Lancaster University

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

Yifan Wang

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Declaration

This thesis is entirely my own work and has not been submitted in full or in part for the award of a higher degree at any other educational institution.

No sections of this thesis have been published.

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Table of Contents

Abbreviations

- 4E-BP: 4E-binding protein
- Akt: protein kinase B
- ANOVA: analysis of variance
- *C. elegans*: *Caenorhabditis elegans*
- CA: corpora allata
- CC: corpora cardiac
- CNS: central nervous system
- *D. melanogaster*: *Drosophila melanogaster*
- d2GAL/UAS-rpr: ablation of IPCs fly model
- daGAL4/UAS-InR^{DN}: ubiquitous expression of dominant negative insulin receptor fly model
- DAPI: 4′,6-diamidino-2-phenylindole (a fluorescent dye)
- EB: ellipsoid body
- elavGAL4/+: elavGAL4 driver crossed with white Dahomey flies
- elavGAL4/UAS-InR^{DN}: neuronal specifically expression of dominant negative insulin receptor fly model
- elavGAL4/UAS-PTEN: neuronal specifically expression of overexpression PTEN phosphatase fly model
- elavGAL4: pan-neuronal driver that specifically expressed in all neurons
- elavGS/+: elavGS driver crossed with white Dahomey flies (without drug treatment)

- elavGS/UAS-PTEN: elavGS driver crossed with UAS-PTEN flies (without drug treatment)
- FB: fan-shaped body
- FOXO: forkhead transcription factor (dFOXO: *Drosophila* forkhead transcription factor)
- GAL4/UAS system: GAL4/upstream activating sequence system
- GH: growth hormone
- GS: GeneSwitch system
- hESC: human embryonic stem cells
- HSC: hematopoietic stem cells
- IGF: insulin/insulin-like growth factor
- IIS pathway: Insulin/IGF-like signalling pathway
- ILPs: insulin-like peptides (DILPs: *Drosophila* insulin-like peptides)
- InR/IR: insulin/IGF-1 receptor (dInR: *Drosophila* insulin/IGF-1 receptor)
- IPCs: insulin-producing cells
- IRS: insulin receptor substrate
- JNK pathway: Jun-NH_2-terminal kinase pathway
- LNds: dorso-lateral neurons
- LNvs: PDF-positive ventrolateral neurons
- MBONs: MB output neurons
- MBs: mushroom bodies

- MNCs/mNSCs: median neurosecretory cells
- Mth: Methuselah
- NAD: nicotinamide adenine dinucleotide
- NO: noduli
- PB: protocerebral bridge
- PBS: phosphate buffer saline
- PDPK1: phosphoinositide dependent kinase 1
- PFA: paraformaldehyde (fixation buffer)
- PI3K: phosphatidylinositol 3-kinase
- PIP_2: phosphatidylinositol (4,5)-biophosphate
- PIP_3: phosphatidylinositol (3,4,5)-triphosphate
- ROS: reactive oxygen species
- RU486: mifepristone (an inducible drug for GS system)
- RU486-elavGS/+: elavGS driver crossed with white Dahomey flies and treated with RU486 drug
- RU486-elavGS/UAS-PTEN recovery: adult-specific neuronal expression of overexpression PTEN phosphatase fly model with a 5-day recovery period before each testing time point
- RU486-elavGS/UAS-PTEN: adult-specific neuronal expression of overexpression PTEN phosphatase fly model
- SEM: standard error of mean
- SNP: single nucleotide polymorphisms

- SY: sugar and yeast
- TGF- β: transforming growth factor-β
- TOR pathway: target of rapamycin signalling pathway
- TSH: thyroid-stimulating hormone
- UAS-PTEN/+: UAS-PTEN driver crossed with white Dahomey flies
- UAS-PTEN: PTEN overexpression UAS driver
- Ub: ubiquitin molecule
- UPS: ubiquitin-proteasome system
- w^{Dah}: white Dahomey

1. Abstract

With the steady increase of human lifespan in many countries, understanding the underlying mechanisms of aging has become a crucial research interest in order to promote human health and longevity and prevent age-associated diseases. Over the last few decades, many studies have shown that ageing is modulated by the evolutionarily conserved nutrient-sensing Insulin/IGF-like signalling (IIS) pathway. IIS interacts with multiple cellular processes, including cell growth, development, metabolic homeostasis, fecundity and stress resistance. Evidence has revealed a strong connection between systemically reduced IIS and extension of lifespan, ranging from invertebrate model organisms, *Drosophila melanogaster*, and *Caenorhabditis elegans*, to mammalians, mice and even humans. Systemic mediation of components in the IIS pathway has resulted in reduced fecundity, increased lifespan and accumulation of carbohydrates and lipids. However, there is still much to be discovered regarding the regulation of different IIS components in specific tissues or organs and their roles in ageing and longevity. Overexpression of dPTEN or dFOXO (the antagonistic regulators of the IIS pathway), systemically or tissue-specifically, is reported to extend lifespan or delay specific tissue or organ ageing such as muscle and cardiac ageing. However, little is known about the role of IIS in neuronal ageing. The effects of altered IIS on all neurons in *Drosophila* was recently tested using the insulin receptor dominant-negative model (elavGAL4/UAS-InR^{DN}). This project aims to continue investigating the role of reduced IIS in neurons on brain functions, ageing, and longevity using inducible overexpression of PTEN to reduce IIS in the *D. melanogaster* model and to determine the role of reduced IIS during development and adulthood. The effects are tested through lifespan, behavioural senescence (negative geotaxis, exploratory walking, and sleeping behaviours), biochemistry and cellular experiments including stress resistance, glycogen, and lipid content assays, and apoptosis assay. Our results confirm that IIS modulated lifespan and healthspan independently and reducing IIS in neurons caused detrimental functional effects in flies.

2. Literature Review

2.1 Introduction – Ageing Research

Since the mid-nineteenth century, human life expectancy has significantly increased globally, mainly because of the largely improved public health measures, better sanitation, refrigeration, vaccination, and change of lifestyles (Partridge, 2010). With the still steadily increased human life expectancy, the majority of the global population has shifted to people at older age and population aged 60 or above is growing faster than all younger age groups. However, increased life expectancy is not always accompanied by a better healthy life expectancy and in fact, health expectancy is not increasing at the same rate as life expectancy. Infectious diseases are no longer the major causes of death while cardiovascular diseases, cancer, stroke, dementia, and so on have taken over the place and contribute to the mortality rate in an exponentially increasing rate (Wilmoth, 2000). Ageing is the major risk factor of those major causes of death. With more people living long enough to develop those diseases and experience age-related loss of functions, ageing has become a heated and challenging research area.

With the development of ageing theories and the research into ageing mechanisms, ageing is now usually defined as a complex process of the accumulation of molecular, cellular, and organ damages, leading to significant substantial functional declines, reduced fertility, and increased chance of death (Fontana et al., 2010). Yet ageing shows significant differences across species: different species age at different rates and some species do not show age-related changes in mortality and fertility (Kirkwood & Austad, 2000). The big variabilities across different tissues, individuals, and species are thought to be that ageing is caused by a complex combination of internal individual-specific mutations and different external factors (environmental exposure etc.) (Flatt and Schmidt, 2010). The complexity and variability of the ageing phenotype led to the conclusion that ageing is not an adaptive process but an unprogrammed side effect in evolution (Partridge & Gems, 2002). It is also concluded that ageing is a polygenic trait and no single gene is responsible specifically for the cause of ageing (Partridge & Gems, 2006).

Since then, further research has changed directions and identified that evolutionarily conserved single genes and pathways can modulate ageing. In recent decades, it has been discovered that mutations in single genes and pathways can extend the lifespan of model organisms.

2.2 Lifespan Extension in Model Organisms

The possibilities of lifespan modulation were first discovered during mutagenesis screening in *Caenorhabditis elegans* (worms) model back in the 1980s (Klass, 1983; Friedman & Johnson, 1988). It revealed that a single gene mutation not only can extend the length of *C. elegans*'s lifespan up to 2-fold but also accompany with improved health and senescence (Kenyon et al. 1993). Piper et al. (2008) summarized interventions of several insulin signalling (IIS) ligands (INS-1, INS-7, and INS-18) and insulin receptors (DAF-2) to extend lifespan several times more than normal lifespan in *C. elegans*. Following those ground-breaking discovery of age-1, daf-2, and daf-16 involving in lifespan extension in *C. elegans*, other mutations with similar effects on lifespan in other model organisms were soon identified. Kaeberlein et al. (1999) identified that sir2 alone, a nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylase, can positively modulate lifespan in *Saccharomyces cerevisiae* (budding yeast). In *Drosophila melanogaster* model, Methuselah [(mth), a G-protein coupled receptors] was identified that can extend lifespan and modulate stress response (Lin et al. 1998); Clancy et al. (2001) identified the loss *of* Chico*,* an insulin receptor substrate protein, can extend lifespan.

Researchers soon moved onto mammalian model organisms as well. In mice, multiple mutant models were discovered that live significantly longer than normal mice. For example, Ames dwarf mice, with a deficiency of anterior pituitary cells and thus a lack of the production of growth hormone (GH), prolactin, and thyroid-stimulating hormone (TSH) (Brown-Borg et al. 1996). Although it remains controversial, another mouse model snell dwarf mice with a *Pit1* gene mutation was also found to have a longer lifespan and more resistant to stress, but subject to experimental conditions (Liang et al. 2003). In more recent studies, Taguchi et al. (2007) have reported mice with a heterozygous deletion of insulin receptor substrate 2 (IRS2) were long-lived and present symptoms of ameliorated ageing. Homozygous deletion of the insulin receptor, heterozygotes or homozygous deletion of IRS2, and heterozygous knockout of IGF1 (insulin/insulin-like growth factor 1) all result in long-lived mice model (Pan and Finkel, 2017). Together with other studies, it has been firmly confirmed that the lifespan of different model organisms can be modulated by mutations in single genes. With those findings, there are some studies reported to test monkeys and also human volunteers with similar mutations. In a study of the biochemical and genetic variations of Ashkenazi Jewish centenarians, an overrepresentation of heterozygous mutations in the IGF1 was discovered among female centenarians (Suh et al. 2008). Genetic variants of forkhead (FOXO) transcription factors (FOXO1a and FOXO3a) show influences on human lifespan in the population-based Leiden 85-

plus Study (Kuningas et al. 2007). The GH1 SNP (growth hormone 1 single nucleotide polymorphisms) is associated with growth hormone deficiency and decreased IIS signalling which is also reported to have a significant benefit in old age survival in human (Van Heemst et al., 2005).

More importantly, age-1, daf-2, daf-16, Chico, IRS-2, FOXO, and so on are all important components of Insulin/insulin-like growth factor (*IGF*)-1 signalling (IIS) pathway in different model organisms (Fig. 2), leading to the investigation of the evolutionarily conserved role of the IIS pathway in longevity modulation. Evidence has confirmed that ageing is hormonally regulated by this evolutionarily conserved nutrient-sensing IIS pathway (Fontana et al., 2010)

2.3 The Insulin Signalling Pathway

The IIS pathway is a complex fundamental pathway that is involved in multiple cellular processes, such as the storage and synthesis of lipids, protein, carbohydrates, gene expression, cell growth, apoptosis, and so on (Fig. 1) (Riehle and Abel, 2016). It is initiated by the binding of insulin or insulin-like peptides to insulin receptor or insulin-like growth factor (IGF) tyrosine kinase receptor. The signal then triggers the activation of a network of intracellular signalling pathways such as PI3K/Akt (phosphatidylinositol 3-kinase/ the protein kinase B) directly or indirectly through insulin receptor substrates (IRS) proteins. PI3K catalyses the conversion of phosphatidylinositol (4,5)-biophosphate (PIP_2) to phosphatidylinositol (3,4,5)-triphosphate (PIP_3), leading to the cascade phosphorylation of phosphoinositide dependent kinase 1 (PDPK1), kinase Akt, and other downstream factors. Akt is a central mediator in various cellular processes (Fig. 1) such as the transcriptional activity of Bcl2 (inhibits apoptosis), mTOR (promotes somatic growth and protein synthesis), eNOS (promotes vasodilation) (Riehle and Abel, 2014). Akt can also mediate the phosphorylation of members of the forkhead transcription factor (FOXO) family in the cytoplasm and inhibit its translocation into the nucleus. FOXO is the downstream factor in the IIS pathway that has been identified to modulate longevity in reduced IIS conditions (Mathew et al. 2017).

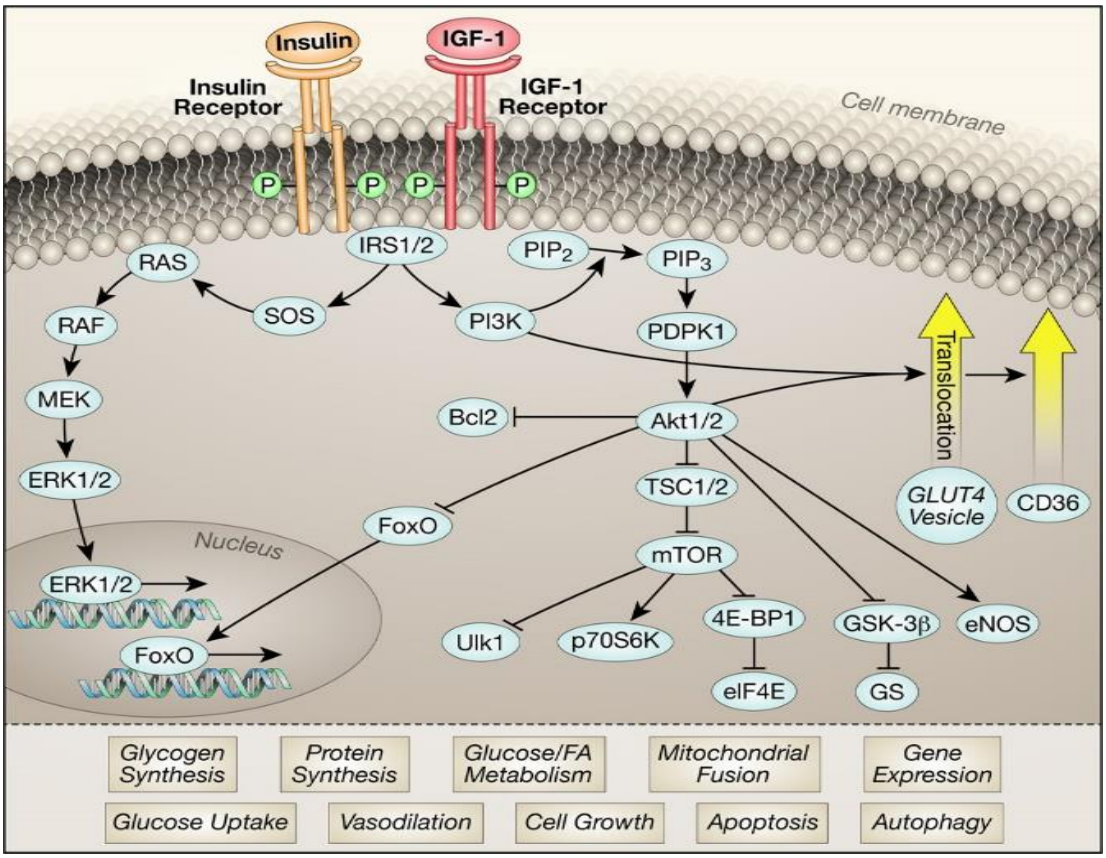


Figure 1. The network of insulin/IGF-1 (IIS) signalling pathway

A summary of the key elements involved in insulin/IGF-1 (IIS) signal transduction network and its role in different cellular processes. The pathway is initiated by the binding of the ligand (insulin or insulin-like peptides) to insulin receptor or IGF-1 receptor including receptor dimerization and autophosphorylation of the cytoplasmic domain. This results in the increase tyrosine kinase activities and the activation of IRS (IRS1/2) and PI3K. PI3K mediates the generation of PIP_3 from PIP_2 and leads to the phosphorylation of Akt and the cascade phosphorylation of different downstream targets. (Taken from Riehle and Abel, 2016).

IIS pathway is evolutionarily conserved in different model organisms ranging from invertebrates to mammalians with different levels of complexity. Different model organisms consist of different number of the components in the IIS pathway: fly and worm model contain a single insulin-like receptor, multiple insulin-like peptide ligands (7 or 38 respectively), and mainly single isoforms of the other downstream factors; mammals (mice for example) have two isoforms of insulin receptor (IR-A and IR-B), an insulin-like receptor (IGF-1), one insulin and two IGF (IGF-1,2) ligands, and many isoforms of other components (Fig. 2) (Broughton and Partridge, 2009).

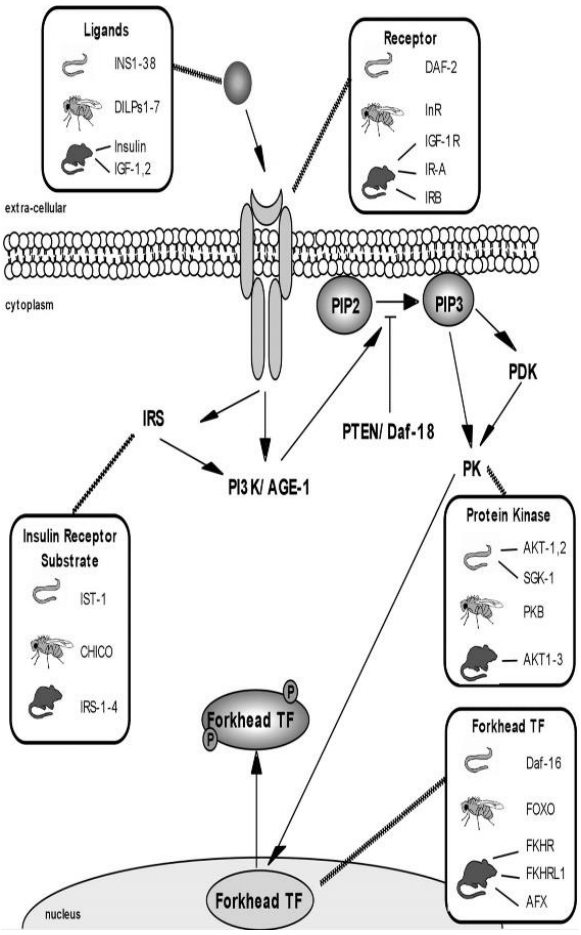


Figure 2. The insulin/IGF-1 (IIS) signalling pathway in different model organisms (worms, flies, and mice)

IIS pathway is conserved in different model organisms but is consisted of different numbers of the components. Signalling begins with the binding of ligands to the insulin receptor. The signal is then transferring either directly or indirectly through IRS to the downstream factor PI3K (or age-1 in worms) which promotes the conversion from PIP_2 to PIP_3. PTEN (or daf-18 in worms) function as the antagonist reverses PIP_3 to PIP_2. The signal then passes from PIP_3 to PDK and PK (protein kinase) which phosphorylates forkhead transcription factor (FOXO or daf-16 in worms) and leaves the nucleus. Different model organisms have different components of the pathway and it is detailed indicated as in the figure. (Taken from Broughton and Partridge, 2009).

IIS pathway also interacts with other pathways to regulate lifespan, such as the target of rapamycin (TOR) signalling pathway and the Jun-NH_2-terminal kinase (JNK) pathway (Fig. 3) (Broughton and Partridge, 2009). TOR pathway is an evolutionarily conserved pathway that controls and regulates protein synthesis and growth in response to nutrient levels (amino acids and growth factors) and reduced TOR mimics effects similar to dietary restriction and extends lifespan (Mair and Dillin, 2008). The IIS/TOR network is not yet thoroughly investigated but reduced activity of this network can extend lifespan, improve some measures of health and function during ageing, and also reduce the potential risks of specific ageing-related diseases (Partridge et al. 2011). On the other hand, JNK pathway responds to environmental-induced stress changes (such as UV irradiation, oxidative stress, and DNA damage) and the transforming growth factor-β (TGF- β) signalling pathway and it is involved in FOXO-dependent lifespan extension (Wang et al. 2005). JNK pathway functions as a regulator of IIS pathway that it restricts IIS activity, induces FOXO nuclear localization, and thus promotes lifespan extension and stress resistance (Karpac et al., 2011).

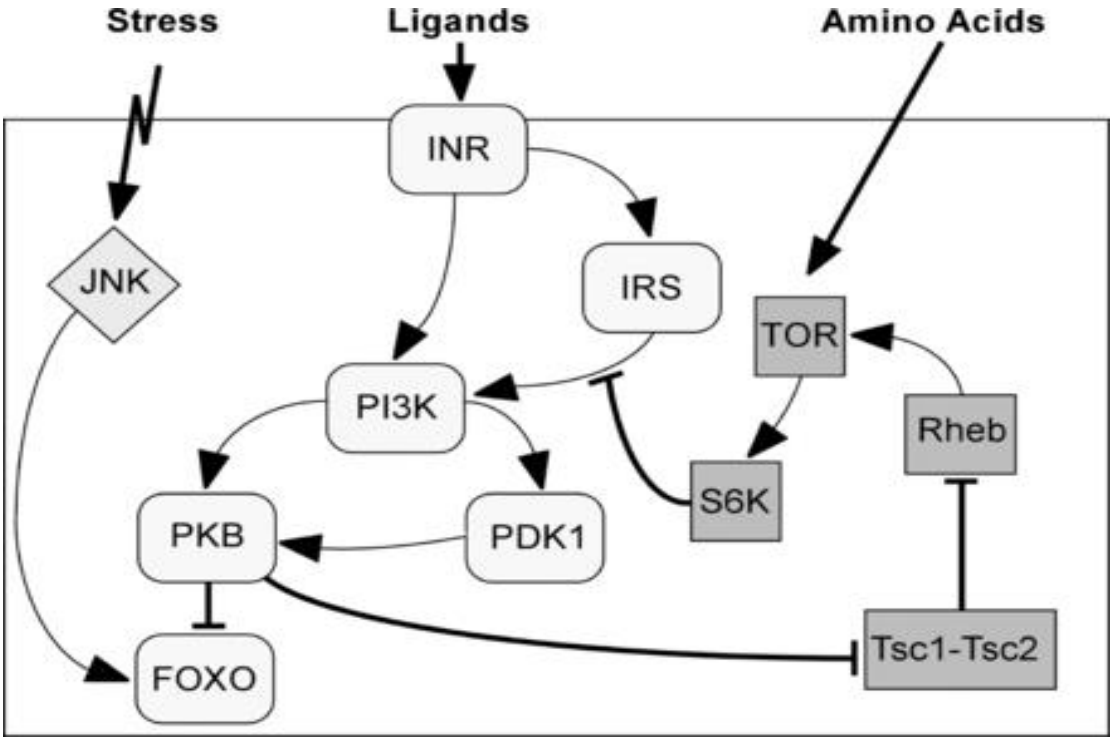


Figure 3. The IIS pathway interacts with other pathways

(1) TOR pathway is an evolutionarily conserved nutrient-sensing pathway in response to nutrient uptake (amino acids and growth factors). TOR pathway interacts with IIS pathway at multiple points: the activation of the S6 kinase (S6K) by TOR inhibits the activation of PI3K; the activation of PKB by PI3K inhibits TSC1-TSC2 (tuberous sclerosis complex 1 and 2) and forms a positive feedback loop to activate TOR pathway. (2) JNK is another conserved pathway in response to environmental stress such as UV radiation, oxidative stress, DNA damage, heat, and inflammatory cytokines. JNK pathway antagonizes the IIS pathway and promotes FOXO nuclear localization. (Taken from Broughton and Partridge, 2009).

FOXO proteins play the most critical role in IIS pathway and they are a subgroup of the Forkhead family of transcription factors which is characterized by a conserved DNA-binding domain (the Forkhead box or FOX) (Mathew et al., 2017). In the absence of growth factor or under cellular stress, FOXO transcription factors translocate into the nucleus and promote gene expressions mainly by binding the consensus core recognition motif TTGTTTAC (Martins et al., 2016). By activating the expression of target genes, FOXOs promote different cellular processes: initiating apoptosis by activating FasL ligand of the Fas-dependent cell death pathway and activating Bim of the pro-apoptotic Bcl-2 family; regulating stress resistance by upregulating Catalase and MnSOD; promoting DNA repair by upregulating GADD45 and DDB1; facilitating cell cycle arrest by upregulating the cell-cycle inhibitor p27 and GADD45; regulating glucose metabolism by expressing gluconeogenic genes such as G6Pase and PEPCK; also involves in muscle atrophy, energy homeostasis, and so on (Fig. 4) (Carter and Brunet, 2007; Ma et al., 2018; Nakae et al., 2008). FOXO proteins are tightly regulated to ensure the precise transcription of specific target genes. Apart from phosphorylation by Akt in response to growth factors or JNK upon cellular stress, FOXOs are also regulated by various post-translational modifications including acetylation or deacetylation, ubiquitination, and methylation, direct protein-protein interactions, and other binding partners (Daitoku et al., 2011).

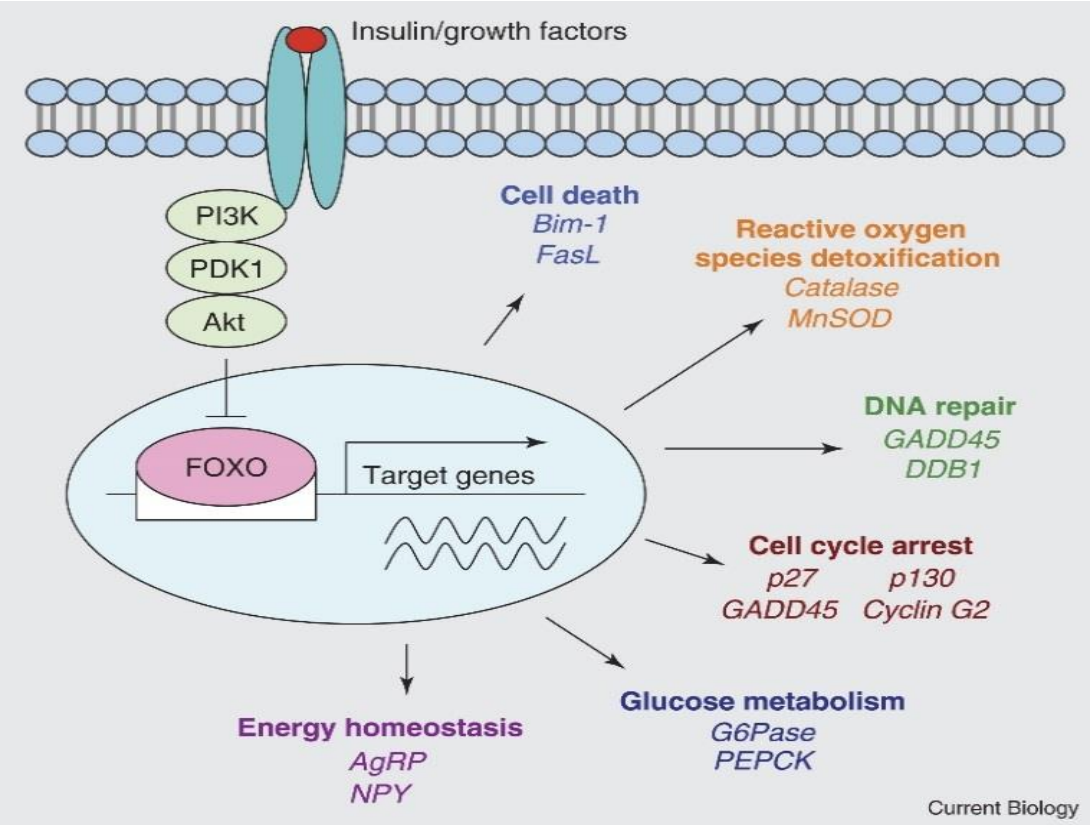


Figure 4. The cellular processes and gene expression programs regulated by FOXO

In the absence of growth factor signalling or under cellular stress, FOXOs translocate into the nucleus and initiates the expression of target genes. FOXO target genes play a role in cell death, reactive oxygen species (ROS) detoxification, DNA repair, cell cycle arrest, glucose metabolism, energy homeostasis, cellular differentiation and so on. (Taken from Carter and Brunet, 2007).

FOXOs have long been associated with longevity and homeostasis and a recent study has reported a dramatic decline in the number of FOXO-bound genes during ageing: dropping from 2617 at young ages to 224 at old ages (Birnbaum et al., 2018). However, the mechanisms by which FOXOs modulate lifespan extension still remains unclear but several mechanisms have been suggested. One of the mechanisms proposed was through the maintenance of proteostasis as FOXOs are key regulators of autophagy and the ubiquitin-proteasome system (UPS) (Webb and Brunet, 2014). Autophagy occurs to degrade and recycle cytoplasmic proteins and organelles in response to starvation and environmental stress (Simonsen et al., 2008). Reduced autophagy is one of the hallmarks of ageing and age-related disorders with the accumulation of cellular damaged cytoplasmic constituents (Toth et al., 2008). FOXOs can regulate the expression of autophagy genes in different cell types including muscle, neurons, cardiomyocytes, renal tubular cells, and hematopoietic stem cells (HSC) from flies (dFOXO) to mammals (FOXO1 and FOXO3) (Webb and Brunet, 2014). Reduced proteotoxicity is associated with the prevention of age-related damage accumulation and the promotion of longevity that the overexpression of dFOXO or 4E-BP (4E-binding protein) in muscle was sufficient to extend lifespan in flies and improve some extent of protein homeostasis in other tissues (Demontis and Perrimon, 2010). UPS is another clearance mechanism responsible for degrading including many regulated, short-lived, abnormal, and denatured proteins; through a three-step cascade mechanism, a ubiquitin molecule (Ub) is passed on from E1-activating enzyme, E2-conjugating enzyme, and E3 ubiquitin ligases to attach to the targeted protein for degradation (Lilienbaum, 2013). FOXOs are regulators for the transcription of atrophy-related muscle-specific *E3* genes, such as *atrogin-1* and *MuRF-1* (Sandri et al., 2004; Bodine and Baehr, 2014). In addition to upregulation of ubiquitin ligases, FOXOs regulate proteolysis by controlling the composition of the proteasome: FOXO4 is sufficient for the expression of the proteasome component PSMD11, the mediator of proteasome assembly and activities in human embryonic stem cells (hESC) (Vilchez et al., 2012).

Two other proposed mechanisms of FOXO-modulated longevity are based on the role of FOXOs in cellular responses to oxidative stress and stem cell regenerations (Martins et al., 2016). Oxidative stress is the dynamic equilibrium between the generation of ROS such as OH, O_2^-, H_2O_2, or O_2, and antioxidant defence mechanisms (Sander et al. 2004). Oxidative stress is one of the key factors that limit lifespan as it contributes to lifetime-accumulated damages in ageing and age-related diseases. FOXOs may influence ageing by increasing the antioxidant capacity of cells that FOXOs are regulated by oxidative stress to upstream pathways (such as JNK) and FOXOs regulate the key detoxification enzymes (such as MnSOD, GADD45, and

catalase in Fig. 4) (Storz, 2011). On the other hand, longevity is also dependent on the maintenance of tissue homeostasis, the balance of removal, regeneration, and differentiation of adult stem cells in tissues (Boyette and Tuan, 2014). FOXOs maintain the renewal of HSC through different aspects: regulating cell cycle mediators (such as p130, p27, p57, p21, cyclin G2, and cyclin G2) to prevent aberrant entry into next cycle; inhibiting apoptosis (upregulating FasL and Bim) to maintain the stem cell pool; mediating resistance to oxidative stress to maintain potential self-renewal (Tothova and Gilliland, 2007). In addition to adult stem cells, FOXOs can maintain the pluripotency of hESC directly that FOXO1 directly control the expression of OCT4 and SOX2 (key factors in the feedback circuit regulating ESC pluripotency during development (Zhang et al., 2011).

Due to the diverse functions of IIS in cell growth, development, metabolic homoeostasis, fecundity and stress resistance, manipulations of IIS can be a double-edged sword. Therefore, research is aiming to identify the optimal time and space of IIS reduction through the right manipulation of the component in the right tissue for lifespan extension and health and function improvement with the least negative peripheral effects (Broughton and Partridge, 2009).

2.4 The IIS in *Drosophila* Model

For ageing research, it is difficult to study in human as human has a longer lifespan and ethical issues involved. Therefore, enormous research into the underlying mechanisms of ageing has been using a variety of model organisms to study the evolutionary conservation of genes and pathways. The aim is to study the functions and mechanisms of those genes and pathways in longevity in model organisms and to transfer to human in the future. Fruit fly (*Drosophila melanogaster*), nematode *C. elegans*, and mouse *Mus* musculus have been largely used as model organisms that each has its advantages and limitations. *Drosophila* is a good model to use in ageing research because of its short lifespan (usually 50-80 days in laboratory conditions), fast development to adulthood, cheap maintenance, fully sequenced genome, and well-established genetic tools for gene manipulation (Groteweil, et al. 2005). It is also closer to mammal comparing to *C. elegans* that flies have a well-differentiated brain, complex behaviours, and organs including a heart, homologues of kidneys, and dioecy (Partridge et al. 2011).

The IIS pathway of *Drosophila melanogaster* starts with the binding of insulin-like peptides (DILPs) to insulin/IGF-1 receptor (dInR), triggering a cascade of intracellular phosphorylation of downstream factors and phosphorylating the transcription factor (dFOXO) through the dPI3K/dAkt pathway (Fig. 5). dPTEN functions as antagonize enzymes that negatively regulate IIS by reversing the transformation of PIP_2 to PIP_3. In *Drosophila*, the IIS pathway contains mainly single isoforms of its components including the insulin-like receptor (dInR), the insulin receptor substrate Chico, the kinase PI3K (Dp110/p60 two subunits), the protein kinase dPDK1, the protein kinase dAkt1, and the transcription factor dFOXO (Broughton et al., 2010). The diverse functions of IIS in *Drosophila* may be the result of diversified IIS ligands (DILPs). The *Drosophila* genome encodes eight DILPs that each is expressed in a distinct expression pattern that is tissue-specific and dependent on developmental stage (Fig. 6) (Nässel and Broeck, 2015).

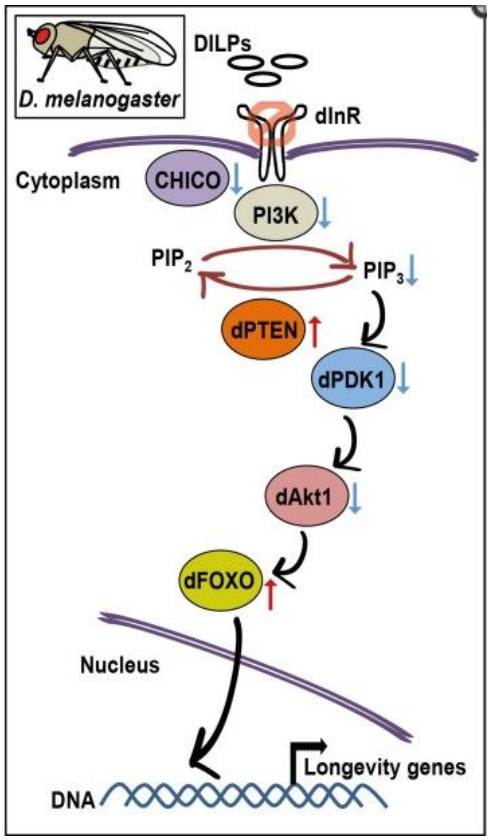


Figure 5. Conserved insulin/IGF-1 signalling pathway in *Drosophila melanogaster*
Signalling begins with the binding of insulin-like peptides, DILPs, to insulin/IGF-1 receptor, dInR. The activation of the insulin receptor directly or indirectly through Chico, activates PI3K, following by promoting the transformation of PIP_2 into the second messenger PIP_3. Elevated levels of PIP_3 subsequently phosphorylates a series of downstream factors, from dPDK1, dAkt1 to downstream transcription factor dFOXO. Phosphorylated dFOXO results in its inactivation and exclusion from the nucleus, thus, the inhibition of target longevity genes expression. Conversely, dPTEN functions as an antagonize enzymes to decrease the level of PIP_3 and its

downstream factors, leading to the activation of dFOXO and the upregulation of longevity genes expression (Taken from Altintas et al., 2016).

DILP2, DILP3, and DILP5 are the most studied peptides that they are produced by a set of median neurosecretory cells (MNCs/ mNSCs) [the insulin-producing cells (IPCs)] in the brain, which are then released into the open circulation from axon terminations in neurohemal areas of the corpora cardiac (CC) and corpora allata (CA), as well as on the surface of the anterior aorta and intestine (Liu et al., 2016). These DILPs are expressed throughout the lifespan from the embryo (mesoderm) to adults and they are produced in other cells as well. DILP2 is the closest homolog of human insulin and it is also expressed in imaginal discs and salivary glands in larva (Brogiolo et al., 2001). DILP3 is abundantly expressed in the muscle cells of the midgut in adults while DILP5 is expressed in principal cells of the renal tubules and follicle cells of the female ovary (Veenstra et al., 2008; Soderberg et al., 2011). A compensatory regulation also exists between these three DILPs that the knockdown of DILP2 leads to the upregulation of DILP3 and DILP5 while DILP3 is required for the expression of DILP2 and DILP5 (Broughton et al., 2008). DILP6 is an IGF-like peptide that it is mainly produced in the larval and adult fat body to regulate growth upon starvation; it is also expressed in larval salivary glands, surface glial cells in the ventral nerve cord (Slaidina et al., 2009; Chell and Brand, 2010). DILP7 is produced by about 20 neurons in the abdominal neuromeres of the ventral nerve cord of larvae and adults and it is important in the regulation of gut functions, tracheal growth, and fecundity (Miguel-Aliaga et al., 2008; Yang et al., 2008; Linneweber et al., 2014). DILP8 is found in the imaginal discs of larvae and plays a role in the regulation of developmental timing and growth (Colombani et al., 2012; Garelli et al., 2012). DILP8 is also released from the adult female ovary and may be important for reproductive function by signalling to Lgr3 (a G protein-coupled receptor for DILP8)-expressing neurons (Meissner et al., 2016). The expression of DILP1 and DILP4 is limited to development stages that DILP4 is only found in the embryonic midgut and mesoderm and DILP1 is expressed in MNCs in the larval brain only (Nässel and Broeck, 2015).

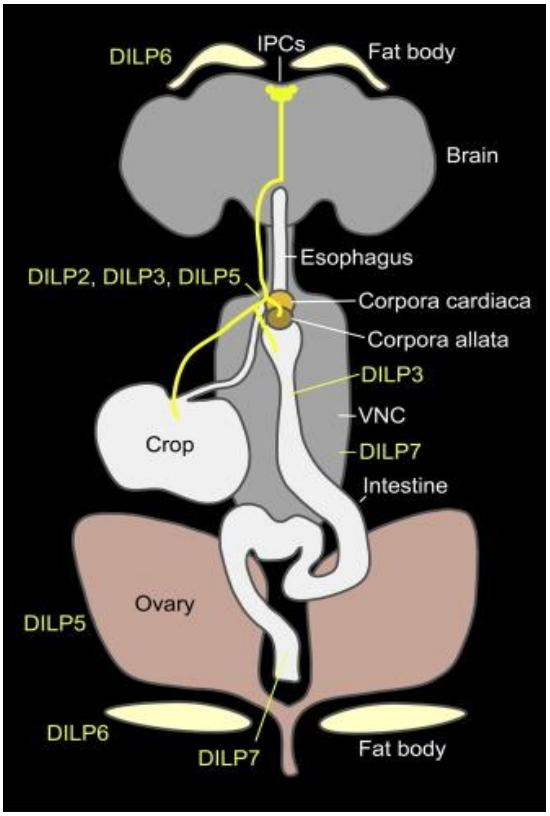


Figure 6. The summary of the production and releasing of DILPs in *Drosophila melanogaster*
DILP2, DILP3, and DILP5 are produced in the insulin-producing cells (IPCs) of the brain and released from axon terminations in the corpora cardiac (CC) and corpora allata (CA) as well as the surface of the anterior aorta and intestine. DILP3 is also found in the muscle cells of midgut while DILP5 in the ovaries and renal tubules. DILP6 is mainly expressed in the fat body as well as surface glial cells. DILP7 is produced by neurons in the abdominal ganglia then released up to the central nervous system (CNS), and down to the posterior intestine and oviduct. DILP8 is found in imaginal discs in the larva and ovaries of adults. DILP1 and DILP4 are mainly expressed in developmental stages. (Taken from Nässel et al., 2015).

Each factor in the IIS pathway plays a critical role and previous research has found that systemically reduced IIS by different points of the IIS is sufficient to extend *Drosophila* lifespan and improve some measures of health and function. Reduced IIS signalling by downregulation of dInR or loss of Chico significantly extends the lifespan of *Drosophila* by 36-85% (Tatar et al., 2001; Tu et al., 2002). The knockdown of three DILPs (DILP2, 3, and 5) individually or in combination can alter the systemic insulin signalling, resulted in reduced fecundity, increased lifespan and accumulation of carbohydrates and lipids (Broughton et al., 2005; Nässel et al., 2015). For the antagonistic regulators of the IIS pathway, like dPTEN or dFOXO, the upregulation or overexpression of them also increase the longevity and delay specific tissue or organ ageing such as muscle and cardiac ageing (Hwangbo et al., 2004; Wessells et al., 2004; Demontis and Perrimon, 2010). dFOXO also forms a feedback regulatory loop that regulates dInR receptor directly to mediate IIS signalling level (Puig et al., 2003).

Tissue-specific modulation of IIS is also sufficient to increase longevity. Hwangbo et al. (2004) demonstrated that upregulating dFOXO in the adult pericerebral fat body was enough to regulate ageing, reduce the expression of DILP2, and repress endogenous IIS signalling in the peripheral fat body. Their findings also suggested that IIS modulates ageing through both autonomous and non-autonomous ways. 4E-BP is the target of dFOXO and plays a role in proteostasis by removing damaged protein aggregates through autophagy and the upregulation of dFOXO/4E-BP can preserve physical functions at least partially by promoting the autophagy/lysosome system (Rubinsztein, 2006). The increased activity of dFOXO/4E-BP in muscles only slowed muscle functional decline and was sufficient to extend *Drosophila* lifespan; dFOXO/4E-BP signalling can mimic the protective effects of decreased nutrient intake and regulate proteostasis, the release of insulin, and the expression of 4E-BP organism-wide (Demontis and Perrimon, 2010). Giannakou et al. (2004) confirmed tissue-specific modulation of IIS can increase lifespan by the overexpression of dFOXO in adult fat body but also suggested that IIS reduction in adults was sufficient to influence longevity and fecundity. The ablation of IPCs in adult fly brains also achieved lifespan extension with additional physiological outcomes such as increased resistance to starvation (increased level of glycogen and triglyceride), reduced female fecundity, and decreased mortality rates (Broughton et al, 2005; Haselton et al., 2010). More recent results have pointed out that the life-extension effects induced by FOXO overexpression in the gut/fat body or in neuroendocrine cells are independent from the presence of FOXO in the rest of the body (Alic et al., 2014). Whether or not tissue-specific modulation of IIS can extend lifespan depending on the tissue involved and through localized FOXO activation signalling to other downstream factors (Alic et al., 2014).

Tissue-specific modulation of IIS extending lifespan was also established in other models as well such as in the worm and the mouse (Bluher et al., 2003; Alic et al. 2004). It is also suggested that IIS reduction in adulthood only is sufficient to influence longevity and avoid any potential detrimental effects during development. This has led the research focusing on tissue-specific and time-specific IIS modulation to promote longevity with minimal detrimental effects on the peripheral effects (Broughton and Partridge, 2009).

2.5 IIS and the Central Nervous System (CNS)

Little is known about the role of reduced IIS in the central nervous system (CNS) on ageing and functions. IIS plays diverse roles in the CNS and IIS reductions in the CNS can be controversial as lowered IIS can extend lifespan but may also comprise the integrity of the CNS (Broughton and Partridge, 2009).

It has been established that IIS pathway can function cell non-autonomously in the regulation of ageing (mutations in one cell type can alter the phenotype of the whole organism); the fat-specific IIS reductions in worms, mice, and flies are sufficient to increase lifespan (Russel and Kahn, 2007). The role of the CNS in IIS-induced lifespan extension is also predominately through endocrine signalling by secreting DILPs from the mNSCs and acting as a positive regulator of IIS (Broughton et al., 2005). DILP2, DILP3, and DILP5 are released from the mNSCs into the circulatory system, thus activating IIS in peripheral tissues and regulating IIS in the whole organism (Brogiolo et al., 2001). The ablation of mNSCs reduces one or more of DILPs to lower IIS systemically and extend fly lifespan, increase resistance to oxidative stress and starvation, and increase lipid and carbohydrate storage (Broughton et al., 2005). The selective alteration in some of the IPCs rather than all neurons achieved the same extent of lifespan extension, accomplished with the reduction of DILP2 mRNA levels and the level of IIS (PI3K activity) in the peripheral tissues (Fat body) (Bauer et al., 2007). As JNK pathway is involved in FOXO-dependent lifespan modulation, increases in the JNK pathway in neurons using the pan-neuronal driver (elavGAL4, a specific driver expressed in all neurons) was sufficient enough to extend lifespan and increase the tolerance of oxidative stress in neurons (Wang et al., 2003). This JNK-induced lifespan extension further indicates a role of the IPCs via the downregulation of DILP production in response to oxidative stress and JNK activation (Wang et al., 2005).

On the other hand, the cell-autonomous effects in the CNS may also play a role in IIS-mediated lifespan extension. IIS pathway has been found to play roles in CNS development and function, including axonal guidance, neurogenesis, neuronal survival, protection from apoptosis, and

cognition (Broughton and Partridge, 2009). Therefore, reducing IIS can affect its downstream target and whole-organism survival via the CNS and the neuronal survival and health.

It is possible that IIS reductions induce lifespan extension but have negative or neutral effects to CNS function and behavioural senescence. In fact, currently, there is evidence shown both negative and positive effects on behavioural functions by IIS reductions. Reduced IIS lowered the learning capacity of nematodes while increased IIS Improved their learning performance (Vellai et al., 2006). Another IIS-induced long-lived worm model did not show improvement in their long-term memory at older ages but present improved memory and improved learning capacity at young ages (Tomioka et al., 2006). The deletion of IRS-2 in mice CNS specifically has shown a decrease in NMDA receptor-dependent synaptic plasticity in the hippocampus at young ages (Costello et al., 2012). In contrast, long-lived Ames dwarf mice with deficiencies in GH showed no age-related decline in locomotor behaviours and improved age-related memory retention (Kinney et al., 2001). Similarly, long-lived fly models either Chico mutant or the overexpression of FOXO in muscles have also shown an amelioration in their negative geotaxis (a locomotor behaviour), mainly as a result of the improvements in muscle function and the effects on walking speed with age (Martin and Grotewiel, 2006; Demontis and Perrimon, 2010). Recent data has demonstrated that neural-restricted IIS reduction was sufficient to increase female lifespan in flies but show neutral or detrimental effects on the behavioural decline, suggesting the disconnection between the role of IIS in lifespan extension and behavioural senescence (Ismail et al., 2015).

2.6 UAS-GAL4 System and GeneSwitch System

To study the role of IIS signalling in specific tissues or cells, a system is needed to manipulate the level of IIS spatially and temporally. The GAL4/upstream activating sequence (UAS) system is one of the most powerful genetic tools for targeted gene manipulation, developed by Brand and Perrimon (Brand and Perrimon, 1993). This system is based on the yeast GAL4 transcription factor which can promote the transcription of the target genes by binding to UAS cis-regulatory sites (Busson and Pret, 2007). One DNA plasmid construct contains the gene-specific promoter region for the gene of interest driving the expression of the GAL4 gene, controlling the expression of GAL4 in the specific tissue based on its specific promoter; the second DNA construct contains the GAL4 binding sites (UAS) driving the expression of any given transgene that is able to express the transgene when bound by GAL4 (Fig. 7) (Nichols, 2006). Through standard embryo injection procedures, these two DNA construct can create two transgenic fly strains. Once these GAL4 and UAS strains mated with each other, the

offsprings contain both constructs, the expression of the transgene in the tissue of interest determined by the GAL4 promoter.

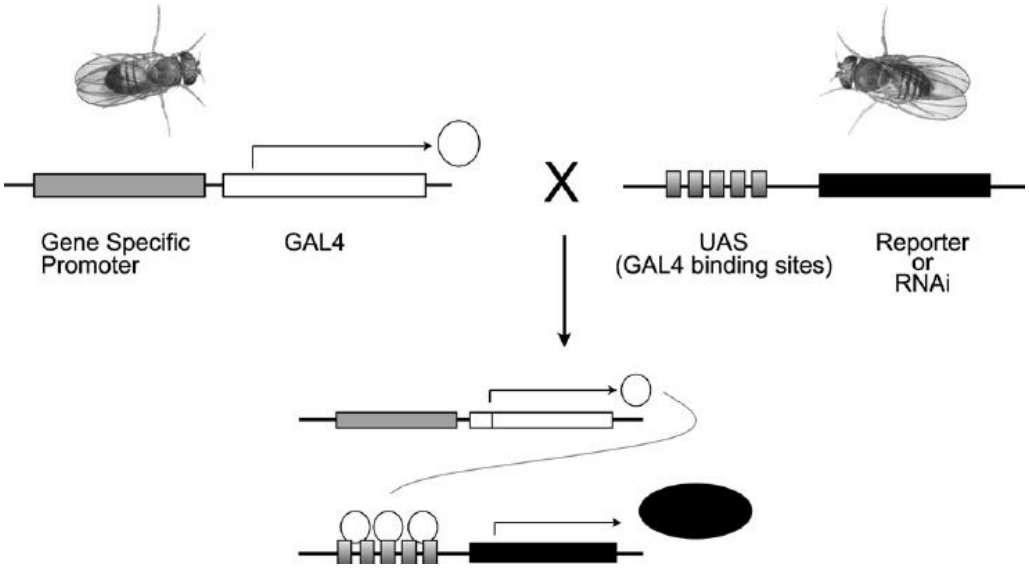


Figure 7. The diagram of the UAS-GAL4 system

This system is based on the yeast GAL4 transcription factor and its upstream activating sequence (UAS) which can remain silent until the binding of GAL4. One fly strain contains GAL4 factor driven by a gene-specific promoter that can only be expressed in specific tissue; another fly strain contains GAL4 binding sites (UAS) and a responder transgene (eg reporter gene or RNAi). By crossing the two strains, the progeny expresses the gene of interest exclusively in the tissues determined by the gene-specific promoter (Taken from Nichols, 2006).

To develop temporal control over UAS-transgene expression on top of its spatial control, a usage of conditional RU486-dependent GAL4 protein (GeneSwitch) in *Drosophila* was established (Osterwalder et al., 2001). This system is achieved by replacing GAL4 with the GeneSwitch protein that it is based on a chimeric GAL4 protein that contains the GAL4 DNA binding domain, the human progesterone receptor ligand-binding domain, and the activation domain from the human protein p65 (Roman et al., 2001). GeneSwitch transcriptional activity depends on the presence of its activator RU486 (mifepristone) that the expression of the target gene can be detected 5h after being treated with RU486 food and the transgene expression levels are dependent on the concentration of RU486 (Osterwalder et al., 2001). There are also other methods for temporal control over tissue-specific expressions in *Drosophila*, such as the tetracycline-dependent 'tet-off' or 'tet-on' transactivation systems or the GAL80 target system. However, both are limited and have adverse effects on the flies either by the requirement of continuous doxycycline or temperature, thus, unsuitable for ageing studies (Barwell et al., 2017).

2.7 Preliminary Data

To investigate the role of IIS in the ageing and function of the *Drosophila* Brain, previously Ismail et al. (2015) used three reduced IIS transgene models to study the changes in behavioural senescence (daGAL4/UAS-InR^{DN} – ubiquitous expression of dominant negative insulin receptor; d2GAL/UAS-rpr – ablation of IPCs; elavGAL4/UAS-InR^{DN} – neuronal specifical expression of dominant negative insulin receptor). Ageing research in model organisms has been largely using behavioural analysis to study the changes of functional healthspan in long-lived models. In this paper, they have compared the effects of IIS reduction ubiquitously or neuron-specifically on lifespan and locomotor behaviours. Negative geotaxis is a reflex motor behaviour as a part of escape behaviours in flies that flies respond to sudden movements and tend to climb higher. It is controlled by motor neurons, giant fibre neurons, and possibly other neurons in the CNS (Ismail et al., 2015). Exploratory walking is a more complex locomotor behaviour and controlled by the central complex and mushroom bodies of the fly brain. Different parameters of this behaviour indicate motor neurons and muscle functions such as speed and walking distance; and decision-making processes, and thus CNS function, such as orientation (direction of walking), bout structure (length and frequency of bouts), and changes in walking direction (Ismail et al., 2015).

Both systemic IIS mutants were long-lived, and results have shown ameliorated negative geotaxis senescence in both models (Ismail et al 2015). By comparison, neuronal-restricted IIS

reduction was sufficient to extend female lifespan only, suggesting that downregulating IIS in the CNS may be sufficient for lifespan extension. Despite the extension in female lifespan, there was no improvement in negative geotaxis in neuronal IIS reduction (Fig. 8). This suggests that the improvement in negative geotaxis is due to the effects on peripheral tissues rather than neural tissues (Ismail et al., 2015).

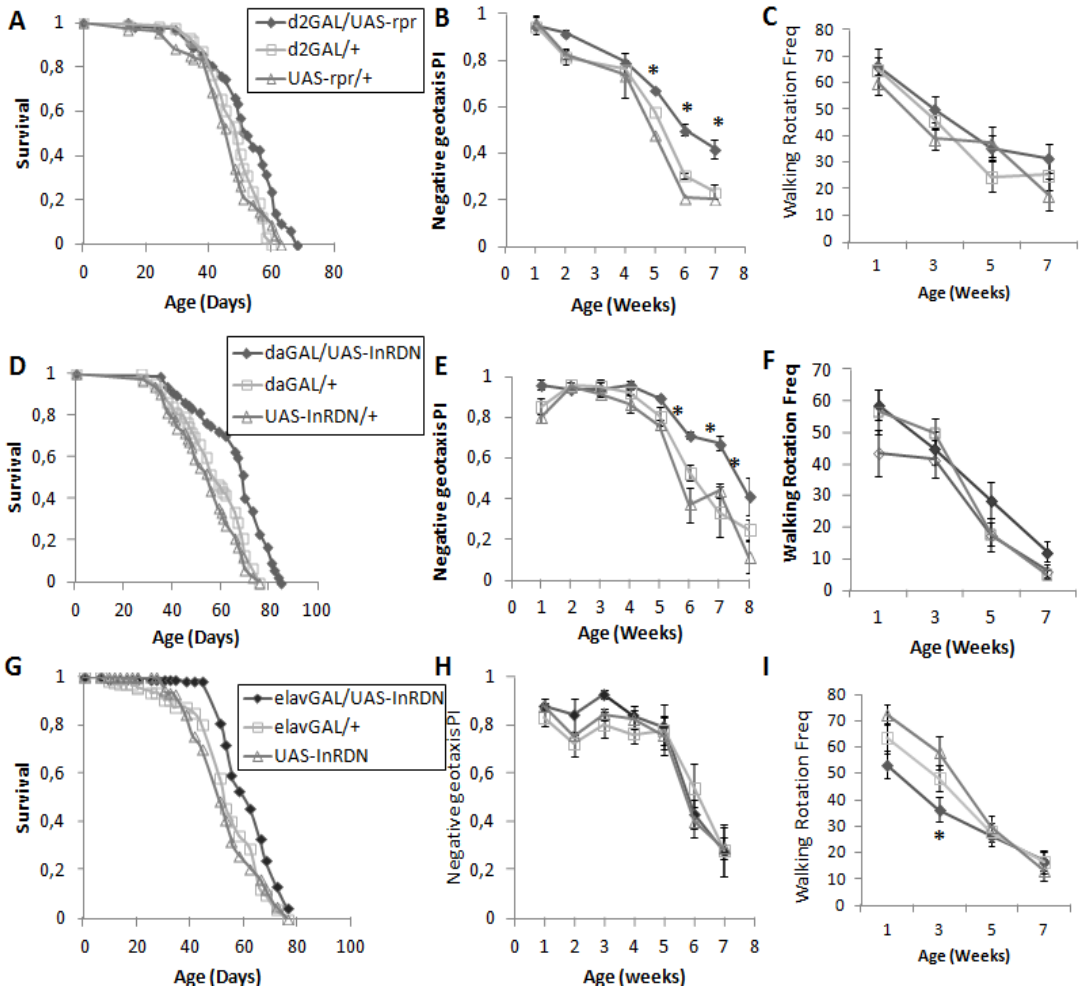


Figure 8. Lifespan and behaviour senescence of female flies with ubiquitous (d2GAL/UAS-rpr and daGAL4/UAS-InR^{DN}) or neuron specific (elavGAL4/UAS-InR^{DN}) reductions in IIS

A-C: The long-lived flies with d2GAL/UAS-rpr mutant have shown improved negative geotaxis decline but no difference in their exploratory walking decline. D-F: The long-lived flies with daGAL4/UAS-InR^{DN} mutant have shown improved negative geotaxis decline but no change in their exploratory walking decline. G-I: The long-lived flies with neuronal-specific IIS reduction (elavGAL4/UAS-InR^{DN}) has shown no change in negative geotaxis senescence but accelerated exploratory walking senescence. Rotation frequency is one parameter for exploratory walking and significant difference ($p<0.05$) is indicated by * at specific age points (Taken from Ismail et al., 2015).

For exploratory walking, there was no improvement of this behavioural decline in long-lived systemic IIS flies while there was a detrimental effect on behavioural decline in the neuronal IIS reduction models (Fig. 8). The results have shown that IIS-regulated lifespan extension can occur in the presence of normal, ameliorated, or accelerated locomotor senescence and different locomotive behaviours are independently regulated by IIS (Ismail et al., 2015). However, the exact role of IIS in CNS function and ageing still remains unclear and further research is needed to answer the question whether the negative effects on behaviour senescence is a result of accelerated ageing or detrimental functional effects on neurons.

On the other hand, sleep is another universally conserved behaviour and it is considered to be controlled by the CNS (Cirelli and Bushey, 2008). Sleep has been extensively investigated in *Drosophila* and shows fundamental characters of sleep that are similar to mammals. The important similarities include the requirement for dopaminergic and GABAergic signalling, a circadian timing mechanism, the impact of homeostatic factors, extended periods of quiescence, and elevated arousal threshold (Catterson et al., 2010). Ageing is associated with reduced duration of sleep and reduced sleep efficiency, thus, ageing-related changes in sleep patterns are intensively under investigation. Flies show ageing-related changes in sleep that are similar to humans including the increase in sleep fragmentation (more walking periods during sleep), the reduction in total sleep time, the decline in the arousal threshold (easier to wake up), and the decline in the recoverability of sleep after sleep deprivation (Vienne et al., 2016). Moreover, insulin-producing cells (mNSCs) are involved in the regulation of nutrition and sleep and in fact, day and night sleep are differentially regulated by nutrient levels and distinct mechanisms (Broughton et al., 2010). *Drosophila* sleep is characterised as a bimodal pattern that peaks both at midday and midnight and can be divided into daytime sleep and nighttime sleep. Two other type of neurons, dorso-lateral neurons (LNds) and PDF-positive ventrolateral neurons (LNvs), are also DILP-producing cells that regulate nighttime sleep and daytime sleep respectively; and daytime sleep is more sensitive to insulin signalling (Cong et al., 2015). A recent study has reported that lowered IIS can ameliorate age-related sleep fragmentation in DILP2-3, 5 mutants and daGAL4/UAS-InR^{DN} flies, suggesting that reduced IIS improves different aspects of age-induced deterioration through multiple pathways (Metaxakis et al., 2014). However, like humans, fly sleep also shows significantly interindividual variability within the same fly population and sleep can be influenced by many environmental and genetic factors, leading to the complexity of sleeping study (Cirelli and Bushey, 2008).

2.8 Aims and Objectives

The aim of this project is to continue the investigation of the role of IIS in the neuronal ageing and function of *Drosophila* by using an alternative transgene model (PTEN) in comparison to the previous results on InR^{DN} model.

The objectives are to determine the role of reduced IIS in development and adulthood in the modulation of longevity and behavioural senescence by reducing IIS in neurons throughout lifespan or in adulthood respectively. The objectives are also to answer the question of whether the effects of reduced IIS on behavioural senescence are due to functional changes or ageing changes.

2.9 Research Design

Based on previous studies, it is hypothesized that reduced IIS is not always beneficial to the neural circuitry underlying locomotor behaviours despite increasing lifespan. Therefore, this project aims to answer if the effects of neuron-specific IIS reduction on behavioural senescence are restricted to the modulation of the insulin receptor or not by using an alternative transgenic model (elavGAL4/UAS-PTEN). To investigate whether the IIS-induced effects on behavioural senescence are due to effects on neuronal function or neuronal ageing, an inducible system, GeneSwitch system, was used to control the period of IIS reduction and allow for the restoration of IIS function prior to behavioural testing. By using the inducible system, IIS reduction was restricted to adulthood only (RU486-elavGS/UAS-PTEN). The role of IIS in neuronal function and ageing during development and adulthood was determined by comparing the effect of constitutive IIS knockdown (elavGAL4/UAS-PTEN) with adult specific knockdown (elavGS/UAS-PTEN+/-RU486) and adult-specific knockdown with recovery (elavGS/UAS-PTEN+RU486 and 5-day recovery without RU486).

Lifespan was measured of each genotype flies, along with female fecundity, negative geotaxis, exploratory walking, and sleeping behaviours were measured every 10 days throughout their lifespan as the indications of their behavioural and functional senescence. To identify the effects of neuronal IIS reduction elsewhere in the body, stress resistance test (starvation and oxidative stress) and body content (glycogen and lipid content) were also measured in each genotype. For further cellular identification, an apoptosis assay was performed where fly brain tissues were dissected and fixed to observe apoptotic neurons under a confocal microscope.

3. Material and methods

3.1 The Preparation and Maintenance of *Drosophila Melanogaster*

3.1.1 Genetic Background and Maintenance of *Drosophila Melanogaster* Stock

In this project, the UAS-GAL4 system was used to modulate the level of insulin signalling in *Drosophila Melanogaster*. To study the effect of reduced insulin signalling throughout lifespan or in adulthood only, two different GAL4 drivers were used and the UAS-GAL4 crosses were set up as shown in Tab. 1.

Throughout lifespan		Adulthood only	
Experimental	Control	Experimental	Control
elavGAL4 (♀) X UAS-PTEN (♂)	elavGAL4 (♀) X w^{Dah} (♂)	elavGS X UAS-PTEN (on drug)	elavGS X w^{Dah} (on drug)
	w^{Dah} X UAS-PTEN	elavGS X UAS-PTEN (off drug)	elavGS X w^{Dah} (off drug)

Table 1. The UAS-GAL4 crosses in the experiment.

The different fly stocks used throughout the experiments are shown as in Tab. 2. All lines were backcrossed into a white Dahomey (w^{Dah}) wild-type for six generations before the experiments to control the genetic background. This wild-type background strain is an outbred strain, originally obtained in Africa (Benin) in the 1970s, maintained, then mutated with a partial deletion in the *white* (*w*) gene which leads to white eyes (Ziehm et al., 2013). All fly stocks were maintained in individual *Drosophila* bottles with sponge bungs plugged in. The bottles were changed every 3 weeks and kept at room temperature in natural light.

Type	Name	Acronym	Source	References
UAS	PTEN overexpression	UAS-PTEN	Ernst Hafen	Ikeya et al (2009)
GAL4	Pan-neuronal	elavGAL4	Bloomington stock no. 25750	(Ismail et al., 2015)
	Gene switch elav-GAL4	elavGS	Partridge lab, UCL	Rogers et al (2012)
Wild-type	White Dahomey	w^{Dah}	Partridge lab, UCL	Broughton et al (2005)

Table 2. The list of different fly stocks used throughout the project.
Stock number refers to Bloomington Stock Centre.

3.1.2 The Setup of Experimental *Drosophila Melanogaster* Flies Using UAS-GAL4 Crosses

As female flies tend to store sperms for days after copulation, to control the production of required genotype offspring for this experiment, virgin female flies were collected from the

stock first for all genetic crosses. Within 6-8 hours after eclosion, female flies are usually immature and unreceptive to male courtship that can be distinguished from older flies based on their pale colour. Collected virgin females were kept as 10 in each vial for 3-4 days to check for any fertilized eggs. 3-4 days old virgin females were mated with different males as shown in Tab. 1 in vials for 4 hours before transferring into *Drosophila* cages. For optimal egg collection, grape juice plates (Tab. 3) with live yeast paste in the centre were used as food and changed every 24 hours for 3 days. Eggs from these plates were collected with 1% PBS and transferred into *Drosophila* bottles (180µL/bottle) with a widened pipette tip. As soon as eclosion, flies were sorted into vials from the bottles with 10 flies per vial and females and males separately. Therefore, all flies used in all behavioural assays should be un-mated females and males. Different groups of flies were kept for lifespan and each behavioural assay and fresh flies were used for analysis at each time point.

3.1.3 The Maintenance of Experimental *Drosophila Melanogaster* Flies and *Drosophila* Media

All flies during the experiment were maintained in an incubator with 25°C temperature, 70% humidity, and a 12h dark/light lighting cycle. All vials were changed every 3-4 days and kept flat on their side to avoid flies sticking in the food. Unless otherwise stated, the standard sugar/yeast food (Tab. 3) was used in the maintenance of fly stocks and experimental flies both in *Drosophila* bottles and vials throughout the project. For elavGS flies, they were maintained on standard *Drosophila* food then transferred into RU486 food (Tab. 3) on day 4. Based on previous studies (Osterwalder et al., 2001), to achieve the optimal effects of RU486 in GeneSwitch system on lifespan extension, this project aimed for the concentration of RU486 at 200mM. Through calculations, 50µl of 3.2mg/ml RU486 solution was added into each fly vial containing approximately 2-3ml of standard *Drosophila* food. All RU486 food was prepared at least 48 hrs before usage. To test the behaviours of elavGS flies with a recovery period, flies were removed from RU486 food and transferred back to Standard food 5 days before testing.

	Standard *Drosophila* food 0.5xS/1.0Y	Starvation experiment food 0xSY	H_2O_2 oxidative stress experiment food	RU486 *Drosophila* food	Grape juice plate
Water (ml)	700	1000	160	700	1000 (grape juice)
Agar (g)	15	15	3	15	16
Sugar – sucrose (g)	50	0	10	50	0
Yeast – MP Biomedicals (g)	100	0	0	100	0
Water at the end (ml)	170	0	6.7	170	0
30% H_2O_2 (ml)	0	0	33.3	0	0
Nipagin (ml)	30	30	0	30	0
Propionic Acid (ml)	3	3	0	3	0
RU486 (µl)	0	0	0	50	0

Table 3. The recipes of all *Drosophila* media in the project

As for the procedures of standard *Drosophila* media, agar powder was firstly added into lukewarm water then boiled for full dissolution. 0.5xS/1.0Y (50g/l sugar and 100g/l yeast) was the ratio of sugar and yeast used in this recipe and the sugar-yeast mixture was constantly stirred and fully mixed with boiled agar solution before boiling again. After boiling, the well-mixed solution was removed from heat and cold water was added. Further nipagin and propionic acid were added as preservatives when the mixture was cooled down to approximately 60°C (nipagin was prepared by dissolving 100g nipagin powder in 1L ethanol). The mixture was then poured into vials or bottles and covered with a breathable fabric to set at room temperature for at least 1 day before using.

3.2 Lifespan

After eclosion in *Drosophila* bottles, un-mated flies of each genotype were sorted into vials as 10 flies per vial (females and males separately). N=100 or 150 was used in the lifespan experiments. Flies were transferred into fresh food vial every 3 days and dead flies were counted each time. The survival curve was plotted as the proportion of surviving flies vs age.

3.3 Fecundity

The number of eggs laid by female flies in 24 hours was recorded for the indication of their fecundity function. Before counting, female flies were transferred into the fresh vial and the

number of eggs laid in each vial was counted after 24h. N=100 was used in this assay and fecundity was measured every 10 days throughout the lifespan. The data was presented as the average number of eggs laid by individual female per day.

3.4 Negative Geotaxis

Flies were transferred from each vial into a serological pipette (25cm long and 1.5cm diameter), aiming for 10 flies in each serological pipette and 3 pipettes for each genotype (N=30). Negative geotaxis was tested every 10 days throughout the lifespan in the 25°C temperature and 70% humidity incubator at 2pm each time. Flies were banged down to the bottom of pipettes at the start and observed for 45 seconds. The number of flies reaching to the 'top' (more than 10cm from the bottom) and the number of flies staying at the 'bottom' (less than 1cm from the bottom) were counted (both were indicated by the red lines on the serological pipette). Three repeats were carried out for each pipette and the data was presented as their performance index ($^1/_2 * \frac{N_{total} + N_{top} - N_{bottom}}{N_{total}}$) (as described in Rival et al., 2004).

3.5 Exploratory Walking

This behaviour assay was performed every 10 days throughout the lifespan in the 25°C temperature and 70% humidity incubator at 1 pm every time. In the experiment, individual flies (N=15 or 16) were aspirated from their vials into a 40mm diameter and 10mm height circular area (Ismail et al). 2% agar plates were underneath and their activities inside the restricted area were video recorded for 15mins. The videos were then analysed with EthoVision XT video tracking software (Noldus) and different parameters of their walking behaviours were plotted into graphs.

3.6 Sleeping Behaviour

Sleeping behaviours were also tested every 10 days throughout the lifespan in the 25°C temperature and 70% humidity incubator with 12h light/dark cycle for 4 days. Flies were knocked out by CO_2 and individual flies (N=15) were transferred into a Trikinetics *Drosophila* Activity Monitor tubes. Different types of *Drosophila* media were provided at the end of the monitor tubes based on their genotypes. Their activities inside the incubator were monitored with a 1min bins for 4 days and no movement within 5 consecutive bins was classified as sleep.

The data of day 2-3 were analysed using the BeFLY! Excel plug-in and different parameters of their sleeping behaviours were plotted into graphs.

3.7 Starvation Resistance

To test fly's resistance to starvation, flies were kept as 10 / vial in standard food or RU486 food vial as normal and transferred into starvation experiment food with 0xSY (Tab. 3) on day 10. N=100 was used in this experiment and dead flies were counted every day. The survival curve was plotted as thr proportion of surviving flies vs age.

3.8 Oxidative Stress Resistance

Flies were kept as 10 / vial in standard food or RU486 food vial as normal and transferred into H_2O_2 oxidative stress experiment food (Tab. 3) on day 10. N=100 was aimed in this experiment and dead flies were counted twice a day (10 am and 5 pm respectively). The survival curve was plotted as the proportion of surviving flies vs age.

3.9 Lipid and Glycogen Assay

The flies were sorted into 10 / vial after eclosion and kept on standard or RU486 food vial until frozen at day 10 with liquid nitrogen (20 flies / Eppendorf tube). Firstly, individual flies were weighed and transferred into a separate Eppendorf tube. 100µl of the saturated Na_2SO_4 solution was added into each tube to homogenise the fly, absorb and precipitate glycogen. 1ml of chloroform: methanol (1:1) solution was added then to extract lipids out. The mixture was inverted several times gently and centrifuged 5mins at 6000 rpm to separate the lipid supernatant and glycogen pellet.

Secondly, for lipid extraction and determination, the supernatant containing lipids was transferred into new Eppendorf tubes and evaporated on 70°C heat block under a fume hood. Fully dried oily residues were resuspended in 200ml of conc H_2SO_4 and then heated at 90°C for 10mins. After briefly cooled on ice, 1ml of vanillin solution (Tab. 4) was added into each tube and mixed well for full-colour development. 200µl of each sample was loaded onto a microassay plate and the absorbance was measured at 490nm using a Tecan infinite M200Pro spectrophotometer within 30mins of the colour development. The concentration of each sample was determined against a standard curve of soybean oil serial solutions (prepared as Tab. 4).

Thirdly, Eppendorf tubes containing only glycogen pellets were fully dried in air or heating at 90°C briefly before further steps to determine glycogen. 1ml of anthrone solution (Tab. 4) was added into the glycogen pellet and then incubated at 90°C for 20mins. The mixture was inverted several times in between to achieve the full dissolution of all pellets and the colour was developed during that period. 200µl of each sample was loaded onto a microassay plate and the absorbance was measured at 620nm using a Tecan infinite M200Pro spectrophotometer. The concentration of each sample was determined against a standard curve of glycogen serial solution (prepared as Tab. 4).

Solution	
Saturated Sodium sulphate	Add sodium sulphate into 1L distilled water until saturated
Chloroform: methanol solution	Chloroform: methanol was 1:1 ratio in this solution
Vanillin solution	1.2% vanillin in 68% orthophosphoric acid
Anthrone solution	75mg anthrone in 53ml 70% H_2SO_4 or 75mg anthrone in 10ml MQ H_2O and 39ml 98% H_2SO_4
Soybean oil	A concentration series of soybean oil (0.92g/ml) by diluting in chloroform: methanol (1:1), concentration at 0µg to 500µg range
Glycogen	A concentration series of glycogen solution prepared by dissolving and diluting glycogen powder in MilliQ water, concentration at 0µg to 500µg range

Table 4. The preparation of all solutions used in this assay and all solutions were prepared fresh for each assay.

3.10 Apoptosis Assay

Chemicon ApopTag Fluorescein Direct In Situ Kit (S7160) was used to perform this assay. Fly brains were freshly prepared each time and dissected into 1% PBS on ice. Brains were fixed in 1% PFA (diluted in PBS) for 10mins at room temperature then washed in PBS three times for 5mins each time. For permeabilization, brains were post-fixed in cooled ethanol: acetic acid (2:1) for 5mins at -20°C and washed in PBS twice for 5mins each afterwards. Equilibration Buffer (13µl per cm^2) was applied for at least 10 seconds before removing and Working Strength TdT enzyme (Tab. 5) was immediately added to incubate in dark and humidified chamber at 37°C for 1hr. The mixture was then agitated for 15secs in Working Strength Stop/Wash Buffer (Tab. 5), incubated for 10mins at room temperature, and washed in PBS three times for 1mins each. In the meantime, an aliquot of Anti-digoxigenin conjugate was removed from the freezer and warmed up to room temperature in dark. Warmed Working Strength Anti-Digoxigenin conjugate (13µl per cm^2) was added into the tissues and incubated in dark and humidified chamber at room temperature for 30mins. Finally, brains were washed

in PBS four times for 2mins each, mounted with DAPI, sealed with a coverslip, and stored at -20°C before further use. All brains were observed under a confocal microscope where Z-stacks were performed. All images were further analysed by Image J and the whole area of apoptotic cells was measured in each brain and the average area of apoptotic cells was calculated.

Solution	
Working Strength TdT enzyme	Mix 77µl Reaction Buffer with 33µl TdT (glycerol stock in -20°C), vortex, and store on ice for up to 6hrs
Working Strength Stop/Wash Buffer	Mix 2ml Stop/Wash Buffer with 34ml dH_2O and store at 4°C for up to a year
Working Strength AntiDigoxigenin Conjugate	Mix 68µl Blocking Solution with 62µl Anti-Digoxigenin Conjugate, vortex, and store on ice for up to 3hrs (protect from light)

Table 5. The preparation of all solutions used in this assay and all solutions were prepared fresh for each assay.

3.11 Statistical Analysis

Lifespan data were subjected to survival analysis (Log-rank tests) in Excel and plotted as survival curves (survival flies vs age). All statistical analyses of other data (fecundity, negative geotaxis, exploratory walking, and sleeping behaviour) were performed in the JMP (version 8) software (SAS Institute). The distribution of studentized residuals of each set data was tested for normal distribution and the outliers were removed if appropriate. Two-way analyses of variance (ANOVA) comparing by genotypes and by age were performed and planned comparisons of means performed using T-test (between 2 pairs, $p<0.05$) or Tukey-Kramer HSD (3 or more groups, $p<0.05$). Data are presented as means of raw data +/- SEM.

4. Results-The Effect of Constitutive IIS Reduction Using elavGAL4/UAS-PTEN on *Drosophila* Ageing and Behavioural Senescence

4.1 Introduction

Previous studies have found that systemic reduction of IIS can extend lifespan and improve some measures of health and function in different model organisms, ranging from invertebrates to mammalians (Fontana et al., 2010). The evolutionary role of the IIS pathway in ageing has been confirmed, yet, little is known about the role of IIS in neuronal function and ageing (Broughton and Partridge, 2009). However, there is growing evidence that reducing IIS is not always beneficial to behavioural senescence. A recent study in our lab using an Insulin Receptor dominant negative transgene to reduce IIS in neurons found that long-lived flies did not show an amelioration of locomotor senescence (Ismail et al., 2015). In this project, an alternative transgene to reduce IIS in neurons was used (elavGAL4/UAS-PTEN) to further study the role of IIS in the nervous system and behavioural senescence. As mentioned above, elavGAL4 is a pan-neuronal driver and drives the expression of UAS-PTEN. PTEN functions as a phosphatase which downregulates IIS signalling via dephosphorylation of PIP3 to PIP2. The effect of reduced IIS on flies was measured by lifespan, fecundity, and behavioural senescence including locomotor behaviours (negative geotaxis and exploratory walking) and sleeping patterns. The performance of the experimental flies (elavGAL4/UAS-PTEN) was compared with the performance of the two control genotypes (elavGAL4/+ and UAS-PTEN/+) and to exclude any negative effects caused by the drivers (elavGAL4 or UAS-PTEN). In addition, previously in our lab, the validations of each genetically modified *Drosophila* lines were performed. For GAL4 drivers, the virgin GAL4 females were crossed with UAS-MCD8-GAL4 males. Their brains were dissected, fixed, and the GFP expression pattern was examined under a confocal microscopy validating the GAL4 lines only expressed in the appropriate neurons. For UAS drivers, UAS-PTEN flies were crossed with the systemic daGAL4 driver to ubiquitously express UAS lines in the whole body. All flies weighed less than the control flies and females had lower fecundity, consistent with previous results of IIS effects on fly growth and fecundity and confirming the effectiveness of PTEN overexpression.

4.2 Lifespan and Fecundity

Pan-neural IIS reduction shortened the lifespan of males but had no effect on females.

Two replicate lifespan experiments were performed for both females and males with N=100 for each experiment and each replicate presented similar results. The fecundity of the females was measured every 10 days alongside each lifespan experiment (N=100). In both experiments, experimental female flies with reduced IIS in neurons showed a normal decline in the survival rate compared to controls (Fig. 9A&C). Female fecundity showed a significant decline with age in control flies and there was no difference between the experimental flies and control flies (Fig. 9B&D). In contrast, reduced IIS in neurons significantly shortened the lifespan of males in both replicate experiments (Fig. 9E&F).

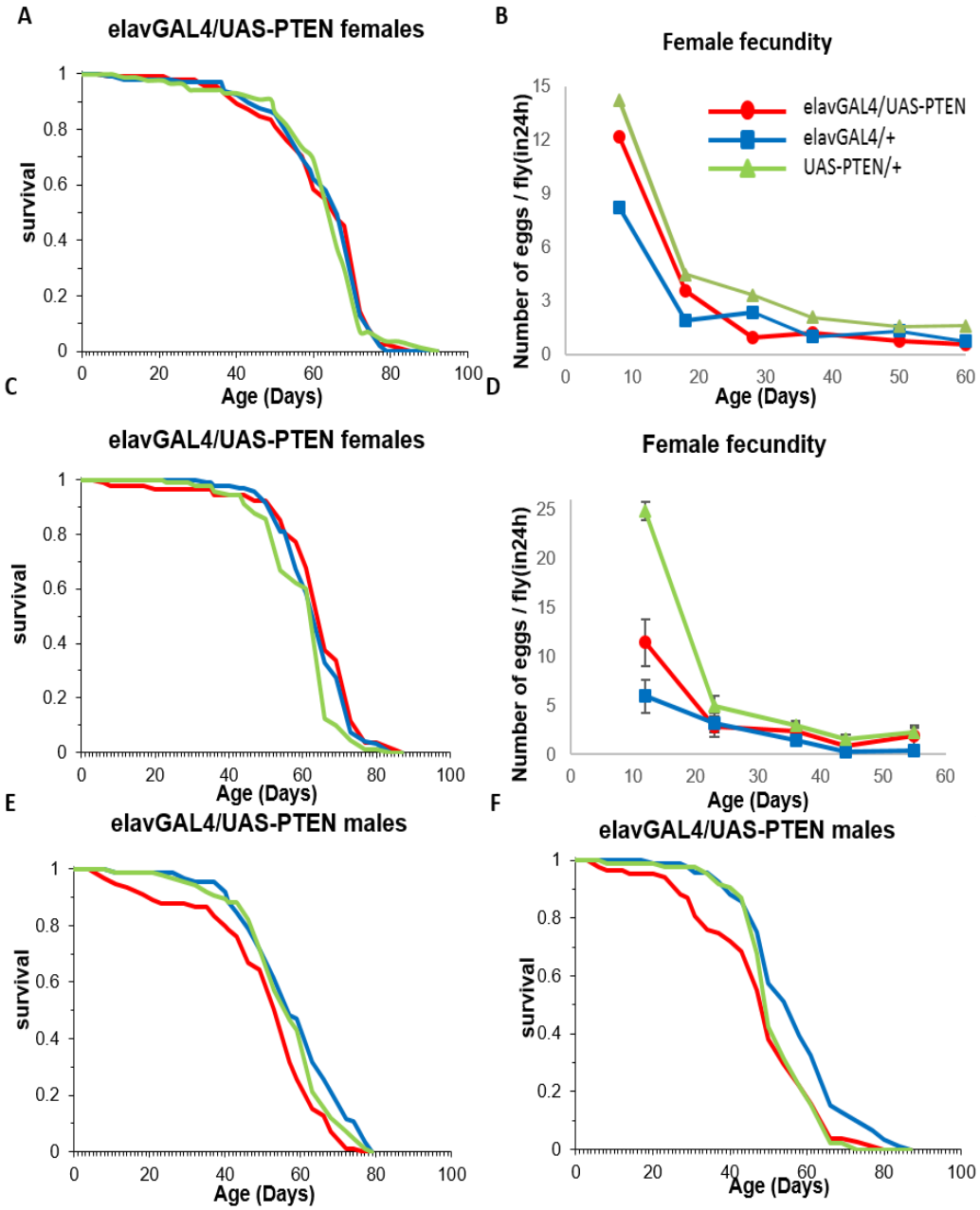


Figure 9. Lifespan and fecundity of elavGAL4/UAS-PTEN flies.

A-D: The lifespan and fecundity of elavGAL4/UAS-PTEN females (N=100) in 2 replicate experiments. Transgenic elavGAL4/UAS-PTEN females with IIS reductions in all neurons had

normal lifespan and fecundity compared to control flies ($p>0.05$ by log-rank test). E-F: The lifespan of elavGAL4/UAS-PTEN males (N=100) in 2 replicate experiments. elavGAL4/UAS-PTEN males with IIS reductions in all neurons were significantly short-lived compared to control flies ($p<0.05$ by log-rank test). Lifespan data was analysed by log-rank test and fecundity data at individual time points by genotype or by age were analysed by two-way ANOVA followed by post hoc means comparisons using Tukey HSD.

4.3 Locomotor behavioural Senescence – Negative Geotaxis and Exploratory Walking

Reduced IIS in neurons had no effect on behavioural senescence in both males and females.

Negative geotaxis is a reflex motor behaviour of the fruit flies and it is a part of their escape behaviour. Once flies are startled and dropped down to the bottom, they respond by climbing up against gravity. This behaviour can be used to indicate the effects of treatments on physical strength and brain function. Exploratory walking is a more complex locomotor behaviour of flies with multiple quantifiable parameters that indicate not only muscle strength (such as total distance or velocity) but also decision-making processes and thus CNS function (such as rotation frequency or duration in central zone) (Ismail et al., 2015). Both locomotor behaviours show age-related changes indicating declines in function. The performances of negative geotaxis and parameters of exploratory walking including total distance, walking duration, rotation frequency, and velocity decline significantly with age.

In control females, negative geotaxis and exploratory walking showed the expected age-related declines (Figure 10). When reducing IIS in the neurons, elavGAL4/UAS-PTEN female flies showed a normal decline in both locomotor behaviours compared to the control flies (Fig. 10A-H). There was a small negative effect on experimental flies at young age points but the difference between the experimental flies and control flies was not significant (observed in Fig. 10 A, C, E, & F). On the other hand, exploratory walking experiments and negative geotaxis were also performed with elavGAL4/UAS-PTEN males every 10 days throughout the lifespan. Despite the shortened lifespan of experimental flies (Fig. 9E&F), there was no effect on behavioural senescence of the experimental flies compared to the control flies in both exploratory walking and negative geotaxis (Fig. 11 A-H).

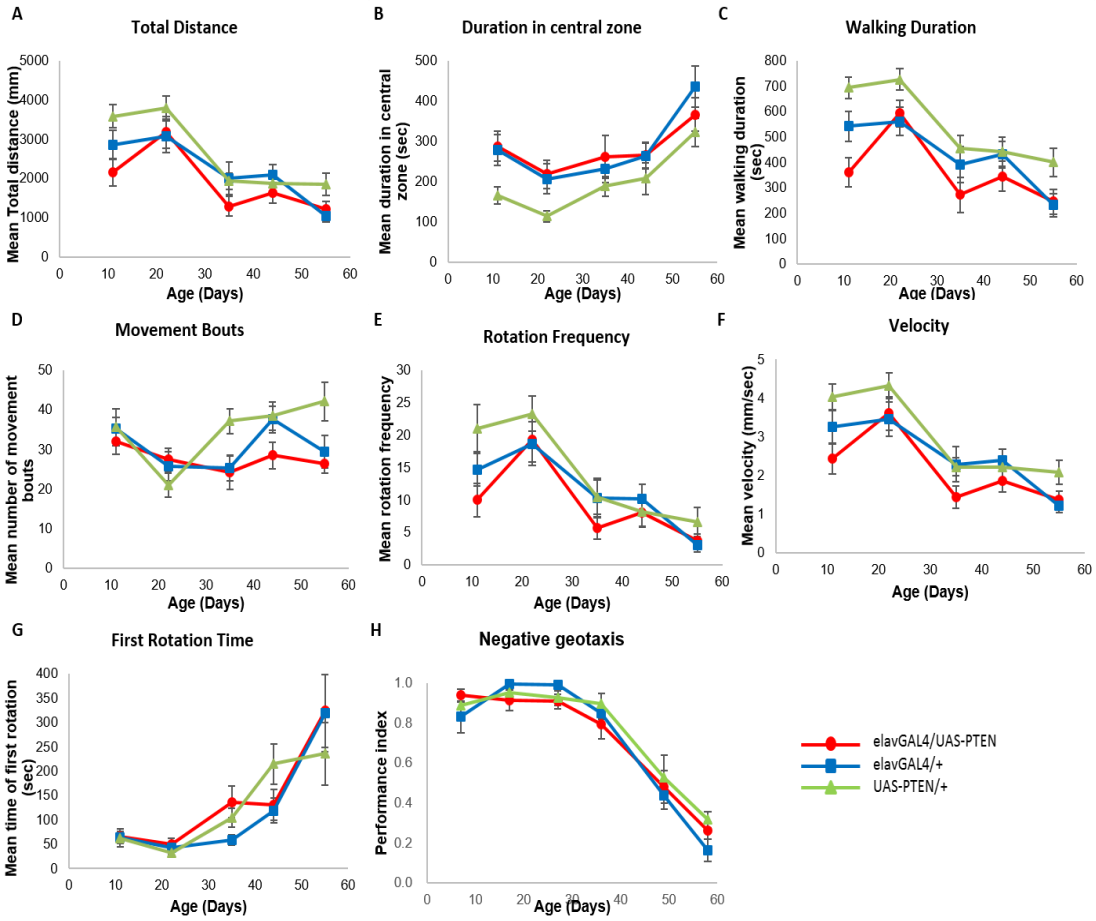


Figure 10. Exploratory walking and negative geotaxis of elavGAL4/UAS-PTEN females.
A-G: Exploratory walking senescence for a cohort of female flies of the indicated genotypes (N=15). A: Female mean total distance walked (mm) vs age. B: Female mean duration in central zone (secs) vs age. C: Female mean walking duration (secs) vs age. D: Female mean number of movement bouts vs age. E: Female mean frequency of rotations (change in walking direction) vs age. F: Female mean velocity (mm/sec) vs age. G: Female mean latency of first rotation (first change of walking direction) (secs) vs age. H: Negative geotaxis performance index (PI) over the lifespan of elavGAL4/UAS-PTEN female flies compared to elavGAL4/+ and UAS-PTEN/+ controls, N=3 (groups of 10 flies) for each genotype. All data are shown as mean value for each parameter ±SEM. All data at individual time points by genotype or by age were analysed by two-way ANOVA followed by post hoc means comparisons using Tukey HSD and no significant effects were found ($p>0.05$).

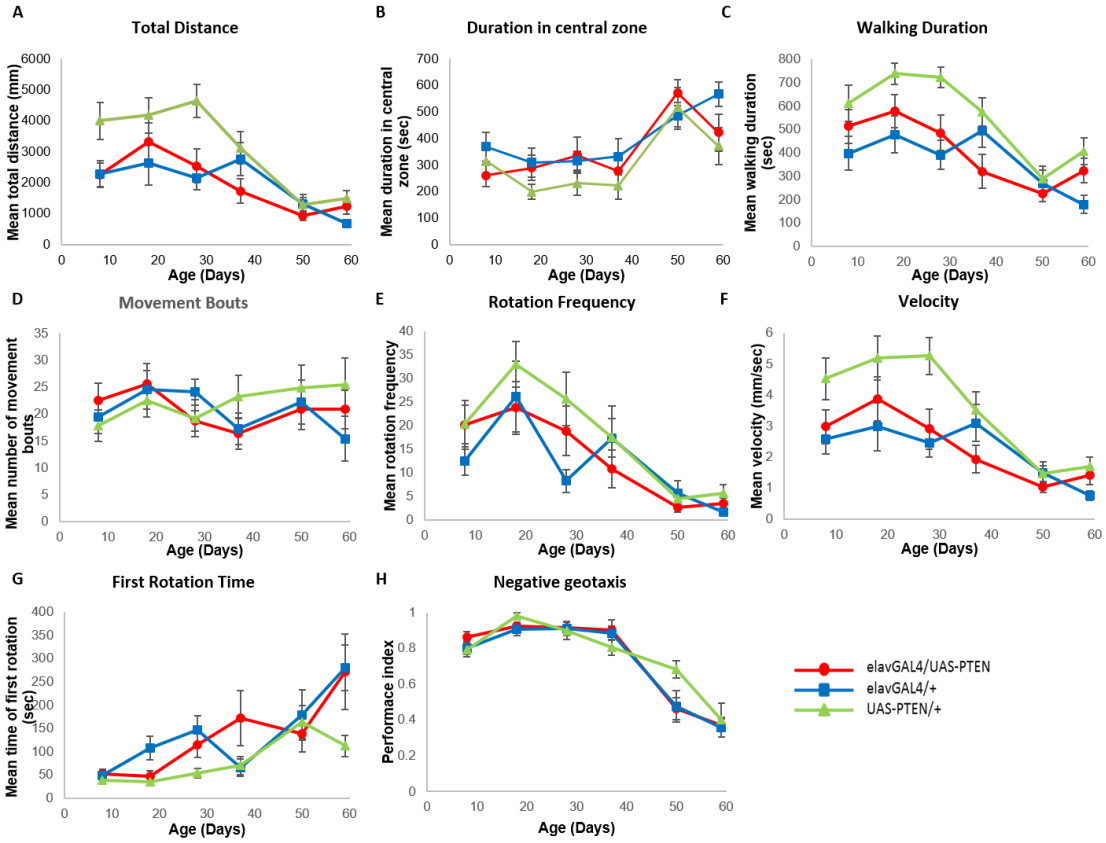


Figure 11. Exploratory walking and negative geotaxis of elavGAL4/UAS-PTEN males

A-G: Exploratory walking senescence for a cohort of male flies of the indicated genotypes (N=15). A: Male mean total distance walked (mm) vs age. B: Male mean duration in central zone (secs) vs age. C: Male mean walking duration (secs) vs age. D: Male mean number of movement bouts vs age. E: Male mean frequency of rotations (change in walking direction) vs age. F: Male mean velocity (mm/sec) vs age. G: Male mean latency of first rotation (first change of walking direction) (secs) vs age. H: Negative geotaxis performance index (PI) over the lifespan of elavGAL4/UAS-PTEN male flies compared to elavGAL4/+ and UAS-PTEN/+ controls, N=3 (groups of 10 flies) for each genotype. All data are shown as mean value for each parameter ±SEM. All data at individual time points by genotype or by age were analysed by two-way ANOVA followed by post hoc means comparisons using Tukey HSD and no significant effects were found ($p>0.05$).

4.4 Sleeping Behaviours

Reduced IIS in neurons had an early age negative effect on males' night-time sleep.

Sleep is another behaviour that is controlled by the CNS and it is essential for all species and influences different physiological processes (Cirelli and Bushey, 2008). Flies' activities are tightly regulated by circadian rhythms in a 12:12 hr light: dark (LD) cycle such that they exhibit two peaks of activity, morning and evening (Dubowy and Sehgal, 2017). Based on the bimodal pattern of *Drosophila* sleep, their sleep can be divided into daytime sleep and night sleep (Cong et al., 2015). The sleep patterns in flies have also been shown to deteriorate with age and become more fragmented with age like in humans – sleep duration becomes shorter and more often being interrupted by waking periods (Robertson and Keene, 2013). However, this senescence is very variable and different studies have not always seen the same kind of fragmentation with age. In this project, this behaviour is mainly used to assess the senescence of sleep parameters as measures of brain function with age and to compare the brain function of different genotypes.

Two replicate sleep behaviour experiments were performed on elavGAL4/UAS-PTEN females and the results are presented in Figures 12 and 13. In the first sleep experiment, the sleep fragmentation with age phenotype was not clearly shown in flies and only a few parameters showed age-related changes (Fig. 12). In control flies, total daily activity showed a significant increase with age; total sleep in light, average bout length in dark, and average bout length in light showed a significant decline with age. Other parameters including total sleep per day, total sleep in dark, bouts of sleep in dark, and bouts of sleep in light showed fluctuations over the five age points but no significant changes with age. The experimental flies showed a normal senescence of sleeping behaviours indicating that expression of PTEN in neurons had no effect on the ageing of sleep behaviour. However, interestingly it was observed that experimental flies appeared to be less active and slept more at young ages (Fig. 12 A, I, & J). The significant difference in the total sleep per day at 18 days old was a result of a specific effect on daytime sleep which showed an increase in sleep bout length but not the number of bouts of sleep.

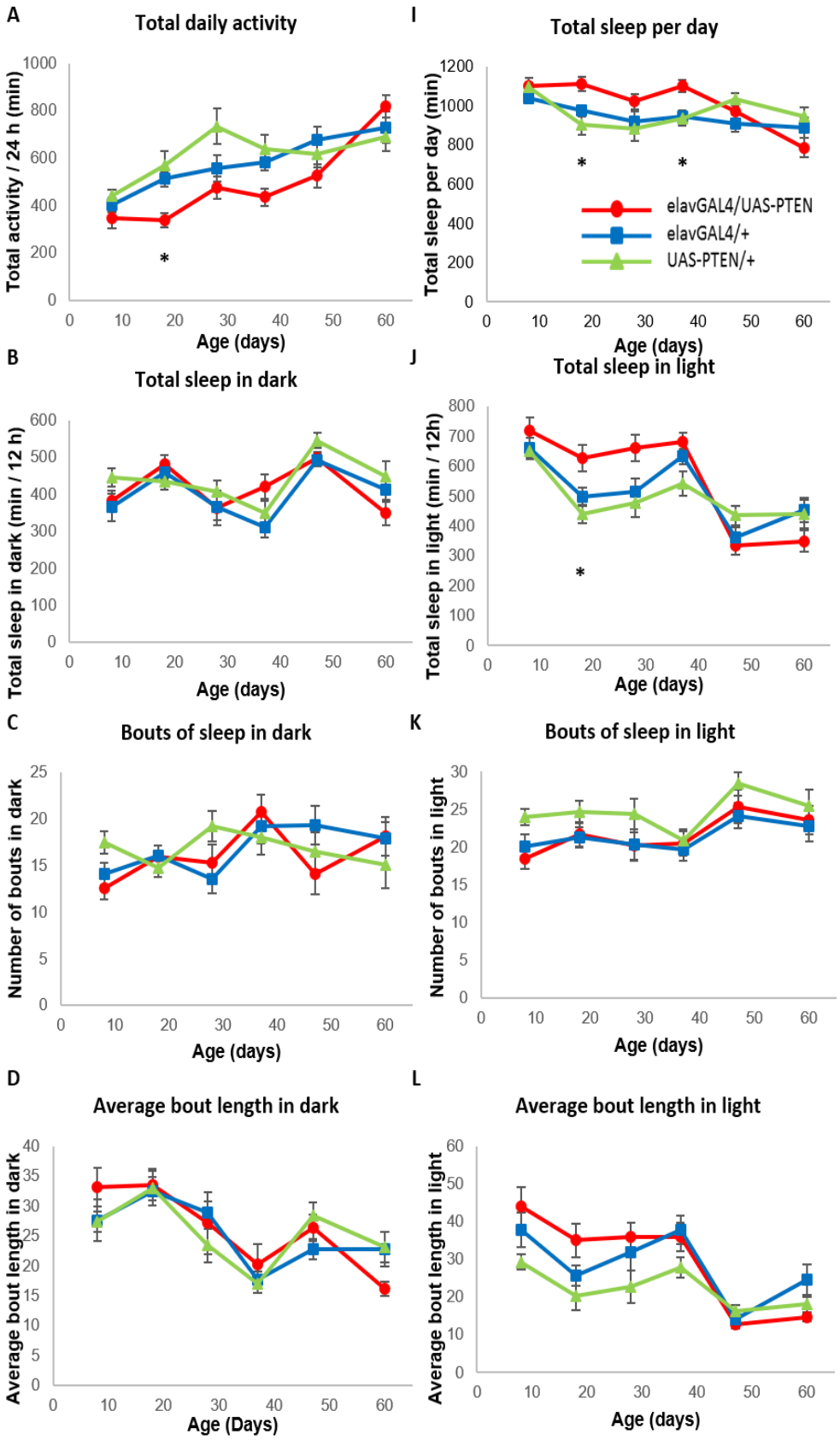


Figure 12. Daily activity and sleep pattern of elavGAL4/UAS-PTEN females.
A: Female mean total activity in 24hrs (mins) vs age. I: Female total sleep in 24 hrs (mins) vs age. B-D: Nighttime sleep of female flies of the indicated genotypes. J-L: Daytime sleep of

female flies of the indicated genotypes. B: Female mean total sleep in 12hrs dark (mins) vs age. C: Female mean total number of sleep bouts in 12hrs dark vs age. D: Female average bout length in 12hrs dark (mins) vs age. J: Female mean total sleep in 12hrs light (mins) vs age. K: Female mean total number of sleep bouts in 12hrs light vs age. L: Female average bout length in 12hrs light (mins) vs age. N=15 and all data are shown as mean value for each parameter ±SEM. All data at individual time points by genotype or by age were analysed by two-way ANOVA followed by post hoc means comparisons using Tukey HSD. * indicates significant differences ($p<0.05$) between the elavGAL4/UAS-PTEN and both controls.

In the second sleep pattern experiment, the age-related deteriorations in sleep behaviour were clearer and sleep became fragmented with age (Fig. 13). Total daily activity and bouts of sleep in light showed a significant increase with age; significant age-related declines were observed in parameters including total sleep in light, bouts of sleep in dark, and average bout length in light. The senescence of activity and sleep patterns of the experimental females does not appear to be significantly different from the control groups in any parameters and the early-age negative effect in experimental flies was not observed in this experiment.

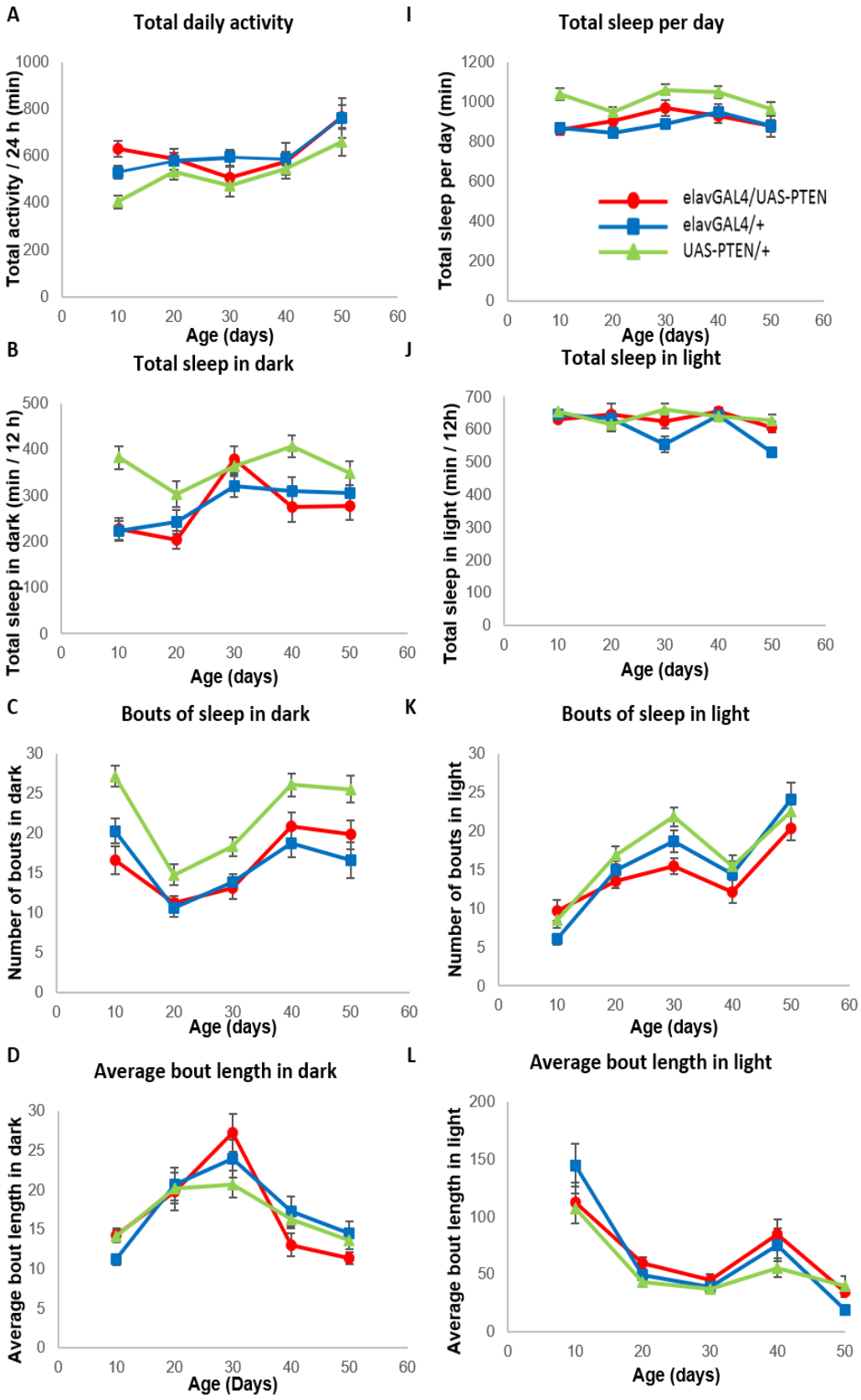


Figure 13. Daily activity and sleep pattern of elavGAL4/UAS-PTEN females.
A: Female mean total activity in 24hrs (mins) vs age. I: Female total sleep in 24 hrs (mins) vs age. B-D: Nighttime sleep of female flies of the indicated genotypes. J-L: Daytime sleep of

female flies of the indicated genotypes. B: Female mean total sleep in 12hrs dark (mins) vs age. C: Female mean total number of sleep bouts in 12hrs dark vs age. D: Female average bout length in 12hrs dark (mins) vs age. J: Female mean total sleep in 12hrs light (mins) vs age. K: Female mean total number of sleep bouts in 12hrs light vs age. L: Female average bout length in 12hrs light (mins) vs age. N=15 and all data are shown as mean value for each parameter ±SEM. All data at individual time points by genotype or by age were analysed by two-way ANOVA followed by post hoc means comparisons using Tukey HSD and no significant effects were found ($p>0.05$).

In the sleeping behaviour experiment of males, clear, age-related deteriorations were observed in control males for some sleep parameters including the decline in total sleep in dark, total sleep in light, average bout length in dark, and average bout length in light; and the increase in bouts of sleep in dark and bouts of sleep in light (Fig. 14). The activity and sleep patterns of elavGAL4/UAS-PTEN males do not appear to differ significantly from the control groups at each age points. However, in males, the expression of PTEN in neurons resulted in a specific effect on nighttime sleep; total sleep in dark and average bout length in dark did not show a normal decline with age. An early age effect was found that the night-time sleep of elavGAL4/UAS-PTEN males was significantly shorter than the control groups and the average bout length in the dark appeared to be shorter as well (Fig. 14 B&D). This lack of age-related decline may be the result of this young age negative effect observed in these two parameters.

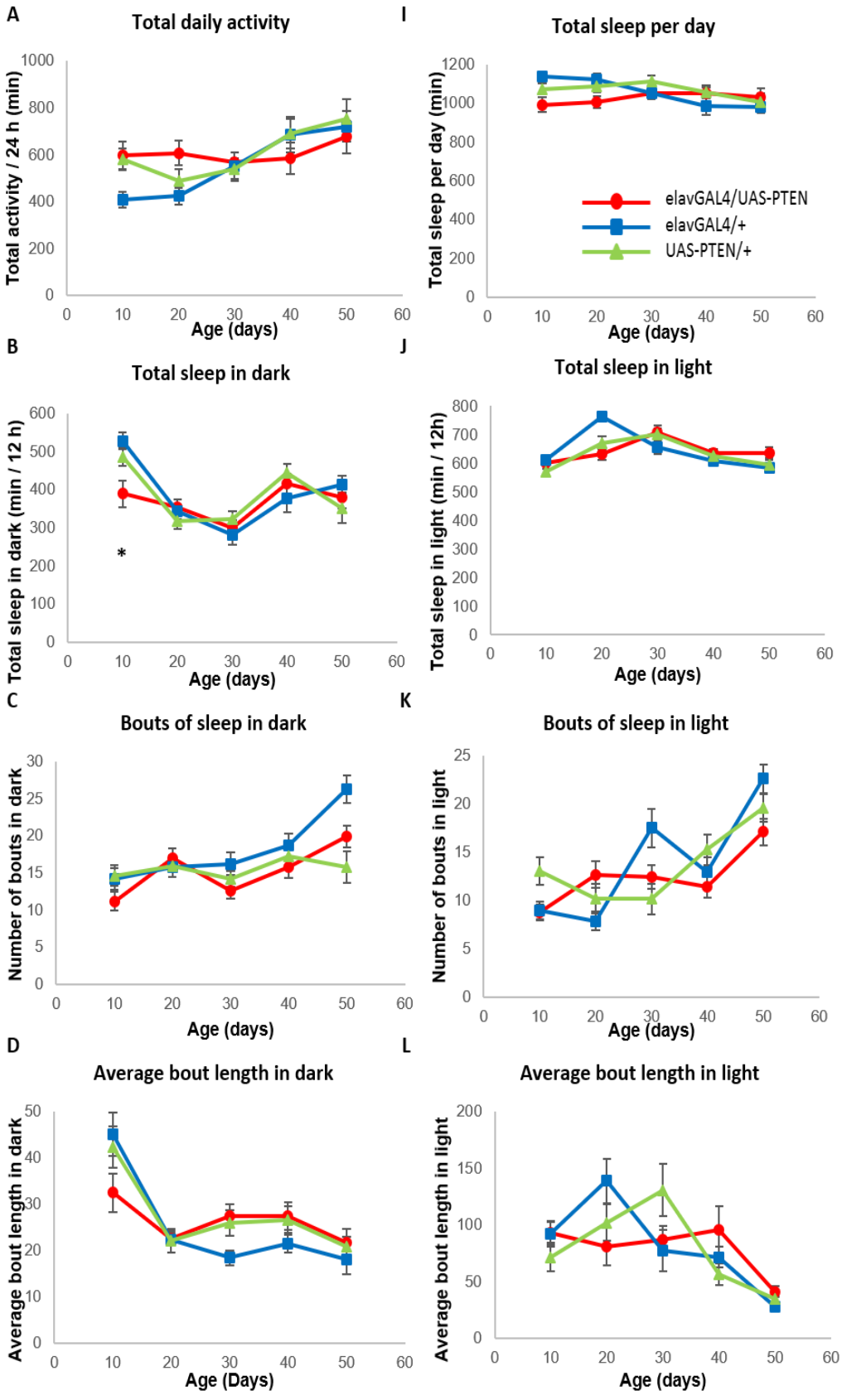


Figure 14. Daily activity and sleep pattern of elavGAL4/UAS-PTEN males.
A: Male mean total activity in 24hrs (mins) vs age. I: Male total sleep in 24 hrs (mins) vs age. B-D: Nighttime sleep of male flies of the indicated genotypes. J-L: Daytime sleep of male flies of

the indicated genotypes. B: Male mean total sleep in 12hrs dark (mins) vs age. C: Male mean total number of sleep bouts in 12hrs dark vs age. D: Male average bout length in 12hrs dark (mins) vs age. J: Male mean total sleep in 12hrs light (mins) vs age. K: Male mean total number of sleep bouts in 12hrs light vs age. L: Male average bout length in 12hrs light (mins) vs age. N=15 and all data are shown as mean value for each parameter ±SEM. All data at individual time points by genotype or by age were analysed by two-way ANOVA followed by post hoc means comparisons using Tukey HSD. * indicates significant differences ($p<0.05$) between the elavGAL4/UAS-PTEN and both controls.

4.5 Summary

In summary of the results on the elavGAL4/UAS-PTEN transgenic flies, females with constitutive IIS reduction in neurons showed a normal lifespan, fecundity, and behavioural senescence. However, an early-age detrimental effect was observed in some behavioural parameters. In contrast, males with a constitutive IIS reduction in neurons were significantly short-lived compared to control flies. Despite the shortened lifespan, males presented a normal senescence of most behavioural parameters. In some parameters, males did not show the normal age-related decline which may have been the result of a potential young-age negative effect. Together, the data show that reducing IIS in neurons via expression of UAS-PTEN is not beneficial to survival and actually shortens the lifespan of males, in contrast to the effect of expression of the UAS-InR^{DN} transgene. However, the data indicate that similar to previous studies, lifespan and behavioural senescence can be disconnected and reduced IIS in neurons can have detrimental effects on behavioural function.

5. Results – The Effect of Inducible IIS Reduction Using RU486-elavGS/UAS-PTEN on *Drosophila* Ageing and Behavioural Senescence

5.1 Introduction

Considering the role of IIS signalling in cell growth, cell division, gene expression, and other fundamental functions, it was suspected that IIS reduction throughout lifespan may cause some negative effects during developmental stages and may have been the cause of the young-age behavioural effects observed in elavGAL4/UAS-PTEN flies and the lifespan effects. In the following experiments, the reduction of IIS was restricted to adult neurons only to exclude any negative effects during the developmental period and to compare the role of IIS signalling in neuronal function and ageing during adulthood and development. As mentioned above, the RU486-dependent inducible system, the GeneSwitch driver (elavGS), was used to control the timing of IIS reduction. To study the role of IIS signalling in adulthood period, experimental flies (RU486-elavGS/UAS-PTEN) developed into adulthood on normal food and were placed onto RU486 drug-containing food to reduce IIS after that to compare to normal flies without IIS reduction (elavGS/UAS-PTEN).

In addition, Ismail et al. (2015) found a detrimental effect on locomotor senescence in the presence of lifespan extension in flies (elavGAL4/UAS-InR^{DN}), suggesting that reduced IIS is not always beneficial to the neural circuitry underlying locomotor behaviours. Their results have raised the question of whether the effect of reduced IIS in neurons on behavioural senescence is the result of changes to neuronal ageing or neuronal function. To answer this question, a recovery experiment was conducted by giving RU486-elavGS/UAS-PTEN flies a recovery period before behavioural testing, allowing them to restore IIS function. Recovery periods were given in the recovery experiments by removing flies from the RU486 inducer 5 days before each time point of testing. Chronic experiments (flies without IIS restoration) and recovery experiments (flies with IIS restoration) were run separately but in parallel at the indicated time points to determine the effect of reduced IIS on neuronal function and ageing process. To exclude the effects on flies caused by the drug RU486, two additional control groups were tested in parallel (RU486-elavGS/+ and elavGS/+).

5.2 Lifespan and Fecundity

Adult-specific neural IIS reduction shortened the lifespan of males but not always of females

Lifespan experiments on RU486-elavGS/UAS-PTEN flies were repeated twice with N=100 and N=500 respectively, shown as Fig. 15 and 16. The fecundity of the females was measured every 10 days alongside each lifespan experiment (N=100). In the first lifespan experiment, females with IIS reduction during adulthood was significantly short-lived (Fig. 15A). A detrimental effect was found at the young age on female fecundity, but females also showed a recovery from the negative effect at the older age and a normal decline in fecundity with age (Fig. 15B). The effect of the RU486 drug on flies was not detected in this experiment as flies on the drug were normally lived and had a normal female fecundity (Fig. 15C&D). The fecundity of flies on the drug at the first-time point appeared to be lower than normal flies but the difference was not statistically significant. Their fecundity declined normally with age, but a slightly higher fecundity was observed at the last age point.

In the second lifespan experiment with a larger number of flies (N=500), experimental flies with IIS reduction during adulthood had a normal lifespan but their fecundity declined faster than the control flies and was significantly lower than control flies at several age points (Fig. 16A&B). A drug effect on the flies was found in the control groups as flies on the drug were significantly short-lived and their fecundity appeared to decline faster than normal flies (Fig. 16C&D). The difference between the two groups on fecundity was significant at several age points, similar to the negative effects seen in Fig. 16B.

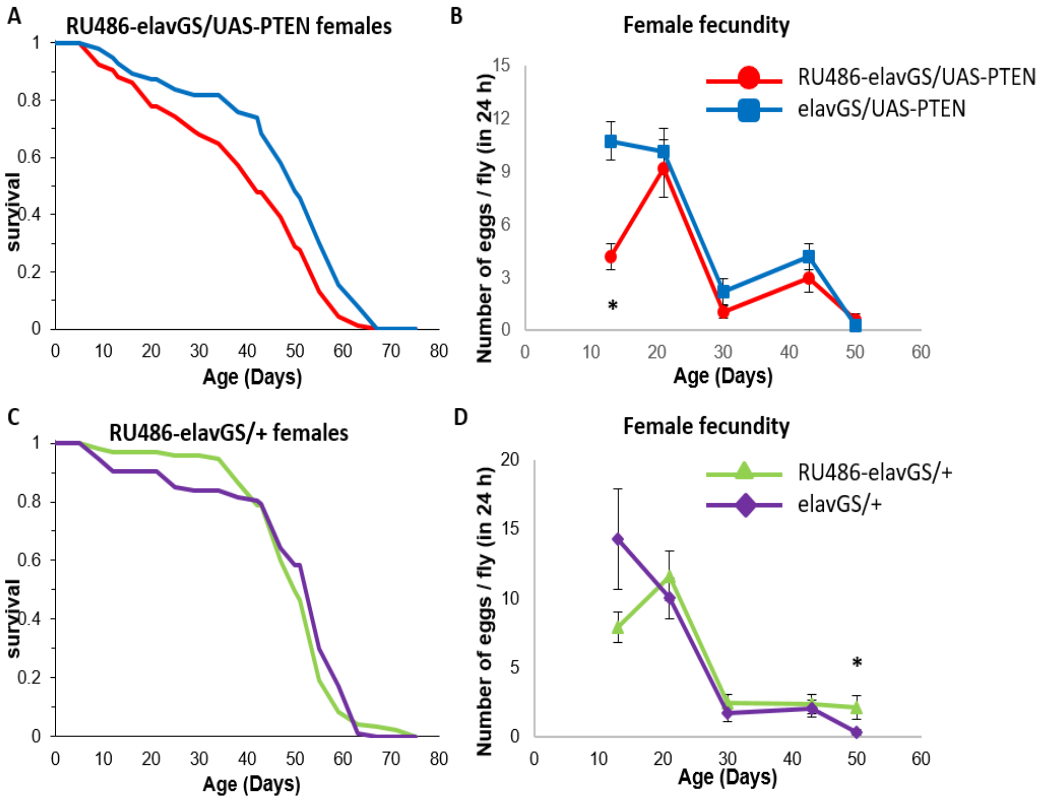


Figure 15. Lifespan and fecundity of RU486-elavGS/UAS-PTEN females.
A-B: The lifespan and fecundity of RU486-elavGS/UAS-PTEN females (N=100). Transgenic RU486-elavGS/UAS-PTEN females with IIS reductions in all neurons had a shorter lifespan and reduced fecundity compared to control flies. C-D: The lifespan and fecundity of RU486-elavGS/+ females (N=100). RU486-elavGS/+ females had a normal lifespan but reduced fecundity compared to elavGS/+ flies. Lifespan data was analysed by log-rank test and fecundity data at individual time points by genotype or by age were analysed by two-way ANOVA followed by post hoc means comparisons using student's t test. * indicates significant differences ($p<0.05$) between the RU486-elavGS/UAS-PTEN and elavGS/UAS-PTEN or between the RU486-elavGS/+ and elavGS/+.

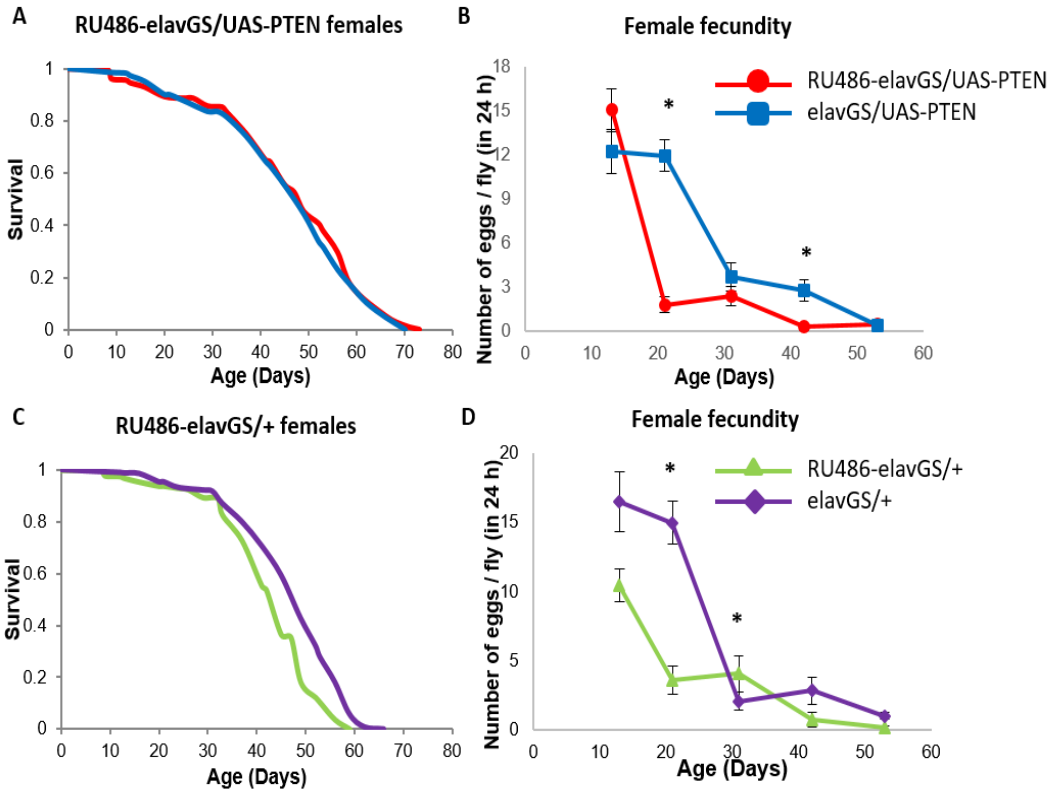


Figure 16. Lifespan and fecundity of RU486-elavGS/UAS-PTEN females.

A-B: The lifespan and fecundity of RU486-elavGS/UAS-PTEN females (N=500). Transgenic RU486-elavGS/UAS-PTEN females with IIS reductions in all neurons had a normal lifespan but reduced fecundity compared to control flies. C-D: The lifespan and fecundity of RU486-elavGS/+ females (N=500). RU486-elavGS/+ females had a normal lifespan but reduced fecundity compared to elavGS/+ flies. Lifespan data was analysed by log-rank test and fecundity data at individual time points by genotype or by age were analysed by two-way ANOVA followed by post hoc means comparisons using student's t test. * indicates significant differences ($p<0.05$) between the RU486-elavGS/UAS-PTEN and elavGS/UAS-PTEN or between the RU486-elavGS/+ and elavGS/+.

On the other hand, two lifespan experiments were also performed on RU486-elavGS/UAS-PTEN male flies with N=100 and N=500 respectively. In both experiments, the lifespan of male flies with reduced IIS during adulthood was significantly shortened compared to control flies (Fig. 17A&B). To test any drug effects on flies, two lifespan experiments were performed in parallel on flies with or without the RU486 drug. However, one experiment showed no drug effect (normal-lived RU486-elavGS/+) while the other experiment showed a negative drug effect (short-lived RU486-elavGS/+) (Fig. 17C&D).

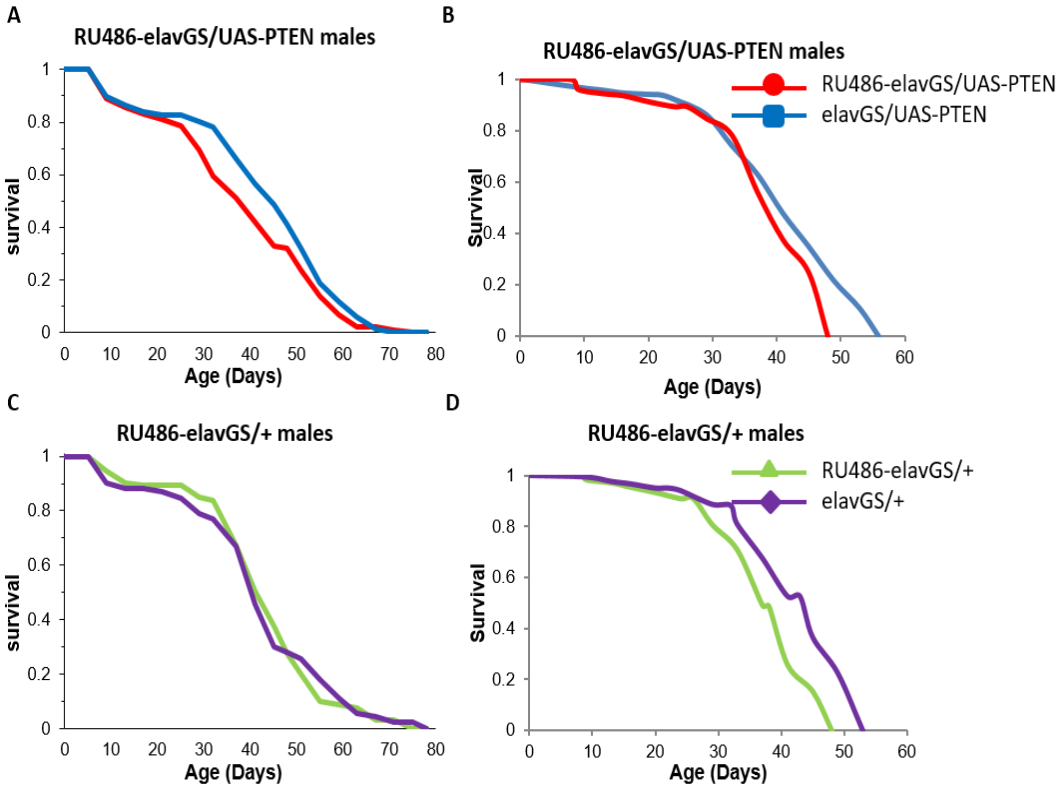


Figure 17. Lifespan of RU486-elavGS/UAS-PTEN males.

A-B: Two replicate lifespan of RU486-elavGS/UAS-PTEN males (N=100, N=500). Transgenic RU486-elavGS/UAS-PTEN males with IIS reductions in all neurons had a shorter lifespan compared to control flies ($p<0.05$ by log-rank test). C-D: Two replicate lifespan of RU486-elavGS/+ males (N=100, N=500). RU486-elavGS/+ males had a normal lifespan in C but shorter lifespan in D compared to elavGS/+ flies ($p<0.05$ by log-rank test).

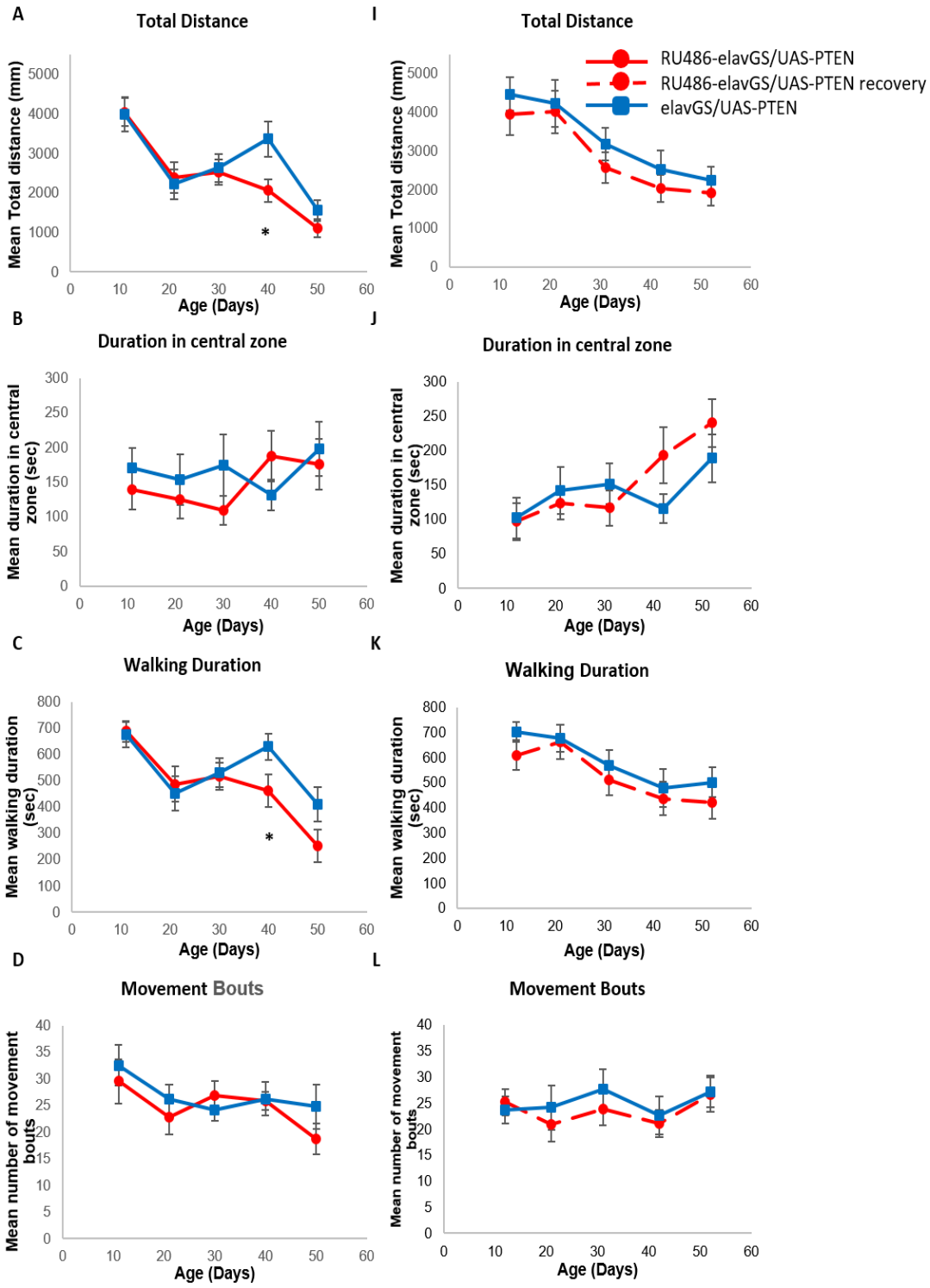
5.3 Locomotion Behavioural Senescence – Negative Geotaxis and Exploratory Walking

Reduced IIS in adult neurons had detrimental effects on behavioural senescence but can be recovered from.

Negative geotaxis and exploratory walking experiments were performed to study the effect of IIS reduction on neuronal ageing and function during adulthood. To answer the question about the detrimental effects on behavioural senescence as the result of ageing or function effects, recovery behavioural experiments were performed in parallel. In the recovery experiments, flies were placed onto RU486 once developed into adulthood and were removed from the inducing drug 5 days before behavioural testing to allow a period of restoration of IIS function. The results of flies on RU486 drug consecutively or with recovery period are compared in parallel.

The performance of flies in exploratory walking and negative geotaxis declines with age such that old flies are less active and have a longer period to respond. As is shown in Fig. 18, significant age-related declines were observed in total distance, walking duration, rotation frequency, velocity, and negative geotaxis and the latency to first rotation time steadily increased with age. Age-related changes were not constant in the duration of flies staying in the central zone and their movement bouts. In females, flies with reduced IIS in adult neurons (RU486-elavGS/UAS-PTEN) had a normal behavioural senescence at early ages (up to 30). The performance of experimental females declined faster than control flies at older ages and the differences between the experimental and control flies were significant in some parameters of exploratory walking and negative geotaxis (Fig. 18A-H).

On the other hand, by giving experimental flies a 5-day recovery period of IIS function, flies (RU486-elavGS/UAS-PTEN recovery) appeared to have recovered from the detrimental effects of IIS reduction and had a normal behavioural senescence compared to control flies (Fig. 18I-P). There is a potential improvement on the latency to first rotation time in recovery flies: the deterioration in this parameter was slower in recovery flies but the differences between the recovery and control flies did not rise to statistically significant (Fig. 18O). The negative geotaxis performance of recovery flies was still worse than control flies at the older age but the difference between the recovery and control flies was less significant compared to flies without recovery period (Fig. 18H&P).


A
Total Distance
Mean Total distance (mm)
Age (Days)
*
I
Total Distance
RU486-elavGS/UAS-PTEN
RU486-elavGS/UAS-PTEN recovery
elavGS/UAS-PTEN
Mean Total distance (mm)
Age (Days)
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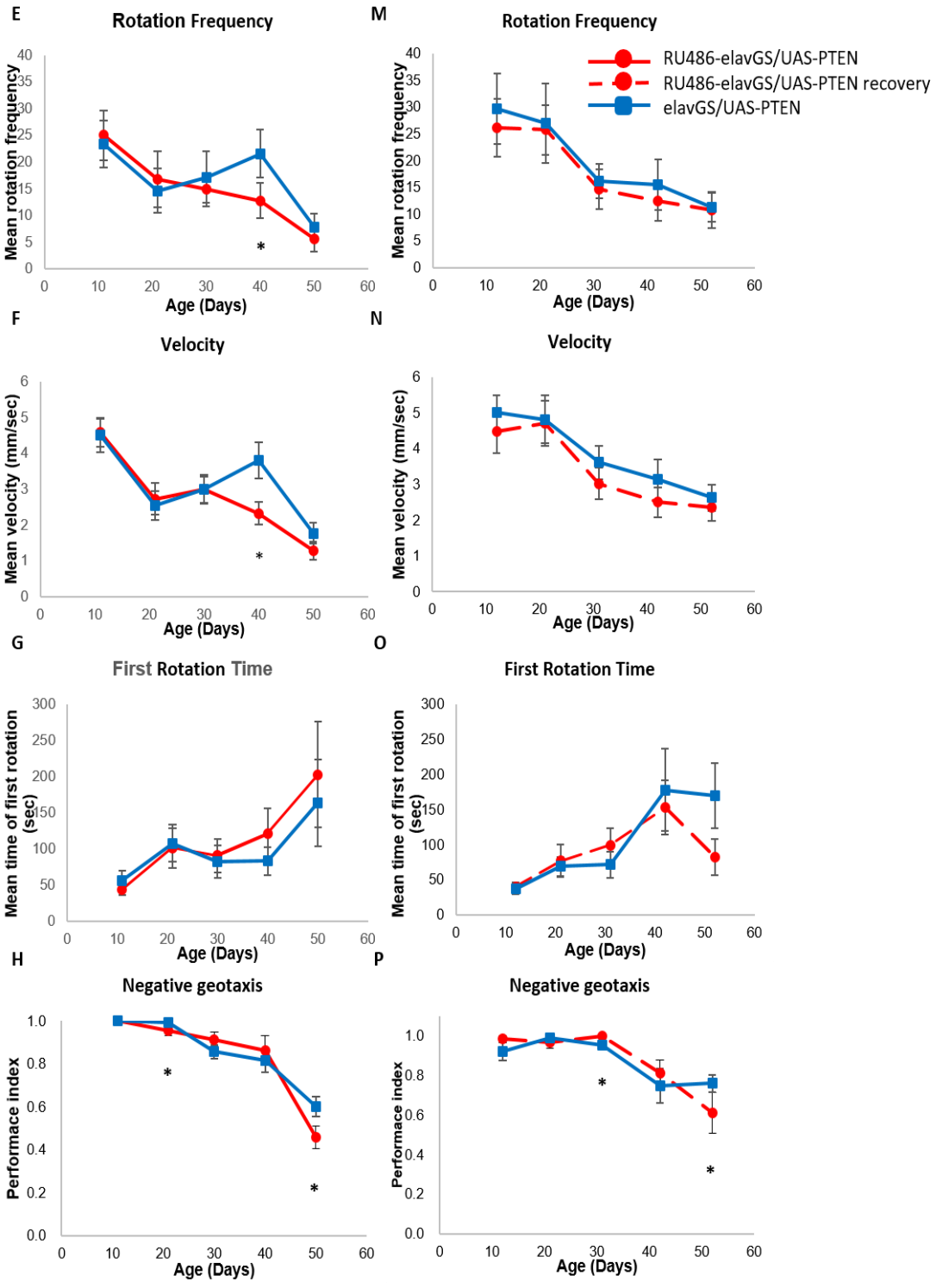


Figure 18. Exploratory walking and negative geotaxis of RU486-elavGS/UAS-PTEN females with and without a recovery period.

A-H: Exploratory walking and negative geotaxis senescence for a cohort of female flies of the indicated genotypes (without recovery period) (N=16). A: Female mean total distance walked (mm) vs age. B: Female mean duration in central zone (secs) vs age. C: Female mean walking duration (secs) vs age. D: Female mean number of movement bouts vs age. E: Female mean frequency of rotations (change in walking direction) vs age. F: Female mean velocity (mm/sec) vs age. G: Female mean latency of first rotation (first change of walking direction) (secs) vs

age. H: Negative geotaxis performance index (PI) over the lifespan of RU486-elavGS/UAS-PTEN female flies compared to elavGS/UAS-PTEN controls, N=3 (groups of 10 flies) for each genotype. I-P: Exploratory walking and negative geotaxis senescence for a cohort of female flies of the indicated genotypes (with 5-day recovery period) (N=16). I: Female mean total distance walked (mm) vs age. J: Female mean duration in central zone (secs) vs age. K: Female mean walking duration (secs) vs age. L: Female mean number of movement bouts vs age. M: Female mean frequency of rotations (change in walking direction) vs age. N: Female mean velocity (mm/sec) vs age. O: Female mean latency of first rotation (first change of walking direction) (secs) vs age. P: Negative geotaxis performance index (PI) over the lifespan of RU486-elavGS/UAS-PTEN female flies compared to elavGS/UAS-PTEN controls, N=3 (groups of 10 flies) for each genotype. All data are shown as mean value for each parameter ±SEM. All data at individual time points by genotype or by age were analysed by two-way ANOVA followed by post hoc means comparisons using student's t test. * indicates significant differences ($p<0.05$) between the RU486-elavGS/UAS-PTEN and elavGS/UAS-PTEN control.

Experiments were also performed on control groups with or without the RU486 drug and presented as in Fig. 19. Flies on RU486 drug (RU486-elavGS/+) showed a normal decline in their behavioural performance with age comparing to the control group (elavGS/+). There were differences between the two groups found at one age point (age 30) in several parameters. However, there is no significant difference between the two groups at any other age points suggesting a normal behavioural senescence of flies on the drug.

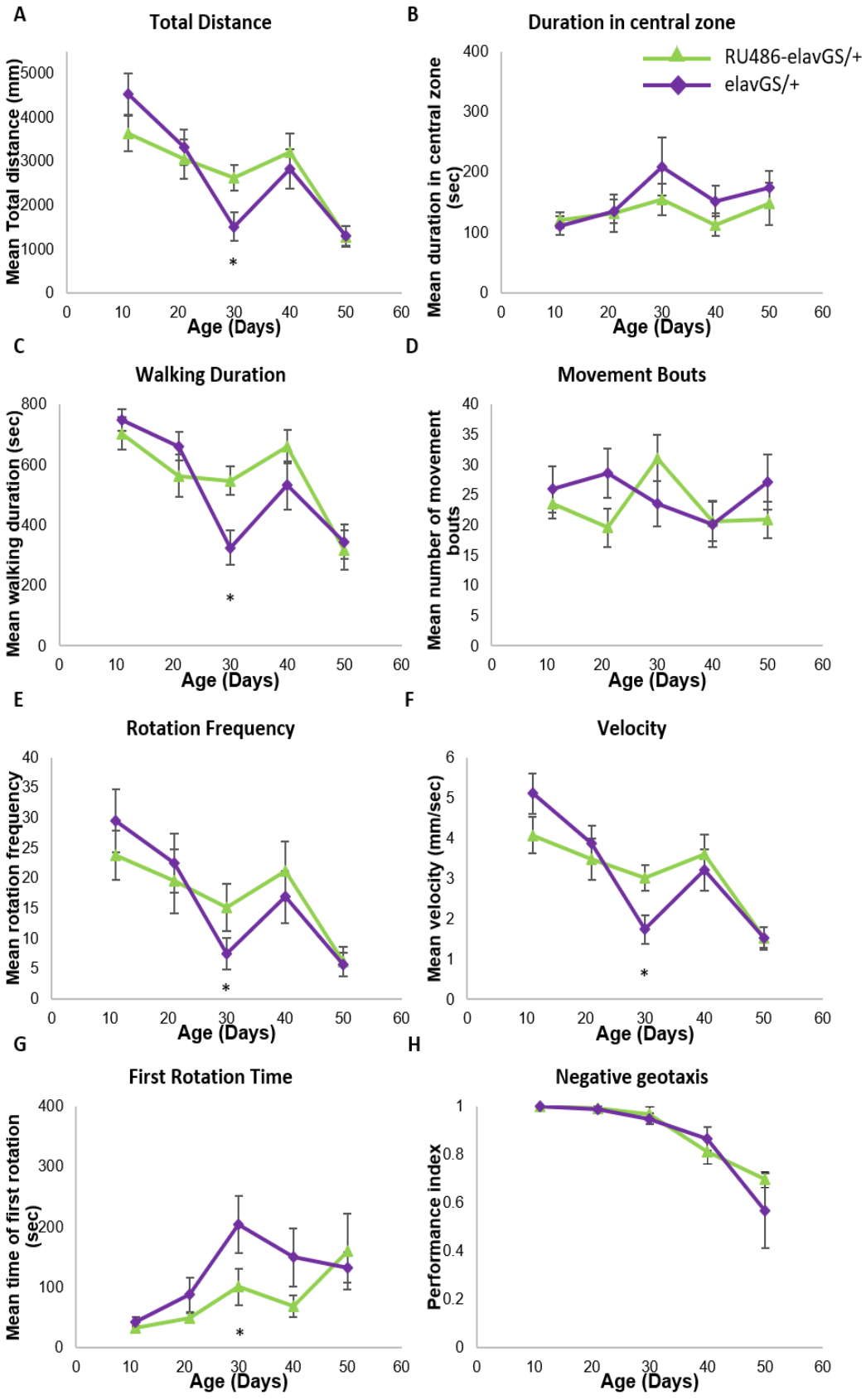
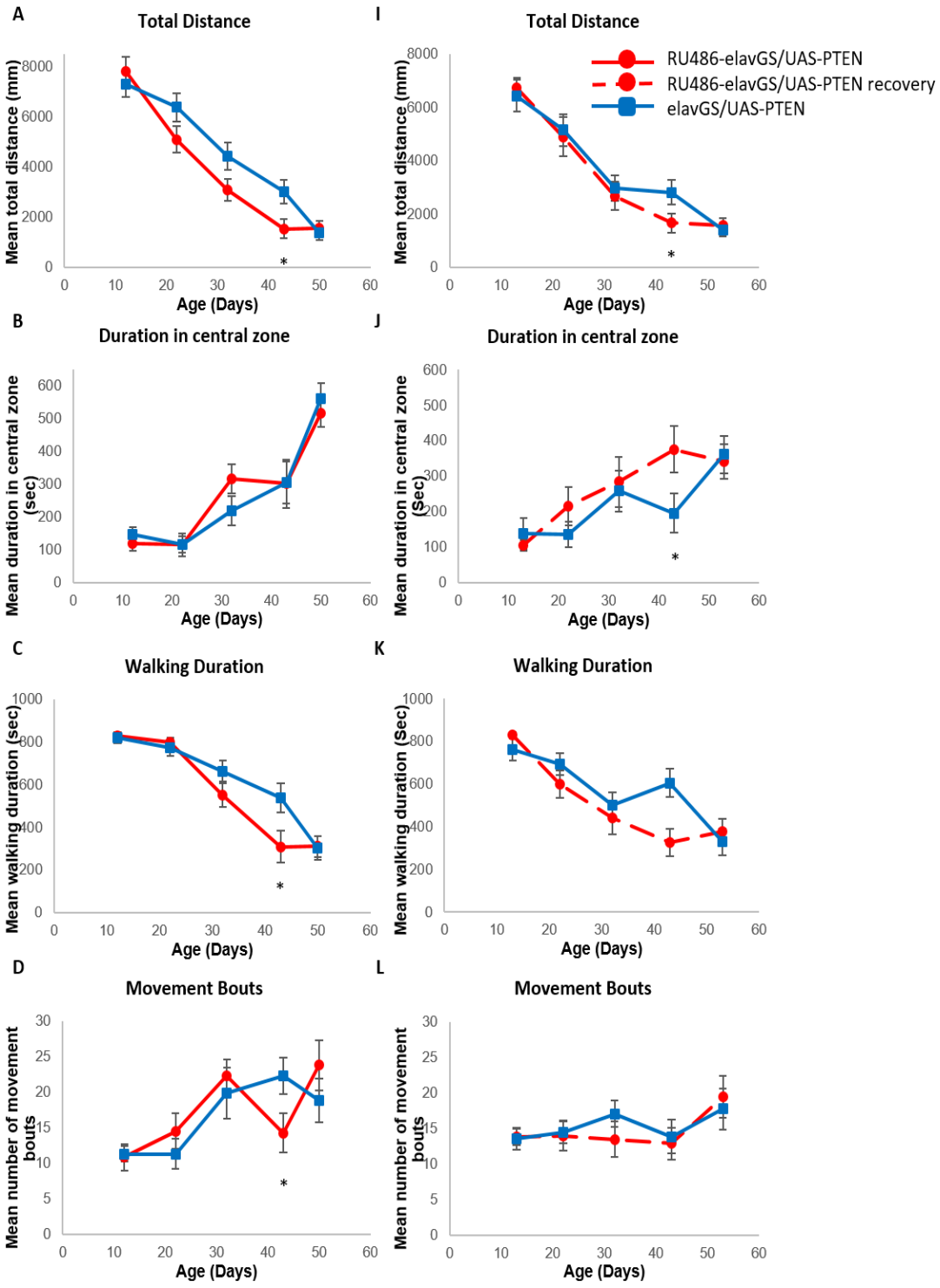


Figure 19. Exploratory walking and negative geotaxis of RU486-elavGS/+ (control) females.

A-H: Exploratory walking and negative geotaxis senescence for a cohort of female flies of the indicated genotypes (N=16). A: Female mean total distance walked (mm) vs age. B: Female mean duration in central zone (secs) vs age. C: Female mean walking duration (secs) vs age. D: Female mean number of movement bouts vs age. E: Female mean frequency of rotations (change in walking direction) vs age. F: Female mean velocity (mm/sec) vs age. G: Female mean latency of first rotation (first change of walking direction) (secs) vs age. H: Negative geotaxis performance index (PI) over the lifespan of RU486-elavGS/+ female flies compared to elavGS/+ controls, N=3 (groups of 10 flies) for each genotype. All data are shown as mean value for each parameter ±SEM. All data at individual time points by genotype or by age were analysed by two-way ANOVA followed by post hoc means comparisons using student's t test. * indicates significant differences ($p<0.05$) between the RU486-elavGS/+ and elavGS/+ controls.

In male flies, age-related changes were found in all parameters of exploratory walking and negative geotaxis: flies are less active with age and significant declines were found in total distance, walking duration, rotation frequency, velocity, and negative geotaxis; flies tended to stay longer in the central zone with age and the latency of first rotation time increased with age (Fig. 20). Experimental flies with IIS reduction throughout adulthood experienced accelerated declines in their behavioural senescence. The differences between the experimental flies and control flies became significant at older age points in nearly all parameters (Fig. 20A-H). On the other hand, in the parallel recovery experiments, a complete or partial recovery was observed in all parameters and the differences between the recovery flies and normal flies were not significant or less significant at older age point (Fig. 20I-P). A slight improvement in negative geotaxis performance was observed in recovery flies at the last age point (Fig. 20P).

In the control groups testing the RU486 drug effects, control flies on the drug (RU486-elavGS/+) appeared to have a normal behavioural senescence comparing to flies without the drug. There were significant differences observed between the two groups at the older age point (Fig. 21). However, the overall faster declines in behavioural performance in Fig. 20A-H were not found in those flies, suggesting that the detrimental effects on behavioural senescence were not drug effects.


A
Total Distance
Mean total distance (mm)
Age (Days)
I
Total Distance
RU486-elavGS/UAS-PTEN
RU486-elavGS/UAS-PTEN recovery
elavGS/UAS-PTEN
Mean total distance (mm)
Age (Days)
B
Duration in central zone
Mean duration in central zone (sec)
Age (Days)
J
Duration in central zone
Mean duration in central zone (Sec)
Age (Days)
C
Walking Duration
Mean walking duration (sec)
Age (Days)
K
Walking Duration
Mean walking duration (Sec)
Age (Days)
D
Movement Bouts
Mean number of movement bouts
Age (Days)
L
Movement Bouts
Mean number of movement bouts
Age (Days)

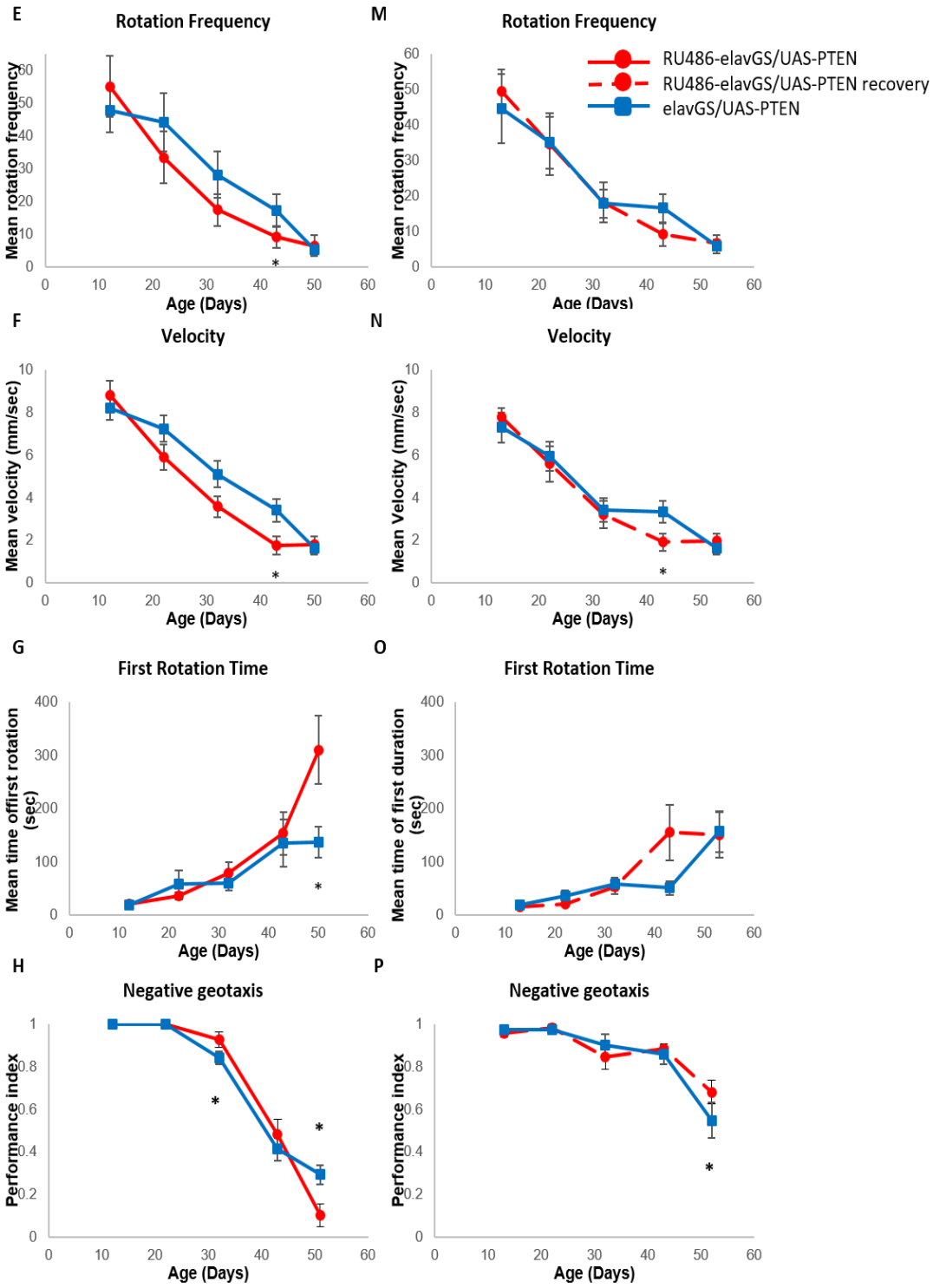


Figure 20. Exploratory walking and negative geotaxis of RU486-elavGS/UAS-PTEN males with and without a recovery period.

A-H: Exploratory walking and negative geotaxis senescence for a cohort of male flies of the indicated genotypes (without recovery period) (N=16). A: Male mean total distance walked (mm) vs age. B: Male mean duration in central zone (secs) vs age. C: Male mean walking duration (secs) vs age. D: Male mean number of movement bouts vs age. E: Male mean frequency of rotations (change in walking direction) vs age. F: Male mean velocity (mm/sec) vs age. G: Male mean latency of first rotation (first change of walking direction) (secs) vs age. H:

Negative geotaxis performance index (PI) over the lifespan of RU486-elavGS/UAS-PTEN male flies compared to elavGS/UAS-PTEN controls, N=3 (groups of 10 flies) for each genotype. I-P: Exploratory walking and negative geotaxis senescence for a cohort of male flies of the indicated genotypes (with 5-day recovery period) (N=16). I: Male mean total distance walked (mm) vs age. J: Male mean duration in central zone (secs) vs age. K: Male mean walking duration (secs) vs age. L: Male mean number of movement bouts vs age. M: Male mean frequency of rotations (change in walking direction) vs age. N: Male mean velocity (mm/sec) vs age. O: Male mean latency of first rotation (first change of walking direction) (secs) vs age. P: Negative geotaxis performance index (PI) over the lifespan of RU486-elavGS/UAS-PTEN male flies compared to elavGS/UAS-PTEN controls, N=3 (groups of 10 flies) for each genotype. All data are shown as mean value for each parameter ±SEM. All data at individual time points by genotype or by age were analysed by two-way ANOVA followed by post hoc means comparisons using student's t test. * indicates significant differences ($p<0.05$) between the RU486-elavGS/UAS-PTEN and elavGS/UAS-PTEN control.

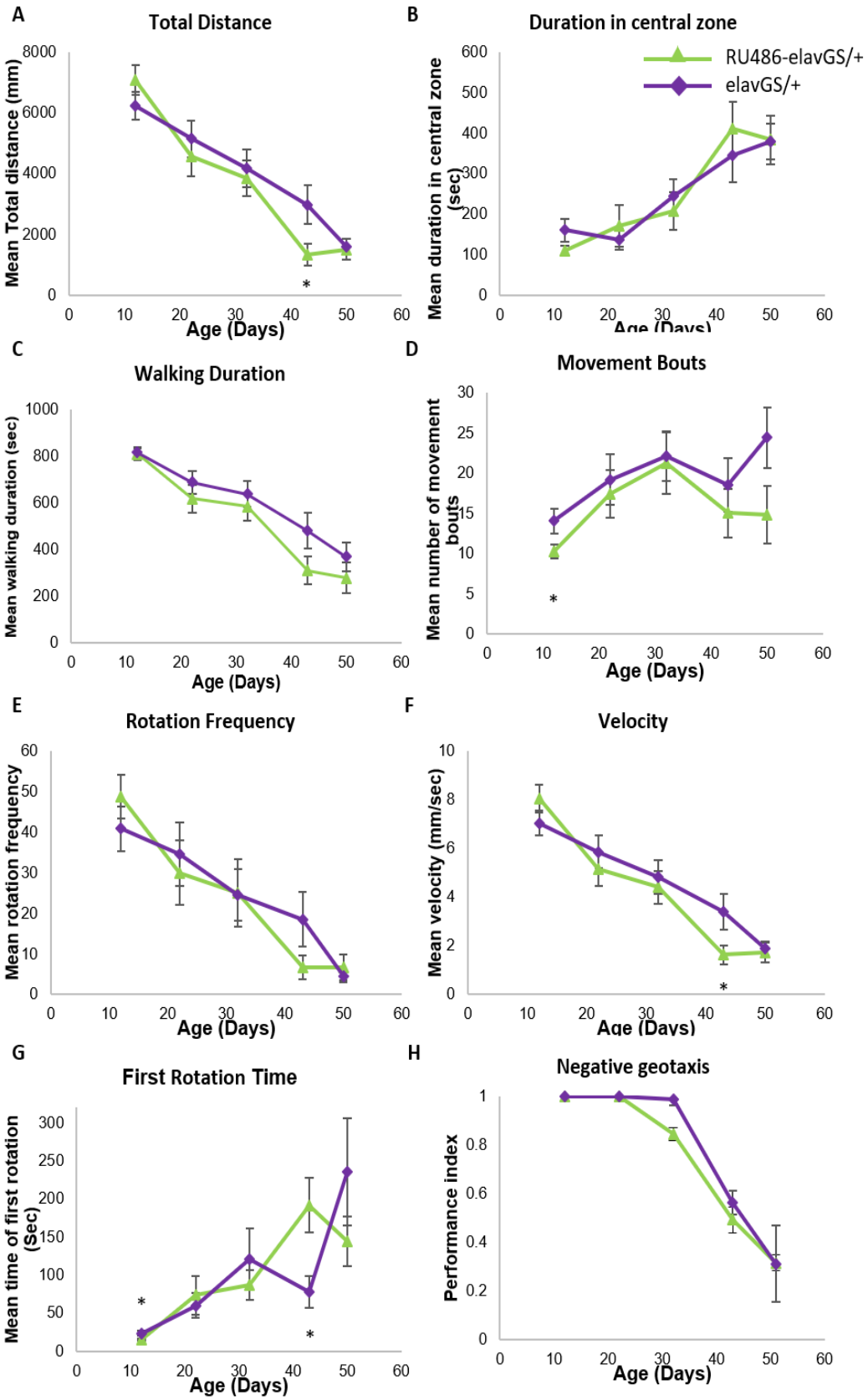


Figure 21. Exploratory walking and negative geotaxis of RU486-elavGS/+ (control) males.
A-H: Exploratory walking and negative geotaxis senescence for a cohort of male flies of the indicated genotypes (without recovery period) (N=16). A: Male mean total distance walked

(mm) vs age. B: Male mean duration in central zone (secs) vs age. C: Male mean walking duration (secs) vs age. D: Male mean number of movement bouts vs age. E: Male mean frequency of rotations (change in walking direction) vs age. F: Male mean velocity (mm/sec) vs age. G: Male mean latency of first rotation (first change of walking direction) (secs) vs age. H: Negative geotaxis performance index (PI) over the lifespan of RU486-elavGS/UAS-PTEN male flies compared to elavGS/UAS-PTEN controls, N=3 (groups of 10 flies) for each genotype. All data are shown as mean value for each parameter ±SEM. All data at individual time points by genotype or by age were analysed by two-way ANOVA followed by post hoc means comparisons using student's t test. * indicates significant differences ($p<0.05$) between the RU486-elavGS/+ and elavGS/+ controls.

5.4 Sleeping Behaviours

Reduced IIS in adult neurons resulted in transient young age detrimental effects.

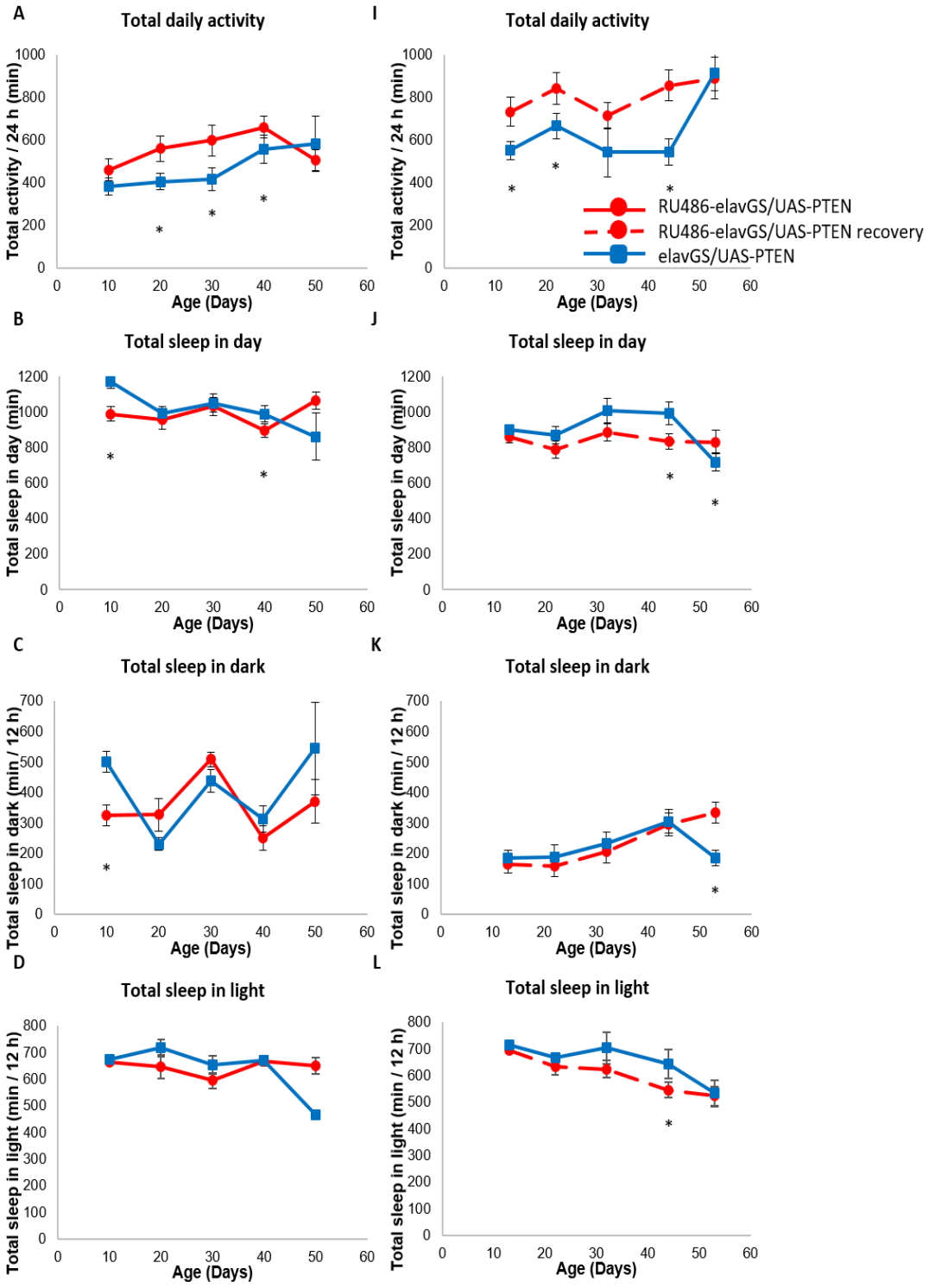
Sleeping behaviours have shown age-related decline as flies' sleep becomes more fragmented with age and more easily interrupted by their walking period. However, it is a complex behaviour and age-related change in each parameter has shown variations in different studies (Cirelli and Bushey, 2008).

The parallel sleeping experiments with or without recovery period of females are presented on the left and right side respectively in Fig. 22. In the experiment of constitutive IIS reduction in adult neurons (RU486-elavGS/UAS-PTEN), age-related changes were not clearly shown in all parameters but in the decrease of bouts of sleep in dark and average bout length in light, and the increase of bouts of sleep in light (Fig. 22A-H). Comparing with the control flies, experimental flies were more active and shown a significant increase in total daily activity and a decrease in total sleep per day at several age points (Fig. 22A&B). An early age detrimental effect was observed in several parameters and the result of decreased total sleep per day may be the combination of decreased total sleep in dark and shorter bout length in dark. Despite the unclear trend of ageing effect on sleeping patterns, there were also differences between the experimental and control flies observed at older age points and some reached statistically significant.

On the other hand, in the recovery experiment (RU486-elavGS/UAS-PTEN recovery), age-related declines were found in total sleep in light and mean bout length in light; and age-related increases were found in total sleep in dark, bouts of sleep in dark, and bouts of sleep in light (Fig. 22I-P). A certain recovery from the detrimental effect at the young age are shown in nearly all parameters. The differences between flies with IIS reduction and control flies at older age point became less significant or not significant in recovery flies. However, flies with a recovery period appeared to show a slightly faster deterioration in their activity and sleep patterns, shown as the differences in total sleep in dark, total sleep in light, bouts of sleep in dark, bouts of sleep in light, and average bout length in light.

In the control experiment of drug effects, flies on the drug (RU486-elavGS/+) appeared to be more active in the total daily activity and have a less total amount of sleep per day (Fig. 23A&B). Such differences were observed in the experimental groups as well (Fig. 22A&B), suggesting a negative effect of the RU486 drug on sleeping patterns. In other parameters of

sleeping, differences between the two control groups were found in a few time points while the majority of their activity and sleep patterns showed no difference. Taken together, some of the detrimental effects seen on experimental flies are the result of drug influence while others are the effect of IIS reduction.


A
Total daily activity
Total activity / 24 h (min)
Age (Days)
I
Total daily activity
Total activity / 24 h (min)
Age (Days)
RU486-elavGS/UAS-PTEN
RU486-elavGS/UAS-PTEN recovery
elavGS/UAS-PTEN
B
Total sleep in day
Total sleep in day (min)
Age (Days)
J
Total sleep in day
Total sleep in day (min)
Age (Days)
C
Total sleep in dark
Total sleep in dark (min / 12 h)
Age (Days)
K
Total sleep in dark
Total sleep in dark (min / 12 h)
Age (Days)
D
Total sleep in light
Total sleep in light (min / 12 h)
Age (Days)
L
Total sleep in light
Total sleep in light (min / 12 h)
Age (Days)

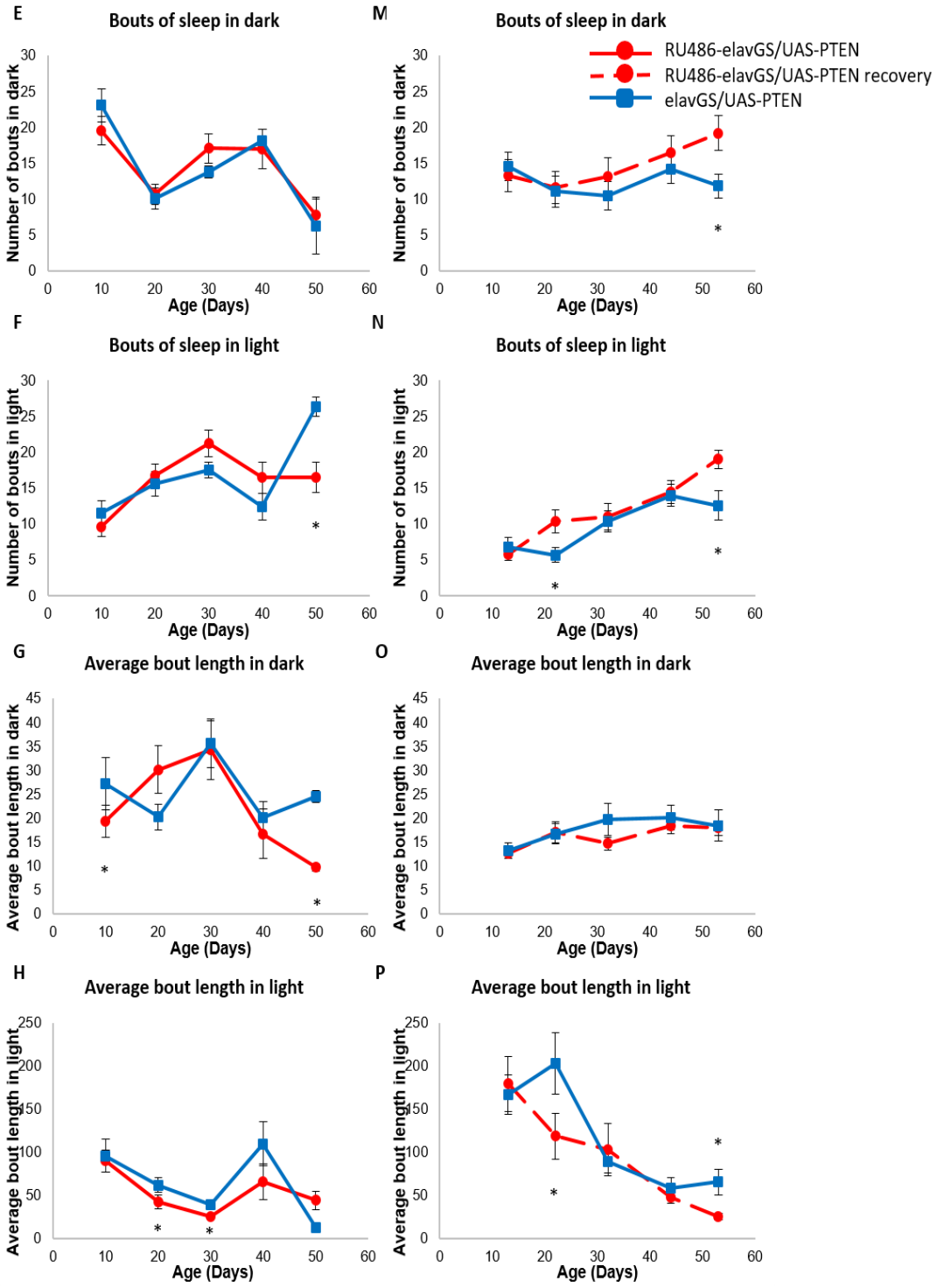


Figure 22. Daily activity and sleep pattern of RU486-elavGS/UAS-PTEN females with and without a recovery period.

A-H: Sleep behaviour senescence for a cohort of female flies of the indicated genotypes (without recovery period) (N=15). A: Female mean total activity in 24hrs (mins) vs age. B: Female total sleep in 24 hrs (mins) vs age. C: Female mean total sleep in 12hrs dark (mins) vs age. D: Female mean total sleep in 12hrs light (mins) vs age. E: Female mean total number of sleep bouts in 12hrs dark vs age. F: Female mean total number of sleep bouts in 12hrs light vs age. G: Female average bout length in 12hrs dark (mins) vs age. H: Female average bout length in 12hrs light (mins) vs age. I-P: Sleep behaviour senescence for a cohort of female flies

of the indicated genotypes (with 5-day recovery period) (N=15). I: Female mean total activity in 24hrs (mins) vs age. J: Female total sleep in 24 hrs (mins) vs age. K: Female mean total sleep in 12hrs dark (mins) vs age. L: Female mean total sleep in 12hrs light (mins) vs age. M: Female mean total number of sleep bouts in 12hrs dark vs age. N: Female mean total number of sleep bouts in 12hrs light vs age. O: Female average bout length in 12hrs dark (mins) vs age. P: Female average bout length in 12hrs light (mins) vs age. All data are shown as mean value for each parameter ±SEM. All data at individual time points by genotype or by age were analysed by two-way ANOVA followed by post hoc means comparisons using student's t test. * indicates significant differences ($p<0.05$) between the RU486-elavGS/UAS-PTEN and elavGS/UAS-PTEN controls.

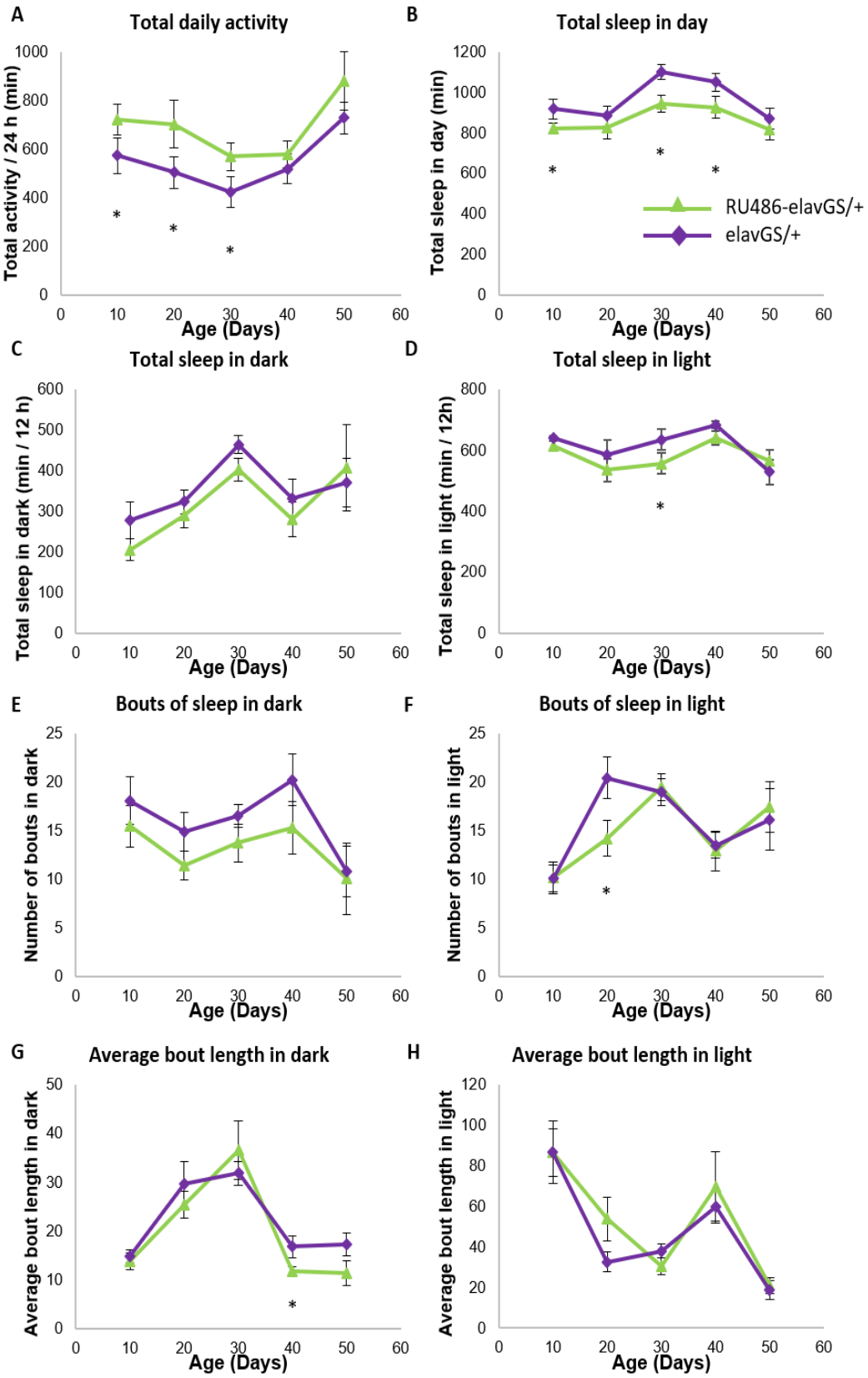


Figure 23. Daily activity and sleep pattern of RU486-elavGS/+ (control) females.

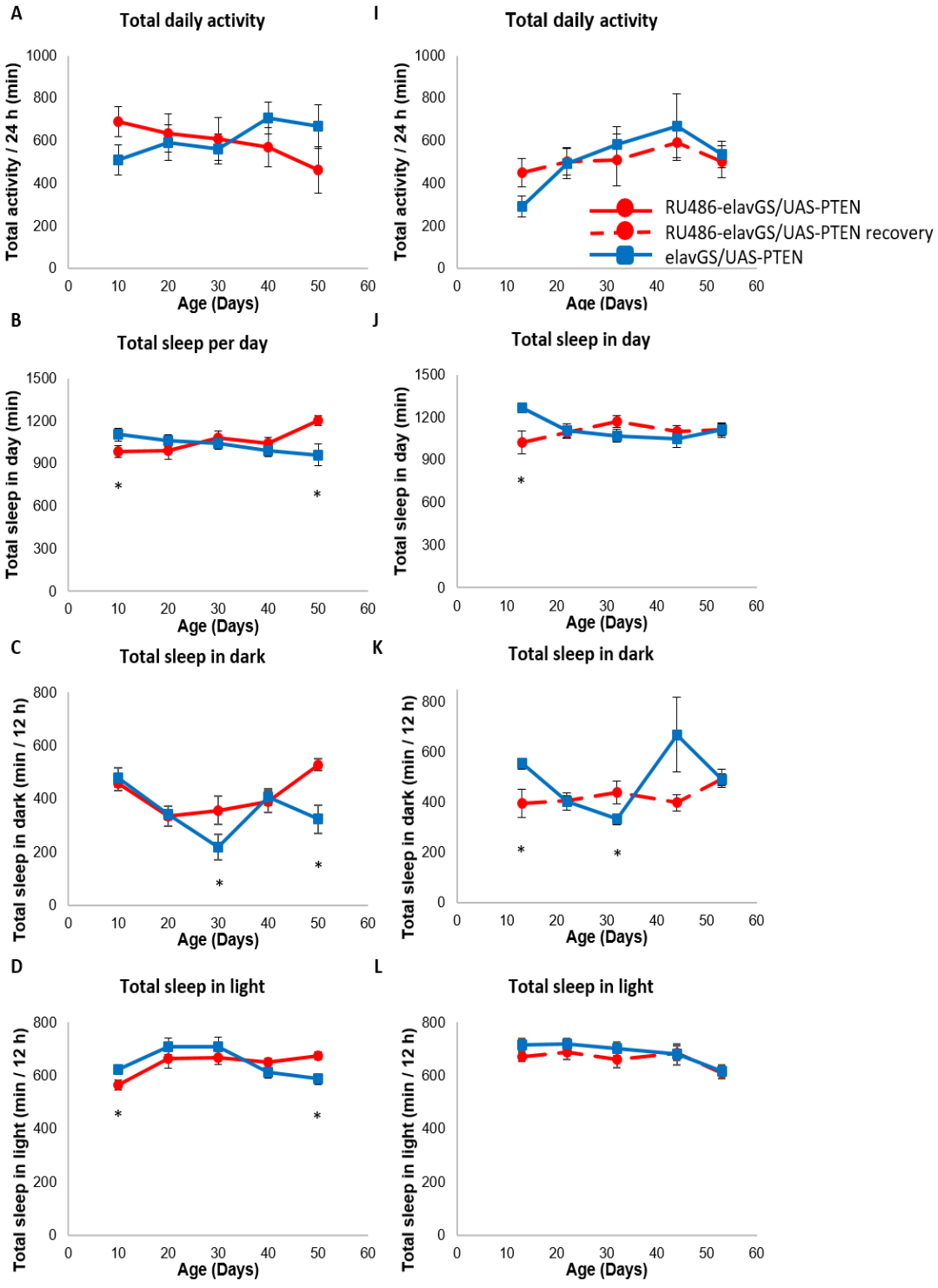
A-H: Sleep behaviour senescence for a cohort of female flies of the indicated genotypes (N=15). A: Female mean total activity in 24hrs (mins) vs age. B: Female total sleep in 24 hrs (mins) vs age. C: Female mean total sleep in 12hrs dark (mins) vs age. D: Female mean total sleep in 12hrs light (mins) vs age. E: Female mean total number of sleep bouts in 12hrs dark vs

age. F: Female mean total number of sleep bouts in 12hrs light vs age. G: Female average bout length in 12hrs dark (mins) vs age. H: Female average bout length in 12hrs light (mins) vs age. All data are shown as mean value for each parameter ±SEM. All data at individual time points by genotype or by age were analysed by two-way ANOVA followed by post hoc means comparisons using student's t test. * indicates significant differences ($p<0.05$) between the RU486-elavGS/+ and elavGS/+ controls.

Turning to the sleeping experiments in males with IIS reduction throughout the adult period (RU486-elavGS/UAS-PTEN), robust ageing-related changes were only seen in the sleep bouts in light and bout length in both dark and light (Fig. 24A-H). The same early-age detrimental effects were found in males as well and the differences were especially significant in sleep bouts in dark and light, and mean bout length in light. In contrast to the control groups, a lack of ageing-induced changes was observed in the experimental flies, especially in bouts of sleep in light and average bout length in light, resulting in significant differences between the two groups at older age points (Fig. 24F&H).

To answer the question whether this is ameliorated ageing or detrimental functional effects at the young age that flies cannot recover from, the recovery experiments were performed. RU486-elavGS/UAS-PTEN recovery flies showed a complete recovery from the negative effects in total sleep in light and partial recovery in bouts of sleep in dark and in light, and average bout length in light (Fig. 24I-P). The ageing-related changes were partially restored in some parameters and there is no difference between the two groups at older age points anymore. However, interestingly, a negative effect at the first age point was still found in some parameters, including total sleep per day, total sleep in dark, and average bout length in dark.

In the examination of drug effects, the RU486-elavGS/+ flies behaved and aged similarly to the control flies overall and there were differences found between the two groups only at some age points (Fig. 25). The differences between RU486-elavGS/+ and elavGS/+ flies in total sleep per day and total sleep in light are similar to the differences seen in the experimental groups, suggesting a potential drug influence on these two parameters. However, other differences observed in the experimental groups were not found in these control flies, suggesting that they were the result of IIS reduction rather than a drug effect.


A
Total daily activity
Total activity / 24 h (min)
Age (Days)
I
Total daily activity
Total activity / 24 h (min)
Age (Days)
RU486-elavGS/UAS-PTEN
RU486-elavGS/UAS-PTEN recovery
elavGS/UAS-PTEN
B
Total sleep per day
Total sleep in day (min)
Age (Days)
J
Total sleep in day
Total sleep in day (min)
Age (Days)
C
Total sleep in dark
Total sleep in dark (min / 12 h)
Age (Days)
K
Total sleep in dark
Total sleep in dark (min / 12 h)
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Age (Days)
L
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Total sleep in light (min / 12 h)
Age (Days)

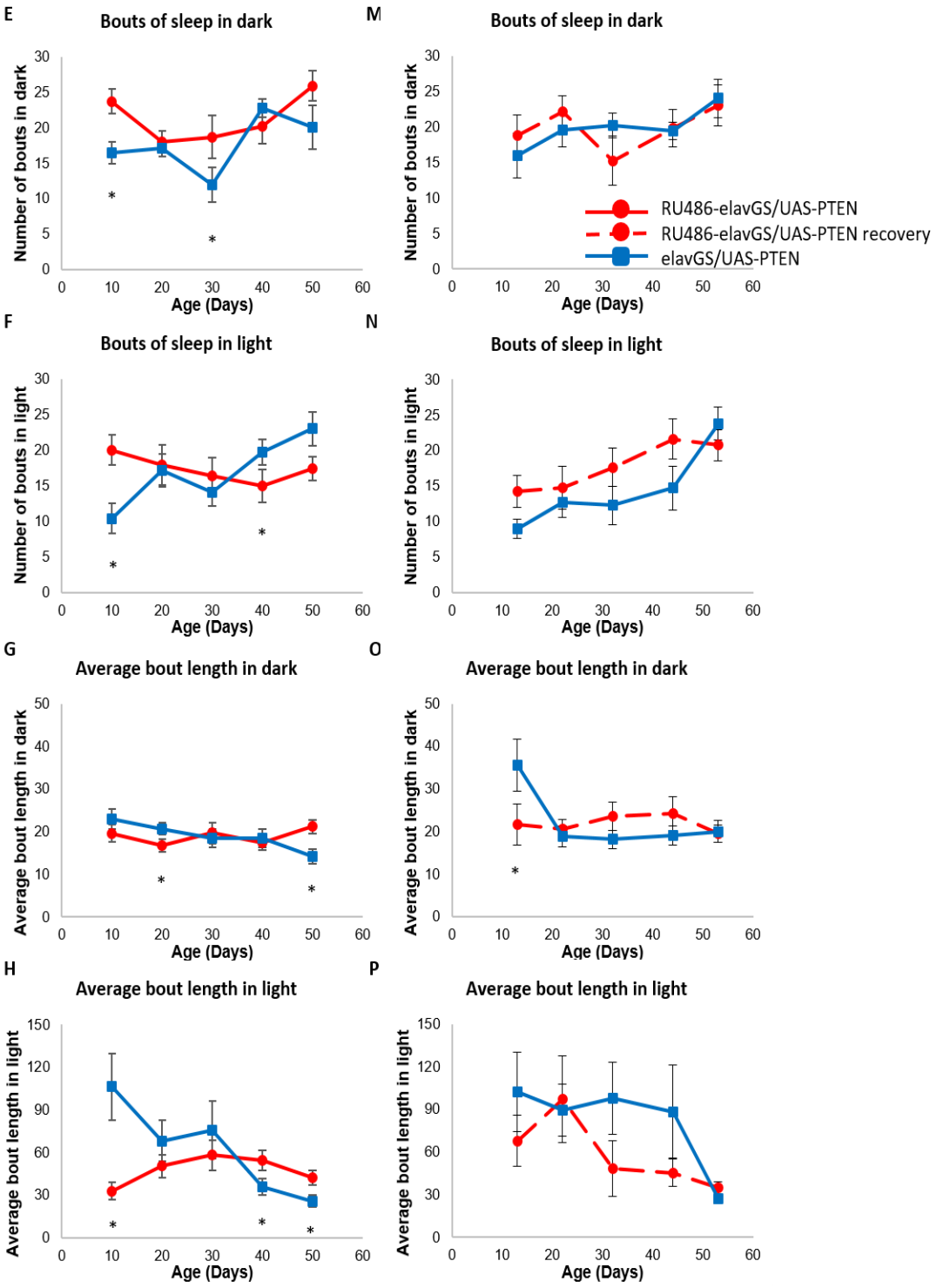

Figure 24. Daily activity and sleep pattern of RU486-elavGS/UAS-PTEN males with and without a recovery period.

A-H: Sleep behaviour senescence for a cohort of male flies of the indicated genotypes (without recovery period) (N=15). A: Male mean total activity in 24hrs (mins) vs age. B: Male total sleep in 24 hrs (mins) vs age. C: Male mean total sleep in 12hrs dark (mins) vs age. D: Male mean total sleep in 12hrs light (mins) vs age. E: Male mean total number of sleep bouts in 12hrs dark vs age. F: Male mean total number of sleep bouts in 12hrs light vs age. G: Male average bout length in 12hrs dark (mins) vs age. H: Male average bout length in 12hrs light (mins) vs age. I-P: Sleep behaviour senescence for a cohort of male flies of the indicated

genotypes (with 5-day recovery period) (N=15). I: Male mean total activity in 24hrs (mins) vs age. J: Male total sleep in 24 hrs (mins) vs age. K: Male mean total sleep in 12hrs dark (mins) vs age. L: Male mean total sleep in 12hrs light (mins) vs age. M: Male mean total number of sleep bouts in 12hrs dark vs age. N: Male mean total number of sleep bouts in 12hrs light vs age. O: Male average bout length in 12hrs dark (mins) vs age. P: Male average bout length in 12hrs light (mins) vs age. All data are shown as mean value for each parameter ±SEM. All data at individual time points by genotype or by age were analysed by two-way ANOVA followed by post hoc means comparisons using student's t test. * indicates significant differences ($p<0.05$) between the RU486-elavGS/UAS-PTEN and elavGS/UAS-PTEN controls.

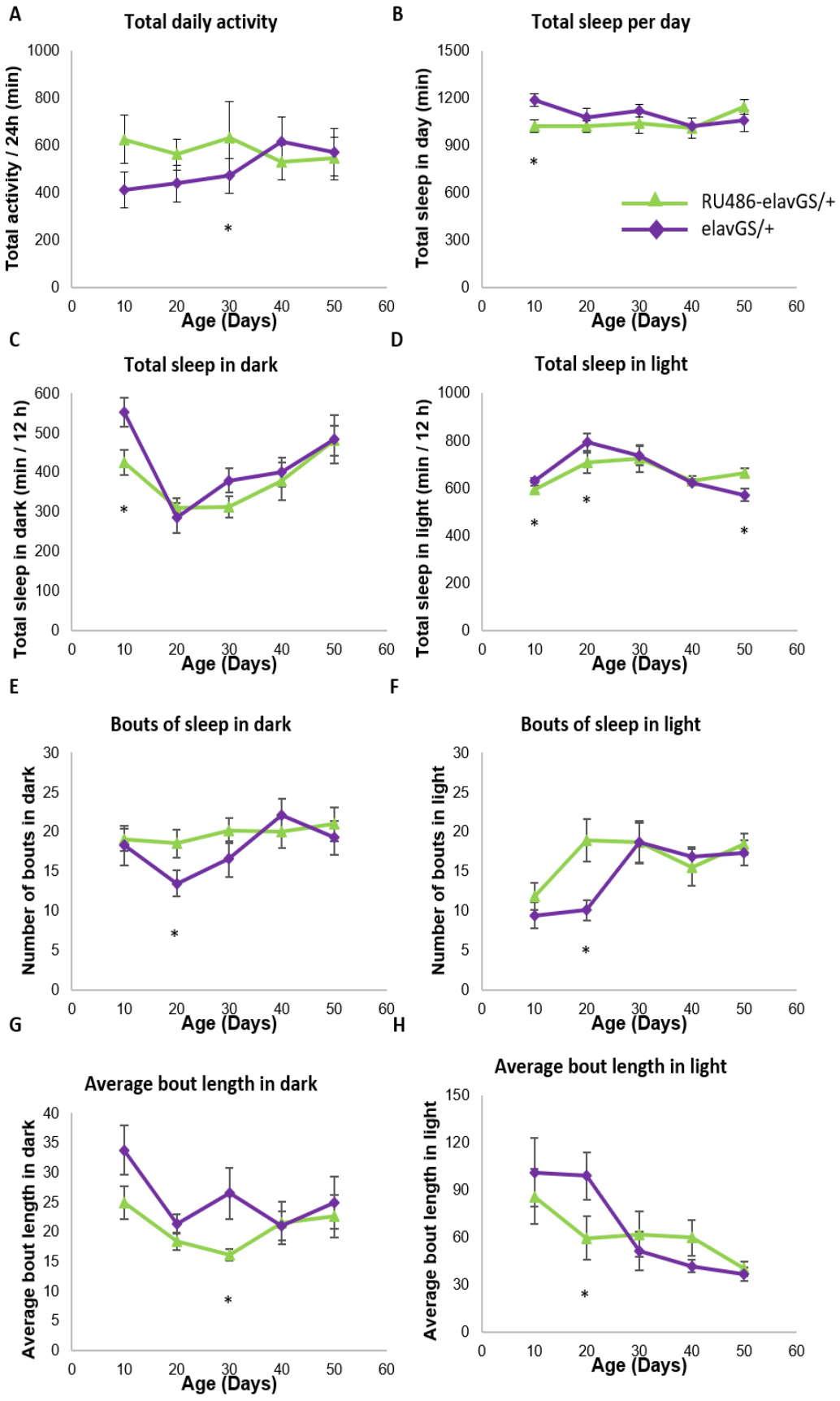

Figure 25. Daily activity and sleep pattern of RU486-elavGS/+ (control) males.

A-H: Sleep behaviour senescence for a cohort of male flies of the indicated genotypes (N=15). A: Male mean total activity in 24hrs (mins) vs age. B: Male total sleep in 24 hrs (mins) vs age. C: Male mean total sleep in 12hrs dark (mins) vs age. D: Male mean total sleep in 12hrs light (mins) vs age. E: Male mean total number of sleep bouts in 12hrs dark vs age. F: Male mean total number of sleep bouts in 12hrs light vs age. G: Male average bout length in 12hrs dark (mins) vs age. H: Male average bout length in 12hrs light (mins) vs age. All data are shown as mean value for each parameter ±SEM. All data at individual time points by genotype or by age were analysed by two-way ANOVA followed by post hoc means comparisons using student's t test. * indicates significant differences ($p<0.05$) between the RU486-elavGS/+ and elavGS/+ controls.

5.5 Summary

To conclude the results on the inducible IIS reduction in adult neurons, RU486-elavGS/UAS-PTEN females were normal-lived or slightly short-lived with normal fecundity (the apparent reduction in fecundity was RU486 drug-induced). RU486-elavGS/UAS-PTEN males were significantly short-lived in both experiments. Adult specific expression of PTEN resulted in an earlier decline in some parameters of locomotor behaviours (including total distance, walking duration, rotation frequency, velocity, latency to first rotation time, and negative geotaxis) compared to controls in both females and males. The differences between the performance of RU486-elavGS/UAS-PTEN and elavGS/UAS-PTEN flies were larger in males but both in females and males, the differences became significant at older age points. When flies were allowed to recover IIS function prior to behavioural testing, the detrimental effects were no longer observed or at least ameliorated in all parameters.

On the other hand, the effect of inducible IIS reduction on sleeping behaviours is more complex and age-related changes in sleep patterns were not clear in our experiments. RU486-elavGS/UAS-PTEN flies presented a drug-induced hyperactivity and reduced amount of sleep in both females and males. Adult specific expression of PTEN resulted in an early age detrimental effect observed in several parameters - different between females and males - including total sleep in dark, total sleep in light, bouts of sleep in dark, bouts of sleep in light, average bout length in dark, and average bout length in light. A lack of ageing-related changes was observed in RU486-elavGS/UAS-PTEN flies in some parameters and resulted in the significant differences at older ages between the RU486-elavGS/UAS-PTEN and elavGS/UAS-PTEN flies. When flies were allowed to recover IIS function before behavioural testing, such young age effects were completely or at least partially recovered, and the age-related changes were observed in RU486-elavGS/UAS-PTEN flies again.

Taken together, these data suggest that the detrimental effects of reduced IIS in neurons are due to functional changes that flies can recover from. However, no beneficial effects of reduced IIS on behavioural senescence were observed, even following a recovery period. This suggests that either PTEN expression resulted in long-lasting detrimental effects on neuronal function or that PTEN expression did not delay or slow neuronal ageing.

6. Results – The Effect of IIS Reduction Using elavGAL4/UAS-PTEN and elavGS/UAS-PTEN on Stress Resistance, Energy Storage, and Cell Apoptosis

6.1 Introduction

The effect of IIS reduction in neurons on behavioural function and senescence during development and adulthood has been investigated above in lifespan, fecundity, and different behavioural analysis. However, in contrast to the lifespan extension induced by reduced IIS in literature, overexpression of PTEN in flies' neurons throughout lifespan or adulthood is not beneficial to their lifespan, especially in males. Strong systemic reductions in IIS are known to be detrimental to lifespan due to the pleiotropic nature of the pathway (Ikeya et al., 2009; Karpac and Jasper, 2009). To investigate if over-expression of PTEN in neurons resulted in systemic effects in flies, phenotypes known to be influenced by IIS such as energy storage and stress resistance were measured.

The IIS pathway is involved in glycogen synthesis, energy storage, glucose metabolism, and stress resistance. The pathway also interacts with other pathways such as TOR or JNK in response to nutrient levels and environmental stress (Broughton and Partridge, 2009). Previous research has reported that long-lived *Drosophila* mutants (IPC-ablated flies and dFOXO mutant) with systemic IIS reduction exhibited increased resistance to starvation and oxidative stress (Broughton et al., 2005; Slack et al., 2011). In addition to increased stress resistance, IPC-ablated flies also presented higher levels of trehalose, lipid and glycogen storages (Broughton et al., 2005). In other models, the downregulation of DILP2 was associated with an increased level of trehalose and resistance to starvation (Broughton et al., 2008); while DILP2 loss-of-function mutant did not show any changes in lipid or glycogen levels (Grönke et al., 2010). Grönke et al. (2010) also reported the increased starvation resistance in DILP1-4 loss-of-function mutants but not in DILP2-3, 5 deletion mutants.

Therefore, two stress resistance tests – starvation and oxidative stress – were performed with elavGAL4/UAS-PTEN and RU486-elavGS/UAS-PTEN flies to understand if the CNS is involved in the regulation of stress resistance or the role of neuronal-restricted IIS reduction on peripheral tissues. The levels of glycogen and lipid in the whole body were measured as another indication of the effects of IIS reduction in neurons on peripheral tissues. An apoptosis assay

was used to directly observing brain structure and apoptotic cells to examine the molecular changes caused by IIS reduction in neurons.

6.2 Stress Resistance Test – Starvation and Oxidative Stress

Pan-neural IIS reduction had no effect on stress resistance; adult-specific neural IIS reduction increased males' starvation resistance.

For starvation and oxidative stress test, 10-day old elavGAL4/UAS-PTEN flies were placed onto agar only or hydrogen peroxide food to induce starvation or oxidative stress. The lifespans of flies were measured as the indication of their resistance to environmental stress. The lifespans of elavGAL4/UAS-PTEN flies were compared with the lifespan of two control genotypes. In both females and males, there were no significant differences in their resistance to either starvation or oxidative stress (Fig. 26).

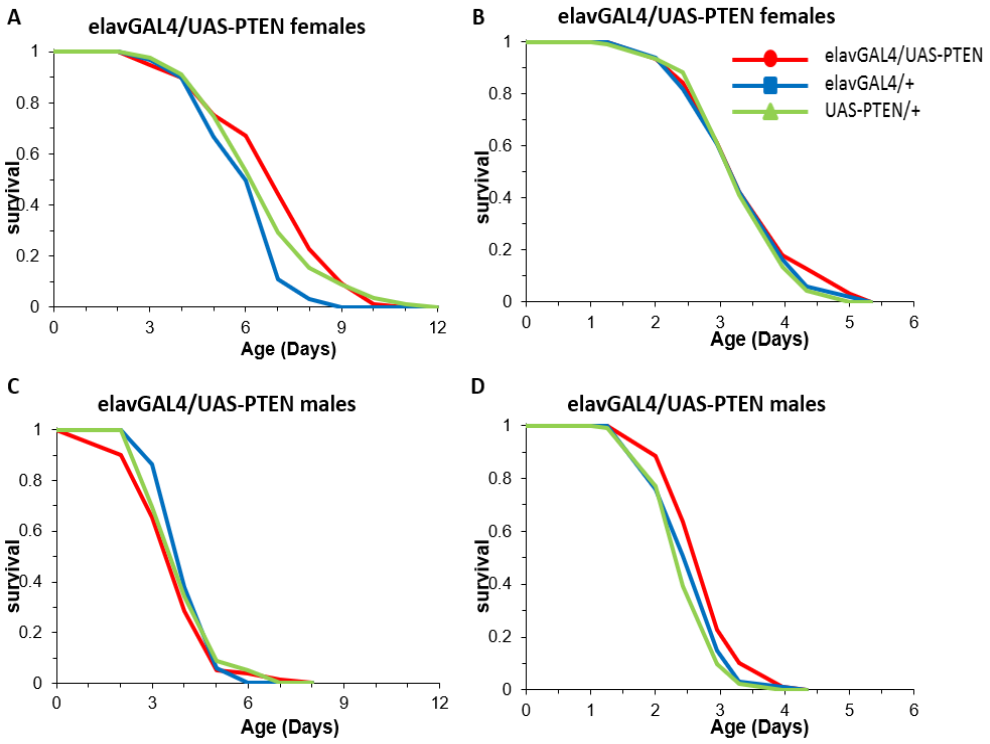

Figure 26. Starvation and oxidative stress survival curve of elavGAL4 /UAS-PTEN flies.
A-B: The survival curve of elavGAL4/UAS-PTEN females on starvation and hydrogen peroxide (oxidative stress) food (N=100). There was no significant difference between the experimental flies and the two control groups ($p>0.05$ by log-rank test). C-D: The survival curve of elavGAL4/UAS-PTEN males on starvation and hydrogen peroxide (oxidative stress) food (N=100). There was no significant difference between the experimental flies and the two control groups ($p>0.05$ by log-rank test).

For experiments on transgenic flies with the elavGS driver with adult specific PTEN expression, flies were allowed to develop normally from embryo to adult, and then RU486-elavGS/UAS-PTEN and RU486-elavGS/+ adult flies were placed onto RU486 drug from day 1. All flies were then transferred to starvation or oxidative stress food accordingly on day 10. The starvation and oxidative stress experiments were performed twice for each gender with N=100 for each experiment.

In terms of starvation resistance in females, in the first experiment, there was no significant change between the RU486-elavGS/UAS-PTEN and elavGS/UAS-PTEN flies (Fig. 27A). A slight difference induced by the RU486 drug was found between the RU486-elavGS/+ and elavGS/+ control groups (Fig. 27B). In the second repeat of this experiment, there was still no significant change of starvation resistance in RU486-elavGS/UAS-PTEN flies comparing to control flies (Fig. 27C). The drug-induced difference was not found in the second repeat (Fig. 27D). Turning on to the oxidative stress experiment in females, in the first experiment, a significant reduction of their resistance to oxidative stress was observed in the RU486-elavGS/UAS-PTEN flies (Fig. 28A). However, such reduced tolerance was not observed in the experimental flies in the second experiment (Fig. 28C). There was no difference caused by the RU486 drug in both experiments (Fig. 28B&D).

In comparison, RU486-elavGS/UAS-PTEN male flies showed a significant increase in starvation resistance in both experiments (Fig. 29A&C) and this difference was not caused by the RU486 drug as there was no difference between the two control groups (Fig. 29B&D). However, the resistance to oxidative stress in RU486-elavGS/UAS-PTEN males was not improved in both experiments (Fig. 30A&C). RU486-elavGS/+ was significantly short-lived and less resistant to oxidative stress in the first experiment (Fig. 30B) but appeared to be normal in the second experiment (Fig. 30D).

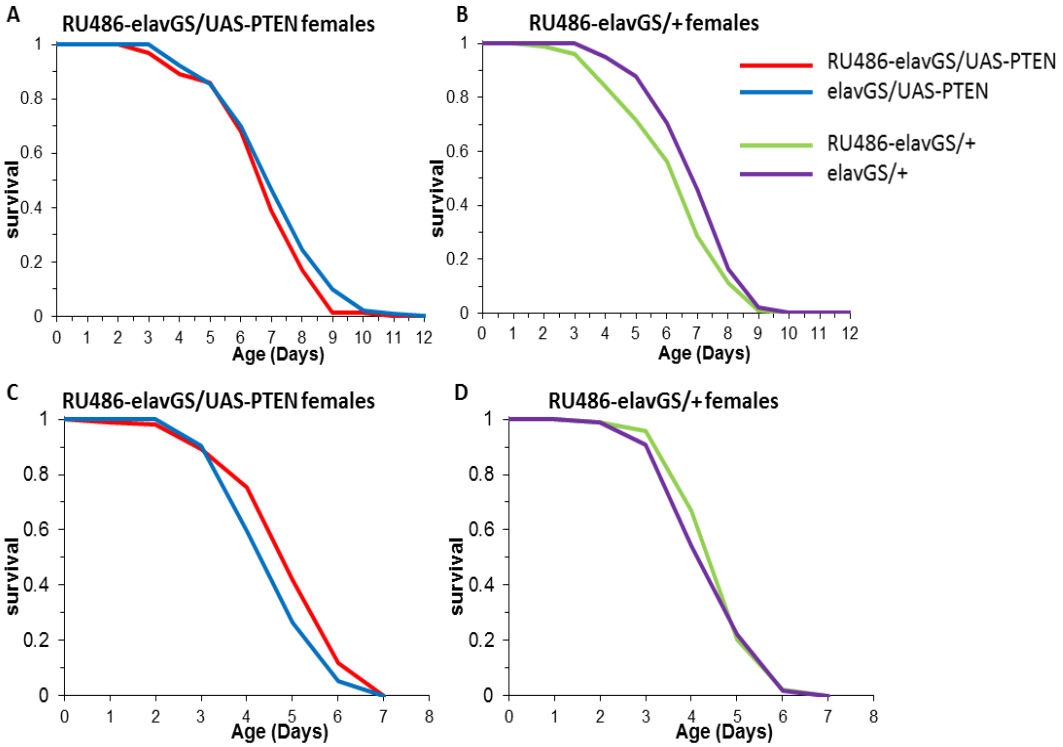

Figure 27. Starvation survival curve of RU486-elavGS/UAS-PTEN and RU486-elavGS/+ (control) females.

A-B: The first starvation experiment on RU486-elavGS/UAS-PTEN females and the control groups for drug test (N=100). There was no significant difference between the RU486-elavGS/UAS-PTEN and elavGS/UAS-PTEN flies but there was a significant difference between the RU486-elavGS/+ and elavGS/+ flies ($p<0.05$ by log-rank test). C-D: The second starvation experiment on RU486-elavGS/UAS-PTEN females and the control groups for drug test (N=100). There was no significant difference shown in both graphs ($p>0.05$ by log-rank test).

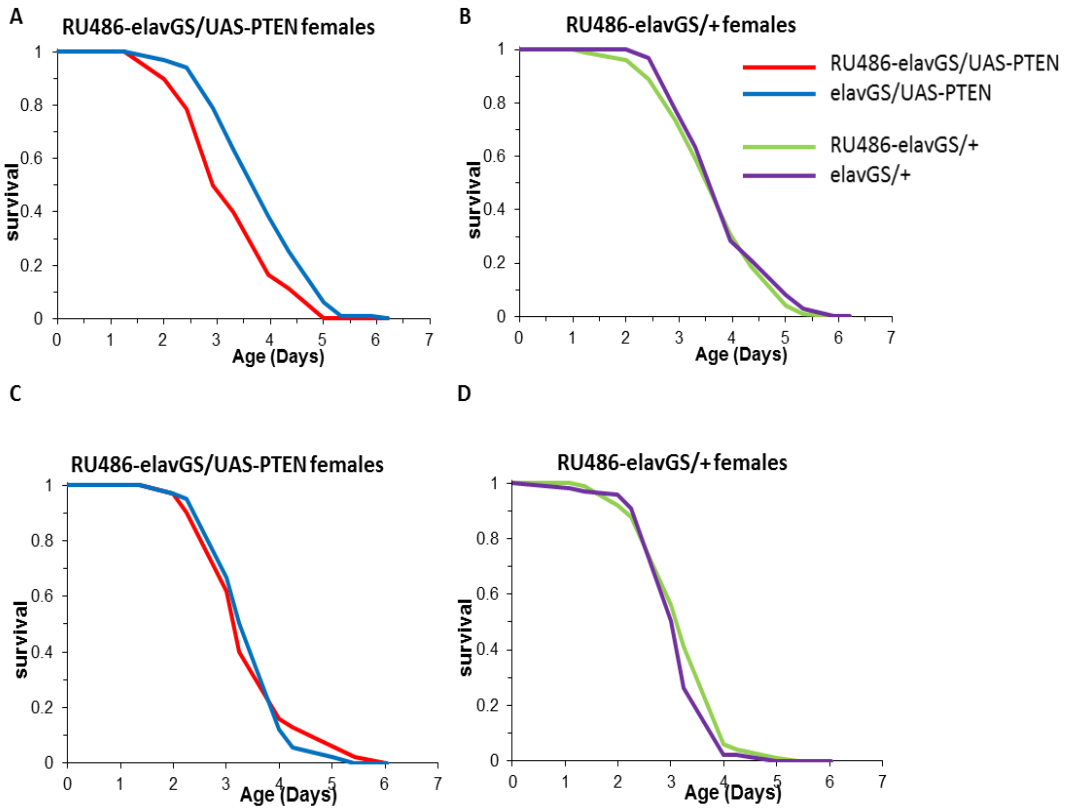

Figure 28. Oxidative stress survival curve of RU486-elavGS/UAS-PTEN and RU486-elavGS/+ (control) females.

A-B: The first oxidative stress experiment on RU486-elavGS/UAS-PTEN females and the control groups for drug test (N=100). RU486-elavGS/UAS-PTEN flies were significantly less resistant to oxidative stress than elavGS/UAS-PTEN flies ($p<0.05$ by log-rank test). There was no difference caused by RU486 drug between the RU486-elavGS/+ and elavGS/+ flies. C-D: The second oxidative stress experiment on RU486-elavGS/UAS-PTEN females and the control groups for drug test (N=100). There was no significant difference shown in both graphs ($p>0.05$ by log-rank test).

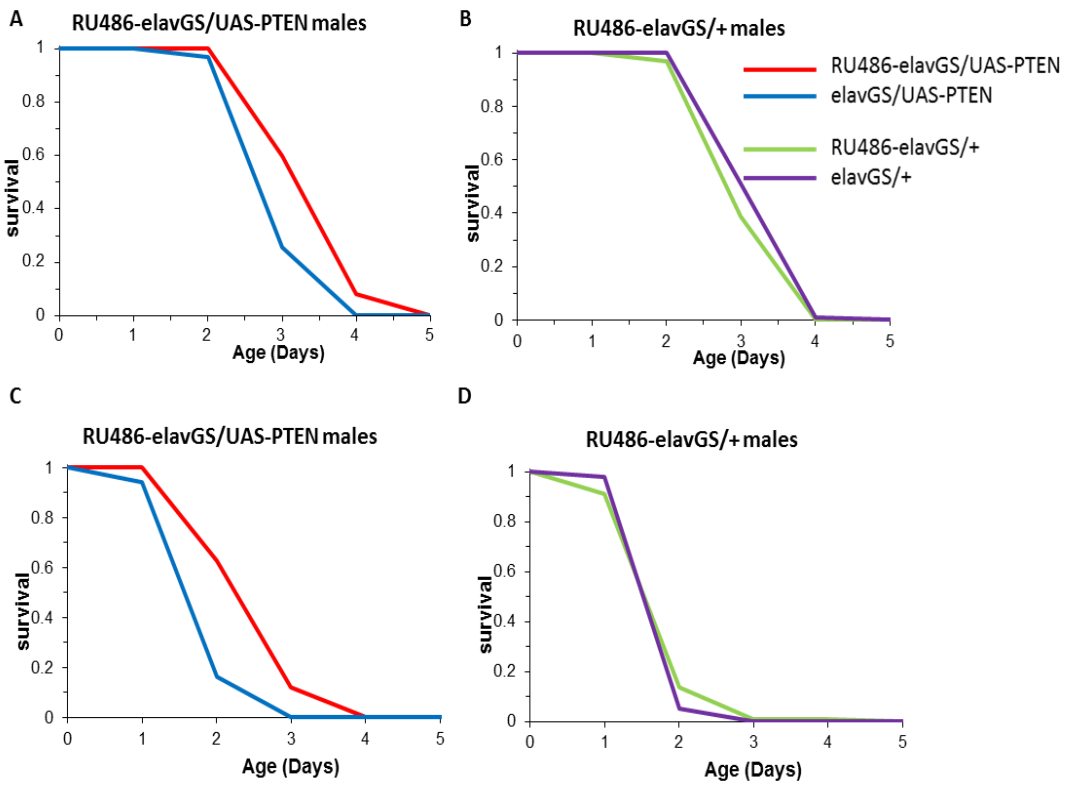

Figure 29. Starvation survival curve of RU486-elavGS/UAS-PTEN and RU486-elavGS/+ (control) males.

A-B: The first starvation experiment on RU486-elavGS/UAS-PTEN males and the control groups for drug test (N=100). There was a significant difference between the RU486-elavGS/UAS-PTEN and elavGS/UAS-PTEN flies ($p<0.05$ by log-rank test) and there was no significant difference between the RU486-elavGS/+ and elavGS/+ flies. C-D: The second starvation experiment on RU486-elavGS/UAS-PTEN males and the control groups for drug test (N=100). There was a significant difference between the experimental flies ($p<0.05$ by log-rank test) and no drug-induced difference shown in the last graph.

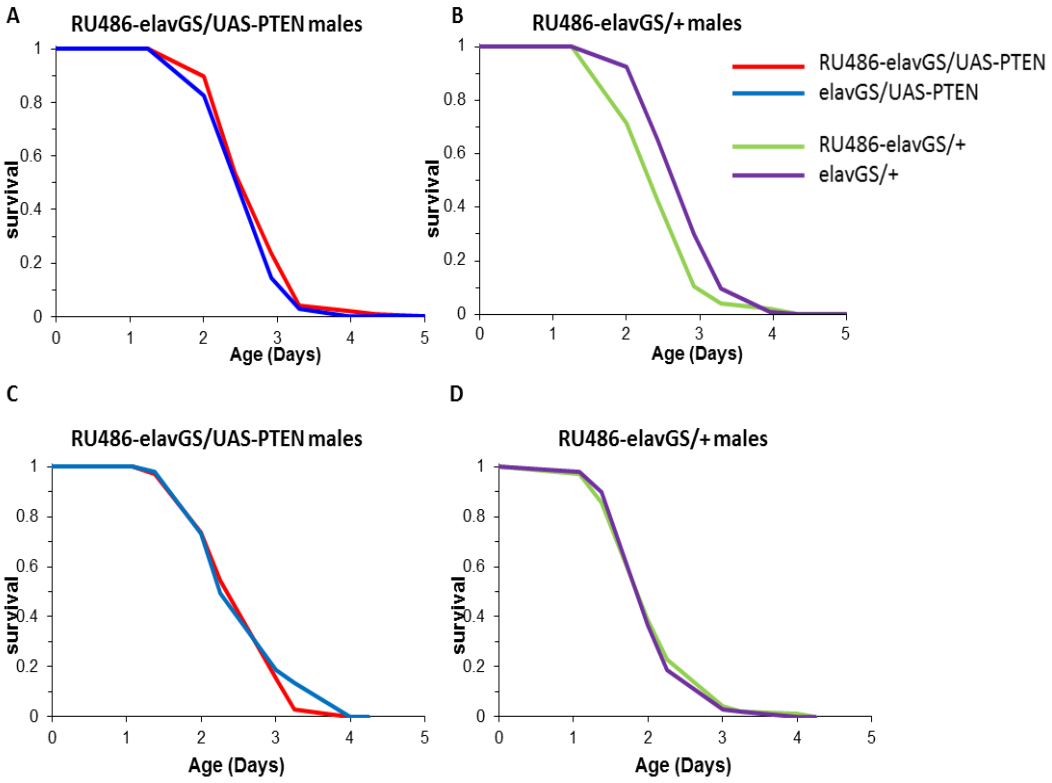

Figure 30. Oxidative stress survival curve of RU486-elavGS/UAS-PTEN and RU486-elavGS/+ (control) males.

A-B: The first oxidative stress experiment on RU486-elavGS/UAS-PTEN males and the control groups for drug test (N=100). There was no significant difference between RU486-elavGS/UAS-PTEN flies and flies off the drug but there was a significant drug effect on RU486-elavGS/+ ($p<0.05$ by log-rank test). C-D: The second oxidative stress experiment on RU486-elavGS/UAS-PTEN males and the control groups for drug test (N=100). There was no significant difference shown in both graphs ($p>0.05$ by log-rank test).

6.3 Glycogen and Lipid content assay

Pan-neural IIS reduction increased lipid content in females; adult-specific neural IIS reduction had no effect on energy storage.

In terms of the glycogen and lipid concentration in elavGAL4/UAS-PTEN flies, the concentration of lipid per fly in the elavGAL4/UAS-PTEN females was significantly higher than in the two control groups; the level of glycogen in the experimental group was not significantly different to both of the controls (Fig. 31A&B). Interestingly, despite the shortened lifespan of elavGAL4/UAS-PTEN male flies, there was no significant difference in the glycogen and lipid content between those flies and the two control flies (Fig. 31C&D).

As for the measure of glycogen and lipid content in RU486-elavGS/UAS-PTEN female flies, there was no difference in the glycogen concentration between RU486-elavGS/UAS-PTEN and elavGS/UAS-PTEN flies but a significant difference was found between RU486-elavGS/+ and elavGS/+ flies (Fig. 32A&B). On the other hand, the level of lipid content in RU486-elavGS/UAS-PTEN did not differentiate from that in elavGS/UAS-PTEN flies and no drug-induced difference was detected either (Fig. 32C&D). As for the concentration of glycogen and lipid per fly in RU486-elavGS/UAS-PTEN males, there was no difference found between RU486-elavGS/UAS-PTEN and elavGS/UAS-PTEN flies (Fig. 33A&B) and no difference between the control flies with or without drug (Fig. 33C&D).

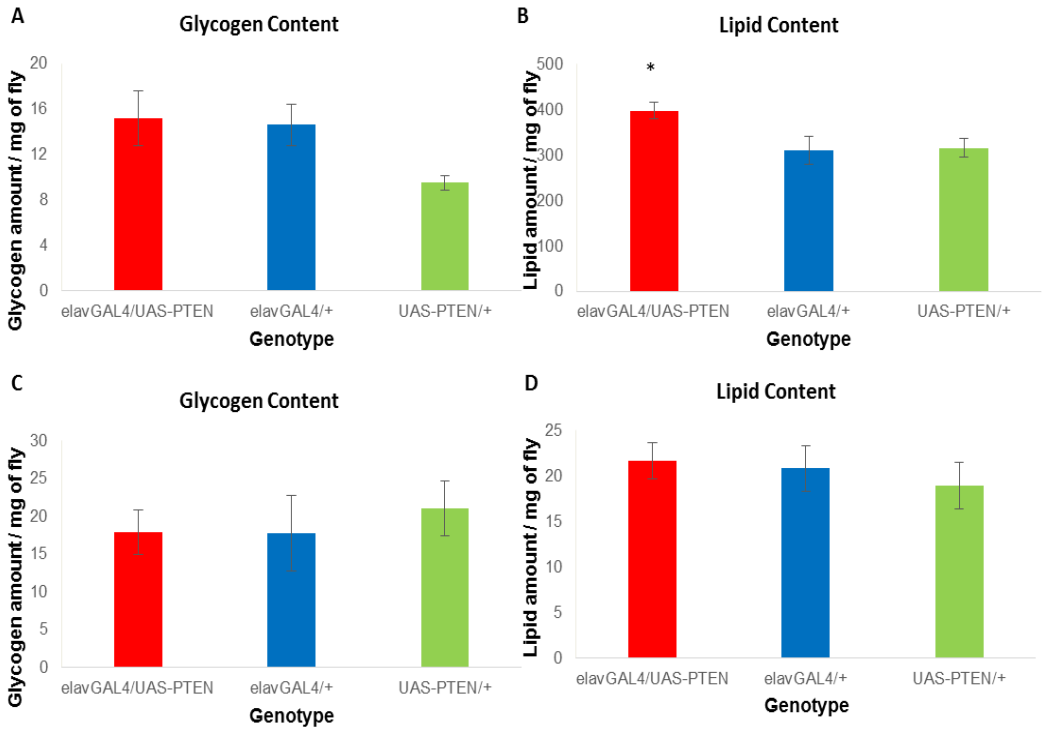

Figure 31. Glycogen and Lipid content of elavGAL4/UAS-PTEN females and males.
A-B: The average concentration of glycogen and lipid per fly in elavGAL4/UAS-PTEN females (N=10). C-D: The average concentration of glycogen and lipid per fly in elavGAL4/UAS-PTEN males (N=10). Data are shown as mean value ±SEM. Data were analysed by two-way ANOVA followed by post hoc means comparisons using Tukey HSD. * indicates significant differences ($p<0.05$) between elavGAL4/UAS-PTEN and the two control group flies.

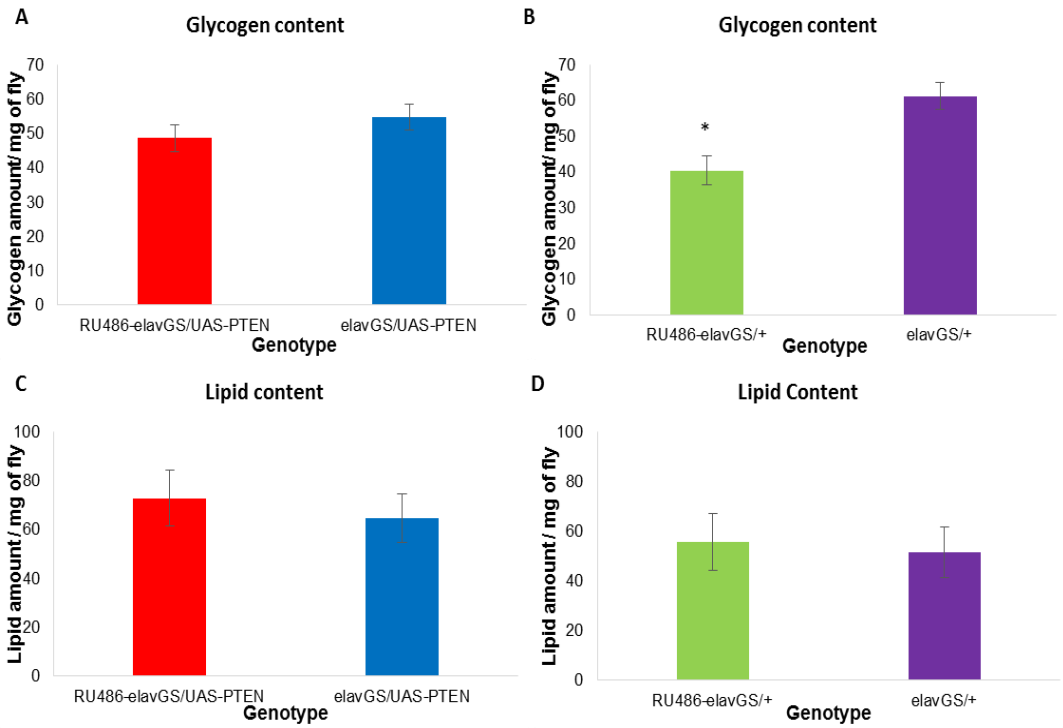

Figure 32. Glycogen and Lipid content of RU486-elavGS/UAS-PTEN and RU486-elavGS/+ (control) females.

A-B: The average concentration of glycogen and lipid per fly in RU486-elavGS/UAS-PTEN and RU486-elavGS/+ (control) females (N=20). C-D: The average concentration of glycogen and lipid per fly in RU486-elavGAL4/UAS-PTEN and RU486-elavGS/+ (control) females (N=20). Data are shown as mean value ±SEM. Data were analysed by two-way ANOVA followed by post hoc means comparisons using student's t test. * indicates significant differences ($p<0.05$) between each pair.

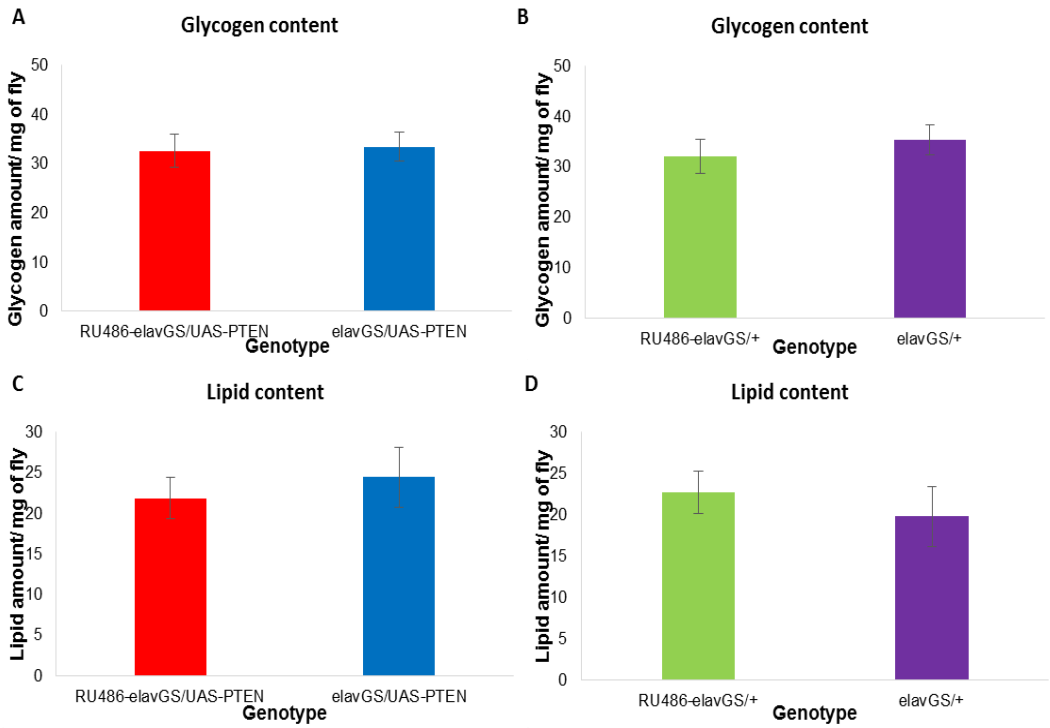

Figure 33. Glycogen and Lipid content of RU486-elavGS/UAS-PTEN and RU486-elavGS/+ (control) males.

A-B: The average concentration of glycogen and lipid per fly in RU486-elavGS/UAS-PTEN and RU486-elavGS/+ (control) males (N=20). C-D: The average concentration of glycogen and lipid per fly in RU486-elavGAL4/UAS-PTEN and RU486-elavGS/+ (control) males (N=20). Data are shown as mean value ±SEM. Data were analysed by two-way ANOVA followed by post hoc means comparisons using student's t test. * indicates significant differences ($p<0.05$) between each pair.

6.4 Apoptosis of *Drosophila* Brain cells

No difference was found between the *Drosophila* brains at 30 days old.

Reducing IIS with the overexpression of PTEN in neurons significantly shortened flies' lifespan and mainly males' lifespan. It was suspected that the reason for the shortened lifespan was due to the detrimental effects caused by PTEN overexpression in the neurons, such as cell apoptosis and neurons loss. Therefore, an apoptosis assay was performed on the brain of the transgenic Drosophila to observe the differences in the brain structure and the level of apoptosing cells between each group. Due to the time limitation of this project, apoptosis assay was only performed on RU486-elavGS/UAS-PTEN males at 30 days. The differences in the levels of apoptosing cells at this time point between different groups were not significant.

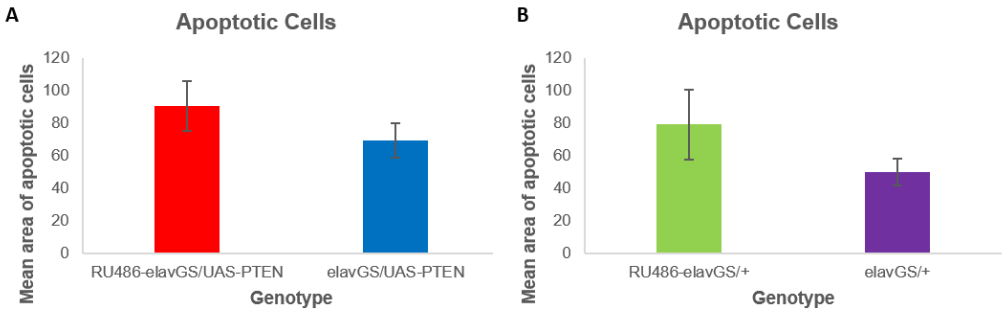

Figure 34. Apoptosis of brain cells in 30-days-old RU486-elavGS/UAS-PTEN and RU486-elavGS/+ (control) males.

A: The average relative area of apoptotic cells per brain in RU486-elavGS/UAS-PTEN and elavGS/UAS-PTEN males (N=7). B: The average area of apoptotic cells per brain in RU486-elavGS/+ and elavGS/+ (N=7). Data are shown as mean value ±SEM. Data were analysed by one-way ANOVA and no significant effects were found.

6.5 Summary

In conclusion, reducing IIS signalling in neurons throughout the lifespan (elavGAL4/UAS-PTEN) did not have any effect on flies' resistance to environmental stress (starvation or oxidative stress) in both females and males. Interestingly, reducing IIS signalling only in adult neurons (RU486-elavGS/UAS-PTEN) appeared to be able to alter flies' resistance to environmental stress but results varied in different batches. Overall, RU486-elavGS/UAS-PTEN females showed no change in starvation resistance and a significant decline in oxidative stress resistance in one experiment; RU486-elavGS/UAS-PTEN males were significantly more resistant to starvation but normal resistant to oxidative stress. As for the level of glycogen and lipid in flies, an increase in the level of lipid concentration was detected in elavGAL4/UAS-PTEN females only and there was no other difference observed in either elavGAL4/UAS-PTEN or RU486-elavGS/UAS-PTEN flies. For the preliminary apoptosis experiment performed in this project, there was no difference in the level of apoptosing cells between different genotypes of males at 30 days old.

7. Discussion

The lifespan-extending effects of ubiquitous IIS reductions have been well-established in different model organisms but there is a lack of understanding of the tissue-specific effects of IIS reductions especially in the nervous system. To investigate the neuronal specific effects of IIS reductions on health and functional senescence, following the results on elavGAL4/UAS-InR^{DN} flies (Ismail et al., 2015), this project studied the effects of brain-specific IIS reduction using an overexpression *PTEN* model to reduce IIS signalling. The effects of reduced IIS signalling were measured on two different types of locomotor functional senescence, sleep patterns senescence, and on survival and fecundity.

7.1 Constitutive Reduction of IIS Signalling with elavGAL4/UAS-PTEN Transgenic *Drosophila*

Previously Ismail et al. (2015) investigated the role of neural IIS with elavGAL4/UAS-InR^{DN} flies by targeting a mutated insulin receptor (UAS-InR^{DN}) to neurons to reduce IIS signalling. Despite the extended lifespan of females, there was no improvement on negative geotaxis and neuronal-specific IIS reduction actually had detrimental effects on several parameters of exploratory walking at specific age points (Ismail et al., 2015). Their results indicated a disconnection between the regulation of lifespan and behavioural senescence by IIS and such IIS-induced lifespan extension can occur in the presence of normal, ameliorated, or declined behavioural function. Considering the importance of IIS signalling, reducing IIS by targeting the insulin receptor may not be the ideal approach and may have detrimental effects on other downstream functions. To further investigate the role of IIS in neuronal ageing and function and to find the optimal time and space for IIS reduction, this project has chosen a downstream factor (the PTEN phosphatase) to reduce IIS.

PTEN is a lipid and protein phosphatase that dephosphorylates PIP_3 to PIP_2, removing a critical docking site for IIS downstream proteins such as Akt and PDK1, thus, acting as an antagonist of PI3K activity and the phosphorylation cascade (Tamguney and Stokoe, 2007). Overexpression of dPTEN in the adult fat body was sufficient to increase the lifespan of flies by about 20% (Hwangbo et al., 2004). However, overexpression of dPTEN in neurons in this study resulted in a normal lifespan and fecundity in females but a significantly shortened lifespan in males (Fig. 9). It is suspected that elavGAL4/UAS-PTEN is a stronger knockdown of IIS and resulted in long-term detrimental changes in the neurons. The effects of IIS reduction are dose-dependent and strong systemic reductions in IIS are known to be detrimental to

lifespan due to the pleiotropic nature of the pathway (Ikeya et al., 2009). It is thought that there is a different optimal level of IIS reduction for lifespan extension in females and males. Sex differences in the study of ageing have long been observed but not fully understood, thus, females and males are not directly compared in this project. Severely reducing IIS has detrimental effects on flies: mutants, for example, PKB^{3} homozygotes, Inr^{GC25}/Inr^{E19}, and Chico homozygous males were short-lived and the complete deletion of IIS are lethal in *Drosophila* (Clancy et al., 2001). In addition, PTEN regulates the activity of Akt substrates FOXO, other substrates such as the E3 ubiquitin ligase and Bcl2, and the TOR and NOTCH1 signalling to control cell proliferation, cell growth, cell differentiation, and apoptosis (Proshkina et al., 2015). dPTEN mediates both cell number and cell size in flies and regulates the subcellular organization of the actin cytoskeleton (Goberdhan et al., 1999). Therefore, the overexpression of dPTEN in neurons might cause negative effects on peripheral pathways that outweighed the benefits of IIS reduction on ageing and resulted in shortened lifespan in males. However, to firmly confirm the knockdown level of IIS in elavGAL4/UAS-PTEN comparing to elavGAL4/UAS-InR^{DN} transgenic flies, further experiments are needed such as western blots to measure the ratio of phosphorylated Akt vs total amount of Akt in the brains, heads, and bodies. On the other hands, it remains unclear the specific mechanism of the effects of neuron-specific IIS reduction in the brain and in peripheral tissues that leads to such lifespan reduction.

As a reflection of functional healthspan in long-lived models, negative geotaxis is one of the most widely used locomotor behaviour assays, measuring the speed of a fly climbing vertically against gravity and its reflex movement ability. It is mostly a measure of neuromuscular function that long-lived flies with systemically reduced IIS or muscle-specifically reduced IIS both shown improvements on negative geotaxis via improved muscle function (Demontis and Perrimon, 2011). Although this locomotor behaviour is controlled by the CNS, long-lived flies with neuronal-specific IIS reduction showed no improvement on this behaviour, suggesting IIS-mediated improvement on this behaviour is through peripheral tissue rather than neural tissue (Ismail et al., 2015). Moreover, a more complicated locomotor behaviour, exploratory walking, was proposed as a measure of not only neuromuscular function but also fly's navigation ability and decision-making processes (CNS function) (Martin, 2004). Individual flies in small chambers were videoed for 15mins and performance of a range of walking parameters (the walking distance, the number of episodes of activity and inactivity, the duration of activity, changes in direction, the pattern of exploration, and walking speed) were obtained. Most of the parameters present robust age-related changes in normal flies (elavGAL4/+ and

UAS-PTEN/+ in Fig. 10): a decrease of walking distance, walking duration, rotation frequency, and velocity; and an increase of the duration in the central zone and first rotation time. Ismail et al. (2015) also excluded the potential effects caused by the senescence of sensory systems (olfaction and vision) on exploratory walking behaviour.

Unlike elavGAL4/UAS-InR^{DN} flies, reducing IIS pan-neurally throughout the lifespan (elavGAL4/UAS-PTEN) showed no effect on locomotor behaviour senescence in both females and males (Fig. 10&11). The normal behavioural senescence of negative geotaxis and exploratory walking observed in short-lived males support the previous results that lifespan and behavioural senescence are independently regulated by IIS signalling in *Drosophila*. Although the elavGAL4/UAS-PTEN females show no significant reductions in locomotor behaviours, small non-significant reductions at first age point (10days) of several parameters were observed. Such reductions at young age point are in line with elavGAL4/UAS-InR^{DN} flies and it is possible that IIS signalling is required for the age-specific neuronal function such as during developmental stages.

Apart from locomotor behaviours, sleep patterns are often used as a measure of functional healthspan in long-lived models as well. Sleep in flies shares the fundamental characters of sleep with sleep in humans and both show an age-related reduction in sleep quality and efficiency (Cirelli, 2009). Ubiquitously reduced IIS in daGAL4/UAS-InR^{DN} or DILP2-3, 5 mutants flies has been shown to ameliorate the age-related decline in sleep such that reduced IIS increases and consolidates night sleep and decreases day sleep and increases day activity (Metaxakis et al., 2014). With the neuron-specific reduction of IIS (elavGAL4/UAS-PTEN flies), there were no changes in sleep senescence between the experimental flies and control flies in both females and males (Fig. 12-14). In contrast to previous results, a significantly reduced daily activity and increased day sleep were observed in elavGAL4/UAS-PTEN females (Fig. 12) at the young age. Although not significant at all age points, the experimental flies appeared to be less active and sleep longer during the day up to 40 days old but declined to the same level of function at older ages comparing to control flies. However, due to the individual variability of sleep, such differences between the experimental flies and control flies in females were not observed in the second sleeping experiments (Fig. 13). On the other hand, despite shortened lifespan, male flies had a normal senescence of sleeping behaviours (Fig. 14). Interestingly, there was a lack of age-related decline in the night sleep (total night sleep length and bout length in dark) of experimental males (Fig. 14C&G). There was also a small non-significant reduction at young age observed in experimental flies in the night sleep but the sleep quality

at older ages was not different between the experimental flies and control flies. It is possible that the observed lack of age-related decline in elavGAL4/UAS-PTEN males was the result of the detrimental effects at the young age rather than ameliorated ageing.

Taken together, the results from elavGAL4/UAS-PTEN flies so far have supported the earlier studies on elavGAL4/UAS-InR^{DN} flies. The IIS pathway modulates lifespan and behavioural senescence independently. Significantly short-lived male flies presented a normal senescence of locomotor behaviours and daytime sleeping behaviours but showed a negative young age effect on nighttime sleep only. The effects of IIS mutation are dose-dependent, and it is speculated that PTEN overexpression may result in a stronger knockdown that exceeds the optimal level of IIS reduction. When reducing IIS from an embryo, a slightly negative effect on behaviours at the young age is often observed and it is thought to be due to the role of IIS in development stages.

7.2 Adult Period Only Reduction of IIS Signalling with elavGS/UAS-PTEN *Drosophila*

Constitutive downregulation of IIS could affect the development of the nervous system as well as other peripheral tissues and result in the negative effects in behavioural performance observed at the young age. To address these possibilities and to investigate the effect of IIS reduction in neurons during development and adulthood, lowered IIS was manipulated to specifically in the adult nervous system of *Drosophila* using the inducible GeneSwitch system. Work on *C. elegans* has already indicated that insulin signalling plays different roles during the lifecycle that the pathway influences ageing during adulthood only (Dillin et al., 2002). Adult-only downregulation of IIS was sufficient to increase the lifespan of *C. elegans* up to 2-fold without any detrimental effects to reproduction (Lind et al., 2018). The timing of the effect of reduced IIS on lifespan was also studied in *Drosophila* that overexpression of dFOXO in the adult fat body increased female lifespan by 20 to 50% but had negative effects on fecundity (Giannakou et al., 2004).

When reducing IIS in adult neurons, the lifespan of RU486-elavGS/UAS-PTEN females was shortened in one experiment (Fig. 15A) but normal in a larger population experiment (Fig. 16A). As flies are from different batches in the two experiments, such difference in lifespan may be the result of variability in the health of parental strain or environmental factors (such as food quality, bacteria, or drug interventions) (Linford et al., 2013). A drug-induced lifespan difference was observed in the second lifespan results (Fig. 16C). Furthermore, RU486-elavGS/UAS-PTEN females presented a consistent reduction in fecundity (Fig. 15B & Fig. 16B)

but this is most likely a result of RU486 itself and not a result of reduced IIS in neurons (Fig. 15D & Fig. 16D). The inducer for the GeneSwitch system, RU486 (mifepristone), is a progesterone receptor antagonist and glucocorticoid receptor antagonist used for birth control and abortion (Landis et al., 2015). The effects on fertility in GeneSwitch transgenic flies are subject to the concentration of RU486 (Osterwalder et al., 2001; Copeland et al., 2009). Therefore, further adjustments on the concentration of RU486 should be made to induce gene alterations without negative effects. On the other hand, RU486-elavGS/UAS-PTEN males were significantly short-lived (Fig. 17A&B), confirming that adult-restricted IIS manipulation in neurons was sufficient to influence lifespan and suggesting that the UAS-PTEN driver may result in a stronger downregulation of IIS. In addition, PTEN is a central regulator that plays an essential role in the control of cell growth, cell division, cell number, and organ size and apoptosis (Gao et al., 2000). The overexpression of PTEN can lead to the reduction of cell number, cell size, and the induction of cell apoptosis (Song et al., 2007). Therefore, apart from the downregulation of IIS, the shortened lifespan of UAS-PTEN transgenic flies may also be the results of disrupted PTEN functions in other aspects.

To further investigate whether the detrimental effects on exploratory walking in elavGAL4/UAS-InR^{DN} flies is the effects on neuronal ageing or neuronal function, in addition to the normal behavioural testing, a recovery behavioural experiment was designed by controlling the inducible drug to adjust the timing and level of IIS signalling. In parallel to normal behavioural testing, a 5-day recovery period was given to flies to allow a recovery of insulin function before testing. This experiment was to observe if an improvement in behavioural senescence following a recovery of IIS function was presented.

In RU486-elavGS/UAS-PTEN females, the experimental flies also showed a normal performance of the locomotor behaviours at young ages but their performance deteriorated faster at older ages and significantly at age 40 days (Fig. 18A-H). An overall faster decline appeared in RU486-elavGS/UAS-PTEN males and the differences were significant at older ages (age 40 or 50 days) (Fig. 20A-H). The detrimental effects of adult-specific PTEN overexpression in neurons on behavioural function support earlier studies on elavGAL4/UAS-InR^{DN}, showing that reduced IIS in neurons is not beneficial to behavioural function (Ismail et al., 2015). By giving a 5-day recovery period, both females and males showed either a normal behavioural senescence or an improved senescence compared to flies without a recovery period (Fig. 18I-P & Fig. 20I-P). These data suggest that a 5-day recovery was sufficient to restore IIS function and bring behavioural performance back to the normal range for most parameters but was

not sufficient for some parameters. Moreover, the recovery period only restored their normal behavioural functions but did not improve any behavioural senescence following recovery. Such rehabilitation from the detrimental effects but lack of improvement in locomotor behavioural senescence following recovery from PTEN overexpression may indicate that reduced IIS is detrimental to neuronal function and does not influence neuronal ageing. It is possible that if IIS function is required for the normal performance of locomotor behaviours, the requirement of IIS function to maintain normal behaviours may be increasing with age as the differences between RU486-elavGS/UAS-PTEN and elavGS/UAS-PTEN flies became significant with age. It is also possible that over-expression of PTEN resulted in either long-lasting detrimental effects on neurons via its modulation of other pathways. We attempted to determine if levels of apoptosis in the brain were altered by PTEN expression but due to time restraints, only males at age 30 days were analysed, with no significant differences observed. Further analysis of apoptosis and other downstream effects of PTEN expression should be made on male and female flies at multiple ages throughout the lifespan.

Over the past few decades, researches have established that plasticity mechanisms such as long-term potentiation (LTP) and synapse formation are altered at older ages, contributing to the decline in cognitive function during normal ageing (Barnes, 2011). The disruption of normal synapse formation of ageing neurons is thought to alter connectivity and higher-order integration and in fact, significant changes in the expression of synaptic genes have been found across ageing mouse, monkey, and human brains (Bishop et al., 2010). In *Drosophila*, PI3K regulates the number of synapses in both larval motor neurons and adult brain projection neurons that PI3K activation is required for inducing synaptogenesis in aged adult neurons and synapse maintenance (Martin-Pena et al., 2006). It is possible that IIS mediates effects on neuronal ageing and function via PI3K and the requirement of IIS/PI3K function increases with age in the maintenance of cognitive function. Additionally, brain-specific ageing analysis has revealed conserved mechanisms of progressive mitochondrial dysfunction and oxidative damage that IIS may mediate effects on age-specific function and behaviour via the regulation of such as protein clearance, oxidative stress, DNA repair, and apoptosis (Bishop et al., 2010). Given the beneficial effect of reduced IIS on lifespan and peripheral cell ageing, it is plausible to assume that the detrimental functional effects on walking behaviour and negative geotaxis caused by the overexpression of PTEN outweigh any positive effects of IIS reduction on neuronal ageing. It is possible that reduced neuronal IIS by PTEN overexpression results in long-term detrimental changes in the brain, such as apoptosis or neuron loss that could be induced by PTEN overexpression, that the fly cannot recover from, as mentioned above. It has

also been suggested that different types of cognitive and behavioural functions have different levels of sensitivity to IIS changes with age (Ismail et al., 2015). Thus, some parameters of the behaviour are more sensitive to changes in IIS with age and shown significant decline while other parameters show no differences overall. Because of the variable sensitivity, the 5-day recovery period may not be sufficient for some parameters and the differences persist. Interestingly, there is a small significant improvement on negative geotaxis of RU486-elavGS/UAS-PTEN recovery males at old age (Fig. 20P) which is similar to the long-lived daGAL4/UAS-InR^{DN} and Chico mutant (Ikeya et al., 2006; Martin and Grotewiel, 2006). It is more likely to be that the recovery of IIS function counterpoised the detrimental effects of strong IIS reduction and revealed the positive effects of mild IIS reduction. The improvement may be a positive functional effect as the overall senescence of the two locomotor behaviours was not improved. Overall, based on these results and previous studies, it is clear that reduced IIS is detrimental to behavioural function. However, to make firm conclusions about the role of IIS in neuronal ageing, the effect of reduced IIS on factors such as cell death throughout the lifespan, needs to be determined.

One of the difficulties of using inducible system GeneSwitch is to rule out the effects of RU486 on *Drosophila*'s behavioural performances. RU486-elavGS/+ and elavGS/+ groups were tested as control groups for drug effects. There were slight differences between the exploratory walking of the two control groups (Fig. 19&21), however, they did not explain the differences between the elavGS/UAS-PTEN +/-RU486 groups, suggesting that the effects seen in RU486-elavGS/UAS-PTEN +RU486 were IIS-induced detrimental effects rather than RU486-induced effects. On the other hand, RU486-elavGS/UAS-PTEN appeared to be hyperactive and sleep less during the day (Fig. 22&24) and similar patterns were found between RU486-elavGS/+ and elavGS/+ groups (Fig. 23&25), suggesting such differences are drug-induced differences rather than lowered IIS-induced differences. However, other differences observed in the experimental groups cannot be explained by drug effects.

When reducing IIS in adult neurons via PTEN expression, significantly detrimental effects were presented at the first time point in both females and males (Fig. 22&24). Such young age effects are similar to the small non-significant negative effects found in elavGAL4/UAS-PTEN, suggesting that the early age effects were not due to developmental deficiency from embryos. In addition, a lack of age-related decline was seen in the daytime sleep pattern of RU486-elavGS/UAS-PTEN males (Fig. 24E-H), leading to some significant differences between the two groups at older ages. By giving a 5-day recovery period, experimental flies appeared to recover

from the early age detrimental effects and the significant differences no longer persisted (Fig. 22&24). Moreover, a restoration of the age-related changes was also seen in the sleep patterns of male flies (Fig. 24M-P) and the significant differences at older ages were recovered. A complete or partial recovery from the detrimental effects of PTEN expression was found in all parameters in RU486-elavGS/UAS-PTEN recovery flies, but there was no improved behavioural senescence. Therefore, in contrast to the speculations in Metaxakis et al. (2014), the results presented here showing no age-related deterioration in flies with reduced IIS do not indicate an amelioration of sleep senescence and may indicate detrimental functional effects at the young age that flies cannot recover from. A 5-day recovery period was sufficient to restore some of the IIS function and their normal behavioural performances, but future studies could investigate different time scales of recovery as well as different doses of RU486 to alter the level of PTEN expression.

Like in mammals, sleep patterns in *Drosophila* are thought to be regulated by the coordination of many different neurons and interconnected brain circuits (Tomita et al., 2017). In the *Drosophila* brain, the mushroom bodies (MBs) are a paired neuropil structure in the central brain, including the dendritic calyx, peduncle, and lobes, and are critical for associative learning and memory (Akalal et al., 2006). MBs also regulate sleep such that the ablation of the MBs reduces sleep and the inhibition of the MBs function via electrical silencing and increasing excitation also alter sleep (Joiner et al., 2006). MBs regulate sleep bi-directionally through MB output neurons (MBONs): the cholinergic MBONs promotes sleep and multiple glutamatergic MBONs promotes wakefulness (Aso et al., 2014). Many neurotransmitters such as dopamine, GABA, or serotonin can regulate sleep through the MBs (Tomita et al., 2017). On the other hand, the central complex (CC) is the central neuropil structures in the *Drosophila* brain and the higher centre regulating locomotor behaviours, consisting of the protocerebral bridge (PB), the fan-shaped body (FB), the ellipsoid body (EB), and the noduli (NO) (Strauss, 2002). Over the years, the involvement of CC in sleep regulation has been reported and the D1-like dopamine receptor expressed in the EB neurons regulates startle-induced and nocturnal arousal and dopaminergic neurons also project to the dorsal FB and promote sleep (Lebestky et al., 2009; Ueno et al., 2002). Sleep-wake cycles are also tightly regulated by the circadian clock and the circadian clock pacemaker neurons in *Drosophila*, LNds and LNvs, regulate nighttime and daytime sleep separately (Cong et al., 2015).

It is clear that the sleep-wake cycle is regulated by different regions of *Drosophila* brain separately but also interactively. The alteration of sleep behaviour could be the results of

global changes in the brain or changes to specific loci that signal to the rest of the brain. Therefore, it is thought that different parameters of sleep behaviour may be differentially sensitive to IIS reductions. Thus, some parameters do not show significant changes when reducing IIS while some may be too sensitive to IIS reductions that the detrimental effects could not be completely recovered. Despite no differences in the sleep patterns with age overall, interestingly, there are several cases where the RU486-elavGS/UAS-PTEN recovery flies appeared to be worse than RU486-elavGS/UAS-PTEN flies at the first or the last time points. As observed in the figures, this may also due to the variable performance of the baseline, the elavGS/UAS-PTEN flies, which are from different batches. Therefore, further adjustments of the experiments are needed where all three groups (RU486-elavGS/UAS-PTEN, elavGS/UAS-PTEN, RU486-elavGS/UAS-PTEN recovery) should be from the same batch for genetic and environmental control and the experiments should be performed on them at the same time to confirm the differences between each group. In addition, in consideration of the variability in the rates and onsets of different functional declines (Grotewiel et al., 2005), it is also suspected that the likely differential sensitivity to IIS manipulation is related to different CNS cell types. The effects on behavioural and functional senescence with pan-neural IIS reduction may be the combination of positive, negative, and neutral effects of IIS reduction on individual neuronal types or circuits in the brain (Ismail et al., 2015). Therefore, future studies are needed to determine the role of each neuronal subtype or structure in the brain in modulating lifespan and behavioural senescence, and to study the changes in the downstream signalling mechanisms in specific tissues in response to IIS reduction.

Taken together, reducing IIS via PTEN expression during the adult period only significantly shortens male lifespan and has detrimental effects on behavioural senescence for both females and males. These results have supported earlier studies on elavGAL4/InR^{DN} that reduced IIS in neurons is not beneficial to the neural circuitry function underlying behavioural senescence. In this study, the lack of improvement in behavioural senescence following a recovery of IIS function suggests reduced IIS is detrimental to neuronal function and does not ameliorate neuronal ageing. The lack of age-related changes in some parameters observed in this study and previous studies may be the result of long-term detrimental changes from the young age that fly cannot recover from rather than delayed ageing. However, further experiments testing the exact detrimental changes in neurons are needed for making firm conclusions about the role of IIS in neuronal ageing. It has also appeared that fly's behavioural senescence when reducing IIS during adulthood (RU486-elavGS/UAS-PTEN) was worse than reducing IIS from the embryo (elavGAL4/UAS-PTEN). It is suspected that elavGS driver is

stronger expression driver than elavGAL4, rather than due to the different roles of IIS in adulthood and development. This will also need further determination using western blots before making firm conclusions.

7.3 Stress Resistance, Energy Storage, and molecular analysis of transgenic *Drosophila*

Reducing IIS in specific tissues such as fat bodies, muscles, or brain is sufficient to alter lifespan, but it still remains unclear the mechanisms of how reducing IIS in specific tissues affects the rest of the body. It is thought that reducing IIS in the neurons may affect insulin signalling elsewhere in the body leading to the shortened lifespan of flies. Apart from longer lifespan, IIS reduction in the model organism is often accompanied with altered metabolism and increased stress resistance (Piper and Partridge, 2018). It is known that genetic manipulations of nutrient-sensing pathways can also mediate responses to environmental stressors such as starvation, oxidative stress, and thermal stress through the similar mechanisms of lifespan modulation (Sepil et al., 2016). Long-lived model *C. elegans* presented increased resistance to thermal and oxidative stress and long-lived *D. melanogaster* appeared increased resistance to oxidative stress and starvation but reduced resistance to thermal stress (Zhou et al., 2011; Broughton et al., 2005).

Surprisingly, constitutively reducing IIS in neurons (elavGAL4/UAS-PTEN) did not improve or reduce resistance to starvation and oxidative stress, despite the shortened lifespan of males (Fig. 26). In RU486-elavGS/UAS-PTEN flies with IIS reduction in adult neurons, females exhibited no changes in starvation resistance but reduced tolerance to oxidative stress in one experiment (Fig. 27&28). On the other hand, males appeared to be more resistance to starvation but showed the same level of tolerance to oxidative stress (Fig. 29&30). On the other hand, an increase in the level of lipid was found in elavGAL4/UAS-PTEN females (Fig. 31B) but neuron-restricted IIS reduction throughout lifespan or during adulthood period only did not alter the level of lipid and glycogen in other cases (Fig. 31-33). It is known that IIS signalling pathway is a complex pathway that controls stress responses and metabolism and the regulation of protein and glycose metabolism is thought through different DILPs. DILP2 regulates the level of blood sugar, DILP5 regulates protein metabolism, while DILP3 is thought to mediate lipid metabolism (Grönke et al., 2010; Broughton et al., 2010; Varghese et al., 2010). It is possible that neuron-restricted IIS reduction was not sufficient to alter the stress resistance or glycogen and lipid metabolism in the whole body and altered metabolism caused by IIS reduction is due to changes in the peripheral tissues rather than in the CNS. It is also

thought that the modulation of stress resistance and energy storage by the IIS pathway is an independent mechanism from the lifespan modulation.

However, it remains unclear the exact mechanisms of the early death of flies led by IIS reduction in neurons until further investigations. Further molecular analysis of the expression of downstream factors of the IIS pathway will bring to light the effects of neuronal IIS reduction in the CNS and in the peripheral tissues. One of the molecular analysis is the apoptosis assay to observe the neuron deaths in different genotypes. However, there was no significant effect on the levels of apoptosing cells between each group at age 30 days (Fig. 34). It is suspected that the major differences between each group were found at older ages and thus, the differences in the brain damage were not clear yet at 30 days old. This analysis needs to be performed at different age points throughout lifespan especially at older age points and also performed with larger numbers of fly brains. In addition, QPCR and western blot could be used to measure the gene expression level of insulin-like peptides (DILPs) in heads and bodies and the level of DILP proteins in insulin-producing cells, to understand the effects on the expression, production, and release of DILPs. Immunohistochemistry assay could be used to examine the level of Akt phosphorylation and FOXO localisation in the neurons.

In conclusion, so far, the results from this project has confirmed that IIS modulates lifespan and behaviour independently and normal behaviour senescence can occur in the presence of shortened lifespan. In comparison to the studies on elavGAL4/UAS-InR^{DN} flies, it is thought that there is an optimal level of reduction of IIS for lifespan extension which is different for females and males, the PTEN transgene results in a stronger knockdown of IIS in neurons compared to the InR^{DN} transgene. Strongly reducing IIS during the adult period only shortens lifespan and has detrimental effects on behavioural senescence. However, such detrimental effects can be recovered when given a recovery period of IIS function, suggesting the theory that the effect of reduced IIS in neurons on behavioural senescence is the result of changes to neuronal function rather than neuronal ageing. Reduced IIS in neurons plays a role in the regulation of sleep and activity but does not alter normal age-related changes. Reduced IIS in neurons can also alter some stress responses but not metabolism level, still, further molecular analysis will be needed to investigate the exact mechanisms of the effects of neuron-specific IIS reduction in brain and in peripheral tissues.

8. References

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