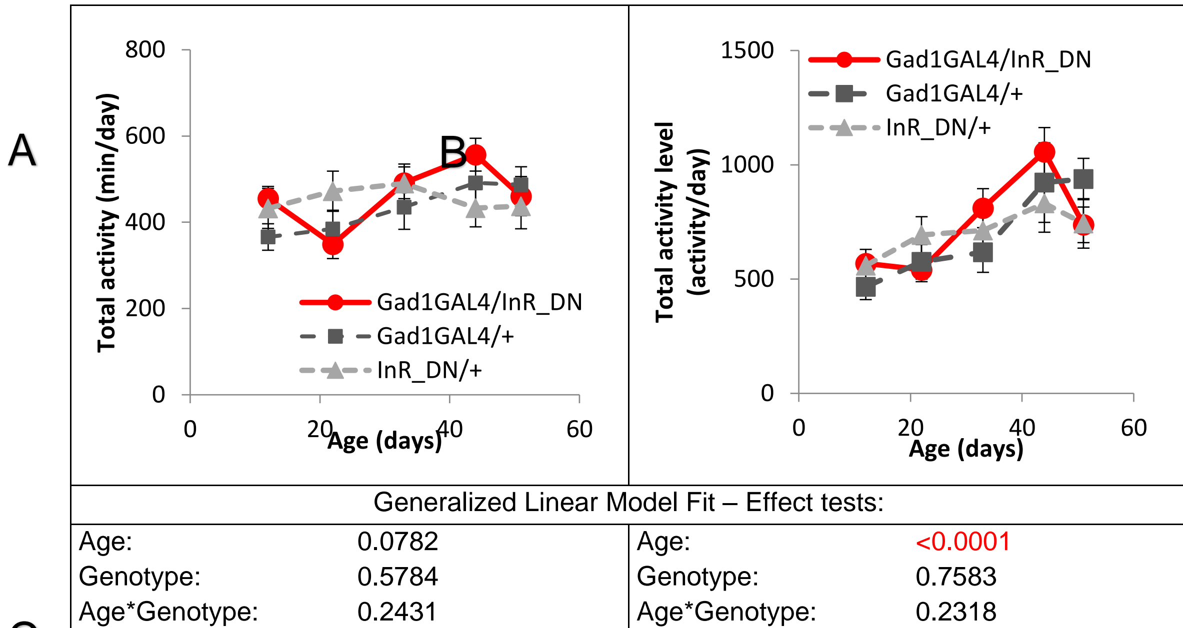
Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.0482
Genotype:	0.3185	Genotype:	0.2776
Age*Genotype:	0.3972	Age*Genotype:	0.7421

Figure 66 - Effect of constitutive IIS reduction in GABAergic neurons on the sleep behaviour of female flies

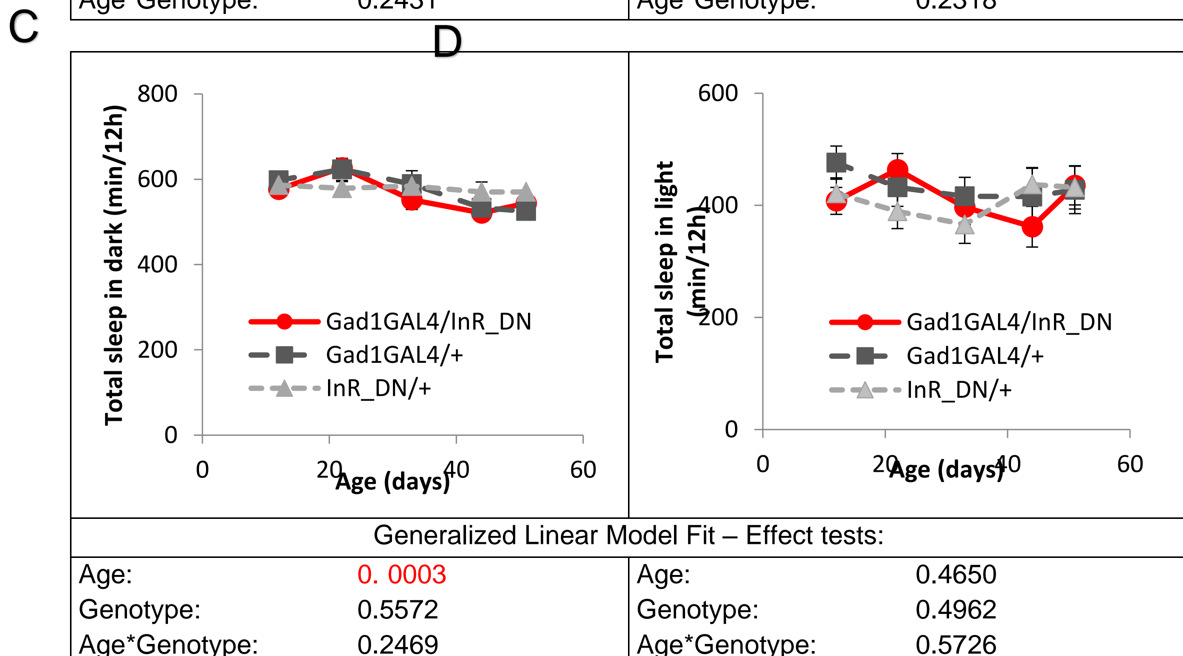
The activity of the mated female flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as 'sleep'. Flies counted as 'dead' if they show less than 100 min activity per day, N=15 for each group and timepoint. The experimental Gad1-GAL4/UAS-InR^{DN} group with constitutive pan-neural IIS reduction was compared to Gad1-GAL4/+ and UAS-InR^{DN}/+ control groups. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and

age*genotype interaction. As there was no significant (p<0.05) genotype or age*genotype effect in this experiment, there was no need for a post hoc test pairwise comparation.
A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

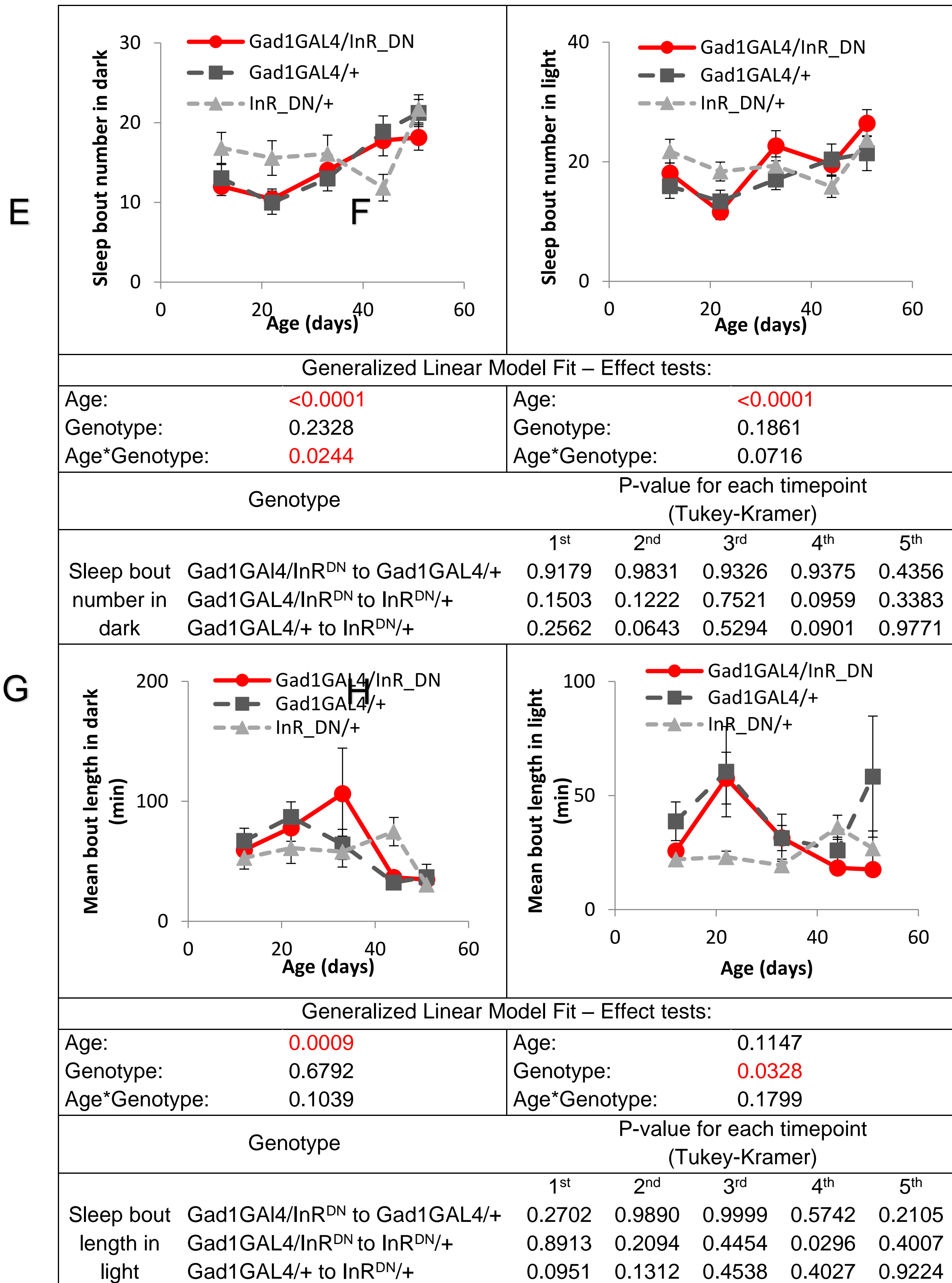
GABAergic males



Generalized Linear Model Fit – Effect tests:			
Age:	0.0782	Age:	<0.0001
Genotype:	0.5784	Genotype:	0.7583
Age*Genotype:	0.2431	Age*Genotype:	0.2318



Generalized Linear Model Fit – Effect tests:			
Age:	0. 0003	Age:	0.4650
Genotype:	0.5572	Genotype:	0.4962
Age*Genotype:	0.2469	Age*Genotype:	0.5726



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	0.2328	Genotype:	0.1861
Age*Genotype:	0.0244	Age*Genotype:	0.0716

	Genotype	P-value for each timepoint (Tukey-Kramer) 1st	2nd	3rd	4th	5th
Sleep bout number in dark	Gad1GAl4/InR^{DN} to Gad1GAL4/+	0.9179	0.9831	0.9326	0.9375	0.4356
	Gad1GAL4/InR^{DN} to InR^{DN}/+	0.1503	0.1222	0.7521	0.0959	0.3383
	Gad1GAL4/+ to InR^{DN}/+	0.2562	0.0643	0.5294	0.0901	0.9771

Generalized Linear Model Fit – Effect tests:			
Age:	0.0009	Age:	0.1147
Genotype:	0.6792	Genotype:	0.0328
Age*Genotype:	0.1039	Age*Genotype:	0.1799

	Genotype	P-value for each timepoint (Tukey-Kramer) 1st	2nd	3rd	4th	5th
Sleep bout length in light	Gad1GAl4/InR^{DN} to Gad1GAL4/+	0.2702	0.9890	0.9999	0.5742	0.2105
	Gad1GAL4/InR^{DN} to InR^{DN}/+	0.8913	0.2094	0.4454	0.0296	0.4007
	Gad1GAL4/+ to InR^{DN}/+	0.0951	0.1312	0.4538	0.4027	0.9224

Figure 67 - Effect of constitutive IIS reduction in GABAergic neurons on the sleep behaviour of male flies

The activity of the male flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as 'sleep'. Flies counted as 'dead' if they show less than 100 min activity per day, N=15 for each group and timepoint. The experimental Gad1-GAL4/UAS-InR^{DN} group with constitutive pan-neural IIS reduction was compared to Gad1-GAL4/+ and UAS-InR^{DN}/+ control groups. Error

bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. As there was no significant ($p<0.05$) genotype or age*genotype effect in this experiment, there was no need for a post hoc test pairwise comparation.
A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

10.2.4: Constitutive reduction of IIS in cholinergic neurons increases the length of sleep bouts in the dark in females, but does not affect males

The experimental group is the ChAT-GAL4/UAS-InR^{DN} expressing the dominant negative insulin receptor selectively in cholinergic neurons. The control groups for the driver and the transgene are ChAT-GAL4/+ and UAS-InR^{DN}/+, both crossed with wild type w^{Dah} background.

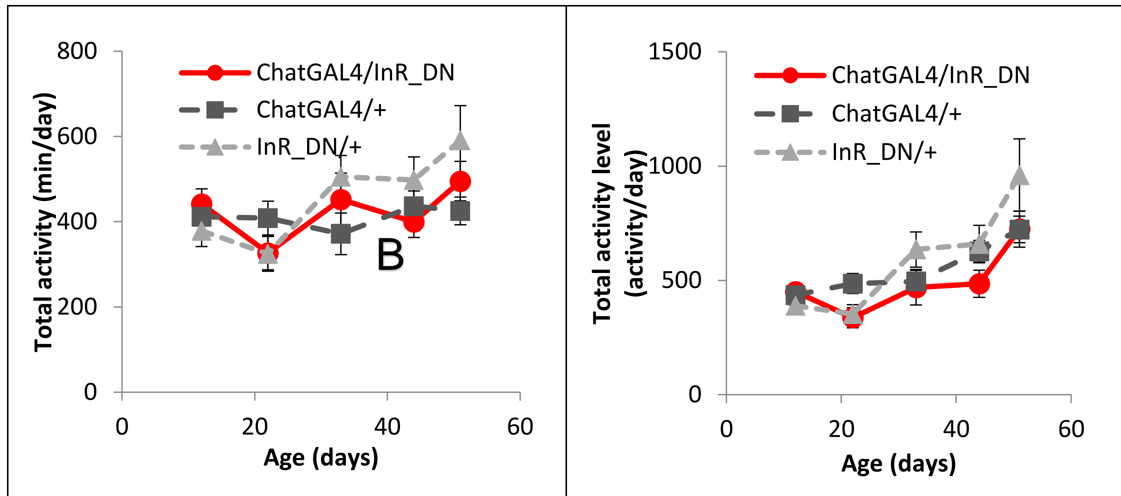
In female flies, age had a significant effect in most parameters apart from total sleep in light according to GLM (**Figure 68**). The Tukey-Kramer HSD test shows that the experimental group with reduced IIS in their cholinergic neurons have longer sleep bouts in dark at age 22 ($p=0.0020$ compared to ChAT-GAL4/+ and $p=0.0067$ compared to UAS-InR^{DN}/+) days and 44 days ($p=0.0210$ compared to ChAT-GAL4/+ and $p=0.0261$ compared to UAS-InR^{DN}/+). The number of sleep bouts was reduced compared to one of the controls between age 12-33 days, but not from both controls at the same time. The other parameters were unaffected.

In males, age had a significant effect on all parameters according to GLM (**Figure 69**). Even though GLM suggested significant Genotype or Genotype*Age effect, the Tukey-Kramer HSD test did not show that the experimental group is significantly different from both controls.

Overall, reducing IIS in cholinergic neurons only affected the length of the sleep bouts in dark in female flies and it had no effect on males.

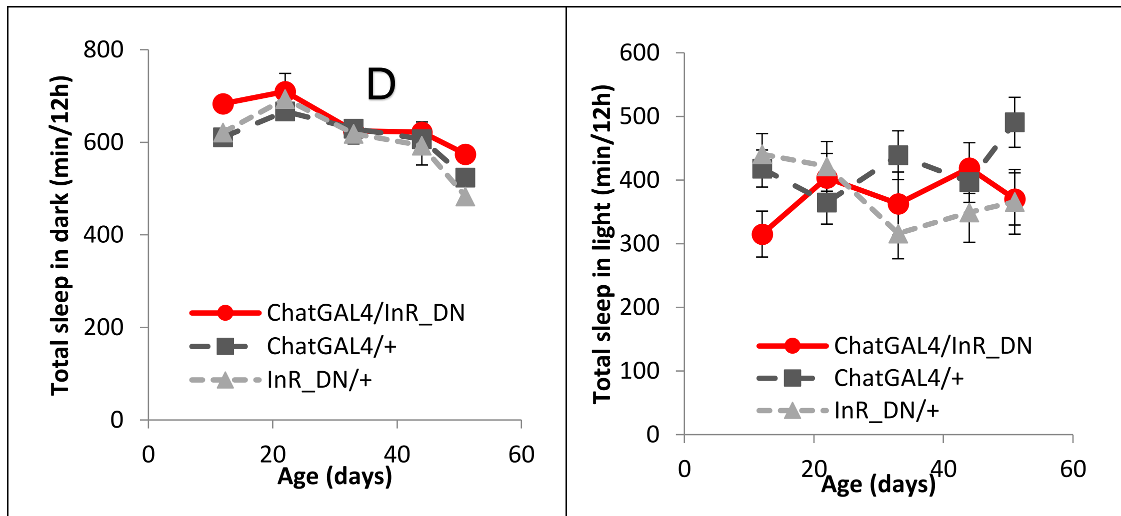
Cholinergic females

A



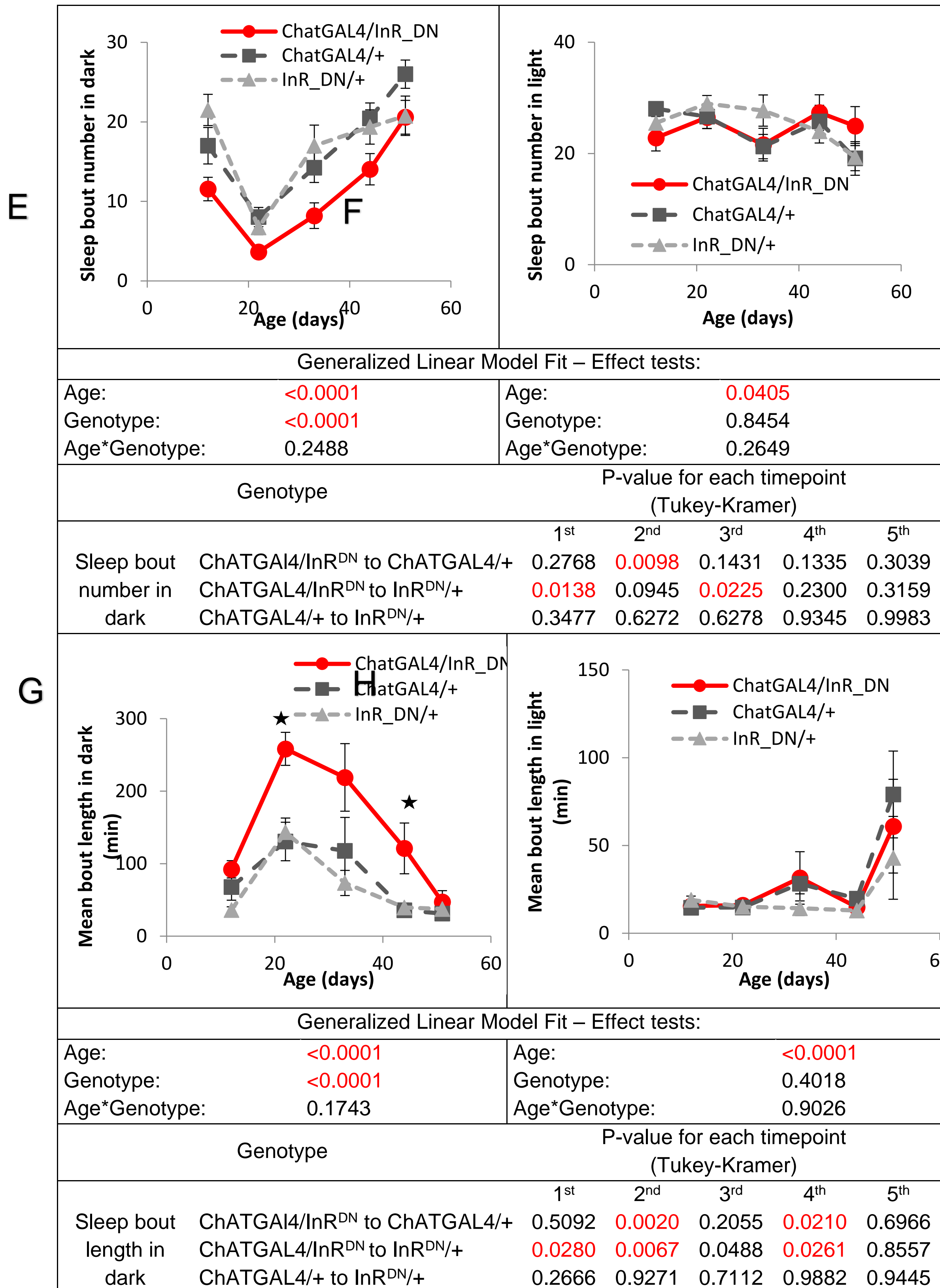
Generalized Linear Model Fit – Effect tests:			
Age:	0.0077	Age:	<0.0001
Genotype:	0.2691	Genotype:	0.0798
Age*Genotype:	0.2043	Age*Genotype:	0.2517

C



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.8768
Genotype:	0.0137	Genotype:	0.1490
Age*Genotype:	0.5600	Age*Genotype:	0.1162

Genotype		P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Total sleep in dark	ChATGAl4/InR^{DN} to ChATGAL4/+	0.0739	0.0007	0.9922	0.8633	0.6025
	ChATGAL4/InR^{DN} to InR^{DN}/+	0.1117	0.3175	0.9866	0.5773	0.1644
	ChATGAL4/+ to InR^{DN}/+	0.9341	0.0358	0.9549	0.8703	0.7099



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.0405
Genotype:	<0.0001	Genotype:	0.8454
Age*Genotype:	0.2488	Age*Genotype:	0.2649

	Genotype	P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Sleep bout number in dark	ChATGAl4/InR^{DN} to ChATGAL4/+	0.2768	0.0098	0.1431	0.1335	0.3039
	ChATGAL4/InR^{DN} to InR^{DN}/+	0.0138	0.0945	0.0225	0.2300	0.3159
	ChATGAL4/+ to InR^{DN}/+	0.3477	0.6272	0.6278	0.9345	0.9983

Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	<0.0001
Genotype:	<0.0001	Genotype:	0.4018
Age*Genotype:	0.1743	Age*Genotype:	0.9026

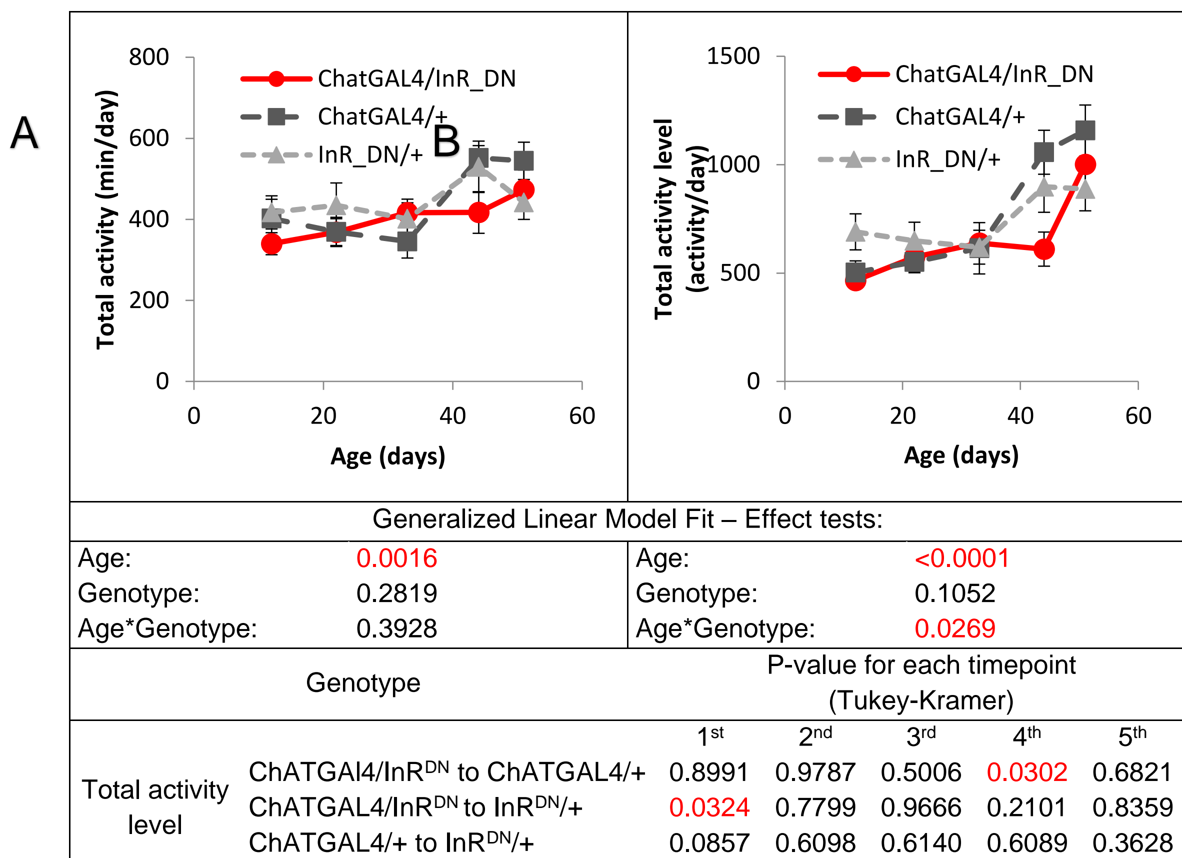
	Genotype	P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Sleep bout length in dark	ChATGAl4/InR^{DN} to ChATGAL4/+	0.5092	0.0020	0.2055	0.0210	0.6966
	ChATGAL4/InR^{DN} to InR^{DN}/+	0.0280	0.0067	0.0488	0.0261	0.8557
	ChATGAL4/+ to InR^{DN}/+	0.2666	0.9271	0.7112	0.9882	0.9445

Figure 68 - Effect of constitutive IIS reduction in cholinergic neurons on the sleep behaviour of female flies

The activity of the mated female flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as 'sleep'. Flies counted as 'dead' if they show less than 100 min activity per day, N=15 for each group and timepoint. The experimental ChAT-GAL4/UAS-InR^{DN} group with constitutive pan-

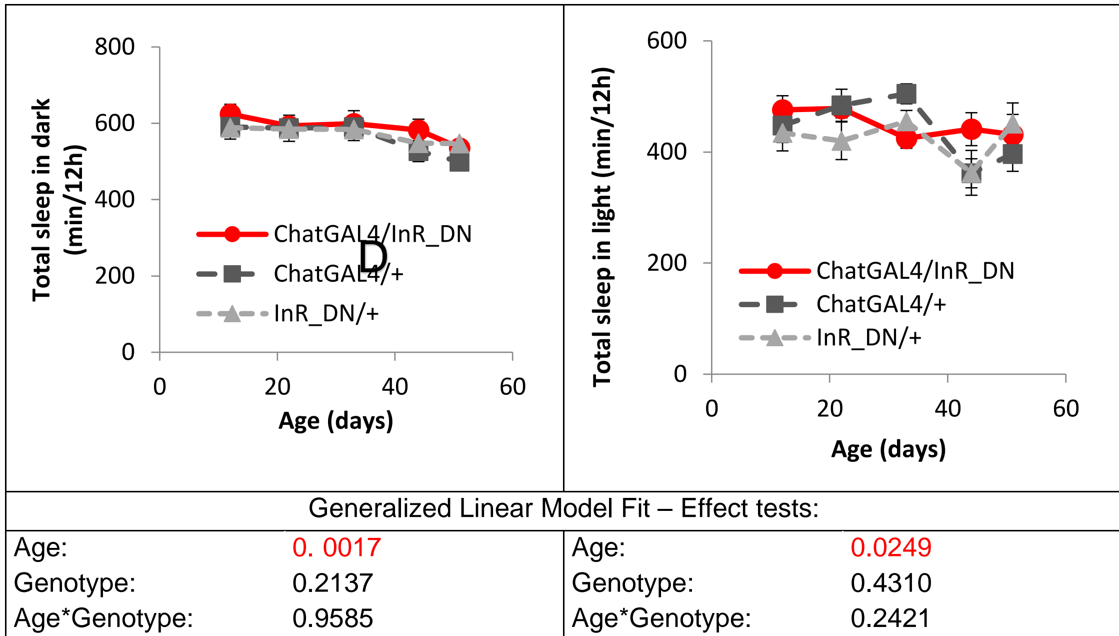
neural IIS reduction was compared to ChAT-GAL4/+ and UAS-InR^{DN}/+ control groups. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. As there was no significant (p<0.05) genotype or age*genotype effect in this experiment, there was no need for a post hoc test pairwise comparation.
A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

Cholinergic males

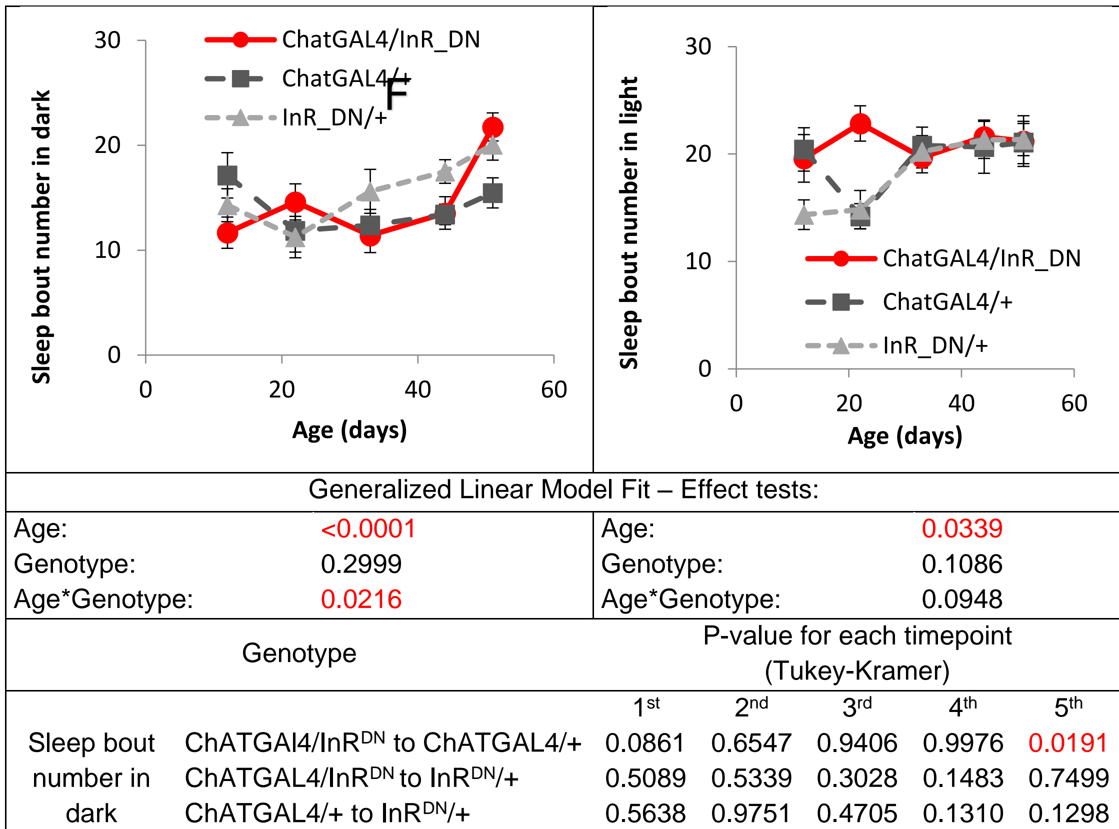


Generalized Linear Model Fit – Effect tests:			
Age:	0.0016	Age:	<0.0001
Genotype:	0.2819	Genotype:	0.1052
Age*Genotype:	0.3928	Age*Genotype:	0.0269

Genotype		P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Total activity level	ChATGAl4/InR^{DN} to ChATGAL4/+	0.8991	0.9787	0.5006	0.0302	0.6821
	ChATGAL4/InR^{DN} to InR^{DN}/+	0.0324	0.7799	0.9666	0.2101	0.8359
	ChATGAL4/+ to InR^{DN}/+	0.0857	0.6098	0.6140	0.6089	0.3628

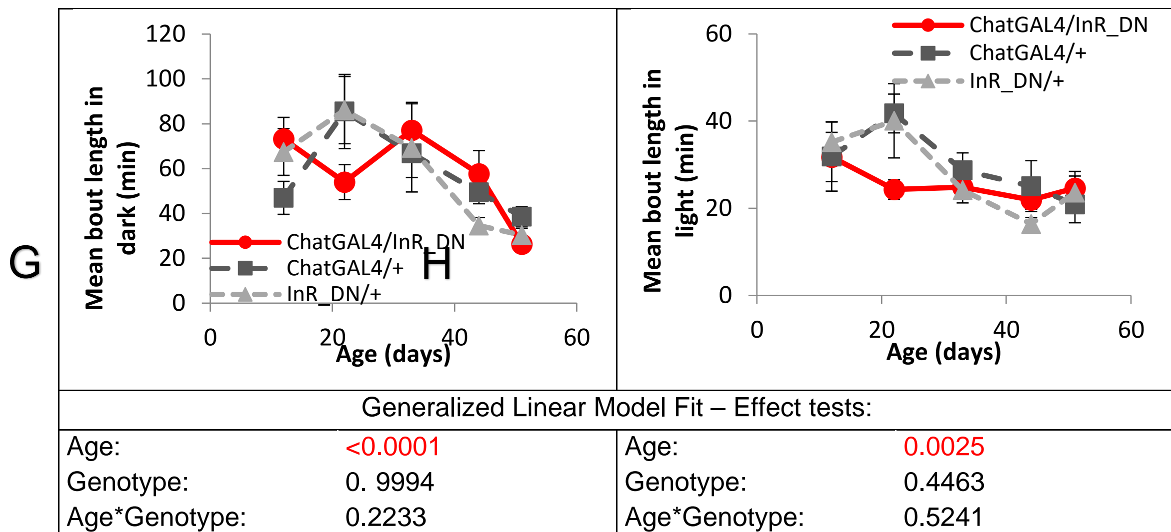


Generalized Linear Model Fit – Effect tests:			
Age:	0. 0017	Age:	0.0249
Genotype:	0.2137	Genotype:	0.4310
Age*Genotype:	0.9585	Age*Genotype:	0.2421



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.0339
Genotype:	0.2999	Genotype:	0.1086
Age*Genotype:	0.0216	Age*Genotype:	0.0948

Genotype		P-value for each timepoint (Tukey-Kramer)				
		1st	2nd	3rd	4th	5th
Sleep bout number in dark	ChATGAl4/InR^{DN} to ChATGAL4/+	0.0861	0.6547	0.9406	0.9976	0.0191
	ChATGAL4/InR^{DN} to InR^{DN}/+	0.5089	0.5339	0.3028	0.1483	0.7499
	ChATGAL4/+ to InR^{DN}/+	0.5638	0.9751	0.4705	0.1310	0.1298



Generalized Linear Model Fit – Effect tests:			
Age:	<0.0001	Age:	0.0025
Genotype:	0. 9994	Genotype:	0.4463
Age*Genotype:	0.2233	Age*Genotype:	0.5241

Figure 69 - Effect of constitutive IIS reduction in cholinergic neurons on the sleep behaviour of male flies

The activity of the male flies was recorded using DAMs for four days and Analysed using DrosoSleep software. Data were recorded as 1 minute bins, and 5 consecutive bins count as ‘sleep’. Flies counted as ‘dead’ if they show less than 100 min activity per day, N=15 for each group and timepoint. The experimental ChAT-GAL4/UAS-InR^{DN} group with constitutive pan-neural IIS reduction was compared to ChAT-GAL4/+ and UAS-InR^{DN}/+ control groups. Error bars represent +/- SEM. The data presented here are the average of day 2 and 3. The effects were tested using Generalised Linear Model Fit, looking for age and genotype effects and age*genotype interaction. As there was no significant (p<0.05) genotype or age*genotype effect in this experiment, there was no need for a post hoc test pairwise comparation.
A) Total activity per day over the lifespan (average number of minutes per day when the fly showed activity). **B)** Total activity level per day over the lifespan (how many times the flies crossed the infrared beam). **C)** Total sleep in dark over the lifespan. **D)** Total sleep in light over the lifespan. **E)** Number of sleep bouts in dark over the lifespan. **F)** Number of sleep bouts in light over the lifespan. **G)** Average length of sleep bouts in dark over the lifespan. **H)** Average length of sleep bouts in light.

10.3: Discussion

While systemic IIS reduction improves sleep at older ages by reducing sleep fragmentation in flies (Metaxakis, et al. 2014), we have shown that constitutive pan-neural IIS reduction did not alter the sleep behaviour of the flies per se or its change with age. However, when IIS was reduced only in adulthood using the elavGS system, female sleep fragmentation was increased at middle age, therefore it had detrimental effects (Chapter 6). The aim of the sleep experiments in this chapter was to investigate the effects of constitutive IIS reduction in specific neuronal subtypes in *Drosophila* on daily activity and sleep fragmentation. Previous studies have shown that all the neuronal subtypes are involved in regulating sleep-wake behaviour in various ways (reviewed by Ly, et al. 2018), thus any effect on sleep behaviour or its senescence in

flies with reduced IIS in specific neuronal subtypes could indicate how IIS influences the function and ageing of those neuronal subtypes.

In summary, we found that reduced IIS in dopaminergic and GABAergic neurons had no significant effect on sleep behaviour or its decline. Reduced IIS in glutamatergic neurons in males significantly increased total activity and total daytime sleep at young ages (12 and 27 days) and reduced IIS in cholinergic neurons in females increased the length of the sleep bouts in the dark at middle ages (22 and 44 days). Thus, constitutive reduction of IIS in specific subsets of neurons effected sleep whereas pan-neural constitutive IIS reduction did not.

Previous studies have shown that dopaminergic signalling is crucial for the regulation of arousal in *Drosophila* (Kume, et al. 2005 and Andretic et.al. 2005) and the loss of function of the D1 dopamine receptor in flies increased night sleep and reduced night sleep fragmentation (Lebestky, et al. 2009). That loss of function in the D1 dopaminergic receptor increases night sleep, raises the possibility that reduced neuronal function resulting from reduced IIS in the dopaminergic neurons could also lead to increased sleep. The sleep behaviour of flies with reduced IIS in their dopaminergic neurons in our study was, however, unaffected and declined normally suggesting that IIS in dopaminergic neurons does not influence neuronal function or ageing.

Reduction of IIS in GABAergic neurons had no effect on sleep, but the Gad1-GAL4 driver itself increased total activity and total activity levels compared to the InR^{DN} control. Since GABAergic neurons promote sleep and regulate sleep latency (Agosto, et.al, 2008), the increased total activity of Gad1-GAL4/+ flies could suggest that the driver itself reduces the function of the GABAergic neurons. This effect of the Gad1-GAL4 driver on activity, however, compromises our interpretation of the effect of InR^{DN} expression in GABAergic neurons.

A study by Yi, et.al. (2013) found that of the two groups of cholinergic neurons in the mushroom bodies of the fly brain, one is sleep-promoting and the other is wake-promoting. In our investigations, IIS reduction in cholinergic neurons resulted in longer sleep bouts in the dark period in females at middle ages compared to controls (i.e. sleep was consolidated) but sleep fragmentation still occurred at older ages similarly to controls. These data indicate that cholinergic neurons involved in modulating sleep in the dark respond to IIS, but the data further suggests that reduced IIS in these neurons does not delay or slow their normal age-related decline in function.

Collins, et.al. (2012) found that Glutamatergic Dorsal Clock Neurons play a role in regulating circadian rhythms by inhibiting light avoidance. We found that reducing IIS in glutamatergic neurons increased total activity and decreased total sleep in the light in males.

Unfortunately, we did not have the time in this project to study the effect of reduced IIS in octopaminergic and serotoninergic neurons. Octopamine has a wake-promoting function and mutations in the biosynthesis pathway of octopamine lead to increased sleep, silencing the cells producing octopamine decreased wakefulness, while stimulating those neurons increased wakefulness (Crocker and Sehgal, 2008). Serotoninergic neurons play a role in promoting sleeping as increasing serotonin levels genetically or pharmacologically enhanced sleep (Yuan, et al. 2006). Serotonin can also promote sleep in some short-sleep mutant flies (Yuan, et al. 2006). It would, therefore, be very interesting to determine how IIS changes in these neuronal types affects sleep behaviour as both neuronal types play an important role in the regulation of sleep-wake behaviour.

To summarise, sleep behaviour showed less characteristic changes with age compared to exploratory walking or negative geotaxis. Although systemic IIS reduction has been shown to reduce sleep fragmentation (Metaxakis, et al. 2014), adult specific pan-neural IIS reduction had detrimental effects on sleep fragmentation in females. Two neuronal subtypes responded to IIS reduction in a sex-specific fashion - cholinergic (females) and glutamatergic (males) suggesting that IIS in these neuronal subtypes is involved in neuronal function. Reducing IIS in GABAergic and dopaminergic neurons had no effect on sleep behaviour suggesting that IIS in these neuronal cell types is not involved in modulating their function or ageing.

Chapter 11: Discussion

The aim of this project was to investigate the role of the Insulin-IGF-like Signalling (IIS) pathway in the *Drosophila* central nervous system (CNS) during ageing by expressing a dominant negative insulin receptor and reducing IIS in all neurons or in specific neuronal subtypes and studying its effect on lifespan and behavioural senescence. Previous data from our lab (Ismail, et al. 2015) along with several previous studies in various model organisms (e.g. Vellai, et al. 2006, Tomioka, et al. 2006, Costello, et al. 2012 and Bhandari, et al. 2007) found a disconnection between lifespan and health-span in response to IIS reduction. We investigated the following two hypotheses in this study. Firstly, the negative effects seen on behavioural decline in response to IIS reduction in neurons may be caused by detrimental effects on the function of the neurons that outweighs the beneficial effects of reducing IIS on the ageing of the neurons. Secondly, it is equally possible that individual neuronal subtypes show a different response to reduction in IIS and the outcomes of IIS reduction in all neurons on behavioural decline is the sum of the positive, negative and neutral effects on each neuronal subtype.

Based on the first hypothesis, we investigated if pan-neural IIS reduction caused any reversible or irreversible changes in neurons that resulted in declines in behavioural function. We began by confirming whether or not the detrimental effects on behavioural function seen in Ismail et al (2015) due to constitutive pan-neural IIS reduction were due to detrimental effects on the CNS during development. We therefore measured the lifespan, locomotor behavioural decline and sleep behaviour of flies with a reduction in neuronal IIS only during the adult stage from the age of 3 days. Next, we tested if flies could recover from reduced neuronal IIS in adulthood with a 3 or 7 day recovery time. Lastly, we visualised apoptotic cells in the fly brain to see if reducing IIS in the neurons induces cell death. We also investigated some possible endocrine effects in response to pan-neural IIS reduction, by measuring effects on insulin-responsive phenotypes such as the expression of *Drosophila* Insulin-like peptides (DILPs) in fly heads and bodies, female fecundity, haemolymph glucose content, starvation resistance and oxidative stress resistance.

Based on the second hypothesis, we investigated the effect of IIS reduction in four of the six neuronal subtypes (dopaminergic neurons, glutamatergic neurons,

cholinergic neurons and GABAergic neurons) on lifespan, locomotor behavioural decline and sleep behaviour.

In order to reduce IIS constitutively in the neurons or specific neuronal subtypes, we used the UAS-GAL4 system. To reduce insulin signalling in adult fly neurons only and for the recovery experiments, we used the inducible GeneSwitch system. In both cases, we expressed an insulin receptor with a dominant negative mutation (UAS-InR^{DN}) that lowers the signalling through the IIS pathway.

We found that adult-specific pan-neural IIS reduction is sufficient to extend female lifespan and reversibly reduces the function of the neurons and may induce apoptosis in the brain, therefore it is not beneficial for health-span. IIS reduction in specific neuronal subtypes either has no effect or has detrimental effects on lifespan and health-span. Furthermore, we found changes in *dilp* expression in response to reduced IIS in neurons, however, we did not yet found evidence that the ageing of the neurons was slowed down and the molecular method of lifespan extension by reduced pan-neural IIS is still to be elucidated.

11.1: The role of IIS in neurons in the modulation of lifespan

It has previously been shown that reducing IIS by the ablation of IPCs (d2GAL4/UAS-rpr) extends lifespan in both male and female flies, while ubiquitous (daGAL4/UAS-InR^{DN}) or neuron specific (elavGAL4/ UAS-InR^{DN}) IIS reduction via expression of a dominant negative insulin receptor only extended female lifespan (Broughton et al, 2005; Ikeya et al, 2009; Ismail et al, 2015). Using the inducible elavGS/InR^{DN} genotype to reduce IIS in neurons from the age of 3 days throughout the lifespan of the flies, we found that female lifespan was extended similarly to that which occurs with constitutive IIS reduction in neurons. Male lifespan was slightly but significantly reduced in one experiment, although RU486 itself had significantly negative effects on longevity in that experiment. Although it is not clear whether the reduction in male lifespan was caused by reduced pan-neural IIS from adulthood or from RU486 itself, given the effect of constitutive neuronal IIS reduction on lifespan and the normal lifespan of elavGS/UAS-InR^{DN} males in a second experiment, it is likely that adult specific neuronal IIS reduction has little effect on male lifespan. Together, these data show that it is not necessary to reduce IIS in neurons throughout the development of flies to achieve its lifespan extending effect in females and reduced IIS

in adult female neurons is sufficient. Male lifespan, however, does not respond to pan-neural IIS reduction (summarised in **Table 7**).

Our results are in line with previous studies, as pan-neural IIS reduction has been shown to promote longevity in flies (Ismail, et al. 2015) and in *C. elegans* worms (Apfeld and Kenyon, 1998, Wolkow, et al. 2000, Alcedo and Kenyon, 2004). Apfeld and Kenyon, (1998) showed that mosaic worms that lost *daf-2* insulin receptor activity in a subset of neurons promotes longevity. Wolkow, et al. (2000) found that systemic mutation of *daf-2* increases lifespan in worms, while the restoring *daf-2* only in neurons reverts to wild-type lifespan, suggesting that the nervous system plays an important role in regulating lifespan. Moreover, ablation of sensory taste neurons can extend lifespan in the presence of *daf-16* (worm FOXO), probably by reducing insulin signalling (Alcedo and Kenyon, 2004).

The transcription factor FOXO is essential for the lifespan extending effect of reduced IIS. In both *C. elegans* and *Drosophila*, removal of DAF-16 or dFOXO blocks the lifespan extending effect of IIS reduction (Kenyon et al., 1993, Slack, et al. 2011). Alic, et al. 2014 studied the possible cell non-autonomous longevity promoting effect of FOXO and found that *dfoxo* to *dfoxo* signalling is not required for the antiaging effect of elevated *dfoxo* levels in the fat body. Increased *dfoxo* expression in the gut/fat body and in the neuroendocrine cells promotes healthy ageing by signalling to various other factors in the various tissues, which process is not fully understood yet. As an example, increased *dfoxo* signalling in the gut/fat body alters the expression of *dilp6* (Bai, et al. 2012) and neuropeptide-like precursor 4 (Alic, et al. 2014). The role of FOXO in pan-neural IIS reduction mediated lifespan extension is not yet known, therefore it would be interesting to measure the effect of reduced IIS in the neurons on lifespan in a dFOXO mutant background.

In order to investigate the mechanism of lifespan extension by pan-neural IIS reduction, we studied some potential endocrine and peripheral effects, namely changes in *dilp* expression, haemolymph glucose content, fecundity, starvation resistance and oxidative stress resistance.

Ismail et al. (2015) did not find any changes in dilp2-7 expression in 10 days old adult fly heads and bodies (N=3) in response to reduced IIS in the neurons using elavGAL4/UAS-InR^{DN}. Our results are in agreement with Ismail, et al. (2015), as there was no significant effect on dilp expression due to expression of UAS-InR^{DN} driven by elavGAL4 in neurons throughout development and adulthood, suggesting that the lifespan extension of elavGAL4/UAS-InR^{DN} was not due to an endocrine regulation of

dilp expression from the IPCs or elsewhere. In response to adult-specific pan-neural IIS reduction using elavGS/UAS-InR^{DN}, we measured a significant reduction in *dilp6* expression in 12 days old female heads (N=5) and bodies (N=6) and we also found a reduction in *dilp2* in the female heads (N=6). In 12 days old males, there was no effect on the *dilp* expression in the body and we found increased *dilp3* and *dilp4* expression in male heads (N=3). These changes are not because of RU486, as it increased *dilp6* in the female bodies, and had no other significant effect in males or females. **Table 8** includes a summary of the *dilp* expression results.

Grönke, et al. (2010) showed that DILPs function redundantly in a negative feedback system and the loss of one DILP can be compensated by the upregulation of other DILPs. Specifically, their research showed that there is a compensatory transcriptional regulation of *dilp*s expressed in the IPCs and there could be a negative feedback system coordinating the expression of *dilp*s between the IPCs in the brain and in the peripheral tissues, such as the fat body. Grönke, et al. (2010) also showed that single *dilp* mutants have normal lifespans except for the *dilp2* mutant, which had significantly extended lifespan in both males and females. *Dilp2–3* mutants also had an extended lifespan, and heterozygous *dilp2-3, 5* mutants were slightly long-lived, but the homozygous *dilp2–3,5* mutants and the *dilp1–4* mutants had normal lifespan. Broughton, et al. (2005) found that ablation of the median neurosecretory cells, that are responsible for producing *dilp2-3 and 5* extended the lifespan of both male and female flies. Broughton, et al. (2008) showed that the knock-down of *dilp2* leads to the compensatory upregulation of *dilp3* and *dilp5* through the IPCs and the reduction of DILP2 is not sufficient to extend lifespan, reduce fecundity or ameliorate oxidative stress resistance. On the other hand, the overexpression of *dilp6* in the adult fat body lengthened lifespan and repressed *dilp2* and *dilp5* expression in the brain and DILP2 release into the haemolymph (Bai et al. 2012).

Based on the literature, the reduced expression of *dilp6* in response to reduced adult specific pan-neural IIS reduction in long lived female heads and bodies is surprising, as *dilp6 null* mutant flies have normal lifespan (Grönke, et al. 2010) and in the studies of Bai et al. (2012), the overexpression of *dilp6* in the fat body extended lifespan and similarly, the overexpression of *dilp6* repressed *dilp2* and *dilp5* expression and DILP2 release, not its reduction. The reduction in *dilp2* expression in female heads in response to adult specific IIS reduction in the neurons could explain the lifespan extension as in the experiments of Grönke, et al. (2010) *dilp2* null mutants had extended lifespan. We did not see a compensatory increase of *dilp3* or *dilp5* in response to *dilp2* reduction as experienced by Broughton et al. (2008), which could

explain why reduced dilp2 did not promote longevity in that study. However, it is not yet confirmed that reduced dilp2 expression in the head is the main cause of extended female lifespan, as the young elvGAL4/UAS-InR^{DN} flies had normal *dilp2* level, but they are also long lived. To support the role of DILP2 reduction in the lifespan extension, we will need to quantify DILP2 protein levels.

In male flies with adult specific pan-neural IIS reduction, the expression levels of *dilp3* and *dilp4* are elevated in the heads, which could explain why males are not long lived, but the levels of *dilp3* and *dilp4* were normal in elavGAL4 flies, which are also not long lived.

Overall, changes in *dilp* expression or DILP production can affect longevity, however the expression of *dilp*s is a complex process with compensatory mechanisms and feedback loops and the detailed mechanism is yet to be understood.

The sexually dimorphic effect of reduced IIS is commonly seen with systemic IIS reductions, as males often show smaller, if any, lifespan extension (Ismail, et al. 2015, Ikeya, et al. 2009). The reason of this sexually dimorphic effect of IIS reduction is still understudied, however there are a lot of theories and research on the causes of sexual dimorphism of lifespan.

This sexual dimorphism in the longevity of organisms is due to differences in reproduction strategies, genetic composition and hormones between males and females (reviewed by Garratt, 2019). In general, the reproduction strategy of males involves high-risk and/or high wear and tear activities, therefore, evolutionarily males benefit from sacrificing longevity for increased mating success. As an example, participating in male-male combats result in cumulative somatic injuries, increasing external mortality. On the other hand, females prefer low-risk mating strategies reducing their external mortality. Based on the evolutionary theories of ageing, increased external mortality in a population can shorten lifespan, because of antagonistic pleiotropy and the accumulation of harmful mutations later in life (Bonduriansky, Maklakov, Zajitschek and Brooks, 2008). As fruit flies are not monogamic, sexual conflict between males and females also play a role in the sexual dimorphism of lifespan (Hollis, et al., 2019). Sexually antagonistic alleles are abundant in the *Drosophila* genome and the X chromosome harbours 97% of the genome-wide sexually antagonistic variations (Gibson, Chippindale and Rice, 2002). Similarly to humans, female fruit flies also have XX sex chromosomes, while males have XY. Since males only have one, “unguarded” X chromosome, recessive deleterious or lifespan shortening mutations can accumulate on the X chromosome that affects males in a

larger extent. Similarly, lifespan shortening mutations on the Y chromosome only affect males, therefore this asymmetric inheritance can lead to sexual dimorphism of lifespan (Maklakov and Lummaa, 2013). The study of Davis, Lobach and Dubal, (2018) created XX or XY chromosomes, either having ovaries of testes. They showed that XX genotype increases survival regardless of the gonads and female gonadal hormones increase lifespan in the presence of two X chromosomes. The mitochondria are also asymmetrically inherited and throughout the evolution they spent more time under female selection, therefore functions more optimally in females than in males (Tower, 2006). The IIS pathway plays a role in sexual dimorphism throughout development. As an example, IIS is required for the development of body size differences between males and females (Rideout, Narsaiya and Grewal, 2015). Belgacem and Martin, (2005) showed that the ablation of IPCs can abolish sexual dimorphism in locomotor behaviour. Transcriptomic studies of Graze, et al. (2018) showed that downregulation of the IIS pathway altered the expression of 50% of the genes, with higher impact on males (higher number of genes affected in larger magnitude). The same study showed that reduced IIS significantly affected longevity regulating pathways in females, but not in males. The sexually dimorphic effect of IIS reduction on gene expression is likely play a main role in the sexually dimorphic effect of IIS reduction on lifespan extension, however the exact mechanism is still to be elucidated.

Reduced female fecundity is a common side effect of lifespan extension by reduced IIS, for example *chico* mutant females have lower fecundity (Clancy, et al. 2001), so do long lived flies with ablated median neurosecretory cells (Broughton, et al. 2005). Ubiquitous IIS reduction is not necessary to reduce fecundity, as the overexpression of dFOXO in the adult fat body increased lifespan and reduced fecundity in females by 50% (Giannakou, et al. 2004). However, it is equally possible to uncouple increased lifespan from reduced fecundity in worms and flies. In *C. elegans*, the knockdown of DAF-2 (the worm insulin receptor) during development decreases fecundity, while the adult-specific knockdown of DAF-2 increases lifespan without affecting fecundity (Dillin et al., 2002). In flies, the overexpression of dFOXO in the adult fat body in the head extends lifespan without reducing fecundity (Hwangbo et al., 2004). In our preliminary fecundity experiment we reduced IIS in the neurons constitutively using the elavGAL4 and in adult flies only using the inducible elavGS driver to express the dominant negative dInR. We found that our long-lived females had normal fecundity, however we only measured one timepoint. So far, reduced pan-neural IIS does not seem to affect fecundity, yet it promotes longevity. However, a larger scale fecundity experiment is needed in the future to confirm our results, either

by measuring cumulative fecundity over a 3-4 week period (as done by Grönke, et al. (2010)) or measuring fecundity every 5 days throughout the lifespan of female flies (as done by Broughton, et al. (2005)). Loss of *dilp2* was shown to reduce fecundity by 25% (Grönke, et al. 2010), so did the ablation of IPCs in fly brains (Broughton, et al.2005). On the other hand, *dilp6* mutant female flies have increased fecundity and reduced juvenile hormone activity, suggesting that *dilp6* negatively regulates juvenile hormone by promoting its degradation and reducing its synthesis (Rauschenbach, et al. 2017). Therefore, reduced *dilp2* can reduce fecundity, while reduced *dilp6* increases fecundity. It is interesting to speculate in our studies that in response to adult specific pan-neural IIS reduction, the effect of the changes in these *dilps* on fecundity possibly counteracts each other and as a result we see no change in fecundity.

IIS reduction by the ablation of median neurosecretory cells increased haemolymph glucose content by two-fold (Broughton, et al. 2005). Haselton, et al. (2010) showed that IPCs are responsible for regulating acute glucose clearance response and partial ablation of IPCs can extend lifespan without insulin resistance. We measured the glucose content of haemolymph in response to constitutive and adult-specific pan-neural IIS reduction in females and found no effect on haemolymph glucose content compared to controls, however our data showed high variability. So far IIS reduction in the neurons does not seem to affect haemolymph glucose levels, but the experiment should be repeated using higher sample numbers to confirm our results.

Lifespan extension is often, but not always linked to improved stress resistance, as an example Giannakou, et al. (2004) showed that dFOXO overexpression in the adult fat body increases female oxidative stress resistance, and Broughton et al. (2005) found that long lived flies with ablated IPCs show resistance to oxidative stress and starvation. However, these flies were also more sensitive to heat shock and showed slower recovery from cold shock (Broughton, et al. 2005). Our results showed no improvement in starvation resistance in response to constitutive or adult specific pan-neural IIS reduction and we found that adult-specific IIS reduction in neurons reduced oxidative stress resistance in both genders (induced by H_2O_2). These data add to the evidence that enhanced starvation and oxidative stress resistance are not necessary for lifespan extension due to reduced IIS. Moreover, the reduced oxidative stress resistance observed in flies with reduced neuronal IIS is interesting and warrants investigation to further our understanding of the mechanisms involved in the effects of reduced IIS in neurons on function and behaviour. Unfortunately, heat and cold shock experiments did not fit into this project, but it would be interesting to test in the future

how elavGAL4/UAS-InR^{DN} and elavGS/UAS-InR^{DN} flies respond to heat and cold shock. The endocrine effects of pan-neural IIS reduction measured so far are summarised in **Table 8**.

Unlike pan-neural IIS reduction, reduced IIS in specific neuronal subtypes did not promote longevity, and in fact in most cases lifespan was shortened. Selective IIS reduction in GABAergic neurons using the Gad1GAL4/InR^{DN} genotype had no effect on the lifespan of the flies, but IIS reduction in dopaminergic (ThGAL4/InR^{DN}), cholinergic (ChATGAL4/InR^{DN}) and glutamatergic (VglutGAL4/InR^{DN}) neurons shortened the lifespan. We only tested four out of the six neuronal subtypes, so in order to see the full picture, the effect of reduced IIS in serotoninergic and octopaminergic neurons on lifespan needs to be measured in the future. As the ChATGAL4 driver affected the health of the flies, the lifespan experiment needs to be repeated with a different cholinergic driver.

Overall, while pan-neural IIS reduction extended lifespan in female flies, the reduction of IIS in specific neuronal subtypes had no or detrimental effects on longevity. Currently the mechanism of lifespan extension by pan-neural IIS reduction is not known, so we can only speculate why reduced IIS in neuronal subtypes could not increase lifespan. Since we did not have the time in this project to test all the neuronal subtypes, it is possible that we did not modulate IIS in the neuronal subtype that is involved in modulating longevity. Ly, et al. (2016) showed that flies lacking octopamine have a shorter lifespan and increased rate of insulin release, therefore octopaminergic neurons are an interesting target for IIS reduction to promote longevity. It is equally possible that reduced IIS in all neurons is required to promote longevity, especially if reduced IIS in neurons slows down the ageing of the neurons and neurons are limiting for lifespan. If this is the case, reducing IIS in only a subset of neurons may not be sufficient to affect the lifespan of the whole organism. Reducing IIS in a subset of neurons may also lead to dysregulation of the health and function of the brain, shortening lifespan. The lifespan measurements are summarised in **Table 7**.

<u>Table 7</u> - Lifespan summary

The effects of ubiquitous (Ismail, et al. 2015), pan-neural and neuronal subtype specific IIS reduction on lifespan in *Drosophila*

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

Table 8 - Some endocrine effects of reduced pan-neural IIS
The effects of pan-neural IIS reduction on *dilp* expression, haemolymph glucose content, female fecundity, starvation and oxidative stress resistance.

Endocrine effect	Constitutive IIS reduction	Adult-specific IIS reduction
dilp expression	• **Bodies:** no effect • **Heads:** no effect	• **Bodies:** Reduced dilp6 in females, no effect in males • **Heads:** reduced dilp6 and dilp2 in females, increased dilp3 and dilp4 in males
Haemolymph glucose	No effect	No effect
Female fecundity	No effect	No effect (RU486 reduces)
Starvation resistance	No effect	No effect (RU486 reduces)
Oxidative stress resistance	No effect	Reduced in both males and females

11.2: Locomotor behavioural decline

Lifespan extension by reduced IIS is sometimes correlated with improved locomotor performance with age. For example, long lived *chico* (insulin receptor substrate) mutant flies show slower age-related negative geotaxis decline (Martin and Grotewiel, 2006), similarly to other fly mutants with ubiquitously reduced IIS, such as pdk-1 (phosphoinositi-dedependent kinase-1), Dp110 (the catalytic subunit of the PI3 kinase) and Akt (protein kinase B) (Jones et al., 2009). Overexpression of *dFOXO* and its target 4E-BP in muscles also extends the lifespan of flies and delays muscle functional decay (Demontis and Perrimon 2010). However, increased lifespan is not always accompanied by improved function, for example long lived DR flies do not have ameliorated negative geotaxis and odour avoidance with ageing (Bhandari, et al. 2007).

The behaviour most commonly used to measure locomotor behavioural decline in *Drosophila* is negative geotaxis, but improvement in negative geotaxis does not indicate improved cognitive function. Amelioration of negative geotaxis by reduced IIS has been shown to be due to the effects of IIS in peripheral tissues and not due to effects on the CNS (Ismail et al, 2015). Increased dFOXO and 4E-BP signalling in muscles delays muscle functional decline and extend lifespan (Demontis and Perrimon, 2010), therefore ubiquitous IIS reduction can increase dFOXO nuclear

localisation in muscles and delay muscle functional decay, increasing climbing speed and negative geotaxis performance.

Another locomotor behaviour, exploratory walking is a better indicator of cognitive function. Some of the exploratory walking parameters can be used as indicators of peripheral function, such as walking speed or total distance walked, while other parameters, like duration in central zone are based on decision making and brain function (Ismail, et al. 2015). Ismail, et al. (2015) showed, that while the ablation of insulin producing cells (IPCs) in the fly brain improved negative geotaxis in both genders, it did not ameliorate the age-related decline in any of the exploratory walking parameters. When IIS was reduced using a dominant negative insulin receptor mutant expressed ubiquitously (daGAL4/InR^{DN}), negative geotaxis only improved in long-lived females, along with slower decline in exploratory walking parameters that are based on peripheral function (e.g. muscle function), namely total distance and velocity. However, normally lived males had no improvement in negative geotaxis or exploratory walking. When IIS was reduced constitutively in fly neurons, there was no improvement in negative geotaxis, and detrimental effects on multiple exploratory walking parameters (both peripheral health and decision-making parameters) (Ismail et al, 2015). This indicates that ubiquitous IIS reduction improves negative geotaxis and some exploratory walking parameters due to delayed ageing of peripheral tissues that influence walking speed (Ismail, et. al 2015). As shown by Demontis and Perrimon (2010), overexpression of FOXO in muscles promotes longevity and improves muscle function with age. Therefore, muscles respond positively to IIS changes and are largely responsible for the improved function. The CNS, however plays little, if any, part in the improved negative geotaxis locomotor function due to systemic IIS reduction.

The lifespan extending effect of reduced IIS is well documented, however, it is essential to fully understand the role and effects of reduced IIS in the CNS in order to find a therapeutic intervention that successfully ameliorates age related functional decline in humans. To further investigate the effect of reduced IIS on the fly CNS, we used an inducible driver (elavGS) to express the dominant negative insulin receptor in neurons from the age of 3 days in adult flies, in order to eliminate any potential negative effect of reduced IIS throughout development. We found that adult specific pan-neural IIS reduction does not affect negative geotaxis, similarly to constitutive pan-neural IIS reduction, and it had detrimental effect on both locomotor and decision-making parameters in exploratory walking. Thus, reduced IIS in adult neurons was sufficient to induce detrimental effects on exploratory walking. Male exploratory walking did not show faster decline, however RU486 itself slightly improved the exploratory walking of

males, so the detrimental effects could possibly have been masked by RU486. These results show that reducing IIS in the brain is not beneficial to behavioural function and reducing IIS in the adult flies is sufficient to induce the detrimental effects on brain function. Therefore, the negative effects of constitutive IIS reduction on behavioural senescence were not caused by developmental effects of reduced IIS. The various effects of reduced IIS on the two locomotor behaviours are summarised in **Table 9**.

Next, we wanted to determine if the detrimental effects on behavioural function were due to negative effects on function of neurons that possibly masked slowed neuronal ageing or if the effects were due to accelerated ageing of neurons. We addressed these questions by testing whether or not it was possible to restore the detrimental effects of reduced pan-neural IIS on exploratory walking by switching back to normal IIS 3 or 7 days before each exploratory walking measurement. Once again, the inducible elavGS line was used to induce and then stop the expression of InR^{DN}. Our results show that functional loss can be recovered from, but behavioural declines were not improved compared to flies with normal IIS. These data suggest that expression of the InR^{DN} transgene negatively affected the function of the neurons. Moreover, the lack of delay or slowing of functional decline in the recovery groups raises the possibility, however, that the underlying ageing of neurons has not changed due to reduced IIS. Given the known tissue specificity of IIS and FOXO in modulating ageing (Alic et al. 2014) it is possible that neuronal ageing is not influenced by reduced IIS, but there are a number of alternative interpretations of these data that require further investigation before firm conclusions can be drawn. It is possible that 7 day recovery time off RU486 is not sufficient to fully recover from the detrimental effects of reduced IIS by the InR^{DN} expression, which is further discussed in 11.4: Limitations and future directions. It is also possible, that reduced IIS has irreversible detrimental effects on neuronal function, that masks slowed neuronal ageing.

We have some preliminary findings showing that both constitutive and adult-specific IIS reduction in females significantly increased the number of apoptotic cells in the brain at old age (35 days old) compared to young flies (10 days old). There was no effect of pan-neural IIS reduction at young age and control flies did not show significantly increased number of apoptotic cells at older age. So far, these results look interesting and raise the possibility that pan-neural IIS reduction may induce apoptosis as the flies are getting older, but it does not promote apoptosis in young flies, even when IIS is reduced constitutively in neurons. As FOXO is a proapoptotic transcription factor and it is upregulated by reduced IIS (Zhang, et al. 2011), increased apoptosis in the brain could serve as an explanation for the detrimental effect of pan-neural IIS on

behaviour and brain function at older ages. Future investigation is needed to confirm these results and find more supporting evidence on neuronal cell death induced by reduced IIS, as discussed in 11.4: Limitations and future directions.

Lastly, we measured negative geotaxis and exploratory walking decline in response to reduced IIS in specific neuronal subtypes. Due to limitations of time and availability of stocks, only four out of the seven neuronal subtypes were measured (we measured dopaminergic, glutamatergic, cholinergic and GABAergic lines, but the serotoninergic, octopaminergic and histaminergic neurons are not included in this study). The results show no effect on negative geotaxis or exploratory walking in response to reduced IIS in dopaminergic, glutamatergic and GABAergic neurons, despite the shortened lifespan of dopaminergic and glutamatergic flies. These data show that shortened lifespan does not necessarily lead to decreased behavioural function, further supporting the disconnection between IIS modulated lifespan and behavioural health-span. Reduced IIS in cholinergic neurons had detrimental effects on both male and female negative geotaxis and negatively affected the exploratory walking of young (13 days old) males. However, the ChAT-GAL4 cholinergic driver itself affected the lifespan and health of the flies, so the detrimental effects on the locomotor behavioural declines are at least partially due to the driver itself, and the experiment needs to be repeated with a better driver for cholinergic neurons. The results of the neuronal subtype specific IIS reduction investigations are included in **Table 9**.

It is interesting that while reduced pan-neural IIS extends female lifespan and detrimental on exploratory walking, reduced IIS in dopaminergic and glutamatergic neurons shortens lifespan with no effect on exploratory walking. Reduced IIS in GABAergic neurons had no effect on the lifespan or negative geotaxis of the flies. As the role of each neurotransmitter on regulating negative geotaxis and exploratory walking behaviour has not yet been elucidated, it is not clear why we did not see more detrimental effects on exploratory walking in response to neuronal subtype specific IIS reduction.

Table 9 - Negative geotaxis and exploratory walking summary
The effects of ubiquitous (Ismail, et al. 2015), pan-neural and neuronal subtype specific IIS reduction on the locomotor behavioural decline of *Drosophila*

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

11.3: Sleep

Metaxakis, et al. (2014) have found that *dilp2-3,5* mutant flies with reduced IIS show ameliorated sleep behaviour as they showed more daytime activity, more sleep at night and less sleep fragmentation with age. Reduced IIS in daGAL4/UAS-InR^{DN} flies did not affect sleep during daytime, but it reduced sleep fragmentation at night and increased night-time sleep. These results show that ubiquitous IIS reduction can improve sleep behaviour and reduce age-related sleep fragmentation. Cong, et al. (2015) showed that DILPs and the dInR regulate sleep behaviour as the *dInR* mutant and all *dilp* mutant flies, except dilp4 have decreased total sleep, while the upregulation of *dilp2* or the *dInR* in the nervous system increased sleep. We found reduced dilp2 expression in elavGS female fly heads, which could therefore explain why elavGS experimental female flies sleep less in the dark.

Since the effect of pan-neural IIS reduction on sleep behaviour in flies had not been measured before, we wanted to determine if reduced IIS in neurons ameliorated sleep fragmentation similarly to ubiquitous IIS reduction. Overall, our sleep experiments did not show such characteristic age-related decline as described by Koh, et al. (2006) and Metaxakis, et al. (2014), but whenever age had an effect on sleep behavioural changes, it showed a similar pattern to that in previous studies, with increased sleep fragmentation indicated by increased number and shorter bouts of sleep with age. However, in contrast to the beneficial effect of systemic IIS reduction on sleep fragmentation, constitutive pan-neural IIS reduction did not affect sleep behaviour and adult specific pan-neural IIS reduction increased female sleep fragmentation. Moreover, unlike the effect on walking behaviour, sleep fragmentation did not improve after recovery from reduced IIS. Therefore, reduced IIS in adult neurons resulted in long-term detrimental effects in females. We were unable to determine how adult-specific IIS reduction affects sleep in male flies due to the detrimental effects of RU486 itself on male sleep behaviour.

As Ly, et al. (2018) described, as all the *Drosophila* neurotransmitters are involved in regulating sleep-wake behaviour, changes in daily activity and sleep of flies with reduced IIS in specific neuronal subtypes can indicate changes in the function of the neuronal subtypes. The wake-promoting neurotransmitters are dopamine, octopamine and histamine, while the sleep-promoting ones are serotonin and GABA. The remaining two, glutamate and acetylcholine have dual function in sleep regulation (reviewed by Ly, et al 2018). We measured the sleep behaviour of flies with reduced

IIS in dopaminergic, glutamatergic, GABAergic and cholinergic neurons and we found that only IIS reduction in glutamatergic and cholinergic neurons affected sleep behaviour, which are the two neurotransmitters with dual function. Currently there is very little information known about the role of insulin signalling in specific neuronal subtypes, so it is not clear why reduced IIS in glutamatergic and cholinergic neurons affected sleep. Unfortunately, we did not have the time in this project to study the effects of reduced IIS in octopaminergic neurons on sleep behaviour. Metaxakis, et. al (2014) showed that increased daytime activity in response to systemic IIS reduction is mediated by octopaminergic signalling. The results of the sleep experiments are summarised in **Table 10**.

Table 10 - Sleep behavioural decline summary
The effects of pan-neural and neuronal subtype specific IIS reduction on the sleep behavioural decline of *Drosophila*

	Genotype		Gender	Effect on sleep
Pan-neural	elavGAL4/ UAS-InR^{DN}	Neuron specific expression of InR^{DN}	Male	No effect
			Female	No effect
	elavGS/ UAS-InR^{DN}	Neuron specific expression of InR^{DN} in adult flies	Male	No effect (RU486 has detrimental effect itself)
			Female	Detrimental at young age: Increased total activity Less sleep in dark More bouts in dark Shorter bout length in dark
Neuronal subtype specific	ThGAL4/ UAS-InR^{DN}	Expression of InR^{DN} in dopaminergic neurons	Male	No effect
			Female	No effect
	VglutGAL4/ UAS-InR^{DN}	Expression of InR^{DN} in glutamatergic neurons	Male	Increased total activity Less sleep in light
			Female	No effect
	ChATGAL4/ UAS-InR^{DN}	Expression of InR^{DN} in cholinergic neurons	Male	No effect
			Female	Longer bout length in dark
	Gad1GAL4/ UAS-InR^{DN}	Expression of InR^{DN} in GABAergic neurons	Male	No effect
			Female	No effect

11.4: Limitations and future directions

One of the main limitations of this project is the lack of a suitable temporal/inducible system for regulating gene expression in *Drosophila*. We decided to use the GeneSwitch System which induces the expression of a transgene in the presence of the steroid drug RU486 (Sofola et al. 2010). Others system, such as the tetracycline-regulated transactivator system uses doxycycline was shown to have a negative effect on the health of the flies, this system is not suitable for lifespan and health-span studies (Roman, et al. 2001) and the temperature sensitive GAL80 system is also unsuitable, as temperature changes highly affect fly lifespan and behaviour. Whereas Alic, et al. (2012) showed that RU486 does not affect the lifespan of female flies, Yamada, et al. (2016) however found that RU486 affects longevity in both genders in a dose- and diet-dependent manner. On a low nutrient diet, RU486 reduces total food consumption probably due to having an aversive taste and negatively affecting longevity, however no detrimental effect of RU486 on lifespan on high nutrient food was found. We have found that RU486 reduces starvation resistance, which could be due to reduced food consumption observed by Yamada, et al. (2016). If flies eat somewhat less from the RU486 food due to its aversive taste, they will survive shorter on starvation diet. Future studies would examine how RU486 effects feeding. However, we have experienced other effects on of RU486 (depicted in **Figure 70**) that are not due to reduced food intake, which further support Poirier et al. (2008) in the need for caution when using RU486 inducible systems in flies. In females, RU486 does did not affect lifespan, locomotor or sleep behaviour, but it reduced female fecundity and elevated the expression levels of dilp6 in female bodies (N=6). In some experiments, RU486 shortened the lifespan of males, but not consistently. RU486 somewhat changed male exploratory walking and had detrimental effect on sleep behaviour, which could recover in the lack of RU486 for exploratory walking but did not show consistent recovery in sleep behaviour. Therefore, RU486 inducible systems are not suitable to study female fecundity and should be used cautiously for lifespan and behavioural experiments, as it may affect behaviour especially in male flies.

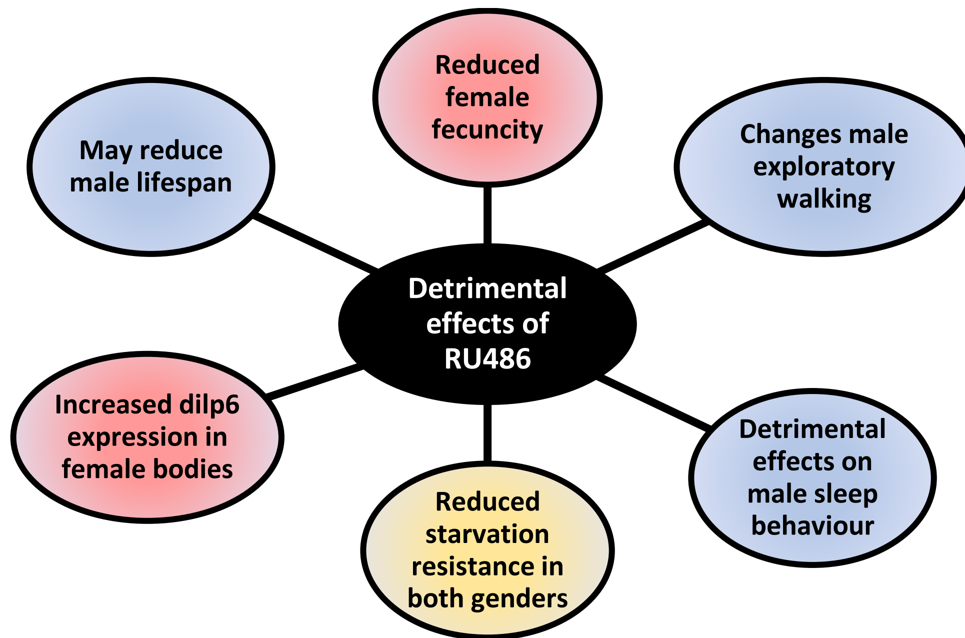


Figure 70 - The detrimental effects on RU486 based on our observations
The red bubbles indicate effects to females, the blue bubbles on males and the yellow bubble is for both genders.

The studies of Poirier, et al. (2008) showed that transgene expression levels by the GeneSwitch system are influenced by the concentration of the inducer, as well as the strain, the age and the sex of the fruit fly. The UAS-lacZ expression of the elavGS driver increased until mid-life in both male and female flies in an RU486 dose dependent manner and it continued to increase throughout the lifespan of females but dropped after the age of 21 days in males. Furthermore, females show higher transgene expression levels in general, compared to male flies (shown in **Figure 71**) (Poirier, et. al., 2008). Leakiness (transgene expression without inducer) of the elavGS driver was also observed at the age of 21 days in males and the age of 35 days in females. The elavGS driver showed leakiness in the digestive system regardless of age and gender but showed strict tissue specificity in the nervous system after induction by RU486 (Poirier, et al., 2008). In the studies of Poirier, et al. (2008) RU486 solution diluted to the appropriate concentration was added on the surface of the food and allowed to dry on room temperature for 12-36 h, while we mixed 200 mM RU486 directly into warm food. Since the administration of RU486 can cause significant differences in transgene expression (Poirier, et al., 2008) the gene expression of elavGS measured by Poirier et al. (2008) cannot be directly applied to our experiments. However, it shows a warning sign that age, sex and RU486 concentration highly affects the expression of the transgene. The lower transgene expression by elavGS in males could give a possible explanation for the different results seen on male and female

lifespan and behaviour. However, different response to IIS reduction between sexes is commonly observed, therefore the varying transgene expression by the GeneSwitch system is not the sole reason for the difference we saw between males and females.

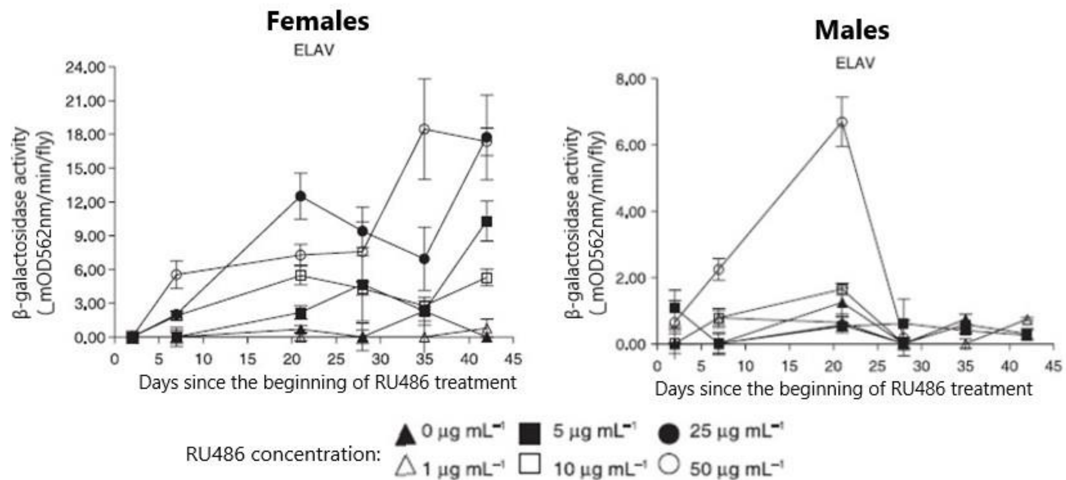


Figure 71 – Concentration, age and sex dependent transgene expression by elavGS
The expression of UAS-lacZ by the elavGS driver measured by β-galactosidase activity. Error bars represent +/- SEM (n=3-5 flies per drug concentration at each age). Figure adapted from Poirier et al. (2008)

We investigated if it is possible for flies to recover from the detrimental effects of reduced pan-neural IIS using an inducible elavGS driver to express UAS-InR^{DN}. We have found that adult specific IIS reduction has detrimental effects on the behaviour, and a 7-day recovery time can restore normal function. These data indicate that pan-neural IIS reduction reduces behavioural performance via an acute effect on neuronal function, and not via an acceleration of neuronal ageing. However, we did not find any improvement in behavioural function compared to controls at older ages suggesting that neuronal ageing was not slowed or delayed by reduced IIS. However, we cannot fully interpret these data until we determine if IIS is fully restored after 7 days of recovery from RU486 treatment. One way to measure the recovery of IIS is to measure the levels of phospho-AKT using western blotting (Wu et al. 2019). However, Western blotting is unlikely to be sensitive enough to visualise changes in AKT phosphorylation in response to reduced IIS in a subset of cells in the brain. As an indirect measure of how well IIS recovers following RU486 treatment, in future experiments phospho-AKT levels before and after 3 and 7 day recovery time will be measured in flies expressing the InR^{DN} driven by the systemic daughterless GAL4 GeneSwitch driver compared to control flies with no IIS reduction.

The expression level and phenotypic effects of a transgene can be variable based on its driver, genetic background, age and sex of the fruit flies (Poirer, et al., 2008 and Ziehm, et al. 2013). In our experiments we used the UAS-InR^{DN} transgene expressed tissue and time specifically by various GAL4 and GeneSwitch drivers. However, the level of UAS-InR^{DN} expression, therefore the level of IIS reduction can be variable based on the driver. Ismail, et al. (2015) found that the expression level of InR^{DN} in the brain by the constitutive daGAL4 driver is lower than its expression by the neuron specific elavGAL4 driver. This may be the reason why constitutive IIS reduction did not affect the locomotive behaviour of the flies detrimentally. Some of our experiments, like the *dilp* expression or sleep measurements did not find any significant effect in response to pan neural IIS reduction by elavGAL4 but did when the experiments were repeated using the elavGS driver. Unfortunately, we did not have time or resources to compare the InR^{DN} expression in the brain by the elavGAL4 elavGS drivers, but different expression level of the transgene could explain these differences and it would be interesting to see in the future. Similarly, we could not measure the expression level of InR^{DN} driven by the neuronal subtype specific GAL4 drivers. As different levels of InR^{DN} expression could be responsible for the varying effect of IIS reduction on lifespan by the neuronal subtype specific GAL4 drivers, the expression of the transgene in each neuronal subtype should be measured and compared.

We have seen that pan-neural IIS reduction in neurons can affect the expression of *dilp*s in the fly body, as females had reduced *dilp6* expression in bodies in response to adult-specific neuronal IIS reduction. It would be interesting to see if pan-neural IIS has any effect on IIS levels in the whole body, which could be done by measuring AKT phosphorylation using western blotting in the bodies of elavGAL4/UAS-InR^{DN} and elavGS/UAS-InR^{DN} flies, comparing the phospho-AKT levels to controls with no IIS reduction.

Currently, all *dilp* expression measurements were carried out using young, 10-12 days old flies. Since the behaviour of young flies were normal and the detrimental effects of reduced pan-neural IIS on exploratory walking and sleep behaviour were present at older ages, we are planning to measure *dilp* expression in 30-35 days old flies using the elavGAL4 and elavGS background to see if any of the accelerated behavioural decline are accompanied by changes in *dilp* expression.

We used an apoptosis tagging kit and fluorescent microscopy to measure the number of apoptotic cells in the fly brains in response to reduced IIS in the neurons.

So far, the results look interesting, as reduced IIS in neurons does not affect young female flies but seemed to increase the number of apoptotic cells at the age of 30 days. However, the experiment needs to be repeated and performed in both sexes to confirm the results. In the future, we are contemplating changing to flow cytometry to count the number of apoptotic cells, instead of using fluorescent microscopy, as this would give more accurate results and would be able to spot smaller differences.

Previous studies (Man, et al. 2000 and Skeberdis, et al. 2001) have found that IIS is beneficial to the function of the CNS as the insulin receptor is involved in synaptic plasticity, therefore playing an important role in cognitive function, learning and memory. Overexpression of Dp110, the catalytic subunit of PI3K in Drosophila increased the number of functional synapses in larvae and adult flies (Martín-Pen˜a et al., 2006). Overexpression of Akt also promotes synaptogenesis, similarly to downregulation of GSK3. On the other hand, overexpression of GSK3 reduces the number of synapses (Franco et al., 2004; Martín-Pena et al., 2006). We are planning to attempt to visualise changes in synapses with age using electron microscopy in response to reduced pan-neural IIS.

In this project, we used cross sectional design for all of our behavioural experiments. Exploratory walking experiments were one of the main focuses of this project and since it measures naïve behaviour in a novel environment, it has to be done cross sectionally. While sleep and negative geotaxis experiments can be done longitudinally and cross sectionally as well, we wanted to stay consistent and carry on with cross sectional studies. Following up the behavioural decline of the same flies as they age would also be really interesting, therefore longitudinal sleep and negative geotaxis experiments could be carried out in the future.

Exploratory walking is a complex behaviour involving decision making that shows robust age-related changes making it a more useful tool than negative geotaxis for measuring declines in brain function (Ismail, et al. 2015). Even though negative geotaxis is a reflex behaviour and has been shown to controlled by the brain, it is strongly dependent on muscle strength and climbing speed and is thus not a good indicator of age-related declines in cognitive function (Demontis and Perrimon 2010). While some of the exploratory walking parameters, such as walking speed are highly based on physical strength, other parameters, such as rotation frequency or duration in central zone, are based on decision making. Exploratory walking behaviour is therefore better at indicating decline in the cognitive function of flies than negative geotaxis. In the future, however, we aim to measure the age-related decline of learning

and memory in the fly model. A commonly used assay of classical conditioning in flies is the olfactory shock learning assay (Tully et al., 1994, Murakami, et al. 2010), in which the fly learns to avoid an odour previously paired with a shock. A disadvantage of this odour-based assay is that flies lose their ability to smell at a fairly young age, whilst their visual acuity is only marginally affected (Zhang, 2016). Ofstad, Zuker and Reiser (2011) developed a new learning and memory assay in *Drosophila*, based on the Morris Water Maze used in mice (Morris, 1981) – the heat maze. Instead of water, flies try to find a colder safe-spot in an uncomfortably warm arena, with the help of visual landmarks on the wall of the arena. Since the flies maintain their ability to walk and see better with age than their olfaction, this assay would be more suitable to measure the age-related decline of learning and memory than the shock/odour assay. In the future, we aim to build our own version of the heat maze to investigate the effect of reduced IIS on age-related learning and memory decline in flies.

As we only tested the response of four out of the seven neuronal subtypes to reduced IIS, it would be interesting to see how serotoninergic, histaminergic and octopaminergic neurons respond to reduced IIS. Next, the serotoninergic neuronal subtypes will be tested using TrhGAL4 (BSC: 38389), expressing GAL4 in the pattern of the tryptophan hydroxylase gene, which is involved in the synthesis of the neurotransmitter serotonin.

The next phase of our research will be focused on identifying the genes downstream of insulin that modulate ageing without affecting neuronal function and we are planning to use RNA-Seq to identify changes in the transcriptome in response to reduced IIS. Some research has already been done on transcriptomic changes in *Drosophila* during ageing but there is not much known about the transcriptional changes in the fly brain in response to IIS changes. Barter et al. (2019) compared the transcriptome of female flies at two different time points, finding that 2.1-15.7% of the expressed genes show changes with age. Birnbaum et al. (2019) measured the changes in FOXO targeting in 2 and 5 weeks old female flies and found a that FOXO-bound genes decreased from 2627 to 224 with age. Furthermore, FOXO-repressing genes are upregulated, and FOXO-activating genes are downregulated with age in fly heads. Birnbaum, et al. (2019) also found large differences between the FOXO- bound genes in wild type and *chico* mutant flies, where 1992 FOXO target genes were unique to wild-type flies and 1393 genes were unique to *chico* mutants. Large number of the genes unique to *chico* mutants were responsible for metabolism or oxidative stress reduction, while genes unique to wild-type flies were playing a role in chromatin organisation, axon guidance, Hippo and MAPK signalling. Davie et al. (2018) created

an atlas of cell types in the adult *Drosophila* brain based on 157,000 single-cell transcriptional profiles of two fruit fly strains. They also measured changes in gene expression brain-wide and found that RNA content is exponentially declining during aging in both neuronal and glial cells. Some of the genes, mainly related to the ribosome, only showed marginal decline with age, while genes involved in oxidative phosphorylation were declining faster than average. Finally, Davie et. al. (2018) was able to accurately predict cell age using machine-learning methods, based on the gene expression profile. Pacifico et al. (2018) measured the brain transcriptome changes with age in both male and female fruit flies, also finding decline in the expression of genes related to the mitochondrial oxidative phosphorylation pathway. They found continuous decline in the expression of neuronal function genes in female flies, which reversed later in life and identified deficits in short term olfactory memory performance in old male and female flies. Moskalev et al. (2019) measured the changes in the transcriptome of long-lived E(z) mutant flies. E(z) is a histone methyltransferase and a suppressor of stress response genes. Its heterozygous mutation leads to increased longevity (with no difference between the sexes), stress resistance and enhanced fecundity; accompanied by sex-specific gene expression changes. Moskalev et al. (2019) found that E(z) mutation mainly changed the expression of genes involved in carbohydrate, lipid, drug and nucleotide metabolism.

Lastly, Graze, et al. (2018) measured the effect of IIS downregulation on gene expression in male and female fruit fly heads using InR^{DN} expressed ubiquitously. They found 662 shared genes between males and females that were affected by IIS reduction, about half of them was upregulated and the other half downregulated. The reduction of IIS had a greater impact on male gene expression compared to females, as 1883 genes were upregulated and 2572 genes were downregulated in males only, while there was 135 up- and 51 downregulated genes in females only. Furthermore, they found not just greater number of expression changes in males, the differences in expression were also greater. Graze, et al. (2018) found sex-specific effects of reduced IIS by InR^{DN} expression on the *dilp* ligands. For example, they found that dilp5 is repressed in both sexes, while dilp6 is upregulated in females and downregulated in males. They also found increased endogenous InR expression in response to InR^{DN} expression in both sexes with greater increase in males compared to females. The expression of the FOXO target genes also change in a sex-dependent manner and Myc and 4E-BP was upregulated in males.

In the future, we are hoping to investigate the changes of gene expression in response to constitutive and adult specific neuronal IIS reduction in the fruit fly brain

and possibly in specific neuronal subtypes in both sexes. Genes identified in this way will then be manipulated genetically and tested for their effect on brain ageing and behavioural function.

11.5: Conclusions

Our study was based on the following three specific research questions:

(1) Are the detrimental effects of pan-neural IIS reduction on behavioural senescence caused by reduced neuronal function or accelerated neuronal ageing?

To answer this first question, we aimed to determine if pan-neural IIS reduction caused any reversible or irreversible changes in neurons that result in declines in neural function or neuronal damage. We have found that adult specific pan-neural IIS reduction causes detrimental effects on some parameters of the exploratory walking decline in females, which are reversible with 7 days recovery, although function was not improved compared to controls. This shows that neuronal ageing was not accelerated, and the detrimental effects were due to reduced function of the neurons. These experiments do not, however, show if the ageing of neurons was slowed or delayed by reduced IIS, and this question is still to be answered. Preliminary results on apoptosis in the brain suggest that there may be permanent damage to the CNS in response to reduced pan-neural IIS, as reduced IIS increased the number of apoptotic cells at the age of 35 days.

(2) How does pan-neural IIS reduction extend lifespan and what endocrine effects does it cause?

The process of lifespan extension by pan-neural IIS reduction is still not understood. Our main finding was that adult-specific IIS reduces *dilp6* expression in long lived adult female heads and bodies, and also reduces *dilp2* expression in adult female heads. Both of these changes could contribute to the lifespan extension seen in females. Reduced pan-neural IIS reduction had no effect on female fecundity, haemolymph glucose content and starvation resistance, but adult-specific IIS reduction in the neurons had detrimental effects on oxidative stress resistance in our study.

(3) Which neuronal subtypes play a role in modulating lifespan and behavioural senescence in response to altered IIS?

We tested four of the seven neuronal subtypes so far, dopaminergic, glutamatergic, GABA-ergic and cholinergic neurons and we did not find any beneficial effect on lifespan or on behavioural decline. Reduced IIS in dopaminergic, glutamatergic and cholinergic neurons shortened lifespan, but the GABAergic flies had normal lifespan. Apart from cholinergic flies where the effect was detrimental, IIS reduction in the other three neuronal subtypes did not affect the locomotor behavioural decline of the flies. The sleep behaviour was found to be normal for dopaminergic and GABAergic flies, but reduced IIS altered some sleep behavioural parameters in cholinergic and glutamatergic flies. Overall, reduced IIS in neuronal subtypes is not beneficial for lifespan or health-span of flies, and some of the neuronal subtypes are more sensitive to changes in IIS than others. The effect of reduced IIS on serotoninergic, octopaminergic and histaminergic neurons is still to be investigated.

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