PGC-1α in neurodegeneration and ageing

Thesis

Li Li, M.Sc., M.B.B.S.

PhD in Ageing and neurodegeneration

Institute of Healthy Ageing

Institute of Neurology

University College London

Gower Street, London. 20th of November 2016
Declaration

I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

___________________

Li Li
PGC-1α in neurodegeneration and ageing

Li Li, M.Sc., M.B.B.S.

PhD Committee:

Prof. Dame Linda Partridge, DBE, FRS, FMedSci

Prof. John Hardy, FRS, FMedSci

Funded by: Parkinson’s UK and the Wellcome Trust
I dedicate this work to my beloved family:

my father Yujun Li,
my mother Jingfen Sun,
my father-in-law Jian Fu,
my mother-in-law Suping Sheng,
my husband Shan Fu, and
my son Li Fu

*I am thankful for your love and support.*
*You are my strong fortress and inspiration.*

I would like to dedicate this work to the pillars of our family

My grandparents:

Guojie Qin†
Wenfang Li†
Yalan Zhang†
Gui Sun†

Thanks to my cousin Dr. Chong Han for all of your advice and support in the UK.

To my extensive family and friends.
Acknowledgements

First of all I would like to thank my mentors Linda Partridge and John Hardy for their support and guidance. It was a definite pleasure to learn from you both. You are great examples of what I would like to achieve in the future.

Both my master's work and the work presented here would not have been possible without the trust, guidance and support of Kerri Kinghorn. Thanks for taking a chance on me and showing me the road to becoming a great scientist. I truly admire you and I hope I can work together again in the near future.

To my dearest friend Jorge Iván Castillo-Quan who was always there for me to guide me, assist me and keep me grounded and focused. I truly enjoyed your mentorship and support.

Thanks to Teresa Niccoli, Nazif Alic, Dobril Ivanov, Anna Tillmann, Yifan Yu, Yuan Zhao, Nikoleta Vavouraki and Vassilios N Kotiadis for helping me when I needed it the most. You are true stars.

A special thank you to my PhD examiners Dr. Alex Whitworth and Dr. Lazaros Foukas for reading and improving the thesis with your comments, suggestions and corrections.

Thanks to Professors Michael Duchen and Andrey Abramov for their advice and support.

Thanks to my office mates, Thanet Sornda, Thomas Moens, Rubika Balendra, Hongyuan Wang, Peter Rennert, Helena Cochemé, Oyinkan Sofola-Adesakin, Fiona Kerr, and Ann Gilliat. It was a great pleasure to share laughter, stress and frustration and overall the good times.

Thanks to the extensive family at the Institute of Healthy Ageing (past and present): Mobina Khericha, Adam Dobson, Nathan Woodling, Anqi Yan, Mingyao Yang, Xiaoli He, Yan Kong, Matthew Piper, Cathy Slack, Hrovje Augustin, Pedro Martinez†, Giovanna Vinti, Ekin Bolukbasi, David Gems, Jenny Regan, James Catterson, Melissa Cabecinha, Jennifer Addcott, Marcia Merrick, Arj Rajasingam, Inge Snoeren, Mumtaz Ahmad, Michael Wright, Julie Black, Danny Filer, Catherine Au, Marina Ezcurra, Daniel Pearce. Fly pushing, molecular lab work, lunch, all those cakes and desserts, and Christmas parties were special because of you. Thanks!

My Chinese Family in and out of London have played an important part in keeping me sane. Thanks to Xi Wang, Joyce Chen, Tianyi Bu, Ge Zhan, Congcong Wang, Yanjie Zhao, Clare Yu and her family, Gloria, Sue, Sophie, Tailin Xu, Josi, Qiang Gang, Yu Tao, Li Li, and Tiandi Yang. Thanks for all the amazing times together.
Contents

Abstract .............................................................................................................................. 11
Research impact ............................................................................................................... 13
List of figures .................................................................................................................. 15
List of tables .................................................................................................................. 21
Publications arising from this thesis ............................................................................. 22
Abbreviations ................................................................................................................ 23

Chapter 1 Introduction .................................................................................................... 26

1.1 Alzheimer’s disease .................................................................................................. 26
   1.1.1 Overview of Alzheimer’s disease .................................................................. 26
   1.1.2 Molecular genetics of AD ....................................................................... 26
   1.1.3 The amyloid cascade hypothesis ................................................................ 28
   1.1.4 Animal models of Alzheimer’s disease ...................................................... 31
   1.1.5 Mitochondrial dysfunction and oxidative stress in Alzheimer’s disease ...... 32
   1.1.6 The unfolded protein response (UPR) in neurodegeneration .................. 34

1.2 Parkinson’s Disease ............................................................................................... 39
   1.2.1 Overview of Parkinson’s disease ............................................................... 39
   1.2.2 Drosophila models of Parkinson’s disease .............................................. 40
      1.2.2.1 Gain-of-function fly models of PD .................................................. 41
      1.2.2.2 Knockout fly models of PD ............................................................. 42
   1.2.3 Mitochondrial dysfunction in PD ............................................................... 43

1.3 PGC-1α: an overview ............................................................................................. 46
   1.3.1 Adaptive thermogenesis ......................................................................... 47
   1.3.2 Mitochondrial biogenesis ...................................................................... 47
   1.3.3 Hepatic gluconeogenesis ...................................................................... 49
   1.3.4 The role of PGC-1α in neurodegeneration ........................................... 50
   1.3.5 The Drosophila homologue of the PGC-1 family .................................. 51

1.4 The problem of ageing ............................................................................................ 54
   1.4.1 The theories of ageing ........................................................................... 56
   1.4.2 The study of ageing in model organisms ............................................. 58
   1.4.3 The molecular basis of the ageing process ............................................ 59
   1.4.4 Mitochondrial dysfunction in ageing ..................................................... 60
   1.4.5 Proteostatic failure in ageing .................................................................. 62
   1.4.6 Anti-ageing interventions ...................................................................... 64

1.5 Thesis outline .......................................................................................................... 73
1.5.1 PGC-1 regulates the ER-unfolded protein response and ameliorates Aβ toxicity. (Chapter 3) .................................................. 74
1.5.2 The role of PGC-1α in Drosophila models of Parkinson’s disease (Chapter 4) 75
1.5.3 PGC-1α-GABPa modulates ageing in Drosophila. (Chapter 5) ................. 76

Chapter 2 General methodology ................................................................. 77

2.1 Drosophila melanogaster: strains and genetics ........................................ 77
   2.1.1 White Dahomey (w^{Dah}) ..................................................... 77
   2.1.2 White 1118 (w^{1118}) ......................................................... 77
   2.1.3 Backcross ........................................................................ 77
   2.1.4 The GAL4-UAS system ....................................................... 78
   2.1.5 The gene-switch system ...................................................... 79

2.2 Drosophila food medium ........................................................................ 80
   2.2.1 Sugar-yeast medium (SY) .................................................... 80
   2.2.2 Grape juice medium ............................................................ 80
   2.2.3 Starvation medium ............................................................. 80
   2.2.4 Dietary restriction ............................................................... 80

2.3 Fly husbandry and culturing ................................................................. 81
   2.3.1 Male and female separation .................................................. 81
   2.3.2 Virgin collection .................................................................. 82

2.4 Drosophila handling and survival .......................................................... 82
   2.4.1 Lifespan assays .................................................................. 82
   2.4.2 Stress assays ....................................................................... 83
     2.4.2.1 Paraquat ........................................................................ 83
     2.4.2.2 Hydrogen Peroxide (H₂O₂) .............................................. 83
     2.4.2.3 Phenobarbital ................................................................. 83
     2.4.2.4 Tunicamycin .................................................................. 83
   2.4.3 Mifepristone (RU486) ............................................................ 84

2.5 Behavioural investigations ................................................................. 84
   2.5.1 Fecundity assay .................................................................... 84
   2.5.2 Climbing assay (negative geotaxis) ......................................... 84

2.6 Biochemistry and molecular biology methods ....................................... 85
   2.6.1 DNA extraction and Single-Fly Polymerase Chain Reaction (PCR) ....... 85
   2.6.2 Gel electrophoresis ............................................................... 86
   2.6.3 Quantitative Real Time PCR (qRT-PCR) ................................... 86
   2.6.4 Western blotting ................................................................... 86
   2.6.5 Protein Quantification ............................................................ 87
Chapter 3 PGC-1 regulates the ER-unfolded protein response and ameliorates Aβ toxicity

3.1 Summary ............................................................................................................................................. 88

3.2 Introduction ........................................................................................................................................ 89

3.3 Results: .............................................................................................................................................. 92

3.3.1 dPGC-1 over-expression ameliorated Aβ42 toxicity ................................................................. 92

3.3.2 dPGC-1 extended the lifespan of Aβ flies beyond the maximum achieved by DR ....................... 98

3.3.3 dPGC-1 over-expression improved mitochondrial function in Aβ flies ............................... 100

3.3.4 dPGC-1 over-expression regulated the mitochondrial stress response to Aβ toxicity and protected Aβ flies against oxidative stress ................................................................. 104

3.3.5 dPGC-1 regulated the UPRER in flies expressing Aβ .............................................................. 108

3.3.6 Rifampicin ameliorated Aβ neurotoxicity via activation of the UPRER ................................. 111

3.3.7 Over-expression of dGABPα or dYY1 did not rescue Aβ toxicity .............................................. 115

3.3.8 PDP1ε ameliorated Aβ neurotoxicity via activation of the UPRER ........................................ 117

3.4 Discussion ........................................................................................................................................ 127

3.5 Materials and Methods .................................................................................................................. 132

3.5.1 Fly Husbandry and stocks ........................................................................................................ 132

3.5.2 Lifespan analyses ........................................................................................................................ 133

3.5.3 Climbing assays .......................................................................................................................... 133

3.5.4 Circadian rhythm ...................................................................................................................... 133

3.5.5 The DR protocol ....................................................................................................................... 134

3.5.6 Paraquat injection ...................................................................................................................... 134

3.5.7 Rifampicin treatment ............................................................................................................... 134

3.5.8 Fly brain imaging using FRET acceptor photobleaching ...................................................... 134

3.5.9 Live cell imaging of the mitochondrial membrane potential ............................................. 135

3.5.10 mtDNA measurement ............................................................................................................ 135

3.5.11 Quantitative PCR ................................................................................................................... 136

3.5.12 ATP assay ................................................................................................................................ 137

3.5.13 Western blot analysis ............................................................................................................. 137

3.5.14 Quantification of Aβ42 ........................................................................................................... 138

3.5.15 Statistics .................................................................................................................................. 138

Chapter 4 The role of PGC-1α in a Drosophila model of Parkinson’s disease

......................................................................................................................................................... 139

4.1 Summary ........................................................................................................................................ 139
4.2 Introduction ........................................................................................................... 139

4.3 Results: ............................................................................................................... 144

4.3.1 Over-expression of dPGC-1 did not rescue the toxic phenotypes of PINK1 null mutants ........................................................................................................... 144

4.3.2 Over-expression of dPGC-1 did not rescue the toxic phenotypes of parkin null mutants .................................................................................................................. 146

4.3.3 Over-expression of dPGC-1 increased the sensitivity to oxidative stress of park null mutant male flies ......................................................................................... 150

4.4 Discussion .......................................................................................................... 153

4.5 Experimental procedures .................................................................................. 154

4.5.1 Fly strains and backcrossing ......................................................................... 154

4.5.2 qRT-PCR ......................................................................................................... 155

Chapter 5 PGC-1α-GABPα modulates ageing in Drosophila .......................... 156

5.1 Summary ............................................................................................................ 156

5.2 Introduction ........................................................................................................ 156

5.3 Results ............................................................................................................... 161

5.3.1 Over-expression of dPGC-1 extended the lifespan of female Drosophila .... 161

5.3.2 Over-expression of dYY1 extended lifespan in female flies ....................... 167

5.3.3 Adult-onset over-expression of dGABPα extended lifespan ..................... 169

5.3.4 The tissue-specific over-expression of dGABPα extended lifespan ........ 172

5.3.5 Energy production was uncoupled from energy consumption in dGABPα-over-expressing flies ........................................................................................................... 174

5.3.6 Over-expression of dGABPα increased the resistance to phenobarbital stress ............................................................................................................................. 177

5.3.7 Over-expression of dGABPα extended the lifespan via mechanisms beyond those of DR................................................................................................................. 181

5.3.8 Next-generation Transcriptome profiling .................................................... 183

5.3.9 Over-expression of dGABPα extended lifespan in overlapped mechanisms with reduced IIS likely through up-regulation of ImpL2 ........................................ 187

5.3.10 dGABPα over-expression and reduced IIS in flies shared overlapping transcriptional responses ........................................................................................................... 190

5.3.11 dGABPα over-expression mediated the lifespan extension of reduced IIS signalling in flies ............................................................................................................. 191

5.3.12 Over-expression of dGABPα reduced AKT signalling ................................ 192
5.3.13 *dGABPα* over-expression inhibited mTORC1 and mimicked the effect of rapamycin on lifespan ................................................................. 193
5.3.14 *dGABPα* modulated intestinal homeostasis ....................................................... 196
5.3.15 Over-expression of *dGABPα* extended lifespan via mechanisms independent of those of lithium treatment .................................................................. 198
5.3.16 Ubiquitous knockdown of AKT increased ER stress resistance ................... 201

5.4 Discussion ........................................................................................................ 202

5.5 Experimental procedures .................................................................................. 208
  5.5.1 Fly strains ..................................................................................................... 208
  5.5.2 Dietary restriction protocol .......................................................................... 209
  5.5.3 Rapamycin preparation and delivery ............................................................. 209
  5.5.4 Lithium preparation and delivery .................................................................. 209
  5.5.5 Stress experiments ....................................................................................... 209
  5.5.6 Fecundity assays .......................................................................................... 209
  5.5.7 Oxygen consumption .................................................................................. 209
  5.5.8 Immunohistochemistry ............................................................................... 210
  5.5.9 Quantitative RT-PCR .................................................................................. 211
  5.5.10 Immunoblot analyses ................................................................................ 211
  5.5.11 Proteasome activity .................................................................................... 212
  5.5.12 RNA sequencing ........................................................................................ 212
  5.5.13 Gene-Ontology (Catmap analysis) ............................................................... 212
  5.5.14 Statistical analysis ...................................................................................... 213

Chapter 6 General Discussion and future plan ....................................................... 214

References ......................................................... 216
Appendix ......................................................... 253
Abstract

Alzheimer’s disease (AD) and Parkinson’s disease (PD) are the most common age-related neurodegenerative disorders, affecting millions of people worldwide. There are currently no effective or curative therapies for the treatment of these diseases. Genetic studies have demonstrated that mitochondrial dysfunction and oxidative stress play key roles in the pathogenesis of both AD and PD. Therefore, genetic or pharmacological interventions targeting these effectors may prove to be useful therapeutic strategies. In my thesis, I focus on the transcriptional cofactor PGC-1α, a key modulator in many biological processes, including mitochondrial biogenesis, the oxidative stress defence system, the circadian clock, and protein quality control. The possible relevance of this gene in the pathogenesis of AD and PD came to light with the demonstration that PGC-1α gene expression levels are decreased in the post-mortem brain tissues of both AD and PD patients.

Consistent with this, I have shown that up-regulation of PGC-1α in the neurons of a Drosophila model of Aβ toxicity rescues lifespan, the behavioural phenotypes and circadian rhythm disruption. I demonstrated that amelioration of Aβ toxicity is associated with improved mitochondrial homeostasis and activation of the ER unfolded protein response (UPRER). In keeping with this, I also found that rifampicin, an antibiotic used in the treatment of tuberculosis and leprosy, also activated the UPRER and rescued the neurotoxicity in Aβ-expressing flies. These findings suggest that stimulating the UPRER, either by increasing intraneuronal PGC-1α levels or through oral administration of rifampicin, may offer significant therapeutic benefits in AD.

Contrary to the results seen using Aβ-expressing flies, I demonstrated that over-expression of PGC-1α did not rescue the neurotoxic phenotypes in Drosophila models of familial PD, lacking PINK1 or parkin.

Given an increasing ageing population coupled with the high prevalence of age-related diseases there is an urgent need for the development of pharmacological agents that can improve healthy aging. PGC-1α has been identified as a novel
modulator of fly ageing. The precise mechanisms involved however have not been fully elucidated.

Here I also identified the transcription factor GA-binding protein (dGABPa), downstream of PGC-1α, as a key modulator of fly ageing. dGABPa gain-of-function extends female lifespan via mechanisms beyond dietary restriction (DR), with an associated reduction in IIS/mTOR signalling. In addition, over-expression of dGABPa improved gut homeostasis by delaying age-related over-proliferation of intestinal stem cells. Additionally, dGABPa gain-of-function flies showed resistance to xenobiotic stress. Finally, I demonstrated that dGABPa up-regulated ImpL2, the insulin ligand binding protein, which down-regulates IIS and extends lifespan. Given the evolutionary conserved effects of reduced IIS/mTOR signalling, these findings suggest that GABPa may be an important modulator of mammalian ageing and a potential therapeutic target in the treatment of age-related diseases.
Research impact

Alzheimer’s disease (AD) is a progressive brain disorder that causes cellular dysfunction and eventually neuronal loss. Clinically this results in memory loss as well as more widespread cognitive dysfunction. In America alone, more than 5 million people are living with AD, and this number is predicted to triple by 2050. AD is the 6th leading cause of death in the U.S., and there is currently no cure or disease-modifying therapy available.

The main objective of this project was to investigate the neuroprotective properties of PGC-1α using the fruit fly Drosophila melanogaster models of Aβ toxicity. PGC-1α, the captain of the mitochondrion, is the key modulator of energy metabolism. The possible relevance of this protein in AD pathogenesis was highlighted by work demonstrating that human post-mortem brains display reduced PGC-1α levels. These findings suggest that elevating PGC-1α expression or activity pharmacologically, or via other means, may prove to be an useful therapeutic strategy.

My work demonstrated that up-regulation of PGC-1α may be neuroprotective, with overexpression of PGC-1α rescuing the neurotoxic phenotypes in Aβ-expressing flies in vivo. My study also elucidated the mechanisms associated with the neuroprotective effect of PGC-1α, including improved mitochondrial homeostasis, activation of the unfolded protein response, both of which have been implicated in AD pathogenesis. I also found that metformin and rifampicin, two drugs already licensed for human use, rescued Aβ toxicity through similar mechanisms of activation as PGC-1α. My findings thus highlight the potential therapeutic benefits of either increasing the intraneuronal levels of PGC-1α, or pharmacologically targeting the UPRER by oral administration of metformin or rifampicin, in treating AD. Future studies are now warranted in mammalian models and in clinical trials.

Genetic manipulations and dietary restriction (DR) in model organisms have proven that lifespan extension is achievable. Most importantly, these same interventions offer protection against a number of age-related diseases and therefore improve general health. Considerable current research efforts are being made to identify novel modulators that mimic healthy lifespan. PGC-1α is one such novel modulator of
ageing that has been shown to extend lifespan in *Drosophila*. In this project, I identified *dGABPα*, as a downstream effector of PGC-1α and a novel modulator of ageing in the fly. I demonstrated that *dGABPα* extended lifespan via mechanisms beyond DR, with an associated reduction in IIS/mTOR signalling. Moreover, activation of *dGABPα* also improved healthy lifespan, including gut homeostasis and resistance to xenobiotic stress. Given the evolutionary conserved effects of reducing IIS/mTOR, this finding suggests that GABPα may prove to be an important modulator of mammalian ageing.
List of Figures

Figure 1.1 Generation of Aβ from the amyloid precursor protein ...................................... 28
Figure 1.2 A schematic representation of the UPR .............................................................. 36
Figure 1.3 The UPR signalling outputs in neurodegeneration ............................................. 37
Figure 1.4 Alpha-synuclein pathology .................................................................................. 41
Figure 1.5 Adaptive thermogenesis and mitochondrial biogenesis ..................................... 48
Figure 1.6 The prevalence of the ageing population ............................................................ 55
Figure 1.7 The incidence of age-related disease in 2006 ..................................................... 55
Figure 1.8 The force of natural selection declines with age ............................................... 57
Figure 1.9 The Drosophila life cycle ..................................................................................... 59
Figure 1.10 The hallmarks of ageing ..................................................................................... 60
Figure 1.11 A model of mitochondrial UPR signalling in C. elegans ................................... 63
Figure 1.12 The role of DR in ageing ................................................................................... 65
Figure 1.13 Testing a genetic intervention that extends lifespan and its interaction with DR ................................................................. 69
Figure 2.1 The GeneSwitch expression systems ................................................................... 79
Figure 3.1 mRNA expression levels of dPGC-1 in Aβ-expressing flies ............................... 93
Figure 3.2 Aβ protein levels were not significantly reduced by dPGC-1 over-expression .......................................................... 93
Figure 3.3 Over-expression of dPGC-1 did not affect Aβ mRNA levels ................................. 94
Figure 3.4 Over-expression of dPGC-1 in neurons extended the lifespan of Aβ-expressing flies ....................................................................... 95
Figure 3.5 Over-expression of dPGC-1 in Aβ-expressing flies partially ameliorated the age-dependent climbing defects ......................................................... 96
Figure 3.6 Over-expression of dPGC-1 in Aβ-expressing flies rescued the defects in circadian rhythm ......................................................................................... 97
Figure 3.7 Over-expression of dPGC-1 in Aβ-expressing flies ameliorated the reduction in ATP ........................................................................................................... 97
Figure 3.8 Over-expression of dPGC-1 increased the lifespan of Aβ flies irrespective of food concentration ................................................................................................. 99
Figure 3.9 Mitochondrial DNA was reduced in Aβ and dPGC-1 expressing flies ....... 101
Figure 3.10 Both Aβ and dPGC-1 over-expression increased the mitochondrial membrane potential in the fly brain ................................................................. 102
Figure 3.11 Aβ and \textit{dPGC-1} had opposing effects on \textit{cyt-c-p} transcription.................103
Figure 3.12 \textit{dPGC-1} over-expression increased ATP levels in Aβ-expressing fly heads ..................................................................................................................................................104
Figure 3.13 Aβ-expressing flies displayed reduced levels of the HSP60 protein.......105
Figure 3.14 Aβ-expressing flies displayed reduced levels of the Grp75 protein.......106
Figure 3.15 Over-expression of \textit{dPGC-1} rescued the survival of Aβ-expressing female flies injected with paraquat ..........................................................................................107
Figure 3.16 The levels of the Grp78 protein were elevated in Aβ-expressing fly heads but returned towards control levels with co-expression of \textit{dPGC-1}.................108
Figure 3.17 The mRNA levels of \textit{Xbp1} splicing were increased in the heads of Aβ-expressing flies co-expressing \textit{dPGC-1} .................................................................109
Figure 3.18 The mRNA levels of \textit{Grp78} were increased in Aβ-expressing fly heads and further increased by the co-expression of \textit{dPGC-1} ......................................110
Figure 3.19 Over-expression of \textit{dPGC-1} in neurons did not change the basal levels of chaperones in young flies.................................................................111
Figure 3.20 Rifampicin increased the lifespan of Aβ-expressing female flies ..........113
Figure 3.21 Rifampicin treatment had no effect on Aβ protein levels.........................114
Figure 3.22 Rifampicin rescued the locomotor ability of Aβ-expressing flies.............114
Figure 3.23 Rifampicin reduced the increased levels of the Grp78 protein in Aβ-expressing fly heads............................................................................................115
Figure 3.24 The co-expression of \textit{dYY1} in the neurons of Aβ-expressing flies lead to a decrease in lifespan .................................................................................................116
Figure 3.25 Co-expression of \textit{dGABPa} in neurons did not rescue the shortened lifespan of Aβ-expressing flies .................................................................117
Figure 3.26 The circadian loop model for the \textit{Drosophila} clock.........................118
Figure 3.27 A model for the FRET donor-acceptor pair ........................................119
Figure 3.28 Acceptor photobleaching within the regions of interest (ROI) ..............120
Figure 3.29 Over-expression of \textit{Pdp1ε} in neurons extended the lifespan of Aβ flies121
Figure 3.30 Co-expression of \textit{Pdp1ε} did not rescue the locomotor ability of Aβ-expressing flies.................................................................................................................122
Figure 3.31 Co-expression of \textit{Pdp1ε} in Aβ-expressing flies did not alter Aβ protein levels ..........................................................................................................................123
Figure 3.32 The mRNA levels of Aβ were increased by the co-expression of Pdp1c in Aβ-expressing flies .................................................................123
Figure 3.33 Over-expression of Pdp1c in Aβ-expressing flies lead to increased DHR96 levels in fly heads .................................................................124
Figure 3.34 Over-expression of Aβ, but not Pdp1c, reduced cyp6a2 mRNA levels in fly heads .......................................................................................125
Figure 3.35 mRNA levels of Xbp1 splicing were increased by the expression of Aβ and Pdp1c in fly heads .................................................................126
Figure 3.36 mRNA levels of Grp78 were increased in flies expressing Aβ and Pdp1c in their heads ..............................................................................126
Figure 4.1 The PINK1-parkin pathway regulates mitochondrial function via PARIS and PGC-1α ..................................................................................142
Figure 4.2 The mRNA expression levels of dPGC-1 were increased in PINK1 loss-of-function flies ........................................................................144
Figure 4.3 The mRNA expression levels of dPGC-1 were raised in dPGC-1-over-expressing flies ...........................................................................145
Figure 4.4 Ubiquitous over-expression of dPGC-1 did not extend the lifespan of PINK1 null mutant male flies ..............................................................145
Figure 4.5 The over-expression of dPGC-1 in PINK1β9 null mutant male flies did not rescue the climbing defects ................................................................146
Figure 4.6 The expression of dPGC-1 was increased in park25 null mutant flies ....147
Figure 4.7 Ubiquitous over-expression of dPGC-1 extended the lifespan of park25 null mutant female flies ...............................................................147
Figure 4.8 Ubiquitous over-expression of dPGC-1 extended the lifespan of park25 null mutant male flies .......................................................................148
Figure 4.9 Ubiquitous over-expression of dPGC-1 in park25 null mutant female flies partially ameliorated the climbing defects ......................................149
Figure 4.10 Ubiquitous over-expression of dPGC-1 did not extend the lifespan of park25 null mutant female flies .......................................................149
Figure 4.11 Ubiquitous over-expression of dPGC-1 did not extend the lifespan of park25 null mutant male flies ..........................................................150
Figure 4.12 Ubiquitous over-expression of dPGC-1 did not alter the sensitivity to H2O2 of park25 null mutant female flies ..............................................151
Figure 4.13 Ubiquitous over-expression of \( dPGC-1 \) increased the sensitivity of \( park^{25} \) null mutant male flies to oxidative stress with \( H_2O_2 \) ..................................................152
Figure 4.14 Ubiquitous over-expression of \( dPGC-1 \) did not alter the sensitivity of \( park^{25} \) female flies to paraquat ...........................................152
Figure 4.15 Ubiquitous over-expression of \( dPGC-1 \) increased the sensitivity to oxidative stress on exposure to paraquat in \( park^{25} \) null mutant male flies .......153
Figure 5.1 Ubiquitous over-expression of \( dPGC-1 \) extended the lifespan of female flies ..................................................................................................................162
Figure 5.2 Ubiquitous over-expression of \( dPGC-1 \) during development and in adulthood did not extend the lifespan of male flies .................................................163
Figure 5.3 Ubiquitous over-expression of \( dPGC-1 \) only in adulthood extended the lifespan of female flies ..................................................................................................................163
Figure 5.4 Ubiquitous over-expression of \( dPGC-1 \) partially ameliorated the locomotor abnormalities of flies ..................................................................................................................164
Figure 5.5 Over-expression of \( dPGC-1 \) in adult neurons extended lifespan ..........165
Figure 5.6 Ubiquitous over-expression of \( dPGC-1 \) increased the sensitivity of female flies to \( H_2O_2 \) ..................................................................................................................166
Figure 5.7 Ubiquitous over-expression of \( dPGC-1 \) did not significantly affect the lifespan of flies exposed to paraquat .................................................................166
Figure 5.8 Ubiquitous over-expression of \( dYY1 \) in adulthood extended the lifespan of female flies ..................................................................................................................167
Figure 5.9 Ubiquitous over-expression of \( Pdp1\epsilon \) in adulthood does not extend lifespan in female flies ..................................................................................................................168
Figure 5.10 Ubiquitous over-expression of \( vri \) shortened the lifespan of female flies ..................................................................................................................169
Figure 5.11 Over-expression of \( dGABP\alpha \) in adulthood extended the lifespan in female flies ..................................................................................................................170
Figure 5.12 Knockdown of \( dGABP\alpha \) in adulthood using RNAi shortened the lifespan of female flies ..................................................................................................................170
Figure 5.13 \( dGABP\alpha \) expression levels in flies either over-expressing \( dGABP\alpha \) or expressing the RNAi construct against \( dGABP\alpha \) ........................................................................171
Figure 5.14 Ubiquitous over-expression of \( dGABP\alpha \) did not affect fecundity .......171
Figure 5.15 Over-expression of \( dPGC-1 \) up-regulated \( dGABP\alpha \) .........................172
Figure 5.16 Over-expression of dGABPα in adult neurons extended the lifespan in female flies ................................................. 173
Figure 5.17 Over-expression of dGABPα in the adult gut and fat body extended the lifespan in female flies ......................................................... 173
Figure 5.18 ATP levels were increased in dGABPα-over-expressing flies ........... 174
Figure 5.19 Over-expression of dGABPα did not alter the mitochondrial mass in young or older flies .......................................................... 175
Figure 5.20 Over-expression of dGABPα did not alter NDUFS3 protein levels ...... 175
Figure 5.21 Oxygen consumption rates were not altered in dGABPα-over-expressing fly heads ........................................................................... 177
Figure 5.22 The effect of ubiquitous over-expression of dGABPα in adulthood on exposure to various stressors in female flies .............................................. 178
Figure 5.23 Over-expression of dGABPα increased proteasome activity ............ 179
Figure 5.24 Ubiquitous over-expression of dGABPα in adulthood did not alter the protein levels of the 26S proteasome .................................................. 180
Figure 5.25 Flies over-expressing dGABPα did not show any change in autophagy 180
Figure 5.26 Ubiquitous over-expression of dGABPα extended the lifespan via mechanisms independent of those of DR ............................................. 182
Figure 5.27 Heat-map of the genes differentially expressed in fly heads over-expressing dGABPα .............................................................................. 185
Figure 5.28 GO categories of fly heads over-expressing dGAPBα ....................... 187
Figure 5.29 Female flies over-expressing INRDN and dGABPα mediated lifespan extension ......................................................................................... 189
Figure 5.30 Comparative analyses of the transcriptional response of IIS down-regulation and dGABPα over-expression ....................................................... 191
Figure 5.31 dGABPα mediated the lifespan extension of reduced IIS ............... 192
Figure 5.32 Over-expression of dGABPα reduced systemic insulin signalling .... 193
Figure 5.33 The ubiquitous over-expression of dGABPα reduced the phosphorylation levels of S6K ................................................................. 194
Figure 5.34 The survival of flies over-expressing dGABPα in response to rapamycin treatment ......................................................................................... 196
Figure 5.35 Over-expression of dGABPα in gut prevented the over-proliferation of stem cells ......................................................................................... 197
Figure 5.36 Ubiquitous over-expression of $dGABPa$ extended the lifespan of lithium-treated flies.

Figure 5.37 Comparative analyses of the transcriptional response of lithium and $dGABPa$ over-expression.

Figure 5.38 Flies lacking AKT are more resistant to the ER stressor tunicamycin.
List of Tables

Table 1 CPH analysis of the survival of Aß-expressing flies on treatment with 500 μM rifampicin .........................................................................................................................113
Table 2 CPH analysis of the survival of flies over-expressing dGABPα on different yeast concentrations of food .........................................................................................183
Table 3 CPH analysis of the survival of flies over-expressing dGABPα on DR and full-feeding food ......................................................................................................................................183
Table 4 CPH analysis of the survival of flies co-expressing dGABPα and INR^{DN} ..............................................................189
Table 5 The CPH analysis of the survival of rapamycin-treated flies co-expressing dGABPα ......................................................................................................................................196
Table 6 CPH analysis of the survival of flies over-expressing dGABPα with and without lithium treatment ........................................................................................................199
Publications arising from this thesis

Appendix 1.................................................................................................................253

Sofola-Adesakin, O., Castillo-Quan, J.I., Rallis, C., Tain, L.S., Bjedov, I., Rogers, I.,
Li, L., Martinez, P., Khericha, M., Cabecinha, M., et al. (2014). Lithium suppresses
Abeta pathology by inhibiting translation in an adult Drosophila model of Alzheimer's
disease. Front Aging Neurosci 6, 190.

Appendix 2....................................................................................................................263

Kinghorn, K.J., Castillo-Quan, J.I., Bartolome, F., Angelova, P.R., Li, L., Pope, S.,
leads to elevated mitochondrial lipid peroxidation and mitochondrial dysfunction.
Brain 138, 1801-1816.

Appendix 3 ....................................................................................................................279

Castillo-Quan, J.I., Li, L., Kinghorn, K.J., Ivanov, D.K., Tain, L.S., Slack, C., Kerr,

Appendix 4....................................................................................................................291

Kinghorn, K.J., Castillo-Quan, J.I., Li, L., Bhatia, K.P., Abramov, A.Y., Hardy, J.,

Appendix 5 ....................................................................................................................293

Niccoli, T., Cabecinha, M., Tillmann, A., Kerr, F., Wong, C.T., Cardenes, D.,

Appendix 6....................................................................................................................304

Kinghorn, K.J., Gronke, S., Castillo-Quan, J.I., Woodling, N.S., Li, L., Sirka, E.,
Gegg, M., Mills, K., Hardy, J., Bjedov, I., et al. (2016). A Drosophila Model of
Neuronopathic Gaucher Disease Demonstrates Lysosomal-Autophagic Defects and
Altered mTOR Signalling and Is Functionally Rescued by Rapamycin. J Neurosci 36,
11654-11670.
Abbreviations

AD  Alzheimer’s disease
4E-BP1  Eukaryotic initiation 4E binding protein 1
ABAD  Aβ-binding alcohol dehydrogenase
ActGS  Actin-GenesWitch
AKT  Protein kinase B
AMPK  5' adenosine monophosphate-activated protein kinase
AP  Antagonistic pleiotropic
APP  Amyloid precursor protein
ARJP  Autosomal recessive juvenile Parkinsonism
ASK1  Apoptotic signal-regulating kinase 1
ATF6a  Transcription factor 6a
Atg  Autophagy related gene
ATP  Adenosine triphosphate
Aβ  Amyloid β
BAT  Brown adipose tissue
Bip  Binding immunoglobulin protein
cAMP  Cyclic adenosine monophosphate
CCCP  Carbonyl cyanide m-chlorophenylhydrazone
CHOP  C/ebpβ homology protein
CncC  Cap'n'collar C
CNS  Central nerve system
CREB  Camp response element binding protein
Cyt-c-p  Cytochrome c proximal
DA  Dopaminergic neurons
daGS  Daughterless-GeneSwitch
DILPs  Drosophila insulin like peptides
DN  Dominant negative
DR  Dietary restriction
eIF2a  Eukaryotic translation initiation factor 2α
elavGS  Embryonic lethal abnormal vision-GeneSwitch
ELISA  Enzyme-linked immunoabsorbent assay
ER  Endoplasmic reticulum
ERAD  ER associated degradation
ETC  Electrical transport chain
FOXO  FOrkhead boX subgroup O transcription factor
FRET  Fluorescence resonance energy transfer
GABPα  GA binding protein α
GADD34  DNA damage-inducible 34
GFP  Green fluorescent protein
Glut  Glucose transporter
Grp78  Glucose regulated protein 78
GS  Gene switch
GSK3  Glycogen synthase kinase 3
GWAS  Genome-wide association studies
H₂O₂  Hydrogen peroxide
HD  Huntington’s disease
HNF-4α  Hepatocyte nuclear factor 4α
HSR  Heat shock response
IF  Intermittent fasting
IIS  IGF-1-like signaling
InR  Insulin receptor
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRE1</td>
<td>Inositol-requiring enzyme 1</td>
</tr>
<tr>
<td>IRS1</td>
<td>Insulin receptor substrate 1</td>
</tr>
<tr>
<td>JNK</td>
<td>JUN amino-terminal kinase</td>
</tr>
<tr>
<td>LB</td>
<td>Lewy body</td>
</tr>
<tr>
<td>LC3-I/II</td>
<td>Microtubule associated protein A 1A/1B-light chain 3</td>
</tr>
<tr>
<td>LiCl</td>
<td>Lithium chloride</td>
</tr>
<tr>
<td>LRRK2</td>
<td>Leucine-rich repeat kinase 2</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEF2</td>
<td>Myocyte enhancer factor-2</td>
</tr>
<tr>
<td>miRNAs</td>
<td>Micro Ribonucleic Acids</td>
</tr>
<tr>
<td>Mitophagy</td>
<td>Mitochondrial autophagy</td>
</tr>
<tr>
<td>mL</td>
<td>Mililitre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mNSCs</td>
<td>Median neurosecretory cells</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic Target of Rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mtor complex 1</td>
</tr>
<tr>
<td>mTORC2</td>
<td>Mtor complex 2</td>
</tr>
<tr>
<td>mtTFA</td>
<td>Mitochondrial transcription factor A</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NFTs</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>NMJ</td>
<td>Neuromuscular junction</td>
</tr>
<tr>
<td>NRF2</td>
<td>NF-E2-related factor</td>
</tr>
<tr>
<td>NRF2</td>
<td>Nuclear respiratory factor 2</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDI1ε</td>
<td>Par Domain Protein 1ε</td>
</tr>
<tr>
<td>PERK</td>
<td>Protein kinase RNA-like ER kinase</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α</td>
</tr>
<tr>
<td>PI</td>
<td>Performance index</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinases</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-induced putative kinase 1</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PRC</td>
<td>PGC-1-related co-activator</td>
</tr>
<tr>
<td>Proteostasis</td>
<td>Proteome homeostasis</td>
</tr>
<tr>
<td>PSEN</td>
<td>Presenilin</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real time PCR</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic Acid interference</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RU/RU486</td>
<td>Mifepristone</td>
</tr>
<tr>
<td>S6Ks</td>
<td>S6 kinases</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia nigra</td>
</tr>
<tr>
<td>SNCA</td>
<td>Alpha-synuclein</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SY</td>
<td>Sugar yeast</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween 20</td>
</tr>
<tr>
<td>TF-EB</td>
<td>Transcription factor-EB</td>
</tr>
<tr>
<td>TFs</td>
<td>Transcription factors</td>
</tr>
<tr>
<td>TOR</td>
<td>Target of rapamycin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream activation sequence</td>
</tr>
<tr>
<td>UCP-1</td>
<td>Uncoupling protein-1</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>UPR&lt;sub&gt;ER&lt;/sub&gt;</td>
<td>Unfolded protein response ER</td>
</tr>
<tr>
<td>UPR&lt;sub&gt;mt&lt;/sub&gt;</td>
<td>Mitochondrial unfolded protein response</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin proteasome system</td>
</tr>
<tr>
<td>W&lt;sup&gt;1118&lt;/sup&gt;</td>
<td>White 1118</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>W&lt;sup&gt;Dah&lt;/sup&gt;</td>
<td>White Dahomey</td>
</tr>
<tr>
<td>XBP1</td>
<td>X-box binding protein 1</td>
</tr>
<tr>
<td>ΔΨ&lt;sub&gt;M&lt;/sub&gt;</td>
<td>Mitochondrial membrane potential</td>
</tr>
</tbody>
</table>
Chapter 1 Introduction

1.1 Alzheimer’s disease
1.1.1 Overview of Alzheimer’s disease
Alzheimer’s disease (AD) is the most common form of dementia in the ageing population, affecting millions of people worldwide. Clinical symptoms of AD include but are not limited to memory loss and cognitive decline, including temporal-spatial disorientation, impairment of judgment and problem solving, and deterioration of language abilities (Faber-Langendoen et al., 1988). Behavioral and personality changes may also be observed with disease progression. In the severe stages of the disease, movement abnormalities often develop, which along with dementia, leave patients often bedridden and dependent on caregivers.

In 1907, Alois Alzheimer first described a dementing illness and identified senile plaques and neurofibrillary tangles (NFTs) in the post-mortem brain tissue (Alzheimer, 1907), the two neuropathological hallmarks still utilized by neuropathologists today to make the histological diagnosis of AD. In 1927, Divry used Congo red staining to describe the senile plaques in the AD brain as extracellular aggregates of amyloid-β (Aβ) (Divry and Florkin, 1927), various species of which are derived from the amyloid precursor protein (APP) (Kang et al., 1987). NFTs, the other classic hallmark of AD pathology, are intracellular aggregates of paired helical filaments composed of hyperphosphorylated tau protein (Brion et al., 1985). NFTs are mainly found in the hippocampus, entorhinal cortex and neocortex (Hyman et al., 1984). Since both Aβ and tau are normal proteins found in healthy neurons, there is a great deal of interest in understanding what triggers their conversion into the abnormal and aggregated forms found in AD.

1.1.2 Molecular genetics of AD
The majority of AD cases are sporadic, while only a small proportion of AD cases (<1%) result from the autosomal dominant inheritance of gene mutations. Since familial cases share similar symptoms and pathology with sporadic AD (Harvey et al., 2003), the identification of the genes involved in these rare forms of AD provides a
powerful tool for the elucidation of disease pathogenesis and therapeutic strategy. Mutations in three genes cause autosomal dominant AD, including the amyloid precursor protein (APP) and the presenilin genes (PSEN1 and PSEN2) (Goate et al., 1991, Levy-Lahad et al., 1995, Rogaev et al., 1995), all of which show altered Aβ production through APP processing (Figure 1.1) (Charier-Harlin, 1991, Citron et al., 1992, Price and Sisodia, 1998, Suzuki et al., 1994). APP is a type I transmembrane protein with a large amino-terminal extracellular domain. To produce Aβ, the extracellular domain is first removed by β-secretase. The remaining carboxy-terminal fragment is then cleaved within the membrane by γ-secretase, which is composed of presenilin and other components (De Strooper et al., 1998, Edbauer et al., 2003). γ-secretase can cleave APP at different sites, producing Aβ peptides of 40 or 42 amino acids. APP gene mutations found in a Swedish family result in increased β-secretase cleavage and thus increased production of both Aβ40 and Aβ42. Among the different species of Aβ, Aβ42 is more prone to oligomerization and is believed to be more toxic than Aβ40 (McGowan et al., 2005). Mutations at the γ-secretase cleavage site found in the familial cases lead to production of more the toxic Aβ42 compared to Aβ40 (Charier-Harlin, 1991, Price and Sisodia, 1998). Moreover, APP mutations found in the Dutch and Arctic families result in increased fibrillogenesis or resistance to proteolysis (Massi et al., 2002, Nilsberth et al., 2001). Identification of these mutants demonstrated a fundamental role of Aβ peptides, especially Aβ42, in the pathogenesis of AD. In addition to point mutations, multiplication in the APP gene can also cause early-onset AD (Cabrejo et al., 2006), indicating the importance of protein aggregation and mis-folding in disease progression. Other disease-related gene mutations have been identified in the presenilin genes, which encode the catalytic subunit of γ-secretase (Levy-Lahad et al., 1995, Rogaev et al., 1995). AD-associated presenilin mutations increase the Aβ42/Aβ40 ratio (Rogaev et al., 1995, Duff, 1997, Oyama et al., 1998), also indicating that Aβ42 is the more toxic species of the Aβ peptides. Taken together, genetic findings from familial AD have helped to establish a basic understanding of the aetiology of AD, in which increased levels of Aβ peptides, especially with an increased ratio of Aβ42/Aβ40 resulting from AD-related gene mutations or duplication, are the main cause of pathogenesis in AD.
Most cases of AD do not exhibit autosomal-dominant inheritance and are termed sporadic AD, in which genetic differences may act as risk factors. The best-known genetic risk factor for AD is the inheritance of the ε4 allele of the apolipoprotein E (APOE) (Strittmatter et al., 1993, Mahley et al., 2006). The APOE4 allele increases the risk of the AD by three times in heterozygotes and by 15 times in homozygotes (Blinnow et al., 2006). How the APOE proteins mediate their effect in AD is not fully elucidated, however the previous studies using APP transgenic mice model that harboured the human APOE genes suggest that APOE4 show reduced efficiency in the clearance of Aβ (Kim et al., 2009).

![Figure 1.1 Generation of Aβ from the amyloid precursor protein.](image-url)

The sites of β- and γ-secretase–mediated cleavage are indicated with arrows, and the transmembrane domain of APP is highlighted in gray. γ-cleavage produces a pool of Aβ fragments that vary in length and hydrophobicity. The mutations in the Aβ region of APP increase the total Aβ production (in blue), alter Aβ biophysical properties (in black), or affect the Aβ spectrum in both quantitative and qualitative ways (in green). Taken from (Benilova et al., 2012).

1.1.3 The amyloid cascade hypothesis

The evidence from pathology, phenotypes mediated by genetic mutations in familiar cases and risk factors conferred by the APOE gene mentioned above appears to facilitate Aβ in the central position of AD pathogenesis. The amyloid cascade hypothesis was first proposed in 1992 by Hardy and Higgins, who postulated that “Aβ peptides are the causative agent in AD pathology, and then give rise to a complex network of downstream events, including neurofibrillary tangles, cell loss, vascular
damage and dementia” (Hardy and Higgins, 1992). Although the majority of research data still supports a role for Aβ as the primary initiator of AD pathogenesis (Musiek and Holtzman, 2015), this hypothesis has subsequently been subject to some criticism.

Firstly, it has become clear that the correlation between dementia or other cognitive alteration and the accumulation of amyloid plaques in the brain is not linear, neither in humans (Price et al., 2009) nor in mice (Games et al., 1995). According to this observation, there is now increasing data to support the modified hypothesis that it is the soluble toxic oligomeric forms of the Aβ peptide that account for the neurotoxicity of Aβ peptide in AD, rather than the monomer or fibrillar aggregates. A variety of cytotoxic oligomeric Aβ species have been identified in AD brain lysates, with pathogenic effects in vitro and in vivo in animal models (Shankar et al., 2008, Walsh et al., 2002, Mucke and Selkoe, 2012). In humans, Aβ oligomers accumulate with age, and directly initiate tau phosphorylation in vitro and in vivo (Jin et al., 2011, Zhang et al., 2014). Mouse models displaying accumulation of Aβ oligomers developed synaptic damage, inflammation, glial activation, cognitive impairment and neuronal loss (Zhang et al., 2014, Tomiyama et al., 2010). Although the precise neurotoxicity of Aβ oligomers in vivo is still unknown, studies have revealed that Aβ can cause oxidative stress, lipid peroxidation, mitochondrial dysfunction and inflammation (Pratico et al., 2001, Benzing et al., 1999, Calkins et al., 2011). Interestingly, abnormal stress responses that can be induced by Aβ, such as oxidative stress (Guix et al., 2012), mitochondrial dysfunction (Kukreja et al., 2014) and inflammation (Liu et al., 2014), can also directly influence APP metabolism and Aβ accumulation, suggesting a confounding feed-forward mechanism to exacerbate Aβ production and toxicity. However, there is still little evidence in humans that genetic mutations that increase mitochondrial dysfunction, oxidative stress, or inflammation cause Aβ pathology. Since the aforementioned pathogenic markers are also seen in other neurological disease, it is likely that these risk factors influence the pathogenic mechanisms downstream of Aβ, but are not unique to AD (Musiek and Holtzman, 2015). Factors that affect Aβ production or aggregation or suppress Aβ clearance can contribute to disease progression. The accumulation of Aβ oligomers is predicted to affect the ability of the brain to maintain proper protein quality control, including autophagy failure, imbalance in protein degradation and protein synthesis. This
eventually prompts further aggregation of Aβ, tau and other intracellular proteins in association with an exacerbation of neurotoxicity (Dasuri et al., 2013, Nixon and Yang, 2011). The sleep disturbance observed in AD patients may also increase neuronal Aβ release via an impaired sleep-wake cycle, thereby leading to abnormal oscillations of extracellular Aβ levels (Kang et al., 2009). Moreover, ageing is a major risk factor for AD. Since proteostatic and cytoprotective pathways in the central nervous system decline with age (Taylor and Dillin, 2011, Lu et al., 2014), age-related disturbances in proteostasis and neuronal stress response signalling pathways may also shift the balance of Aβ metabolism towards aggregation. In addition, age-related disorders such as vasculopathy and atherosclerosis, diabetes, head trauma, or sleep disruption may also affect Aβ accumulation and aggregation, exacerbating AD pathology and stimulating the onset of dementia (Kang et al., 2009, Musiek and Holtzman, 2015). A full understanding of the complex interplay between these processes and Aβ aggregation is still awaited, but it is likely that these cellular pathways may prove to be excellent therapeutic targets in the treatment of AD.

Secondly, the relationship between Aβ plaques and neurofibrillary tangles of tau remains unclear and therefore has been of considerable debate for many years. Previous studies showed that tau pathology occurs first in the transentorhinal cortex, often in the absence of plaque pathology. Moreover, the pattern of tau pathology is highly regular, whereas Aβ plaque pathology is much more varied (Braak and Braak, 1991). In order population, there is significant increase in the presence of neurofibrillary tangles, but no association between age and Aβ plaque was observed (Price et al., 2009). In addition, Gomes-Isla et al. measured the total number of neurons in AD brain compared to age-matched controls, and found that there was a statistically significant correlation between the loss of neurons and the increased in the presence of neurofibrillary tangles with disease progression; however, no correlation was observed between Aβ load and either disease duration or neuronal loss (Gomez-Isla et al., 1997). All the evidence discussed above suggest that there is considerable gaps in our understanding of AD pathology, therefore to some extent debate amyloid hypothesis. The hypothesis that accommodates most of the known data is that Aβ aggregation and deposition triggers a process that leads to neuronal
loss via the formation of neurofibrillary tangles of tau, although the mechanisms are not fully understood.

Thirdly, there are currently three main therapeutic interventions strategies aimed at Aβ: reducing Aβ production, facilitating Aβ clearance and preventing Aβ aggregation. However, all of the Aβ-centric approaches that reached Phase III clinical trials have failed. For example, semagacestat, a well-characterized γ-secretase inhibitor, effectively reduced Aβ deposition in both APP transgenic mouse model (Ness et al., 2004) and human volunteers (Bateman et al., 2009). However, Phase III trails demonstrated that patients who were treated with semagacestat displayed an increased deterioration in cognition and daily activity compared to controls. This failure again challenged the amyloid hypothesis, and also remain questions unanswered: by how much should Aβ production be lowered, and at what stage of the disease process would an Aβ-directed therapeutic approach be likely to show clinical efficacy? Recently, aducanumab, a human monoclonal antibody that selectively targets aggregated forms of Aβ, was reported to reduce both soluble and insoluble Aβ in a transgenic mice model (Sevigny et al., 2016). In addition, AD patients in the early stages of disease injected with aducanumab showed reduced Aβ levels, which was accompanied by a slowing in clinical decline. These ongoing clinical trials, as well offering great promise for the future treatment of AD, also provide compelling support for the amyloid hypothesis.

1.1.4 Animal models of Alzheimer’s disease

A number of genetic models have been developed in the study of AD, including a large number of mouse models, many of which have failed to recapitulate all of the histopathological and behavioral features of AD. Transgenic mice harboring human APP mutations develop amyloid pathology and memory defects without neuronal loss (Mucke et al., 2000, Cheng et al., 2004). In order to isolate the effects of Aβ, transgenic Aβ mouse models were generated, but only amyloid pathology was observed (McGowan et al., 2005). Transgenic mice with mutant presenilin expression demonstrated increased Aβ42 levels, while no AD pathology or cognitive deficits developed (Holcomb et al., 1999, Dewachter et al., 2008). Moreover, a transgenic
mutant tau mouse model demonstrated hyperphosphorylated tau, neurofibrillary lesions and cell loss (Lewis et al., 2000, Andorfer et al., 2003).

In addition, non-mammalian experimental models have been used to study the biology of AD. They are excellent genetic tools suited for drug screening and the study of toxic molecular cascades involved in disease progression. Furthermore they offer advantages over mammalian models with a quick generation time and low cost. The most commonly used invertebrate model organism in neurodegeneration is the fruit fly Drosophila melanogaster given their complex brains, short lifespan and relative ease of genetic manipulation. Several fly models already exist to study AD biology. Flies that over-express Drosophila or human tau have been developed that display several neuronal dysfunction phenotypes, including disrupted neuronal and synaptic function and climbing defects (Mershin et al., 2004, Wittmann et al., 2001, Ubhi et al., 2007). Fly models expressing Aβ peptides have also been developed that show neurodegeneration, reduced lifespan and amyloid deposition (Finelli et al., 2004, Crowther et al., 2005, Iijima et al., 2004). Although an APP orthologue exists in flies, the Aβ sequence is not conserved (Rosen et al., 1989). In keeping with this limitation, the only abnormal phenotypes seen in a fly model of APP over-expression were axonal transport phenotypes (Gunawardena and Goldstein, 2001). Therefore in summary, Drosophila models directly expressing Aβ allow investigation of Aβ toxicity in the absence of any endogenous amyloid production. It is a simple model to study the downstream effects of Aβ neurotoxicity and the potential mechanisms of Aβ degradation, independent of APP processing.

1.1.5 Mitochondrial dysfunction and oxidative stress in Alzheimer’s disease

Mitochondrial dysfunction and oxidative damage have been implicated in the pathogenesis of AD. Studies have shown that oxidative damage and lipid peroxidation occur early in the pathogenesis of AD brains and in an APP mouse model (Nunomura et al., 2001, Pratico et al., 2001). Moreover the genes responsible for mitochondrial metabolism and apoptosis were up-regulated in neurons undergoing oxidative stress, suggesting early cellular change before Aβ deposition (Reddy et al., 2004). Deficiency in cytochrome c oxidase and α-ketoglutarate dehydrogenase has been
PGC-1α in neurodegeneration and ageing

observed in AD patients, suggesting the involvement of mitochondrial dysfunction in AD pathogenesis (Gibson et al., 1988, Parker et al., 1990).

In addition, mitochondrial dysfunction and oxidative damage also contribute causally to AD-related pathology. Indeed treating neurons with hydrogen peroxide, a potent oxidizer, increases intracellular levels of Aβ (Ohyagi et al., 2000). Furthermore, treatment of astrocytes with the mitochondrial uncoupler CCCP (carbonylcyanide m-chlorophenylhydrazone), an inhibitor of oxidative phosphorylation (OXPHOS), promotes amyloidogenic APP processing and intracellular Aβ accumulation (Busciglio et al., 2002). APP mouse models lacking the mitochondrial antioxidant enzyme, MnSOD, or treated with inhibitors of energy metabolism, have also shown dramatically increased levels of β-secretase, Aβ, and senile plaques (Li et al., 2004, Velliquette et al., 2005).

Several studies have suggested that AD pathology can directly affect mitochondria and mitochondrial proteins. APP can directly target mitochondria, and clog the mitochondrial protein importation machinery, leading to mitochondrial dysfunction and metabolic defects (Anandatheerthavarada et al., 2003). Aβ can bind to a mitochondrial-matrix protein named ABAD (Aβ-binding alcohol dehydrogenase), leading to Aβ-induced apoptosis and free-radical generation in neurons (Lustbader et al., 2004). Moreover, Aβ can physically interact with mitochondria, inhibiting cytochrome c oxidase activity, impairing mitochondrial respiration, and increasing free radical generation and oxidative damage in AD neurons (Crouch et al., 2005, Manczak et al., 2006, Casley et al., 2002).

Mitochondrial DNA (mtDNA) is particularly sensitive to free radical damage. Deletions and mutations in mtDNA have been observed in post mortem AD brains (Bender et al., 2008, Coskun et al., 2004), highlighting the likely role of mitochondrial dysfunction in AD pathogenesis. Moreover, oxidative stress has been implicated in AD pathology, with alterations in several signalling pathways identified in AD brain tissue. For example, oxidative damage can increase the levels of β-secretase through activation of p38 mitogen-activated protein kinase (MAPK) and C-Jun amino-terminal kinase (Tamagno et al., 2005), leading to excessive tau
hyperphosphorylation through the activation of glycogen synthase kinase 3 (GSK3) (Lovell et al., 2004).

1.1.6 The unfolded protein response (UPR) in neurodegeneration

Cells are faced with a variety of stressors throughout their lifetime, which leads to protein mis-folding and aggregation (Balch et al., 2008). Maintaining protein homeostasis (proteostasis) requires the appropriate coordination of efficient folding and assembly of proteins and protein quality control systems, in order to minimize the load of unfolded or mis-folded proteins, and to reduce abnormal protein aggregation. Protein folding and assembling occur mainly in the endoplasmic reticulum (ER), where chaperones are induced to ensure the proper folding of proteins. Mis-folded proteins are then recognized by the quality control mechanisms, triggering degradation by the ubiquitin-proteasome machinery or the lysosome-autophagy pathway. Under conditions of cellular stress or pathogenic conditions that alter the protein-folding process, accumulation of mis-folded protein in the ER lumen causes ER stress. In response to ER stress, the folding and degrading capacity of the ER is adjusted by induction of a complex signalling network, known as the unfolded protein response (UPR\textsuperscript{ER}).

Whether the UPR restores proteostasis or induces apoptosis depends on the intensity and duration of the stress stimuli. Moderate accumulation of mis-folded proteins can activate the UPR to augment protein folding, quality control and protein degradation mechanisms (Hetz, 2012, Walter and Ron, 2011, Wang and Kaufman, 2012). Mis-folded proteins can also be re-translocated to the cytosol, where they are cleared via the ER-associated protein degradation (ERAD) pathway, including ubiquitin-proteasome mediated or lysosome-autophagy degradation.

The UPR consists of two central components, a group of ER transmembrane sensors and downstream signalling cascades that enable adaptation to stress or the induction of apoptosis (Figure 1.2). Three well-characterized signalling branches mediate this response: PERK (protein kinase RNA-like ER kinase), inositol-requiring enzyme 1 (IRE1), and transcription factor 6α (ATF6α) (Wu and Kaufman, 2006, Hetz and Mollereau, 2014). Under normal physiological conditions, these three trans-
membrane signaling components are bound and inhibited by Grp78. During ER stress, mis-folded proteins titrate down Grp78, thus activating signaling cascades downstream of the UPR. PERK directly phosphorylates and inhibits ubiquitous eukaryotic translation initiation factor 2α (eIF2α), leading to a rapid and transient attenuation of translation. A reduction in global translation relieves the stress of mis-folded proteins in the ER by reducing the total load of newly synthesized proteins in the ER lumen. Moreover, phosphorylated eIF2α selectively induces the translation of the mRNA encoding the transcription factor ATF4, which then translocates into nucleus and induces the expression of ER chaperones and genes involved in autophagy, redox control and amino acid metabolism (Walter and Ron, 2011). The most conserved UPR signalling branch is initiated by IRE1. Activated IRE1 catalyzes splicing of the mRNA encoding the transcription factor X-box binding protein 1 (Xbp1), leading to a stable and active transcription factor that controls UPR target genes involved in protein folding, ERAD, lipid synthase, ER chaperones, and ER translation (Hetz et al., 2011). Upon ER stress, ATF6α is transported to the Golgi where it is processed to produce a cytosolic fragment, which operates as a transcription factor that regulates Xbp1, ER chaperones, and ERAD genes (Walter and Ron, 2011).
Figure 1.2 A schematic representation of the UPR

The UPR is mediated by 3 trans-membrane signalling components. Under normal physiological conditions, these are bound and inhibited by Grp78. During ER stress, mis-folded proteins titrate down Grp78, thus activating signalling cascades downstream of the UPR. Taken from (Niccoli et al., 2016).

Moreover, chronic or irreversible ER stress in neurodegenerative disease can trigger apoptosis via signalling mechanisms that overlap with those activated in the adaptive response (Figure 1.3). Sustained activation of PERK signalling triggers a series of transcriptional responses mediated by ATF4, which induce C/EBP-homologous protein (CHOP) and its downstream targets, leading to increased DNA damage-inducible 34 (GADD34), BAX-and BAK-dependent caspases. In turn the survival protein BCL-1 is inhibited, leading to cells entering apoptosis (Tabas and Ron, 2011, Shore et al., 2011, Urra et al., 2013). Moreover, the IRE1 pathway also contributes to apoptosis and cell death by inducing the activity of JUN amino-terminal kinase (JNK) and the apoptotic signal-regulating kinase 1 (ASK1) (Urano et al., 2000). In addition, IRE1 can also degrade microRNAs (miRNAs), thereby negatively regulating the expression of ER chaperones (Urra et al., 2013). In summary, the UPR is a complex
signalling network that coordinates adaptation to ER stress or elimination of damaged cells by apoptosis, depending on the intensity and temporal exposure to stress.

![Diagram of UPR signalling outputs in neurodegeneration](image)

**Figure 1.3 The UPR signalling outputs in neurodegeneration**

Disease-related genes can trigger the mis-folding of a particular protein, which can then form different types of aggregates, ranging from small oligomeric species to inclusion bodies. This abnormal aggregation process results in ER stress. Prolonged ER stress overcomes the adaptive responses of the UPR and apoptosis is induced. Various apoptotic pathways have been described, including up-regulation of CHOP through ATF4. This pathway also inhibits the expression of BCL-2 family members, and the up-regulation of BH3-only proteins results in BAK- and BAX-dependent apoptosis. CHOP also induces the expression of growth arrest and GADD34, which increases the levels of ROS and protein synthesis. IRE1α also induces activation of JNK and ASK1, which contributes to cell death. In addition, IRE1α can degrade miRNAs that negatively control the expression of caspases. UPR signalling events can also modulate the expression of various genes involved in the aetiology of disease. Taken from (Hetz and Mollereau, 2014).

The UPR also contributes to ageing and age-related neurodegeneration. The ability of cells to ensure proteostasis by the UPR declines with age (Taylor and Dillin, 2011).
Damaged, mis-folded, and aggregated proteins progressively accumulate with age. At the same time ER chaperones are down-regulated, and activation of the UPR$^{\text{ER}}$ and ER stress is limited during ageing, leading to an overall decline in the capacity of the cell to adapt and protect its proteome against age-related disease (Ben-Zvi et al., 2009).

Neurodegeneration is characterized by the accumulation of mis-folded pathological proteins in the central nervous system (CNS). For example, amyloid plaques and neurofibrillary tangles, the two hallmark pathological lesions in AD, and $\alpha$-synuclein, the main component of Lewy Bodies (LB) in PD, all arise from protein mis-folding. The involvement of ER stress in neurodegenerative disease is well known but the role of the UPR in neurodegeneration is complex and yet to be fully determined. Activation of the UPR can either enhance or reduce neurodegeneration depending on the specific UPR-signalling mechanisms activated. In PD, chronic ER stress has been identified as a pathological event that contributes to dopaminergic neuronal loss (Hoozemans et al., 2007). However, activation of UPR components (ATF6) protects against PD-induced neurotoxicity in cells (Egawa et al., 2011), and up-regulation of GRP78 or eIF2$\alpha$ phosphorylation has shown neuroprotective effects in PD mouse models (Gorbatyuk et al., 2012, Boyce et al., 2005).

In AD, neurons expressing phosphorylated tau show activated PERK signalling, suggesting up-regulation of the UPR (Hoozemans and Schepers, 2012). In addition, Xbp1, the downstream effector in the IRE1 branch of the UPR, has been found to be a risk factor in sporadic AD (Liu et al., 2013). Reduced levels of Xbp1 are also found in AD patients’ brains (Reinhardt et al., 2014), suggesting that elevating Xbp1 may represent an excellent therapeutic avenue. Indeed, over-expression of active Xbp1 rescues the neurotoxic phenotypes in a Drosophila model of A$\beta$ toxicity (Casas-Tinto et al., 2011). Moreover, a C. elegans model of AD confirmed that Xbp1 is critically important for maintaining ER homeostasis in the presence or absence of ER stress (Safra et al., 2013). Moreover, we recently demonstrated that down-regulation of glucose-regulated protein 78 (Grp78; also know as Bip-binding immunoglobulin protein) in fly neurons can ameliorate A$\beta$-induced neurotoxicity. In addition this correlated with increased activation of the UPR (Niccoli et al., 2016). Grp78 is the...
master negative regulator of the UPR, and binds and inhibits the UPR transmembrane sensors under normal physiological conditions. Under ER stress, misfolded proteins titrate down Grp78, leading to activation of UPR sensors and their downstream targets. Our study therefore suggests that decreasing Grp78 protein levels, leading to activation of the UPR, may be neuroprotective in AD.

In addition to the up-regulation of the adaptive response of the UPR in AD, the apoptosis phase of the UPR has been also implicated in AD pathogenesis. JNK3, the downstream effector of IRE1, which activates apoptosis, and eIF2α phosphorylation, the downstream effector in PERK activity, have both been shown to be up-regulated in AD brain and APP mouse models (Yoon et al., 2012, Ma et al., 2013). Activation of JNK3 increases Aβ levels by promoting APP processing, while suppression of eIF2α kinase ameliorates AD-related memory defects, suggesting that chronic or irreversible activation of the UPR could also exacerbate AD pathology. Indeed, down-regulation of Grp78 in fly neurons ameliorates Aβ toxicity via activation of the UPR, to the extent that eIF2α phosphorylation is not detectable (Niccoli et al., 2016). This supports the hypothesis that moderate activation of the UPR may be beneficial in preventing AD pathology. In summary, the impact of the UPR on AD depends on the strength and duration of the stress stimulus. However, a better understanding of this pathway awaits more in vivo studies.

1.2 Parkinson’s Disease

1.2.1 Overview of Parkinson’s disease

Parkinson disease (PD) is the commonest movement disorder and the second most prevalent neurodegenerative disease after AD. The typical clinical presentation of PD includes tremor, bradykinesia, rigidity, gait and balance impairment (Lees et al., 2009). Although the cause of PD is not fully explored, it is believed to involve both genetic and environmental factors. There is an increased risk in people exposed to certain pesticides including rotenone and paraquat (Moretto and Colosio, 2013, Tanner et al., 2011). Moreover, MPTP is a pro-drug to the neurotoxin MPP+, which inhibits Complex 1 activity, leads to mitochondrial dysfunction and causes permanent symptoms of PD by destroying DA neurons in the SN of the brain (Langston et al.,
1983). There is also a reduced risk in tobacco smokers and those who drink coffee (Kalia and Lang, 2015). The predominant pathological features of PD are the selective loss of dopaminergic neurons in the substantia nigra (SN) of the mid brain, in association with intraneuronal inclusions called Lewy bodies (LB), composed of insoluble aggregates of the presynaptic protein α-synuclein (Spillantini et al., 1997, Dickson et al., 2009). The finding of α-synuclein gene (SNCA) mutations in familial PD patients, together with the identification of SNCA as a genetic risk factor for sporadic PD in two genome-wide association studies (GWAS) and a meta-analysis, confirmed the fundamental role of α-synuclein in the pathogenesis of PD (Polymeropoulos et al., 1997, International Parkinson Disease Genomics et al., 2011, Simon-Sanchez et al., 2009, Satake et al., 2009, Martin et al., 2011).

Over the past few years, a number of genes linked to Parkinsonism, known as the PARK genes, have been characterized. Currently 16 PARK loci have been identified, with autosomal dominant genes such as SNCA (PARK1), and leucine-rich repeat kinase 2 (LRRK2) (PARK8) (Paisan-Ruiz et al., 2004), and autosomal recessive genes such as parkin (PARK2) (Kitada et al., 1998), PTEN-induced putative kinase 1 (PINK1) (PARK6) (Valente et al., 2004), DJ-1 (PARK7) (Bonifati et al., 2003), ATP13A2 (Ramirez et al., 2006) and PLA2G6 (PARK14) (Paisan-Ruiz et al., 2009). Unraveling the link between mutations in these genes and neurodegeneration has greatly aided our understanding of the pathogenic mechanisms underlying familial and sporadic PD pathology.

1.2.2 Drosophila models of Parkinson’s disease

Recent research has focused on the development of genetic animal models, including organisms as diverse as yeast, worms, Drosophila and mice, in order to model the cellular and phenotypic features of human PD. Such models allow the dissection of the critical events leading to α-synuclein accumulation and neurodegeneration in PD. Among these animal models, the fruit fly Drosophila melanogaster has produced some of the most significant advances in our understanding of the cellular pathways in PD.
Drosophila is a widely used genetic tool to study neurological and genetic disorders, including PD and AD. One of the greatest advantages of using Drosophila to model PD is that it can recapitulate some of the characteristic phenotypes of patients, such as inclusion-like formation and dopaminergic neuronal loss (Feany and Bender, 2000). Moreover, PD Drosophila models also show locomotor defects that parallel the motor disability of patients. In fact, the climbing phenotype has become one of the best approaches to evaluating neurodegeneration in flies. In addition, the other advantages of using the fruit fly as a model organism, is that it is fast breeding, has rapid growth and easy genetic manipulation, as well as substantial genetic stocks. In addition, approximately 60% of the genes in Drosophila overlap with those of human genes. Therefore, Drosophila has become an important organism for the study of neurodegeneration.

1.2.2.1 Gain-of-function fly models of PD

Drosophila models exist for the study of many of the genetic variants of PD. Of the classic genes involved in PD, SNCA is the only one not conserved in Drosophila. However, by over-expressing human SNCA in the fly neuronal tissue, it has been possible to model the neurotoxicity of α-synuclein in PD in Drosophila. The fly models display LB-like aggregates (Figure 1.4), movement phenotypes, and dopaminergic neuronal loss (Feany and Bender, 2000). Although work in cell and mouse models suggest that mutant α-synuclein leads to the development of LB-like inclusions, as well as mitochondrial dysfunction and increased oxidative stress (Conway et al., 2000, Greenbaum et al., 2005, Masliah et al., 2000), the precise mechanisms by which α-synuclein species result in neurotoxicity is still unclear (Cookson and van der Brug, 2008).

Figure 1.4 Alpha-synuclein pathology
PGC-1α in neurodegeneration and ageing


Work in Drosophila has shown that over-expression of both wild type and pathogenic forms of α-synuclein leads to dopaminergic neuronal loss and neurodegeneration (Feany and Bender, 2000, Karpinar et al., 2009, Chen and Feany, 2005, Periquet et al., 2007). Furthermore expression of mutant forms of α-synuclein, which increase the generation of oligomeric species, leads to greater toxicity, confirming the preferential toxicity of α-synuclein oligomers in vivo (Karpinar et al., 2009).

However, so far all of these PD-like phenotypes are induced through the use of the UAS-GAL4 system in Drosophila, which results in the expression of α-synuclein throughout development as well as adulthood. One concern is that developmental effects may significantly alter brain physiology and neurodegeneration measured in adulthood. It is therefore impossible to disentangle developmental effects from secondary neurodegenerative effects in these models. Similar to other animal models such as rats or monkeys, specific over-expression of α-synuclein in adult neurons avoids any deleterious effects of developmental expression and the possibility of compensatory mechanisms (Waxman and Giasson, 2009). It is therefore necessary to model late-onset sporadic PD through the temporal expression of α-synuclein using the inducible GeneSwitch system. I have shown that over-expression of different variants of α-synuclein (Karpinar et al., 2009) in adult neurons do not shorten lifespan, and only cause climbing defects to a very limit extent (unpublished results). Moreover, I found that mutations that increase the generation of oligomeric species of α-synuclein increase the amount of total α-synuclein protein. It is therefore impossible to conclude whether the toxic phenotypes are due to increased oligomeric species or a greater total load of α-synuclein.

1.2.2.2 Knockout fly models of PD

Unlike SNCA, PARK2 is well conserved in Drosophila. Flies lacking parkin display a wide variety of phenotypes, including locomotor defects, DA neurodegeneration and muscle degeneration, in addition to clear mitochondrial phenotypes (Greene et al., 2003, Cha et al., 2005). In addition, Drosophila PINK1 mutants show almost all of the phenotypes of parkin mutants, presenting with decreased climbing ability, flight
disability, flight muscle degeneration as well as DA neuronal loss (Park et al., 2006, Clark et al., 2006, Whitworth and Pallanck, 2009). Moreover, epistasis experiments using the fruit fly have shown that PINK1 and parkin are linked in a common pathway with parkin acting downstream of PINK1 (Park et al., 2006). The link between PINK1 and parkin and their role in mitochondrial disease will be described in detail in Chapter 4.

Recently we generated the first PLA2G6 knockout fly model, which showed reduced survival, locomotor defects, increased sensitivity to oxidative stress, mitochondrial lipid peroxidation and mitochondrial defects (Kinghorn et al., 2015, Kinghorn et al., 2016). Another known causative gene, DJ-1, is also conserved in Drosophila, and has been shown to mediate a protective role against oxidative stress. DJ-1 alpha and DJ-1 beta double knockout flies have a normal lifespan, but exhibit locomotor defects and increased sensitivity to environmental stressors, including paraquat and rotenone, demonstrating the involvement of DJ-1 in stress response pathways (Menzies et al., 2005, Meulener et al., 2005). All of these genetic fly models enable us to study the pathogenesis of PD, and also provide a useful platform for drug discovery.

1.2.3 Mitochondrial dysfunction in PD

Mitochondrial dysfunction was first implicated in the pathogenesis of PD more than 2 decades ago. The first evidence came from a postmortem study, which described abnormalities of mitochondrial function in complex I and increased free radical mediated damage in the PD brains (Schapira et al., 1989). Since then a number of gene mutations causing familial PD have been identified. The study of how these genes cause dopaminergic neuronal loss has significantly aided our understanding of the mechanisms of pathogenesis of PD. Studies have shown that mitochondrial dysfunction and oxidative damage play an important role in PD pathology. Although proteostatic failure due to protein mis-folding and aggregation is undoubtedly crucial to PD pathogenesis, the importance of mitochondrial dysfunction has been specifically highlighted by work studying PD causing genes. Mutations in SNCA, PINK1, PARK2, and PLA2G6 have all been shown to cause alterations in mitochondrial function, further confirming the role of mitochondrial dysfunction in PD (Schapira and Gegg, 2011, Gadd et al., 2006, Singleton et al., 2003).
As mentioned above, α-synuclein is one of the major components of LB in PD and other synucleinopathies. The finding that point mutations or multiplications in the \( SNCA \) gene were associated with PD provided important insights into the role of α-synuclein in neurodegeneration (Polymeropoulos et al., 1997, Kruger et al., 1998, Farrer et al., 2004, Zarranz et al., 2004, Spillantini et al., 1997). Although the majority of α-synuclein was found localized to the cytoplasm, a small part of the protein was identified localized to the membrane of mitochondria in a mouse brain (Li et al., 2007a). Studies from cell and animal models demonstrated that α-synuclein could directly interact with the mitochondrial membrane to inhibit Complex I activity, reducing ATP synthase and the mitochondrial membrane potential, resulting in mitochondrial dysfunction (Liu et al., 2009, Devi et al., 2008, Nakamura et al., 2008, Banerjee et al., 2010, Kamp et al., 2010, Martin et al., 2006). In turn these mitochondrial abnormalities lead to increased oxidative stress, synaptic defects and eventual cell loss and neurodegeneration.

Autosomal recessive mutations in the \( PINK1 \) gene can cause an early onset juvenile parkinsonism. The PINK1 protein, encoded by \( PINK1 \) gene, is a serine/threonine kinase possessing a mitochondrial targeting sequence at its N-terminus and has an intra-mitochondrial location (Valente et al., 2004). \( PINK1 \) pathology in humans and animal models includes mitochondrial dysfunction, defective oxidative phosphorylation (OXPHOS), and increased free radical damage (Hoepken et al., 2007, Piccoli et al., 2008, Gautier et al., 2008, Gandhi et al., 2009, Gegg et al., 2009). Mutations found in \( PINK1 \) lead to the abnormal phosphorylation of PINK1-targeted mitochondrial proteins, which in turn leads to mitochondrial respiratory and metabolic defects, oxidative damage and neuronal death (Valente et al., 2004).

\( PARK2 \) mutations are the commonest cause of autosomal recessive juvenile parkinsonism (ARJR), with an age of onset around 40 years. This form of PD displays a benign disease course and a good response to L-DOPA. Parkin-associated PD is associated with pure dopaminergic neuronal loss in the SN without LB formation, in contrast to \( PINK1 \)-associated PD neuropathology in which LBs are seen (Kitada et al., 1998, Farrer et al., 2001, Pramstaller et al., 2005). Parkin functions as an E3 ligase,
ubiquitinating proteins for degradation by the proteasome (Shimura et al., 2000, Zhang et al., 2000, Chin et al., 2010). Cell studies have revealed that parkin can translocate and associate with the mitochondrial outer membrane to regulate mitochondrial function (Darios et al., 2003). Human and animal model studies have identified reduced complex I and IV activities in parkin mutants, which lead to reduced ATP production, impaired electron transport chain (ETC) activity, and apoptotic neuronal death (Greene et al., 2003, Darios et al., 2003, Muftuoglu et al., 2004, Mortiboys et al., 2008, Grunewald et al., 2010). Cell studies have demonstrated that PINK1, together with parkin, plays a crucial role in the turnover of mitochondria by autophagy (mitophagy), a mitochondrial quality control program that maintains mitochondrial dynamics and homeostasis (Zhang and Ney, 2010). Damaged mitochondria were firstly recognized by PINK1. PINK1 builds up on the outer membrane of the mitochondria and recruits parkin to target the damaged mitochondria for degradation by lysosomes-autophagy pathway (Zhang and Ney, 2010, Poole et al., 2008, Narendra et al., 2008, Vives-Bauza et al., 2010).

The PL2AG6 gene encodes an 85-kDa group VI calcium-independent phospholipase A2 beta enzyme. PL2AG6, at the PARK14 locus, has been characterized as the causative gene in a subgroup of patients with autosomal recessive early-onset dystonia-parkinsonism. Neuropathological examination of the post mortem brains of affected individuals revealed widespread LB pathology and the accumulation of hyperphosphorylated tau (Paisan-Ruiz et al., 2009, Paisan-Ruiz et al., 2012). PL2AG6 localizes to the mitochondria, and is required for the remodeling of membrane phospholipids and calcium signalling (Gadd et al., 2006). Loss-of-function mutations lead to abnormalities in mitochondrial morphology and synaptic membranes (Beck et al., 2011). In addition, we recently showed that knockout of the Drosophila homologue of PL2AG6 leads to elevated mitochondrial lipid peroxidation and reactive oxygen species (ROS), mitochondrial respiratory chain dysfunction, reduced ATP synthesis and abnormal mitochondrial morphology (Kinghorn et al., 2015). In summary, studies of the mechanisms of pathogenesis of the PARK genes in causing neurodegeneration highlight the central role of mitochondrial dysfunction in PD.
In conclusion, the mechanisms of pathogenesis of both AD and PD described above are tightly linked to mitochondrial dysfunction. Therefore, targeting mitochondrial biogenesis may potentially ameliorate neurotoxicity in these neurodegenerative conditions. In Chapters 3 and 4, I will focus on dissecting the role of PGC-1α, the key regulator of mitochondrial biogenesis, in the pathogenesis of AD and PD using Drosophila models.

1.3 PGG-1α: an overview

Transcription factors (TFs) regulate gene transcription in the control of the myriad of biological signalling pathways necessary for the effective functioning of our cells. Gene transcription must be temporally coordinated through the interplay of protein complexes including TFs, RNA polymerases and related factors. TFs are proteins that bind DNA around the 5’ end at the transcription start site in a sequence-specific manner. Transcriptional coactivators are proteins that can increase the rate of transcription by interacting with TFs, but do not themselves bind to DNA in a sequence-specific manner. Transcriptional coactivators coordinate RNA polymerase recruitment and orientation, and the unwinding of DNA to initiate transcriptional activity. The work in this thesis will focus on Peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α), which is a well-know transcriptional coactivator involved in multiple biological processes in different tissues and physiological states.

The PGC-1α gene is located on chromosome 4 in humans, and encodes a protein containing 798 amino acids. It is mainly located in the cell nucleus and expressed at a high level in metabolically active tissues, where mitochondria are abundant and oxidative metabolism is active, such as in skeletal muscle, brown adipose tissue (BAT), the heart, the brain and kidneys. It is involved in a wide variety of biological functions, including adaptive thermogenesis, mitochondrial biogenesis, glucose/fatty acid metabolism, fibre type switching in skeletal muscle, as well as in the development of the heart.
1.3.1 Adaptive thermogenesis
PGC-1α was first discovered as a transcriptional coactivator in studies that distinguished white and brown fat pathways in adaptive thermogenesis. Adaptive thermogenesis involves the activation of a number of physiological mechanisms to increase heat production in response to environmental challenges such as cold, diet or infections, particularly in brown fat and muscle (Cannon et al., 1998, Rothwell and Stock, 1979). This process is important for energy homeostasis and metabolic defense against obesity. BAT, containing multiple small lipid droplets and a high number of mitochondria, is the main metabolic site for energy dissipation, in contrast to white adipose tissue (WAT), which primarily stores energy. Uncoupling protein-1 (UCP-1), which is exclusively expressed in BAT, is the main component in diet/cold-induced thermogenesis (Enerback et al., 1997), where its gene transcription is controlled by the transcription factors cAMP response element binding protein (CREB) (Kozak et al., 1994), thyroid hormone receptor (Cassard-Doulcier et al., 1994), and nuclear hormone receptor PPARγ (Sears et al., 1996). Interestingly, PGC-1α, whose mRNA levels are dramatically increased in response to cold stress, can elevate the transcriptional activity of PPARγ and the thyroid hormone receptor on the UCP-1 promoter (Puigserver et al., 1998). Indeed, ectopic expression of PGC-1α in brown fat cells activates UCP-1 and the expression of a number of genes necessary for mitochondrial respiratory chain activity. It also leads to the up-regulation of mitochondrial density, and thus has a central role in adaptive thermogenesis and energy metabolism.

1.3.2 Mitochondrial biogenesis
Enhanced mitochondrial biogenesis in skeletal muscle, in which PGC-1α is highly expressed and up-regulated by cold, adrenergic stimuli and exercise, is an important component of adaptive thermogenesis (Figure 1.5). Ectopic expression of PGC-1α in myotubes results in elevated mitochondrial biogenesis, including increased respiration and mtDNA copy number, as well as increases in genes involved in OXPHOS (Wu et al., 1999). In a mouse model of amyotrophic lateral sclerosis (ALS), a disease characterized by loss of motor neurons, over-expression of PGC-1α maintained mitochondrial biogenesis and muscle function, and delayed muscle atrophy, in the absence of any effect on lifespan (Da Cruz et al., 2012). Scarpulla and collaborators
have identified two transcription factors, nuclear respiratory factor (NRF)-1 and -2, that can bind to the promoter regions of nuclear-encoded mitochondrial genes, including ATP synthase, cytochrome-c, cytochrome-c-oxidase subunit IV, and mitochondrial transcription factor A (mtTFA). It is of particular interest that mtTFA can translocate to the mitochondrion and activate mitochondrial DNA (mtDNA) replication and mtDNA-encoded mitochondrial protein expression (Virbasius et al., 1993, Evans and Scarpulla, 1990). This suggests that the NRFs are key nuclear receptors for mitochondrial biogenesis and respiration. Interestingly, PGC-1α physically interacts with NRF-1, therefore co-regulating its transcriptional activity in mitochondrial biogenesis. In addition to NRF-1, transcription factors YY1 and ERRα were later also identified as being co-activated by PGC-1α in mitochondrial biogenesis (Scarpulla, 2008, Cunningham et al., 2007). Moreover, PGC-1α can dramatically induce gene expression of NRF-1 and -2, and ERRα, forming a double – positive–feedback loop that drives the expression of many OXPHOS genes (Wu et al., 1999, Mootha et al., 2004).

Figure 1.5 Adaptive thermogenesis and mitochondrial biogenesis

Mitochondrial biogenesis is highly induced by environmental stressors including cold, diet and infections in adaptive thermogenesis. PGC-1α, the key regulator in mitochondrial biogenesis is
activated by a cytokine signalling pathway through direct phosphorylation by p38 MAPK. In addition, the expression level of PGC-1α is directly controlled by the cAMP-CREB pathway. PGC-1α interacts with the transcription factors NRF-1 and NRF2 which in turn control the expression of genes for mitochondrial function. Taken from (Puigserver and Spiegelman, 2003).

PGC-1α plays a central role in mitochondrial biogenesis, and itself is regulated by several signalling cascades. It is activated by the cytokine signalling pathway through direct phosphorylation by p38 mitogen-activated protein kinase (MAPK) (Puigserver et al., 2001). Moreover, CREB- a transcription factor activated by cAMP, directly activates the expression of PGC-1α by binding to its promoter in hepatic gluconeogenesis (Herzig et al., 2001). In addition, PGC-1α activity can also be directly regulated by insulin-stimulated AKT phosphorylation in hepatic metabolism (Li et al., 2007b).

1.3.3 Hepatic gluconeogenesis
The study of PGC-1α is increasingly an area of interest in diabetes. Blood glucose levels are normally tightly controlled by the balance between peripheral glucose uptake and hepatic gluconeogenesis. The exact role of PGC-1α in the control of glucose uptake and metabolism is controversial. Studies using cultured muscle cells suggest that PGC-1α regulates glucose uptake and metabolism by co-activating the muscle transcriptional regulator MEF2 (myocyte enhancer factor-2), up-regulating the endogenous level of the insulin-sensitive glucose transporter (Glut4), and increasing glucose uptake (Michael et al., 2001). However, another study reported that over-expression of PGC-1α in skeletal muscle down-regulated Glut4 transcription and impaired the glycemic control after insulin administration (Miura et al., 2003). Indeed, it has been demonstrated that glucose uptake and metabolism do not necessarily reflect insulin sensitivity, which is most likely controlled by hepatic gluconeogenesis.

The liver is the major producer of glucose, mainly through gluconeogenesis, which occurs during fasting and is aberrantly activated in diabetes mellitus. PGC-1α is highly induced in type I (insulin-deficient) and type II (profound insulin resistance with a relative lack of insulin) diabetic mouse models, suggesting a key role for insulin as a suppressor of PGC-1α expression in the liver. The diabetic state is also
associated with low insulin-stimulated cAMP-CREB signalling, which directly controls the gene expression of PGC-1α (Herzig et al., 2001, Yoon et al., 2001). Moreover, insulin can suppress PGC-1α activity directly through AKT phosphorylation. This prevents the recruitment of PGC-1α to the cognate promoters, impairing its ability to bind and co-activate the forkhead transcription factor FOXO1 or the hepatocyte nuclear factor 4α (HNF-4α), to promote gluconeogenesis and fatty acid oxidation (Puigserver et al., 2003, Li et al., 2007b). Therefore, insulin inhibits PGC-1α activity, leads to a reduction in hepatic gluconeogenesis and reduced blood glucose levels. However, in type II diabetes, where patients exhibit high blood glucose levels, insulin resistance, and a low level of insulin, activation of PGC-1α and the associated up-regulation of hepatic gluconeogenesis lead to a further increase in glucose production and release. This phenomenon eventually leads to a further reduction in insulin sensitivity and exacerbates insulin resistance.

1.3.4 The role of PGC-1α in neurodegeneration

The physiological functions of PGC-1α have been studied in metabolically active tissues. However, there is an increasing interest in dissecting its role in neurodegeneration. PGC-1α null mice show hyperactivity and other movement disorders representative of basal ganglia pathology, such as stimulus-induced myoclonus, dystonic posturing and frequent limb clamping. They also develop spongiform lesions with axonal loss in the striatum, and exhibit neuronal cell death, suggesting a neuroprotective role for PGC-1α (Lin et al., 2004, Lucas et al., 2014, Leone et al., 2005). Moreover, PGC-1α plays a role in the defence against oxidative stress, a system that is known to contribute to neurodegeneration in ageing cells. Indeed, PGC-1α null mice display a more severe loss of dopaminergic neurons compared to controls when challenged with MPTP (1- methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine), a complex I inhibitor that induces PD in humans and animal models (St-Pierre et al., 2006).

PGC-1α has been shown to be a neuroprotective therapeutic target in animal models of several neurodegenerative diseases caused by abnormal protein aggregation, including Huntington’s disease (Cui et al., 2006, Weydt et al., 2006, Tsunemi et al., 2012) and ALS (Zhao et al., 2011, Liang et al., 2011). Furthermore, amelioration of
neurotoxicity by PGC-1α activation is associated with the improvement in mitochondrial and lysosomal-autophagy function. Human studies have shown that PGC-1α activity negatively correlates with the age of onset of AD (Helisalmi et al., 2008) and PD (Clark et al., 2011). Indeed, human post-mortem studies demonstrated that PGC-1α levels are remarkably decreased in AD and PD patients’ brains (Qin et al., 2009, Shin et al., 2011), suggesting that neuronal up-regulation of this factor may offer therapeutic benefits. However, the effects of elevating PGC-1α in AD animal models are elaborate (Sheng et al., 2012, Katsouri et al., 2011, Dumont et al., 2014), and its precise function in the pathogenesis of AD awaits further investigation. PGC-1α over-expression has been shown to rescue dopaminergic neuronal loss in primary neuronal cultures expressing mutant α-synuclein, in association with up-regulation of mitochondrial respiratory chain complex genes (Zheng et al., 2010). The role of PGC-1α in PD in vivo, however, is yet to be studied. In my thesis, I will focus on dissecting the role of PGC-1α in AD and PD using Drosophila models. A comprehensive review of what is already known with regards to the role of PGC-1α in AD and PD is provided in Chapters 3 and 4 respectively.

1.3.5 The Drosophila homologue of the PGC-1 family

Mammalian PGC-1α is a member of the PGC-1 family, which also includes the closely related homolog PGC-1B (Lin et al., 2002), and PGC-1-related coactivator (PRC) (Andersson and Scarpulla, 2001). All of these proteins have significant functional overlap in mitochondrial biogenesis, and a high degree of homology in the transcriptional activation domain, RNA-binding motif, and serine-arginine-rich motif, which makes it difficult to tease apart their relative roles in vivo. Among invertebrate models, Drosophila carries a single PGC-1 homolog in its genome (Gershman et al., 2007), whereas other invertebrate models such as yeast and C. elegans do not carry any PGC-1 homologous sequences (Lin et al., 2005). The Drosophila homologue of PGC-1α, spargel (CG9808), is the only homologue of the mammalian PGC-1 family, therefore providing a system in which PGC-1 function can be assessed without interference from redundancy.

Drosophila larvae lacking dPGC-1 show that there is 44% of the nuclear encoded mitochondrial proteins have been reduced the expression compared to wild type
controls, together with mitochondrial respiratory chain defects, demonstrating that 
dPGC-1 is required for mitochondrial biogenesis in a similar manner to its 
mammalian counterpart (Tiefenböck et al., 2010). Moreover, dPGC-1 null flies show 
reduced lipid storage and glycogen levels in association with a lean body phenotype. 
These results confirm that the main metabolic functions of PGC-1α described in 
mammals, including mitochondrial biogenesis, fatty acid oxidation and energy 
metabolism, are all conserved in Drosophila.

dPGC-1 has been reported to be a downstream target of the insulin/TOR signalling 
pathway involved in cellular growth, metabolism and nutrition (Tiefenböck et al., 
2010, Mukherjee and Duttaroy, 2013). Insufficiency of either insulin or TOR results 
in developmental growth defects due to low ATP levels. Reduced expression of 
dPGC-1 results in growth retardation with smaller body size and developmental 
delay, while dPGC-1 gain-of-function has no immediate effect on Drosophila growth 
(Rera et al., 2011, Mukherjee and Duttaroy, 2013). dPGC-1 acts to increase 
mitochondrial activity, cell growth and the transcription of target genes in response to 
inulin signalling, and over-expression of dPGC-1 can overcome insulin/TOR-
mediated cell growth defects. However, dPGC-1 does not mediate all of the effects of 
TOR, since dPGC-1 has been shown to have no effect on 4E-BP phosphorylation, the 
key regulator of mTOR-mediated mitochondrial activity and biogenesis (Morita et al., 
2013). In addition, epistasis analysis has shown that over-expression of dPGC-1 does 
not ameliorate the negative effects of over-expression of FOXO, the main IIS 
downstream effector responsible for cellular growth. This thus suggests that dPGC-1 
either works in parallel with FOXO or up-stream of FOXO. Therefore, the exact 
mechanisms linking dPGC-1-mediated cellular growth and mitochondrial function 
under the control of insulin/TOR are yet to be elucidated, though the overall effect of 
dPGC-1 on growth is positive, like other members of the insulin/TOR signalling 
pathway.

An earlier study claimed that activation of insulin signalling is important for the 
transport of the dPGC-1 protein from the cytoplasm to the nucleus (Tiefenböck et al., 
2010). However, reduced insulin signalling by starvation failed to sequester the 
protein to the cytosol (Mukherjee et al., 2014), suggesting that the cellular localization
of dPGC-1 might occur independently of insulin signalling. Interestingly, dPGC-1 has also been found to form a negative feedback loop in the regulation of insulin signalling, suggesting a complex network between dPGC-1 and IIS (Tiefenböck et al., 2010). In addition, a recent study suggested that dPGC-1 can be directly up-regulated by Mitf, the Drosophila homologue of the transcription factor EB (TFEB) (Bouche et al., 2016). Inhibition of mTORC1 induces Mitf translocation to the nucleus and regulates the expression of genes involved in lysosomal biogenesis and lipid metabolism. This therefore highlights a novel role of dPGC-1 in the mTOR-signalling pathway.

Mammalian PGC-1α and its relationship with insulin/mTOR signalling have become more tissue-specific during the course of evolution. In the absence of insulin, PGC-1α expression is elevated in the liver and gluconeogenesis is subsequently initiated (Puigserver et al., 2003). Therefore, liver-specific knock down of PGC-1α in mice results in higher insulin sensitivity (Koo et al., 2004). Conversely, the absence of PGC-1α in skeletal muscles has no effect on insulin sensitivity (Zechner et al., 2010). In vivo studies have demonstrated the functions of dPGC-1 in several distinct fly tissues. Eye-specific knock down of dPGC-1 results in eye phenotypes, including abnormal ommatidia and bristle formation (Merzetti and Staveley, 2015), suggesting a role in neurodegeneration. Over-expression of dPGC-1α in Drosophila muscle tissue improved the climbing ability of the flies (Tinkerhess et al., 2012), although the precise mechanisms underlying these effects require further investigation. With respect to longevity, reduced IIS/mTOR signalling extends lifespan in different species (Fontana et al., 2010). The action of dPGC-1 on longevity is apparent as reduced dPGC-1 expression causes significant shortening of lifespan in Drosophila (Mukherjee et al., 2014). The most comprehensive study of dPGC-1 gain-of-function in vivo to date was that performed by Walker and colleagues. They demonstrated that over-expression of dPGC-1 in the fly gut improved gut homeostasis and extended lifespan (Rera et al., 2011). Improved mitochondrial function and reduced ROS production were cited as the two major underlying mediators of the lifespan extension, however this has yet to be fully proven.
Interestingly, over-expression of dPGC-1 in intestinal stem cells also prevented age-related over-proliferation of cells in maintaining gut homeostasis (Rera et al., 2011). Although this mechanism is not fully elucidated, it is known that a moderate reduction in IIS/mTOR signalling can also reduce age-related cell proliferation (Biteau et al., 2010). Previous studies have suggested that dPGC-1 forms a negative feedback loop in the regulation of insulin signalling (Tiefenböck et al., 2010), which potentially explains why dPGC-1 gain-of-function mimics reduced IIS/mTOR in regulating stem cell proliferation. Further work is required in this area to dissect the precise mechanisms of action of dPGC-1 in insulin/mTOR signalling. It is still controversial as to whether up-regulation of dPGC-1 in animal models is beneficial to lifespan (Tinkerhess et al., 2012), depending on the tissues and the experimental conditions. Research in Drosophila has significantly aided our understanding of the role of the PGC-1 group of proteins in ageing. Since both PGC-1α and IIS are involved in many physiological metabolic processes that contribute to obesity, diabetes, ageing and neurodegeneration, understanding how dPGC-1 modulates IIS is likely to provide new insights into our understanding of mammalian ageing and age-related diseases. It may also lead to the identification of novel biomarkers and therapeutic targets in the treatment of these diseases.

1.4 The problem of ageing

The need to understand the ageing process and to discover new strategies to improve healthy lifespan is becoming more urgent in light of the increasing ageing population and the higher prevalence of age-related diseases (Fontana and Partridge, 2015). By 2050, the number of people over the age of 60 globally will triple (Figure 1.6) (Fontana et al., 2014).
The prevalence of people over the age of 60 in developing and developed countries will dramatically increase by 2050. Taken from (Fontana et al., 2014).

The majority of people over the age of 65 years already report suffering from one or more chronic disorders, such as diabetes, cancer, heart disease, stroke and neurodegeneration (Hung et al., 2011). With an ever-increasing aged population the prevalence of ageing-associated diseases has increased (Figure 1.7). These demographics are increasingly becoming a central issue for public health in developed countries, and bring considerable costs to the individuals as well at the population level. Moreover, studies using animal models suggest that delaying one age-related disease could potentially reduce the prevalence of others (Fontana et al., 2014), suggesting that ageing and age-related disorders are tightly linked.

Figure 1.6 The prevalence of the ageing population

Figure 1.7 The incidence of age-related disease in 2006
1.4.1 The theories of ageing

Ageing is a complex process, involving genetic and environmental factors. Many theories of ageing have been developed to explain why we age and how we age. The altruistic theory of ageing proposes that the existence of ageing is beneficial, since decreasing the burden of older individuals may benefit the young and healthy individuals when natural resources are limited (Kirkwood and Austad, 2000). However it does not explain why ageing exists in the first place.

The evolutionary theory of ageing comes from the observation that the force of natural selection declines with age (Figure 1.8). The variation within the population exists, because random mutations will occur in the genome of individuals. Natural selection is the evolutionary mechanism that determines whether these mutations persist or are eliminated, depending on their contribution to reproductive capacity. If the mutations have a negative impact on development or reproductive ability, they would tend to be removed from the population and will not pass on to the next generation. However, the mutations that do not affect reproductive capacity will persist, and the offspring can inherit such mutations. In other words, harmful mutations that occur during childhood before the age of reproductive capacity are under strong natural selection, and thus are less likely to be passed on. However, the force of natural selection starts to decline after reproduction commences, which means that mutations that occur later will be under low or no natural selection, and will continue to accumulate. Accumulation of late-acting deleterious mutations over the course of the evolution of a population causes what we now understand as ageing, and this is called the mutation accumulation theory of ageing (Medawar, 1957).

This can be better understood by a comparison between progeroid syndromes and Huntington’s disease (De Strooper et al.). Patients with progeroid show phenotypes in early life, limiting their reproductive capacity, giving rise to a low prevalence of this disorder in the population. Conversely, HD is a late-onset neurodegenerative disorder, which enables the defective gene to be passed on to the next generation, giving rise to
a higher prevalence (Rose, 1999, Haldane, 1941). This illustrates how deleterious disease-causing genes are not removed by natural selection.

Figure 1.8 The force of natural selection declines with age

Mutations arising during childhood before the onset of reproductive fitness are under strong natural selection. Mutations whose phenotypes manifest after sexual maturity are under less selective pressure. Therefore, late-manifesting mutations are able to pass on to the next generation. Taken from (Rose, 1999).

The evolutionary theory of ageing arising from the concept of natural selection has been further developed into several differential explanations. George Williams proposed that mutations that ensure reproductive capacity would be favored by natural selection even if they pose a disadvantage later in life, which is referred to as the antagonistic pleiotropic (AP) theory of ageing (Williams, 1957). The AP theory highlights the dual early benefit and late negative effect of such mutations, and provides guidance on translating developmental studies into ageing research. Researchers must bear in mind that genes that are required during development will affect reproductive fitness by natural selection, and their contribution to ageing will therefore remain unknown. Insulin and the mTOR signalling pathways provide good proofs of principle of the AP theory. These two pathways are very important for development and growth, as they promote energy metabolism and protein synthesis. Meanwhile, these pathways are now known to contribute to ageing, as down-
regulation of the insulin/mTOR signalling pathways extends lifespan in diverse organisms (Fontana and Partridge, 2015), highlighting the pleiotropic functions of the insulin/mTOR pathways. They are also important for reproduction and therefore they persist after natural selection.

1.4.2 The study of ageing in model organisms

To understand the biological processes that drive ageing, laboratory animal models have been used to investigate different pharmacologic or genetic interventions. In order to assess the effects of such interventions on lifespan, the median and maximum lifespan of groups of animals are often measured. Median lifespan refers to the time at which 50% of the population remains alive. Maximum lifespan refers to the time when the last individual dies. Each model organism has its advantages and disadvantages for studying ageing and age-related diseases. However, interventions in any specific cellular pathway have to be at least evolutionary conserved in order to effectively translate research findings from these animal model species to humans (Partridge, 2001). In mammalian models, the mouse is the preferred organism with an average lifespan of 3 years and the availability of well-characterised genetic tools. Although mice are costly and require considerable maintenance, especially for lifespan experiments requiring large population numbers (Swindell, 2012), their use is important when testing successful interventions discovered in less complex organisms.

Simple model organisms like *C. elegans* and *Drosophila melanogaster* offer many advantages for ageing research, due to their relatively short lifespan and well-known genomes. *Drosophila* (the fruit fly) is a powerful genetic tool for studying genetic inheritance, development, behaviour, disease and ageing. Moreover, the distinct brain structure with cell types analogous to the human brain, and the presence of a blood brain barrier, make it an excellent model to study neurodegeneration (McGurk et al., 2015, Fernandez-Funez et al., 2015). *Drosophila* has a short life cycle, which is approximately 10 days from egg laying to the eclosion of adult flies at 25°C (Figure 1.9). Under laboratory conditions, *Drosophila* can live for around 3 months, and female flies live slightly longer than male flies. The *Drosophila* genome contains 4 pairs of chromosomes: an X/Y pair, and three autosomes named 2, 3, and 4.
Determination of the sex in *Drosophila* depends on the ratio of X chromosomes to autosomes, which is different from that in humans. Moreover, 60% of the *Drosophila* genes are conserved in the human genome, and 75% of known human disease genes have a recognizable homologue in the *Drosophila* genome. Therefore, *Drosophila* has been successfully used as a genetic model for several human diseases including PD and AD, some of which I have utilized in my research.

![Figure 1.9 The Drosophila life cycle](image)

Fertilized females store sperm in storage organs, and fertilize their eggs just before laying the embryo (egg). The embryo completes its development within 24 hours and hatches as a first instar larva and progresses to a third instar larva. Just before pupariation the third instar larva crawls up the side of the housing bottle and undergoes metamorphosis inside the protective pupal case. During this four-day period most larval tissues are replaced with adult-like tissues. After flies emerge from their pupae, their wings expand, and their exoskeleton hardens and becomes pigmented. Taken from (Hartwell et al., 2011).

### 1.4.3 The molecular basis of the ageing process

Ageing is the progressive loss of physiological integrity, leading to dysfunction and increased vulnerability to death. This deterioration is the main risk factor for human diseases, including cancer, diabetes and neurodegeneration. Ageing research has
achieved huge success during the past few decades, particularly with the discovery that ageing process can, to a certain extent, be delayed or ameliorated. The hallmarks of ageing include mitochondrial dysfunction, dysregulated nutrient sensing, proteostatic failure, alterations in epigenetics, telomere attrition, genomic instability, alterations in intercellular communication, stem cell exhaustion and cellular senescence (Figure 1.10) (Lopez-Otin et al., 2013). The ageing process is complex: not only do the individual components contribute to ageing, but also there is considerable crosslink between different components. Here I will describe the role of mitochondrial dysfunction and proteostatic failure in the ageing process.

![Figure 1.10 The hallmarks of ageing](image)

This diagram depicts the 9 hallmarks of ageing, including mitochondrial dysfunction, deregulated nutrient sensing, proteostatic failure, alterations in epigenetics, telomere attrition, genomic instability, alterations in intercellular communication, stem cell exhaustion and cellular senescence. Taken from (Lopez-Otin et al., 2013).

### 1.4.4 Mitochondrial dysfunction in ageing

The mitochondrion is the energy factory of living cells, playing an important role in maintaining intracellular homeostasis. The role of mitochondrial dysfunction in neurodegeneration has been addressed above. Its involvement in the ageing process, however, is complex and controversial. The physiological functions of mitochondria
include energy metabolism and respiration, which is essential for development and cell growth. The mitochondrial respiratory chain, as well aiding the production of ATP, is also the main site of ROS production. The mitochondrial free radical theory of ageing states that the accelerated mitochondrial dysfunction that occurs with age causes increased ETC leakage and production of ROS. In turn this accumulation of ROS results in free radical damage, including mtDNA mutations or deletions, increased lipid peroxidation, calcium deregulation, further mitochondrial deterioration and eventually cell death (Harman, 1965, Green et al., 2011). However, many studies have suggested that free radical damage and lifespan can be uncoupled. Mitochondrial ROS and oxidative damage in mice did not accelerate ageing (Zhang et al., 2009), while reduction of ROS by increasing antioxidant defence in mice failed to extend lifespan (Van Raamsdonk and Hekimi, 2009). Indeed, several studies showed that moderate increases in ROS can be beneficial and extend lifespan in yeast and C. elegans (Doonan et al., 2008, Mesquita et al., 2010, Van Raamsdonk and Hekimi, 2009). Therefore, the role of ROS in ageing requires re-evaluation: rather than being a direct contributor to ageing, it is more likely a response modulator for stress-elicited survival signalling (Sena and Chandel, 2012).

Mutations and deletions in aged mtDNA may also contribute to ageing (Park and Larsson, 2011). mtDNA is considerably vulnerable to ageing-associated stress and mutation due to the lack of protective histones and the limited efficiency of repair mechanisms (Linnane et al., 1989). Interestingly, most mtDNA mutations in aged cells appear to be caused by replication errors early in life rather than oxidative damage. These mutations may undergo polyclonal expansion and result in ETC dysfunction and mitochondrial ageing (Ameur et al., 2011, Payne et al., 2011). Moreover, mice lacking mitochondrial polymerase gamma showed accumulation of mtDNA mutations and deletions, impaired mitochondrial function, and shortened lifespan (Kujoth et al., 2005, Trifunovic et al., 2004, Vermulst et al., 2008). Therefore, it would be interesting to know whether genetic manipulations that decrease the load of mtDNA mutations can extend lifespan.

PGC-1α, the captain of the mitochondrion, has dual effects in increasing mitochondrial mass and activity and defending against oxidative damage (St-Pierre et
A lack of PGC-1α leads to mitochondrial dysfunction and can cause premature ageing and age-related diseases. Recently a study showed that overexpression of PGC-1 in *Drosophila* could extend lifespan, improve mitochondrial function, including increasing ETC activity and mitochondrial mass, as well as reduce ROS (Rera et al., 2011). Given the controversial roles of these effectors in ageing, further studies are warranted to investigate whether genetic manipulation of the above effectors can directly extend lifespan.

### 1.4.5 Proteostatic failure in ageing

A decline in proteome homeostasis (proteostasis) leads to ageing and age-related neurodegeneration (Hetz et al., 2011). Maintenance of proteostasis requires proper protein folding and assembly and protein quality control. To protect against proteotoxicity, cells have evolved several independent stress responses that result in the induction of chaperones and degradation machineries within selected organelles. Three well-characterized stress response pathways are the endoplasmic reticulum unfolded protein response (UPR<sub>ER</sub>) (Ron and Walter, 2007), the mitochondrial unfolded protein response (UPR<sub>mt</sub>) (Haynes and Ron, 2010) and the heat shock response (HSR) (Akerfelt et al., 2010). The UPR is the stress response pathway that is activated by the accumulation of misfolded proteins in the ER or mitochondria, in order to either re-establish proteostasis or induce apoptosis. However, the ability to maintain proteostasis by activating the UPR<sub>ER</sub> declines with age (Casas-Tinto et al., 2011). Activation of the UPR<sub>ER</sub> by over-expression of an active Xbp1, the downstream effector of the IRE1 branch of the UPR<sub>ER</sub> promotes ER stress resistance, and extends lifespan in *C. elegans* (Taylor and Dillin, 2013). The questions of whether activating the UPR<sub>ER</sub> can extend lifespan in more complex organisms remains unanswered.

In addition, deleterious mitochondrial stress caused by excessive ROS production may be detrimental to the maintenance of the mitochondrial proteome, with elevated mitochondrial lipid peroxidation (Kinghorn et al., 2015), calcium deregulation (Du et al., 2010), and eventually abnormal assembly of the ETC components (Tatsuta and Langer, 2008). Interestingly, mild mitochondrial stress has been shown to be beneficial in terms of lifespan (Merkwirth et al., 2016). The UPR<sub>mt</sub> is a stress-response pathway that adapts and activates the folding capacity of the mitochondrial
matrix in response to mitochondrial dysfunction, leading to up-regulation of nuclear-encoded, mitochondrial-localized chaperones. These in turn promote both protein folding and degradation to either restore mitochondrial proteostasis or to trigger apoptosis (Neupert and Herrmann, 2007, Haynes and Ron, 2010). In mammalian cells, activation of the UPR\textsuperscript{mt} correlates with increased JNK2 signalling, leading to induction of the transcription factors CHOP and C/EBP\textbeta, thus up-regulating the mitochondrial chaperone HSP60 (Aldridge et al., 2007, Zhao et al., 2002).

**Figure 1.11 A model of mitochondrial UPR signalling in *C. elegans***

The mitochondrial UPR is initiated when mis-folded proteins in the mitochondrial matrix exceed the capacity of the mitochondrial chaperones. The ClpXP protease degrades the unfolded protein into peptides, which is then released to the cytosol by the HAF-1 transporter. This peptide efflux activates the translocation of the transcription factor ZC376.7 into the nucleus by unknown mechanisms. ZC376.7, together with UBL-5 and DVE-1, form a transcriptional complex to drive gene expression of nuclear-encoded but mitochondrial-localized chaperones. Transcriptional up-regulation of mitochondrial chaperones in turn relieves the stress and re-establishes homeostasis. Taken from (Haynes and Ron, 2010)

In *C. elegans* (Figure 1.11), the protease ClpXP degrades mis-folded proteins into peptides, which are then exported into the cytosol through the HAF-1alpha transporter, leading to translocation of the transcription factor ZC376.7 into the nucleus. This leads to the up-regulation of the mitochondrial chaperones HSP60 and mHSP70/Glucose regulated protein 75 (Grp75), thus relieving stress and re-establishing homeostasis (Haynes et al., 2007, Yoneda et al., 2004). Recent studies
found that an early perturbation of mitochondria leads to remodelling and silencing of chromatin, and extends lifespan in *C. elegans* (Tian et al., 2016). Therefore, the role of UPR\textsuperscript{mt} in ageing is complex and awaits further investigation.

Another biological stress response pathway involved in ageing is the UPS (Ubiquitin proteasome system), which is also implicated in age-related neurodegenerative diseases such as AD and PD (Morimoto, 2008, Lopez-Otin et al., 2013). The UPS, together with the lysosome-autophagy system and chaperone network, acts to maintain cellular protein homeostasis by removing unwanted or damaged proteins that may become toxic to cells (Jung et al., 2009, Glickman and Ciechanover, 2002). UPS activity is ATP-dependent, which supports the hypothesis that improving energy metabolism may suppress the age-related decline in proteasome activity (Fontana and Partridge, 2015).

**1.4.6 Anti-ageing interventions**

Since ancient times individuals have been seeking ways to prolong youth and avoid the ageing process. The most successful findings in ageing research to date come from the discovery that ageing can be ameliorated by diet, genetic or pharmacological interventions in the nutrient-sensing pathway. Multiple molecular pathways appear to set the pace of physiological ageing. Interventions in these pathways prolong lifespan and healthy ageing in rodents and mammals. Reduced food/caloric intake, simultaneously avoiding malnutrition, can delay ageing and age-related disease in different organisms from yeast to humans (Fontana et al., 2010).
Figure 1.12 The role of DR in ageing

DR improves healthy lifespan through multiple systemic, neuronal, and cellular mechanisms. Taken from (Fontana and Partridge, 2015).

DR in mice and rats improves healthy lifespan, in association with a reduction in the prevalence of age-related pathologies (Fontana et al., 2010, Ikeno et al., 2006, Maeda et al., 1985). Young monkeys subjected to DR show improved metabolism and a lower prevalence of type-2 diabetes, cancer and cardiovascular disease (Colman et al., 2014, Mattison et al., 2012). In humans, long-term DR results in similar metabolic and molecular changes to those seen in animals. DR improves several markers of health and reduces the risk of developing cardiovascular diseases, neurodegeneration, and cancer (Heilbronn et al., 2006, Cava and Fontana, 2013, Masoro, 2003). Due to its robustness and strong conservation across different species, the discovery of the molecular mechanisms involved in DR will provide useful insights into the mechanisms of ageing. The mechanisms by which DR improves healthy lifespan are not fully understood in any species. One of the best known mechanisms of DR that is evolutionarily conserved across species is that, under the conditions of nutrient limitation, there is an adaptive switch in metabolic state from reproduction and growth toward somatic maintenance to ensure survival (Holliday, 1989). DR also extends lifespan in many less-complex and shorter-lived organisms including S. cerevisiae, C. elegans and Drosophila. The wide use of yeast and invertebrates has
enabled researchers, through genetic and pharmacological interventions, to discover the evolutionarily conserved mechanisms that extend healthy lifespan.

One of the molecular mediators of DR on longevity is the mTOR (mechanistic target of rapamycin), which is an evolutionarily conserved nutrient-sensing protein kinase that responds to amino acid levels. It thus regulates growth and metabolism in all eukaryotic cells (Johnson et al., 2013). mTOR functions in two distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Rapamycin inhibits mTORC1 activity, extending lifespan across species from yeast to mammals. It mediates its longevity effects via mechanisms including reduced mRNA translation and induction of autophagy through at least two mTORC1 substrates, ribosomal protein S6 kinases (S6Ks) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (Bjedov et al., 2010, Powers et al., 2006, Robida-Stubbs et al., 2012, Harrison et al., 2009, Medvedik et al., 2007, Miller et al., 2011). Moreover, inhibition of mTORC1 also improves proteostasis and enhances stem cell function (Kapahi et al., 2010, Johnson et al., 2013). mTORC2 is not sensitive to inhibition by rapamycin, and is usually considered not relevant in nutrient sensing. However it is necessary for the phosphorylation and activation of AKT downstream of the IIS (Guertin and Sabatini, 2009). In addition, mTORC1 extends lifespan in mechanisms overlapping with IIS. mTORC1 is activated by insulin through PI3K (phosphatidylinositol 3-kinases) and AKT kinase signalling, while mTORC1 negatively regulates IIS through S6K, which inhibits insulin receptor substrate 1 (IRS1) (Laplante and Sabatini, 2012, Takano et al., 2001). Additional studies have indicated that the mTORC1 target 4E-BP is also a transcriptional target of FOXO in fruit flies (Tettweiler et al., 2005). mTORC1 has also been implicated in several ageing processes including inflammation and mitochondrial dysfunction (Johnson et al., 2013). Hyper-activation of mTOR is often associated with inflammation, and rapamycin has been shown to have anti-inflammatory effects via NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) (Liu, 2006, Nuhrenberg et al., 2005). The activity of NF-kB signalling increases with age (Ren et al., 2007, Libert et al., 2006), and imbalanced NF-kB signalling is a major risk factor underlying ageing and age-related disease (Chung et al., 2009). This highlights the potential therapeutic benefits of limiting NF-kB signalling to improve tissue homeostasis in ageing (Guo et
In addition, regulation of mitochondrial function by mTORC1 is complex. mTOR complex actively promotes mitochondrial biogenesis and metabolism through PGC-1α and YY1 (Cunningham et al., 2007). However, yeast and mouse studies have demonstrated that inhibition of mTORC1 results in a metabolic shift towards greater mitochondrial respiration, thereby increasing chronological longevity (Bonawitz et al., 2007, Polak et al., 2008).

It has been shown in *S. cerevisiae* that decreased mTOR signalling mediates the pro-longevity effects of DR (Kaeberlein et al., 2005). Moreover, the first *C. elegans* model to show a DR-like response with lifespan extension was that carrying an *eat-2* mutation, which directly causes pharyngeal pumping defects (Lakowski and Hekimi, 1998). Several components in the mTOR signalling networks have been shown to mediate the lifespan extension of DR in *C. elegans*. Late-onset knockdown of mTOR using RNAi extends lifespan (Vellai et al., 2003), in a FOXO-independent manner. This suggests that mTOR mediates lifespan regulation through mechanisms other than reduced insulin signalling pathway activity (Vellai et al., 2003, Guarente and Kenyon, 2000). Down-regulation of the components in the mTOR complex or the downstream effectors in the mTOR signalling pathway have been found to mediate lifespan extension by reducing bacterial food concentration in the diet, suggesting a DR-like mechanism (Chen et al., 2009b, Honjoh et al., 2009, Jia et al., 2004). Moreover, inhibition of the mTOR pathway in the DR mimetic *eat-2* mutant background does not further extend lifespan, suggesting that mTOR signalling pathway inhibition and DR share overlapping mechanisms in regulating lifespan (Hansen et al., 2008, Chen et al., 2009b).

DR also extends lifespan in *Drosophila* (Clancy et al., 2002, Mair et al., 2003, Rogina et al., 2002). In *Drosophila*, the method of restricting food intake is different from that in yeast or worms. The diet used in *Drosophila* usually contains a carbohydrate source (sucrose), yeast as a source of amino acids, vitamins, minerals, cholesterol and essential fatty acids. Previous studies showed that it was modulation of the yeast in the diet that mediated the DR-associated lifespan extension, rather than the carbohydrate content or water (Kapahi et al., 2004, Mair et al., 2005). Therefore, the diet in *Drosophila* DR studies is manipulated by reducing the yeast component
without changing the carbohydrate load. Therefore, the evaluation of DR on lifespan is usually performed by varying the amount of yeast from low to high, using at least 3 different concentrations. With an increased gradient of yeast concentration, lifespan gradually increases to a maximum, after which the lifespan drops with any further increases in yeast concentration. When the values of the median or maximum lifespan are plotted against the concentration of yeast, the graph appears as a tent-shaped curve. The DR condition therefore refers to the yeast concentration that shows the highest lifespan (either median lifespan or maximum lifespan). Lower yeast concentrations limit lifespan by malnourishment, while higher concentration limits lifespan by over-nourishment (Bass et al., 2007, Grandison et al., 2009, Metaxakis and Partridge, 2013). The practical use of a DR-tent is to test the potential interactions between different interventions and diet on lifespan (Figure 1.13). When the intervention is truly a DR mimetic, the flies should behave as though they are already to some extent subjected to DR. They should therefore be more prone than controls to starvation at low levels of nutrition. At the food dilution that maximizes control lifespan, the flies with intervention would be malnourished and less long-lived. Lifespan of those flies should peak at a higher food concentration than that of controls dose, and at this and higher food concentrations, the flies with intervention should be longer lived than controls (Clancy et al., 2002) (Figure 1.13A). If the intervention is not a DR mimetic, lifespan extension will occur to a similar degree regardless of the yeast conditions (Figure 1.13B). In addition, if an intervention shares some mechanisms with DR, it might still further extend lifespan under DR conditions, however the response to different yeast conditions will differ and the maximum benefit for this intervention might be within full-feeding (Figure 1.13C). Cox proportional hazard (CPH) analysis makes this comparison much easier, and simply evaluates the effect of an intervention across all the different yeast levels. In other words, CPH will establish whether the shape of the DR-tent is different between interventions and related-controls. A significant interaction suggests that the intervention and DR share similar mechanisms. A non-significant difference suggests that the intervention is a non-DR mimetic or acts beyond DR.
PGC-1α, the transcriptional coactivator in mitochondrial biogenesis, has previously been shown to extend lifespan in *Drosophila* (Rera et al., 2011). However, its mechanisms of lifespan extension are not fully understood. (A) The DR-tent if over-expression of *dPGC-1* is a true DR-mimetic. (B) The DR-tent if over-expressing *dPGC-1* is partially overlapped with DR. (C) The DR-tent if *dPGC-1* over-expression extends lifespan beyond DR. Taken from (Castillo-Quan et al., 2015).

Inhibition of mTOR signalling components extend lifespan in *Drosophila*, but not to a greater extent than that seen with DR, supporting the idea that mTOR mediates the lifespan extension by DR-related mechanisms (Kapahi et al., 2004). Moreover, feeding flies with rapamycin, which inhibits mTORC1 activity, extends lifespan in *Drosophila* (Bjedov et al., 2010). Rapamycin further increases the lifespan of *Drosophila* under different yeast concentrations. However, the responses to diet are different between controls and rapamycin-treated flies across the different food conditions, in which the largest increase in lifespan by rapamycin is under the full feeding. This result suggests that pharmacological inhibition of mTOR signalling by rapamycin shares overlapping mechanisms with DR in lifespan regulation.

In addition, mTOR signalling also mediates mammalian longevity. The long-lived
Ames dwarf mice show reduced protein synthesis via inhibition of mTOR activity (Bartke and Brown-Borg, 2004, Sharp and Bartke, 2005). Moreover, administration of the mTORC1 inhibitor rapamycin extends lifespan in mice, further demonstrating the beneficial effect on lifespan of inhibiting mTOR signalling (Harrison et al., 2009, Chen et al., 2009a).

Insulin/IGF-1-like signalling (IIS) also mediates the lifespan extension of DR, although comprehensive studies are very limited. Reduced insulin/IIS increases longevity in *C. elegans*, fruit flies and mice (Fontana et al., 2010, Kenyon, 2010). Lifespan extension and stress resistance resulting from reduced IIS is mediated by the FOXO family of transcription factors through PI3K and AKT kinase signalling (Laplante and Sabatini, 2012, Tullet et al., 2008). Inhibition of AKT activates FOXO, which then up-regulates several “longevity pathways” controlling DNA repair, autophagy, antioxidant activity, stress resistance and cell proliferation (Webb and Brunet, 2014, Wang et al., 2014). Reducing IIS via partial ablation of the insulin-like peptide-producing median neurosecretory cells (mNSCs) in the adult *Drosophila* brain, which produce three of the seven *Drosophila* insulin-like peptides (dilp2, 3 and 5), reduces IIS signalling and extends lifespan (Broughton et al., 2010). Moreover, a study using *chico* mutants, a long-lived insulin mutant in *Drosophila* showed that reduced IIS and DR extended lifespan via overlapping mechanisms (Clancy et al., 2002). Although the nature of the mechanisms by which *chico* acts like DR requires further analysis, reduced IIS and DR may interact in determination of lifespan because they both alter some common downstream process. Indeed, reduced IIS signalling can inhibit mTORC1 activity through AKT phosphorylation in *Drosophila*, suggesting that mTOR may be one of the common pathways downstream of both interventions.

The other two nutrient sensors, AMPK (5’ adenosine monophosphate-activated protein kinase) and sirtuins, act in the opposite direction to IIS and mTOR. They therefore sense nutrient scarcity and catabolism instead of nutrient abundance and anabolism (Lopez-Otin et al., 2013). In general, activation of AMPK and sirtuins promotes longevity. AMPK activation has multiple effects on metabolism. Activation of AMPK can down-regulate mTORC1 activity, therefore controlling cell growth, autophagy and translation (Alers et al., 2012). Activation of AMPK also activates
PGC-1α, antioxidant defences and fatty acid oxidation (Wu et al., 1999, St-Pierre et al., 2006). Moreover, AMPK mediates longevity induced by DR in *C. elegans* (Burkewitz et al., 2014, Greer et al., 2007). Administration of metformin to worms and mice show lifespan extension mediated by activation of AMPK (Anisimov et al., 2011, Mair et al., 2011, Onken and Driscoll, 2010). In addition metformin does not further extend lifespan in *Drosophila* on DR food, further confirming the idea that it mimics the DR effect in lifespan extension (Slack et al., 2012).

Over-expression of some sirtuins (i.e., SIRT1, 3, and 6) increases lifespan via mechanisms overlapping with DR (Guarente, 2013). Activation of SIRT increases genomic stability, reduces NF-kB signalling, and improves metabolic homeostasis through histone deacetylation (Mouchiroud et al., 2013, Guarente, 2013). Moreover, SIRT1 can deacetylate and activate PGC-1α to regulate glucose homeostasis (Rodgers et al., 2005), therefore sharing similar downstream effectors with AMPK.

Another characterized effector mediating DR in mice is the transcription factor NF-E2-related factor (NRF2), which is the key regulator in several enzymatic pathways involved in anti-oxidant responses (Martin-Montalvo et al., 2011). The beneficial effect of calorie restriction in mice is partially mediated by NRF2. In addition, we recently published research demonstrating that lithium, the most commonly prescribed drug for the treatment of bipolar disorder, extends lifespan in *Drosophila* through inhibition of GSK-3 and activation of NRF-2. Furthermore this lifespan extension was mediated at least partially through DR (Castillo-Quan et al., 2016).

Heat shock factors (HSFs) have been also implicated in the effects of DR on health and longevity (Akerfelt et al., 2010). HSFs mediate the stress responses to environmental/pathological stressors by activating molecular chaperones such as HSP70 and stress proteins. These proteins are therefore essential for all organisms to survive exposure to acute stress. Furthermore the heat shock responses become impaired with age, in addition to numerous other cellular defense systems including the UPR and the removal of mis-folded proteins by the cellular degradation machinery, leading to cellular ageing and neurodegeneration (Powers et al., 2009, Ben-Zvi et al., 2009). Down-regulation of HSF in *C. elegans* leads to lifespan shortening and accelerated protein aggregation. On the other hand, reduced IIS can activate HSF,
improve proteostasis, and extend lifespan (Hsu et al., 2003, Morley and Morimoto, 2004). In addition, age-related proteostatic failure can be ameliorated by over-expression of HSF or daf-16 (FOXO), suggesting an important role of these stress-responsive transcription factors in maintaining global proteome homeostasis (Ben-Zvi et al., 2009). Moreover, HSF1 can be directly deacetylated by SIRT1, which then becomes activated for DNA binding. Indeed a decrease in SIRT1 levels during ageing is also associated with an inhibition of HSF1 DNA-binding activity (Westerheide et al., 2009). Furthermore, an age-related decrease in the HSF1 DNA-binding activity and reduced transcripts of HSP70 are reversed by caloric restriction (Heydari et al., 1996). These results suggest that HSF1 and SIRT1 function together to protect cells from stress stimuli, thereby promoting survival and extending lifespan.

In addition to reducing food intake, the timing of a meal also appears to be important. During evolution, animals and humans ate intermittently. For many invertebrates, long periods of starvation are normal and they have developed conserved responses against starvation (Fontana and Partridge, 2015). For example, intermittent fasting (IF), with 2 days of normal feeding followed by 2 days of fasting extends lifespan in C. elegans through reduced IIS/mTOR signalling (Honjoh et al., 2009). Chronic starvation even extends lifespan in C. elegans with shared mechanisms with IF that include reduced IIS/mTOR signalling and activation of FOXO family transcription factors (Kaeberlein et al., 2006, Lee et al., 2006, Uno et al., 2013, Honjoh et al., 2009). Short-term every-other-day feeding/fasting mimics many positive outcomes of DR, improving the healthy lifespan by up to 30% in mice (Brown-Borg and Rakoczy, 2013). However, the age of initiation of the fasting seems also to be crucial, as old-aged mice are more sensitive to fasting than younger ones, suggesting they are more likely starvation-sensitive (Goodrick et al., 1990). In addition to its pro-longevity effects, IF may also prevent age-related chronic disorders, such as diabetes, neurodegeneration, obesity, cardiovascular disease and hypertension (Mattson et al., 2014). Human trials on the effects of IF are ongoing and appear to show promising results, including reduced body weight, fat mass, serum concentrations of total and LDL cholesterol, triglycerides and arterial blood pressure. Biomarkers for cancer risk were reduced, but total and free IGF-1 did not change (Harvie et al., 2011). In rodents, several molecular changes appear to mediate the benefits of fasting, including a metabolic shift to fat metabolism and ketone production, enhanced adaptive cellular
and molecular stress responses that prevent and repair molecular damage, reduced inflammation and oxidative stress (Mattson et al., 2014). In addition, fasting can also improve mitochondrial function, elevate stress responses by increasing production of the chaperones HSP70 and Grp78. It also leads to the inhibition of the nutrient sensing network AKT/mTOR pathway, increases autophagy and stimulates the DNA repair pathway in different cell types (Brown-Borg and Rakoczy, 2013, Mattson et al., 2014). Prolonged fasting in mice reduced circulating IGF-1, improved stem cell protection, self-renewal and regeneration (Cheng et al., 2014).

However, the effect of IF in cancer or other cachexia disease states is controversial. One study reported that fasting could initiate and promote liver carcinogenesis in rats with increased production of TGF-beta1 (Tessitore and Bollito, 2006). Moreover, *Drosophila* models of tumours secreted ImpL2, the antagonist of insulin signalling, leading to a sustained and dramatic reduction of insulin activity and tissue loss, suggesting that excessive reduction of IIS/mTOR signalling pathway might be causative for the pathogenesis and progression of tumours (Figueroa-Clarevega and Bilder, 2015, Kwon et al., 2015). This cachexia mimics malnourishment and further reducing the insulin signalling by IF will deteriorate this situation. Wasting, starvation, IF, and DR therefore share similar molecular mechanisms in controlling the physiological and pathological state in response to nutrient limitation, although the levels of reduction and outcomes are varied.

### 1.5 Thesis outline

My PhD work was mainly focused on dissecting the role of PGC-1α in neurodegenerative diseases, including AD and PD, in *Drosophila* models. This work also involved the identification of the interaction partners of PGC-1α in regulating metabolic processes in disease states. Moreover, I also characterised the downstream effectors of PGC-1α in the regulation of ageing in the fly. This work provides significant insights into the role of PGC-1α and its interactors in both ageing and neurodegeneration. It also highlights a number of potential therapeutic strategies for promoting healthy ageing and preventing age-related diseases such as neurodegeneration.
Most of my PhD work is yet to be published. However, I have already been a co-author on a number of publications and these can be found in the Appendix.

A summary of each results chapter is detailed below.

1.5.1 PGC-1 regulates the ER-unfolded protein response and ameliorates Aβ toxicity. (Chapter 3)

AD is the most common neurodegenerative disorder affecting millions of people worldwide. Ageing is the greatest risk factor for developing AD (Niccoli and Partridge, 2012). Therefore there is an urgent need to develop therapeutic strategies for AD in the ever-increasing ageing world population. Mitochondrial dysfunction, oxidative stress, proteostatic failure, and circadian rhythm disruption have all been implicated in the pathogenesis of AD (Hetz and Mollereau, 2014, Bender et al., 2008, Nunomura et al., 2001, Harper et al., 2005), suggesting that these processes may provide excellent therapeutic targets for preventing AD. PGC-1α is a key regulator in many biological processes, including mitochondrial biogenesis, oxidative stress responses, circadian clock control, and protein quality control (Puigserver and Spiegelman, 2003, St-Pierre et al., 2006, Liu et al., 2007, Wu et al., 2011). It has also been found to be present at reduced levels in AD post mortem brains (Qin et al., 2009), suggesting that elevating the levels of PGC-1α in neurons might be beneficial in AD. In Chapter 3, I demonstrated for the first time in vivo that up-regulation of PGC-1α in neurons can prevent the neurotoxic phenotypes in a Drosophila model of Aβ toxicity. I then investigated the mechanisms by which activation of dPGC-1 in neurons ameliorates Aβ induced neurotoxicity.

To further explore the downstream effectors of dPGC-1 in ameliorating Aβ neurotoxicity, I performed a small-scale screen of transcription factors previously reported to co-activate dPGC-1 in regulating many metabolic processes. We have previously shown that Aβ neurotoxicity leads to an arrhythmic sleep pattern (Niccoli et al., 2016). I confirmed this by demonstrating that over-expression of Aβ disrupts the circadian clock via increasing the period tau. I also demonstrated that over-expression of dPGC-1 ameliorates this neurotoxic phenotype. Mammalian PGC-1α
PGC-1α in neurodegeneration and ageing

has been implicated in a second feedback loop of circadian clock control by co-activating a transcription factor RORα (Liu et al., 2007). However its role in the *Drosophila* in a similar loop is still unknown. In order to examine this further I investigated the Par Domain Protein 1ε (PDP1ε), the transcription factor involved in second feedback loop of the circadian clock in *Drosophila* (Cyran et al., 2003). I demonstrated that *dPGC-1* and PDP1ε interact to form a complex by Fluorescence Resonance Energy Transfer. Using genetic manipulation, I further showed that overexpression of *Pdp1ε* in neurons can ameliorate Aβ neurotoxic phenotypes in flies.

Activation of *dPGC-1* or *Pdp1ε* in neurons can ameliorate Aβ neurotoxicity through activation of the UPR<sup>ER</sup>. Therefore compounds potentially targeting the UPR<sup>ER</sup> may offer therapeutic avenues for the treatment of AD. I therefore tested three compounds in Aβ-expressing fly models. In collaboration with Dr. Sofola-Adesakin, we found that lithium, the main drug used in the treatment of bipolar disorder, rescued the toxic phenotypes in Aβ-expressing flies by reducing protein synthesis (Sofola-Adesakin et al., 2014). I also found that metformin, the drug for treating type-2 diabetes, could rescue the shortened lifespan of Aβ-expressing flies. I then collaborated with Dr Niccoli to investigate the mechanisms of this amelioration, and found that metformin reduces Grp78 protein levels, leading to activation of the UPR<sup>ER</sup> (Niccoli et al., 2016). Another drug I tested in collaboration with Dr. Castillo-Quan and Dr. Kinghorn is rifampicin, a well-known antibiotic used for the treatment of tuberculosis and leprosy. Previous studies have suggested that rifampicin can reduce Aβ oligomerization and improve the memory defects in an APP mouse model via improved autophagy-lysosomal function (Umeda et al., 2016). In keeping with this I describe the neuroprotective effects of rifampicin in Aβ-expressing flies.

1.5.2 The role of PGC-1α in *Drosophila* models of Parkinson’s disease (Chapter 4)

One of the main aims of my PhD work was to investigate the role of PGC-1α in familial PD. This work followed on from extensive studies suggesting that PGC-1α and mitochondrial dysfunction might play a central role in PD pathogenesis (Whitworth and Pallanck, 2009, Shin et al., 2011). In Chapter 4, I describe my work assessing the role of PGC-1α in *Drosophila* null mutants of *parkin* and *PINK1*.
1.5.3 PGC-1α-GABPα modulates ageing in Drosophila. (Chapter 5)

*Drosophila* models that over-express *dPGC-1* in the intestinal system show extended lifespan with improved intestinal homeostasis and mitochondrial function (Rera et al., 2011). However the mechanisms of lifespan extension by *dPGC-1* have not been fully explored. Mammalian GABPα is induced by PGC-1α in the regulation of mitochondrial biogenesis in skeletal muscle (Yang et al., 2014, Mootha et al., 2004). *Drosophila* GABPα is required for proper mitochondrial gene expression together with *dPGC-1* in a nutrient-sensing manner (Tiefenböck et al., 2010, Baltzer et al., 2009), suggesting the *Drosophila* homologue may share a conserved molecular function with its mammalian counterpart. I hypothesised that *dGABPα* may be regulated by *dPGC-1* and therefore responsible for *dPGC-1*-mediated lifespan extension. In Chapter 5, I describe my work investigating the role of *dGABPα*, the homologue of human GA-binding protein α subunit (also called Nuclear respiratory factor 2α, NRF2α), in regulating longevity and healthy lifespan. I used a genome-wide unbiased approach to interrogate the transcriptomes in response to over-expression of *dGABPα*, in order to aid our understanding of the molecular mechanisms underlying lifespan extension. I performed RNAseq analyses, to assess how the transcriptional response of *dGABPα* activation correlates with other interventions that promote longevity in *Drosophila*. 
Chapter 2 General methodology

2.1 Drosophila melanogaster: strains and genetics

One of the major advantages of working with the fruit fly *Drosophila melanogaster* is that there is a huge wealth of different laboratory stains and mutants. The Partridge laboratory has a large inventory of genetic stocks allowing appropriate use of and generation of different crosses. All stocks were kept at 18°C, while experimental crosses and transgenic flies were kept at 25°C and 65% humidity on a 12:12 light: dark cycle using standard sugar/yeast (SY) medium. Flies were reared at standard larval density (around 300 flies per 200 mL bottles) and eclosing adults were collected over a 4-hour period after which they were mated for 48 hours, before collecting females or males for experiments (Bass et al., 2007).

2.1.1 White Dahomey (w\text{Dah})

The background control stock white Dahomey (w\text{Dah}) has been maintained in large population cages with overlapping generations since 1970. The w\text{Dah} stock was derived by the incorporation of the w\text{1118} mutation into the outbred Dahomey background by backcrossing (Bass et al., 2007). The w\text{Dah} strain is naturally infected by the intra-cytoplasmic bacterium *Wolbachia*, which modulates the lifespan effects of the IIS pathway (Gronke et al., 2010, Ikeya et al., 2009, Negri, 2011).

2.1.2 White 1118 (w\text{1118})

The w\text{1118} Drosophila stock is an inbred and isogenic strain often used for neurodegeneration studies. This strain is also often used as a control in ageing studies to demonstrate that pro-longevity effects found in the w\text{Dah} strain are independent of genetic background (Bjedov et al., 2010).

2.1.3 Backcrossing

Backcrossing of the transgenic lines was necessary to ensure that any differences between mutant and control flies are due to the expression of the transgene alone, and
not due to confounding mutations or insertional mutagenic effects (Partridge and Gems, 2007). Previous studies have demonstrated that insufficient standardization of the genetic background, and inappropriate backcrossing or use of inappropriate controls, produces artificial lifespan extension (Burnett et al., 2011, Toivonen et al., 2007). For this reason, before the transgenic stocks could be used for experiments, they were backcrossed at least 6 generations into the desired background strain. In the first cross, transgenic males were mated with wild type female virgins, which results in the wild type cytoplasmic content (including the mitochondrial genome) being pass to the offspring. The offspring then were selected either by their visible phenotype (eye colour for example) or by PCR analysis following transgene/construct-specific sequences. After this initial cross virgin females were collected and crossed to wild type males, and this cross was repeated at least another five times until six backcrossing events were completed. Transgenic lines were then made homozygous or balanced and kept as a stock for experiments. Transgenic lines were normally refreshed by three generations following a similar protocol before re-use after extended periods of time.

### 2.1.4 The GAL4-UAS system

The GAL4-UAS system is considered one of the most powerful genetic tools to study gene expression and function in the fruit fly (Brand and Perrimon, 1993). Its major advantage is that it can restrict the expression of genes of interest in a cellular or tissue-specific manner using native gene promoters. The system has two parts: the GAL4 gene, encoding the yeast *Saccaromyces cervisiae* transcriptional activator protein GAL4, and the UAS (Upstream Activation Sequence), an enhancer to which GAL4 specifically binds to activate gene transcription. The GAL4 transgene is placed under the control of a native gene promoter, or driver gene, which is then only expressed in cells where the driver gene is usually active. For example, the elav-GAL4 fly line will express the GAL4 protein under the elav promoter. Elav encodes for embryonic lethal abnormal vision, a pan-neuronal protein and is therefore a neuronal driver. Therefore GAL4 should only activate gene transcription where a UAS has been introduced. For example, when green fluorescent protein (GFP) is placed under UAS control, i.e. UAS-GFP, and this line is crossed with an elav-GAL4 line, the
offspring genotype \textit{elavGAL4>UAS-GFP} will express GFP only in neurons (Osterwalder et al., 2001).

2.1.5 The gene-switch system
The gene-switch (GS) system uses the GAL4/UAS system with the restriction that the transcriptional activity depends on the presence of a steroid hormone or a chemically related compound (Osterwalder et al., 2001, Ford et al., 2007, Roman et al., 2001). In the GAL4/UAS system, the binding of the transcription factor GAL4 to UAS, results in the transcription of the UAS-linked transgene (Brand and Perrimon, 1993). In the GS system, GAL4 is fused with the regulatory domain of the human progesterone receptor (Osterwalder et al., 2001, Ford et al., 2007, Roman et al., 2001, Poirier et al., 2008). In the absence of the activator mifepristone (RU486), the GAL4 protein and UAS transgene can coexist in the fly with only leaky weak expression of the transgene under the control of UAS. Only when these flies are fed with RU486, the GAL4-GS is able to bind to the UAS and therefore the transgene is induced (Figure 2.1) (Osterwalder et al., 2001, Roman et al., 2001).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{GeneSwitch_expression_systems.png}
\caption{The GeneSwitch expression systems}
\end{figure}

This diagram illustrates the cross between a gene-switch driver (\textit{elavGS}) and a UAS construct linked to the \textit{SNCA} gene (\textit{UAS-SNCA}) to obtain a line carrying both the driver and UAS line, which results in the following line: \textit{elavGS/+>UAS-SNCA/+}. Flies only express the \textit{\(\alpha\)-synuclein} protein in the nervous system following ingestion of medium supplemented with RU486 (RU+). Flies with the same genotypes, but not fed RU486 (RU-), are used as genetic controls.
2.2 *Drosophila* food medium

2.2.1 Sugar-yeast medium (SY)

Our standard laboratory medium (SY) was used for the development and maintenance of all flies, unless otherwise stated. The SY medium was made in the following way: 15 g of agar were dissolved in 700 mL distilled water by heating until boiling. After this, 100 g of autolysed yeast powder and 50 g of sugar were added until boiling. 170 mL of distilled water were added to the mixture and it was then left to cool down. When the temperature of the mixture was between 50-60°C, 30 mL of nipagin (100 g/L) and 3 mL propionic acid (both anti-fungal preservatives) were added. The food was then dispensed (4 mL per vial) into plastic vials and allowed to set at room temperature before storing at 4°C (Bass et al., 2007).

2.2.2 Grape juice medium

Grape plates were used as a surface for egg laying and collection. Collected eggs were subsequently dispensed into bottles containing SY medium. Grape plates were made as follows: 25 g agar were dissolved in 500 mL distilled water and brought to the boil. To this, 300 mL of red grape juice were added and the medium was brought to the boil again. After adding an additional 50 mL of distilled water the medium was brought to the boil again. After adding an additional 50 mL of distilled water the medium was allowed to cool to below 60°C. As with the SY medium 21 mL of nipagin (100 g/L) were added. The medium was dispensed into plastic petri dishes and allowed to set at room temperature before storing at 4°C.

2.2.3 Starvation medium

The starvation medium was prepared by adding 15 g of agar to 700 mL of distilled water. This was brought to the boil and cooled down before dispensing into vials. No additional ingredients (including anti-fungal) were added to avoid any potential source of nutrients.

2.2.4 Dietary restriction

For DR experiments a yeast dilution protocol was established as detailed below:

Recipe for SY preparation for DR tent (1 litre of medium)
1. Agar was added to the initial volume of water (ddH\textsubscript{2}O) just before heating. Stirring was continuous throughout the preparation process to prevent any of the ingredients sticking to the saucepan.

2. Just after boiling, sucrose was added and the mixture was brought to the boil again.

3. The yeast was added and the mixture was brought to the boil again. The saucepan was then removed from the heating source and the additional ddH\textsubscript{2}O was added to cool down the medium.

4. Only after the medium reached a temperature below 60°C (preferably between 50 and 58°C), nipagin and propionic acid were added.

5. The medium was then ready for dispensing.

### 2.3 Fly husbandry and culturing

#### 2.3.1 Male and female separation

Some anti-ageing interventions that extend lifespan are sex-specific, and are often confined to females in both *Drosophila* (Clancy et al., 2001) and mice (Selman et al., 2009). In other cases the responses show some dimorphism, with the response attenuated in males (Bjedov et al., 2010, Miller et al., 2014). Therefore, it is a common practice in ageing studies to test males and females separately (Partridge and Gems, 2007). Additionally, continuous mating can impact several physiological parameters, including survival (Barnes et al., 2008, Chapman and Partridge, 1996). Female and male flies can be distinguished very easily under a microscope whilst flies are anesthetized with CO\textsubscript{2} (Greenspan, 2004). For most experiments only the female flies were used, though some phenotypes were also corroborated in males to ensure sex-independent effects. Female and male flies were sorted using a fine paintbrush while anesthetized. Flies were usually separated at day 2 post-eclosion after a 48-hour mating period.
2.3.2 Virgin collection

To obtain the desired genotypes, it was often necessary to cross male and female flies of different genotypes. This ensured the transfer of at least one copy of the chosen transgene to the progeny. This was achieved by collecting unmated female flies (or virgins). Virgin collection was carried out for backcrossing and to obtain the desired genotypes for experiments. Female *Drosophila* flies do not respond to courting males during the first 6 hours after eclosion at 25°C (Greenspan, 2004). Therefore newly emerged female flies were collected within 6 hours after clearing rearing bottles.

2.4 *Drosophila* handling and survival

2.4.1 Lifespan assays

An important determinant for ageing analysis is to ensure that the parents of the experimental flies were the same age at egg laying and reared under the same conditions. In this way the effects of parental age on lifespan were controlled (Priest et al., 2002). 'Egg squirt' protocols were undertaken to ensure that all flies in the experiment were raised at similar larval densities (~300 eggs per bottle containing 70 mL of food), thereby avoiding any effects of larval density on lifespan (Priest et al., 2002). Flies were allowed to lay eggs for less than 24 hours on grape medium plates, with live yeast paste to encourage mating. The eggs were then collected from the plate by washing with phosphate buffered saline (PBS) solution and collected into falcon tubes. The eggs were allowed to settle to the bottom of the tube. Using a 100 µL Gilson pipette, ~18-20 µL of egg suspension was dispensed into 200 mL glass bottles containing 70 mL SY medium. This equated to ~300 eggs per bottle.

After 10 days of development flies emerged from their pupae and were then transferred into fresh bottles. The flies were then allowed to mate in these new bottles for 48 hours. After this flies were sorted using a paintbrush under CO₂ anesthesia, and divided into vials (15 flies per vial).

Flies were then transferred to fresh food vials three times a week throughout life. The number of dead flies found during each transfer was recorded. Accidental death and escapees were distinguished from deaths, and were censored from the experiment.
From these data, a survivorship graph was generated, making it possible to compare survival curves over time between different genotypes or experimental conditions. Lifespan curves were analyzed using a log-rank test.

### 2.4.2 Stress assays

For stress assays lifespan experiments were performed using different chemicals to induce cellular stress.

#### 2.4.2.1 Paraquat

Flies were kept under pre-determined conditions similar to those in the lifespan assays for a fixed period of time, after which they were transferred onto food containing paraquat. 1 M paraquat containing food was prepared by dissolving N, N’-dimethyl-4, 4’-bipyridinium dichloride (paraquat, Sigma 856177) into ddH₂O. This was then was added to a 0.0 SY (agar/sugar) medium to achieve a final concentration of 10 mM. All stress assays were tested without yeast in the medium to avoid interaction between yeast and the stressor. The medium used has all the other components of the standard medium including sucrose. Flies were typically assayed with 15 flies per vial. Deaths were scored every 4 hours after the detection of the initial wave of death. Data was plotted as a survival curve and analysed using a log-rank test.

#### 2.4.2.2 Hydrogen Peroxide (H₂O₂)

For the preparation of H₂O₂ containing medium, a 30% H₂O₂ stock solution from Sigma was diluted into a 0.0 SY medium to give a final concentration of 2.5% or 5%. Flies were typically assayed with 15 flies per vial. Deaths were scored at least 3 times per day, plotted as a survival curve and analyzed using a log-rank test.

#### 2.4.2.3 Phenobarbital

Phenobarbital (Sigma P1636) was prepared by directly adding the salt to 0.0 SY medium to obtain a final concentration of 6%.

#### 2.4.2.4 Tunicamycin
Flies were tipped into vials containing 1% agar, 1.5% sucrose, and 10 mg/l of tunicamycin. Dead flies were scored at regular intervals, and lifespan curves were compared using a log-rank test.

2.4.3 Mifepristone (RU486)
As explained above the GeneSwitch system uses mifepristone (RU486) as an inducer of gene expression in the presence of the GeneSwitch driver (Osterwalder et al., 2001, Poirier et al., 2008). Our laboratory has optimised the dose of RU486 that produces effective transgene expression without impairing longevity, fecundity or metabolism. RU486 (Sigma, M8046) was dissolved in 100% ethanol and supplemented to standard SY medium before dispensing into vials. Food supplemented with RU486 was stored at 4°C until use.

2.5 Behavioural investigations
2.5.1 Fecundity assay
Fecundity has historically been heavily linked to longevity (Partridge et al., 2005). Although this association has been uncoupled (Grandison et al., 2009), interventions to prolong lifespan without affecting fecundity are still limited. To assess fecundity female flies were allowed to lay eggs for a period of 24 hours in vials containing standard medium. Vials containing eggs were frozen and kept at -20°C for a short time until eggs were counted using a hand held counter. The total number of eggs laid was divided by the number of flies per vial and a t-test was used to determine statistical value.

2.5.2 Climbing assay (negative geotaxis)
Age-related decline in locomotor ability represents a behavioural measurement of neuronal health. It is an insightful measurement read-out of the functional capacity of flies that is also sensitive to some anti-ageing interventions (Gargano et al., 2005, Jones et al., 2009). The climbing assay involved the placement of 15 flies in a 35 cm
column (1.5 cm diameter) with a conic bottom end. Flies were tapped down and observed during a 45 second period. The column was separated into three areas: top, middle, and bottom by two lines: one was 10 cm from top and the other was 3 cm from bottom. After 45 seconds the flies located in each of these three sections were recorded. Each column was evaluated three times to minimized trial error and a minimum of three vials per genotype/condition was assessed. The numbers of flies in the top (ntop) and at the bottom (nbottom) were used to obtain a motor performance index (PI). This was calculated as \( \frac{1}{2} (\text{ntot} + \text{ntop} - \text{nbottom} / \text{ntot}) \). The PI was plotted against time per each genotype and condition. Statistical analysis was performed in R using ordinal logistical regression, using the individual heights for each fly as data points.

2.6 Biochemistry and molecular biology methods

2.6.1 DNA extraction and Single-Fly Polymerase Chain Reaction (PCR)

To analyse single flies for specific mutations one fly was placed in a 1.5 mL eppendorf and squashed with a blue pestle. 50 µL of squish buffer (990 µL of squish buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 25 mM NaCl) + 10 µL of proteinase K) were then added. The liquid was transferred to a PCR tube and incubated for 1 hour at 37°C and then at 95°C for 10 min. The sample was spun, after which it was either stored at 4°C or prepared for PCR.

The PCR reaction consisted of (per fly) 2 µL of extracted DNA, 2.5 µL 10X PCR buffer (15 mM MgCl₂), 0.5 µL dNTPs (10 mM), 0.5 µL of each primer (10 mM), 0.25 µL TAQ polymerase and 18.75 µL of ddH₂O. All reagents were obtained from Qiagen, UK. The PCR cycles were run using a thermal cycler (Eppendorf UK limited), with the following protocol: 1 cycle of 94 °C for 15 min (initial melting step to denature the hot-start TAQ polymerase), 30 to 35 cycles of 95 °C for 30 sec (for denaturing DNA), 50-60 °C for 30 sec (to anneal the primers), and 72 °C for 2 min (elongation step). Finally, 1 cycle of 72 °C for 10 min was performed for the final elongation phase. Samples were then either stored at 4 °C, -20 °C or run on an agarose gel.
2.6.2 Gel electrophoresis

1 to 2 grams of agarose (Sigma, UK) were dissolved in 100 mL of TAE (Tris base, acetic acid, EDTA) buffer and heated in a microwave, until close to boiling, after which it was slowly cooled down. Once cooled 5 µL of ethidium bromide was added to the agar solution. The mixture was placed in a gel tray and a comb was added to create wells for the sample. The gel was allowed to set for 1 hour. A marker was added to the first well and samples were added in conjunction with 6X loading dye (40% glycerol, 6X TBE buffer, and 0.25% bromophenol blue) at a final volume of 20-25 µL. Electrophoresis was performed between 70-100 V as necessary to separate the DNA bands. The samples were then visualized under the UV light using a UV trans-illuminator.

2.6.3 Quantitative Real Time PCR (qRT-PCR)

Total RNA was extracted from 15 whole flies or 25 fly heads using TRIZol® (GIBCO) according to the manufacturer’s instructions. The concentration of total RNA purified for each sample was measured using Nano Drop2000 (Thermo Scientific). 5 µg of total RNA was then subjected to DNA digestion using DNase I (Ambion), immediately followed by reverse transcription using the Superscript II system (Invitrogen) with oligo (dT) primers. Quantitative PCR was performed using the PRISM 7000 sequence-detection system (Applied Biosystems) and SYBR Green (Molecular Probes) by following the manufacturer’s instructions. Each sample was analyzed in duplicate with both target and reference (RP49) gene primers in parallel. The values are the mean of three or four independent biological repeats ± SEM.

2.6.4 Western blotting

10 female flies/15 fly heads were homogenized in 200 uL/40 uL of 2X SDS Laemmlli loading sample buffer (120mM Tris-HCl pH6.8, 20% glycerol, 4% SDS) containing 5% β-mercaptoethanol. Samples were heated at 95 °C for 5 minutes after which extracts were cleared by centrifugation. 10 µL of protein extract were loaded and
PGC-1α in neurodegeneration and ageing

separated on pre-cast 4%-12% Invitrogen Bis-Tris gels (NP0322). After separation, the proteins were transferred to a nitrocellulose membrane in Tris-glycine buffer supplemented with 10% ethanol. Membranes were saturated in 5% milk in TBS-T (Tris-buffered saline with 0.05% Tween-20) for 1 hour at room temperature (RT) and then incubated with a selected primary antibody in saturation solution overnight. After 5x washes in TBS-T, they were then incubated with an appropriate secondary antibody for 1 hour at RT. Bands were visualized with Luminata Forte (Millipore) and imaged with ImageQuant LAS4000 (GE Healthcare Life Sciences). Quantification was carried out with ImageJ.

2.6.5 Protein Quantification

The Bicinchoninic acid protein (BCA) assay from Thermo Scientific (23225) was used to determine the protein concentration of biological samples. BSA albumin standards were diluted using the same diluent as the samples. The following standard concentrations were prepared: 2000, 1500, 1000, 750, 500, 250, 125, 25, 0 µg/mL. The working reagent (WR) was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B. 25 µL of each standard and sample was dispensed twice into a microplate well. To each well 200 µL of the WR was added by using a multi-channel pipette and mixed thoroughly on a plate shaker for 30 seconds. The plate was then covered and incubated at 37 °C for 30 mins. The plate was then allowed to cool to room temperature. The absorbance was then measured at 562 nm on a plate reader.
Chapter 3 PGC-1 regulates the ER-unfolded protein response and ameliorates Aβ toxicity

3.1 Summary

Alzheimer’s disease (AD) is the most common neurodegenerative disorder and is neuropathologically characterised by the extracellular deposition of aggregates of the Aβ protein. The misfolding of Aβ is associated with cellular defects in brain energy metabolism, hypersensitivity to stress and proteostatic failure (Musiek and Holtzman, 2015). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a transcriptional coactivator that regulates energy homeostasis within the cell, and is the master regulator of mitochondrial biogenesis (Puigserver and Spiegelman, 2003). The possible relevance of this gene in the pathogenesis of AD was identified with the demonstration that PGC-1α gene expression levels are decreased in the post-mortem brain tissue of AD patients (Qin et al., 2009). Consistent with this I have shown that up-regulation of dPGC-1 in the neurons of a Drosophila model of Aβ toxicity rescues lifespan, behavioral phenotypes and circadian rhythm disruption. Moreover I demonstrated that the amelioration of Aβ toxicity is associated with improved mitochondrial homeostasis and activation of the unfolded protein response (UPR). Specifically, dPGC-1 increased electron transport chain (ETC) activity and ATP production. Moreover, expression of dPGC-1 also protected Aβ-expressing flies against oxidative stress and was shown to regulate the levels of mitochondrial chaperones in response to Aβ, thereby modulating mitochondrial UPR (UPR{MT}). In addition, over-expression of dPGC-1 negatively regulated Grp78 protein levels, indicative of increased endoplasmic reticulum UPR (UPR{ER}). Therefore through UPR activation in the mitochondrial matrix and up-regulation of ER-mediated protein homeostasis and quality control, dPGC-1 over-expression ameliorated the Aβ associated neurotoxic phenotypes. Lastly I demonstrated that dPGC-1 physically interacted with Par Domain Protein 1ε (PDP1ε) in the Drosophila brain, and that genetic activation of Pdp1ε in neurons partially rescued the shortened lifespan of Aβ-
expressing flies via activation of UPR\textsuperscript{ER}. In keeping with this, I demonstrated that rifampicin, a well-known antibiotic, also activated the UPR\textsuperscript{ER} and rescued neurotoxicity in Aβ flies. These findings suggest that either increasing intraneuronal PGC-1α levels, or oral administration of rifampicin, could offer therapeutic benefits in AD by increasing the UPR\textsuperscript{ER}.

3.2 Introduction
AD is a progressive neurological disorder characterized by memory loss, cognitive decline and neuronal death predominantly in the cerebral cortex and hippocampus. Typical neuropathology includes extracellular deposits of amyloid-β aggregates and intracellular inclusions of hyper-phosphorylated tau aggregates (Crews and Masliah, 2010). The disease progression is thought to be triggered by accumulation of Aβ oligomer peptides, generated through the abnormal processing of the amyloid precursor protein (APP). This results in a neurotoxic cascade of events, including mitochondrial dysfunction, oxidative stress, proteostatic failure, circadian disruption, inflammation, tau hyper-phosphorylation, and eventual neuronal death. Some of these pathways also directly influence APP metabolism and Aβ accumulation (Hardy and Higgins, 1992, Musiek and Holtzman, 2015). Whether these pathogenic processes are the main drivers of disease in AD is controversial, however, what is clear, is that they all represent possible therapeutic targets in the treatment of AD. Genetic or pharmacological interventions targeting these modulators may provide excellent therapeutic avenues.

Neurodegenerative diseases are characterized by the neuronal accumulation of insoluble aggregates of mis-folded proteins in the endoplasmic reticulum (ER) (Hetz and Mollereau, 2014). Activation of the UPR\textsuperscript{ER} allows cells to either restore protein homeostasis or to enter apoptosis as a protective response. Three well-characterized UPR sensors mediate this response: PERK (protein kinase RNA-like ER kinase), inositol-requiring enzyme 1 (IRE1), and transcription factor 6α (ATF6α) (Wu and Kaufman, 2006, Hetz and Mollereau, 2014). Under normal physiological conditions, these trans-membrane signalling components are bound and inhibited by GRP78. During ER stress, mis-folded proteins titrate down the levels of Grp78, thus activating
the signalling cascades downstream of ATF6α, IRE1 and PERK. ATF6α is transported to the Golgi where it is processed to produce a cytosolic fragment, which operates as a transcription factor, regulating X-box binding protein 1 (Xbp1) and Grp78 mRNA levels. Active IRE1 induces the splicing of Xbp1 mRNA, leading to the expression of the active transcription factor Xbp1. PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α), and induces the expression of ATF4. All of the above leads to the up-regulation of ER chaperones, ER-associated protein degradation, autophagy, redox reactions, and eventually apoptosis.

Increasingly evidence has indicated that disruptions in brain energy metabolism are critically involved in the pathogenesis of AD (Kapogiannis and Mattson, 2011), including mitochondrial damage (Galindo et al., 2010, Wu et al., 1999, Calkins et al., 2011), a decline in glucose metabolism (Mosconi et al., 2008), and oxidative damage (Siegel et al., 2007, Head et al., 2001). The PGC-1 family of transcriptional co-activators, including the founding member PGC-1α (PPARγ co-activator 1), and its homologue PGC-1β and PRC (PGC-1-related co-activator), are important for the normal functioning of mitochondrial biogenesis (Mootha et al., 2004, Scarpulla, 2008), adaptive thermogenesis (Puigserver and Spiegelman, 2003), hepatic metabolism (Puigserver and Spiegelman, 2003, Li et al., 2007b), stress defence (Valle et al., 2005, St-Pierre et al., 2006), and circadian rhythms in metabolically active organs (Liu et al., 2007). Moreover, PGC-1α has been show to modulate ageing and diabetes, the two main risk factors for developing AD (Rogers et al., 2012, Willette et al., 2015). The PGC-1 family was reported in animal models as a novel modulator of lifespan (Rera et al., 2011). Furthermore, PGC-1α-responsive genes involved in oxidative phosphorylation have been shown to be reduced in the muscles of patients with type 2 diabetes mellitus (Mootha et al., 2003, Patti et al., 2003), suggesting its importance in ageing, human diabetes, and possibly in the development of AD. Indeed, the age-related decline in protein homeostasis and neuronal stress response signalling might contribute to AD progression (Taylor and Dillin, 2011, Hipp et al., 2014), and PGC-1α, which has been implicated in the stress defence system, and in protein quality control (St-Pierre et al., 2006, Wu et al., 2011), might therefore represent a promising therapeutic target.
PGC-1α mutant mouse models display neurodegenerative pathology in the brain and behavioural abnormalities, highlighting its protective role in neurons (Leone et al., 2005, Lin et al., 2004, Lucas et al., 2014, St-Pierre et al., 2006). Indeed, PGC-1α is neuroprotective in animal models of several neurodegenerative diseases characterised by abnormal protein aggregation, including Huntington’s disease (Cui et al., 2006, Weydt et al., 2006, Tsunemi et al., 2012) and amyotrophic lateral sclerosis (Zhao et al., 2011, Liang et al., 2011). Human studies report that PGC-1α activity negatively correlates with the age of onset of AD (Helisalmi et al., 2008) and PD (Clark et al., 2011). In addition, PGC-1α and ATP are remarkably decreased in AD patient brains (Kapogiannis and Mattson, 2011, Hoyer, 1996, Qin et al., 2009). However, the effect of elevating PGC-1α in AD animal models is not well established, with some studies showing a protective role of PGC-1α in mitochondrial dysfunction (Sheng et al., 2012) and Aβ accumulation (Katsouri et al., 2011), while another study demonstrated opposite effects (Dumont et al., 2014).

The question of whether reduced PGC-1α is causal in the neurodegeneration of AD is yet to be determined. A reduction in PGC-1α could contribute to disease progression in several ways. It may cause mitochondrial dysfunction and reduced ATP, as PGC-1α is the master regulator of the electron transport chain to generate ATP synthesis. Reduced PGC-1α also leads to increased sensitivity to stressors by ROS, thereby impairing mitochondrial homeostasis (St-Pierre et al., 2006, Lu et al., 2014, Haynes and Ron, 2010). PGC-1α deficiency may also exacerbate age-related disturbances in proteostasis and neuronal stress response pathways (Taylor and Dillin, 2011, Hipp et al., 2014, Wu et al., 2011). Mammalian PGC-1α regulates the circadian clock and therefore a reduction in this transcription factor may contribute to circadian rhythm disruption (Liu et al., 2007). I therefore investigated these pathways to dissect the role of PGC-1α in the pathogenesis of AD.

I studied the role of PGC-1α in the pathogenesis of AD, using a model of Aβ toxicity in the fruit fly Drosophila melanogaster (Sofola et al., 2010). Drosophila is a powerful genetic tool to study neurodegeneration (McGurk et al., 2015, Fernandez-Funez et al., 2015), with distinct brain structures and analogous cell types to those in the human brain, as well as a blood brain barrier. I utilised the fly AD model that
expresses pathogenic Arctic Aβ1-42, which displays a shortened lifespan, behaviour defects as well as neurodegeneration (Sofola et al., 2010). Moreover, *Drosophila* possesses a single homologue of the mammalian PGC-1 family, *Spargel* (*dPGC-1*), (Tiefenböck et al., 2010), therefore providing a system in which PGC-1 function can be analysed in the absence of redundancy.

In order to determine whether neuronal PGC-1 contributes to the pathogenic cascades downstream of toxic Aβ, I genetically over-expressed *dPGC-1* in the neurones of flies over-expressing Arctic Aβ1-42 also in neuronal tissue. I demonstrated that *dPGC-1* over-expression partially rescued the Aβ-induced neurotoxic phenotypes, including lifespan, climbing ability and circadian rhythm, without affecting the levels of Aβ peptides. In addition, over-expression of *dPGC-1* increased mitochondrial membrane potential and ATP levels, compensating for the mitochondrial defects mediated by Aβ. Furthermore, activation of *dPGC-1* ameliorated Aβ-induced hypersensitivity to oxidative stress, and regulated mitochondrial chaperones in response to Aβ, suggesting improved mitochondrial proteostasis. *dPGC-1* also down-regulated the expression of Grp78, the negative master regulator of UPRER, suggesting activation of the UPRER, leading to improved proteostasis and restoration of ER function. In addition, I identified a novel transcriptional complex of PGC-1, the Par Domain Protein 1ε (*PDP1ε*), and demonstrated that they physically interact in fly brain using FRET (Fluorescence Resonance Energy Transfer). Activation of *Pdp1ε* ameliorated the shortened lifespan of Aβ-expressing flies, likely through activation of the UPRER. Consistent with this, treatment of Aβ flies with the antibiotic rifampicin, induced the UPRER by decreasing Grp78 levels, and rescued the toxic phenotypes associated with Aβ *in vivo*. Taken together, our results highlight the potential therapeutic benefits of up-regulating the UPRER in AD, either via increasing intraneuronal PGC-1α or PDP1ε or via the use of pharmacological activators such as rifampicin.

3.3 Results:

3.3.1 *dPGC-1* over-expression ameliorated Aβ42 toxicity

To investigate the role of *dPGC-1* in Aβ-mediated neurotoxicity I assessed the effect of over-expression of *dPGC-1* on a number of Aβ-induced neurotoxic phenotypes.
Firstly I confirmed the over-expression of *dPGC-1* in the heads of Aβ-expressing flies using qRT-PCR (Figure 3.1). In order to confirm that *dPGC-1* was not mediating its effect through a change in Aβ load, I demonstrated that *dPGC-1* over-expression did not affect Aβ protein or mRNA levels in fly heads (Figure 3.2 and 3.3).

**Figure 3.1 mRNA expression levels of *dPGC-1* in Aβ-expressing flies**

qRT-PCR analysis revealed that 30-day-old flies co-expressing Aβ + *dPGC-1* in the neural tissue using the inducible *elav-GS* driver (Aβ; *dPGC-1* RU+) displayed *dPGC-1* mRNA levels (relative to a control endogenous gene, *RP49*) are significantly higher than in non-expressing Aβ + *dPGC-1* flies (Aβ; *dPGC-1* RU-) and induced and non-induced Aβ-expressing flies (Aβ; RU+ and Aβ RU-). The bars represent the mean ± SEM (n=4, ***p<0.001 by two-way ANOVA Newman-Keuls test).

**Figure 3.2 Aβ protein levels were not significantly reduced by *dPGC-1* over-expression**

*dPGC-1* over-expression had no significant effect on Aβ protein levels as measured by an ELISA assay on the heads of 30-day-old flies expressing Aβ (Aβ, red bar, RU+) compared with Aβ + *dPGC-1* co-expressing flies (Aβ; *dPGC-1*, green bar, RU+). Bars represent the means ±SEM (n=3, **p<0.01; ***p<0.001 by two-way ANOVA Newman-Keuls test).
Over-expression of dPGC-1 did not alter Aβ mRNA levels (relative to an internal control gene RP49) in the heads of 30-day-old flies compared to controls as measured by qRT-PCR. Bars represent the means ±SEM (n=3, ***p<0.001 by two-way ANOVA Newman-Keuls test).

Over-expression of dPGC-1 in the neural tissue of adult flies using an inducible elavGS driver significantly extended the lifespan of both female and male Aβ-expressing flies (Figure 3.4 A and B) (median lifespan was extended by 28% in females, p = 1.22354 x 10^{-17} and 8% in males, p<0.001). dPGC-1 over-expression also protected against Aβ-induced climbing defects, a behavioral measurement for neuronal health (Figure 3.5).

Circadian rhythms, which have been found disrupted in AD patients and Drosophila Aβ-expressing models (Harper et al., 2005, Blake et al., 2015, Long et al., 2014), are directly controlled by neuronal activity. Circadian rhythm is a biological process that displays an endogenous, entrainable oscillation that completes one cycle (tau) in 24 hours and is driven by the circadian clock located in the suprachiasmatic nucleus in humans. I demonstrated, in keeping with previous work (Long et al., 2014, Blake et al., 2015), that Aβ-expressing flies show an increase in the period tau compared to non-induced controls, suggesting an impaired circadian clock. Over-expression of dPGC-1, however, restored the period to control levels (Figure 3.6), suggesting an improved circadian clock. Taken together our results suggest that increasing dPGC-1 in neurons ameliorates Aβ-induced toxicity.
Figure 3.4 Over-expression of dPGC-1 in neurons extended the lifespan of Aβ-expressing flies.

Survival curves of female (A) and male (B) flies expressing Aβ or Aβ + dPGC-1 in adult neurons (RU+) and non-induced controls (RU-) (p < 2 x 10^-17 vs. Aβ RU+ and Aβ; dPGC-1 RU+ female flies by log-rank test; p < 0.001 vs. Aβ RU+ and Aβ; dPGC-1 RU+ male flies by log-rank test), n=150 flies per conditions.
Figure 3.5 Over-expression of dPGC-1 in Aβ-expressing flies partially ameliorated the age-dependent climbing defects.

Co-expression of dPGC-1 in Aβ-expressing female flies results in greater climbing ability than in non-induced controls. (P < 2 x 10^{-16} Aβ RU+ vs. Aβ; dPGC-1 RU+ by ordinal logistic regression).
Figure 3.6 Over-expression of dPGC-1 in Aβ-expressing flies rescued the defects in circadian rhythm

(Above) Actograms for the locomotor activity rhythms of 21-day-old male flies expressing Aβ or Aβ + dPGC-1 in adult neurons (RU+) and un-induced controls (RU-). This data was recorded over 5 days under 12:12 LD conditions followed by 5 days in DD conditions in Trikinetics Drosophila Activity monitors. (Below) Mean free-running periods under DD conditions of the same flies were calculated using autocorrelation, plotted as means ±SEM (n=32, *p < 0.05, ***p < 0.001 by Two-way ANOVA Newman-Keuls test). This experiment was performed in collaboration with Dr Anna Tillman.

To further explore the mechanisms by which dPGC-1 improves the health-span of Aβ-expressing flies, I measured the ATP concentrations in whole flies. I found reduced ATP levels in Aβ-expressing flies, with restoration towards control levels when dPGC-1 was co-expressed (Figure 3.7), suggesting an improvement in steady state of homeostasis.

Figure 3.7 Over-expression of dPGC-1 in Aβ-expressing flies ameliorated the reduction in ATP
PGC-1α in neurodegeneration and ageing

ATP levels were reduced in 15-day-old whole flies expressing Aβ in neurons using the elavGS driver (elavGS>UAS-Aβ, red bar, RU+) compared with control non-induced flies (elavGS>UAS-Aβ, black bar RU-). ATP levels measured in flies co-expressing Aβ and dPGC-1 (elavGS>UAS-Aβ/UAS-dPGC-1, green bar, RU+) in adult neurons demonstrated that dPGC-1 gain-of-function partially ameliorated the decreased ATP levels associated with Aβ-expression. The bars are plotted as the means ±SEM (n=7), **p<0.01 by two-way ANOVA Newman-Keuls test. The genotypes used were: elavGS>UAS-Aβ; elavGS>UAS-Aβ/UAS-dPGC-1.

3.3.2 dPGC-1 extended the lifespan of Aβ flies beyond the maximum achieved by DR

DR, an intervention involving the chronic reduction of food intake without malnourishment, is the most robust environmental method known to extend lifespan in a wide range of evolutionarily diverse organisms from yeast to mammals (Fontana et al., 2010, Katewa and Kapahi, 2010, Colman et al., 2009). In addition to its pro-longevity effects, DR has also been shown to improve healthy lifespan in mice, monkeys and humans (Fontana et al., 2010, Ikeno et al., 2006, Maeda et al., 1985, Colman et al., 2014, Mattison et al., 2012). Long-term DR results in an improvement in several markers of health, and reduces the risk of developing age-related disorders such as neurodegeneration (Heilbronn et al., 2006, Cava and Fontana, 2013, Masoro, 2003). As a result of the robust effects of DR, and its strong conservation across different species, unravelling the precise mechanisms linking this intervention to lifespan extension will provide useful insights into the mechanisms of ageing and age-related neurodegeneration.

I showed that over-expression of dPGC-1 in fly neurons extended lifespan in Aβ-expressing flies (Figure 3.4). Other studies also suggested dPGC-1 is a modulator for longevity in Drosophila (Rera et al., 2011), however the mechanism has not been fully elucidated. To investigate whether lifespan extension mediated by dPGC-1 in Aβ-expressing flies involves a DR-like mechanism, I carried out a lifespan analysis using a DR-tent (varying concentrations of yeast in the food). If over-expression of dPGC-1 in Aβ expressing neurons extends lifespan like a true DR mimetic, the lifespan under the dietary conditions where lifespan has already been maximized will not be further extended or may even be shortened. If on the other hand dPGC-1
PGC-1α in neurodegeneration and ageing

extends lifespan through overlapping mechanisms with DR, it might still further extend lifespan in the optimised DR condition, however, the maximum benefit on lifespan may occur with full feeding. Interestingly I found that over-expression of dPGC-1 in neurons extends both median and maximum lifespan in all yeast conditions compared to Aβ-expressing flies (Figure 3.8), suggesting that dPGC-1 extends lifespan via an additive effect to DR. Interestingly, dPGC-1 over-expression achieved the maximum lifespan extension in full-feeding, indicating an overlapping mechanism with DR. Taken together, dPGC-1 over-expression in neurons ameliorates the shortened lifespan of Aβ flies through mechanisms partially overlapping with those of DR.

Figure 3.8 Over-expression of dPGC-1 increased the lifespan of Aβ flies irrespective of food concentration
Over-expression of dPGC-1 in neurons increased (A) the median and (B) the maximum lifespan in Aβ-expressing female flies across different yeast concentrations in SY food. Plotted are median lifespan values for Aβ-expressing flies (blue line) and Aβ + dPGC-1 co-expressing flies (red line) against yeast concentration (0.1 x, 1.0 x, and 2.0 x yeast) in SY food supplemented with 200 µM RU. Flies co-expressing Aβ + dPGC-1 had significantly increased lifespan at each yeast concentration compared to Aβ-expressing flies (**p<0.001, log-rank test). For example, expression of dPGC-1 increased the median lifespan of Aβ-expressing flies by 17% and maximum lifespan by 24% in 1.0 x SY DR food, and increased median lifespan by 19% and maximum lifespan by 37% under 2 x SY full feeding food. The genotypes used were: elavGS>UAS-Aβ; elavGS>UAS-Aβ/UAS-dPGC-1.

3.3.3 dPGC-1 over-expression improved mitochondrial function in Aβ flies

PGC-1α affects many cellular processes, and therefore may rescue Aβ toxicity through a number of different mechanisms. Aβ toxicity has been linked to mitochondrial dysfunction, impaired energy metabolism and oxidative damage (Garcia-Escudero et al., 2013, Lustbader et al., 2004, Abramov et al., 2004). PGC-1α is the master regulator of mitochondrial biogenesis and oxidative phosphorylation through co-activation of nuclear receptors (Mootha et al., 2004, Tiefenböck et al., 2010, Rera et al., 2011, Puigserver and Spiegelman, 2003). PGC-1 regulates mitochondrial density in mammalian primary neurons (Wareski et al., 2009), the fly fat body (Tiefenböck et al., 2010), and the fly gut (Rera et al., 2011). I therefore asked whether dPGC-1 induction increases mitochondrial biogenesis in fly neurons by measuring the amount of mitochondrial DNA (mtDNA) relative to that of nuclear DNA (nDNA). I demonstrated that Aβ expression lead to a significant reduction in mtDNA (Figure 3.9). This result parallels the observation of mtDNA deletions or mutations in AD patients’ brains (Bender et al., 2008, Coskun et al., 2004). Over-expression of dPGC-1 in neurons did not rescue this phenotype (Figure 3.9).
Changes in mitochondrial DNA copy number do not necessarily reflect changes in the activity of the electron transport chain (ETC) (Tiefenböck et al., 2010). I therefore investigated whether up-regulation of dPGC-1 in neurons can increase ETC activity in Aβ-expressing flies. The ETC consists of a series of enzymes that transfer electrons from donors to acceptors via redox reactions, to create an electrochemical proton gradient, the mitochondrial membrane potential ($\Delta\Psi_M$), which is the driving force for the synthesis of adenosine triphosphate (ATP) by the F1F0-ATP synthase. To assess the mitochondrial membrane potential, I measured TMRM fluorescence in Aβ-expressing fly brains, and found a significant increase in $\Delta\Psi_M$ compared to controls (Figure 3.10). Hyperpolarization of mitochondria has previously been described in many neurodegenerative disorders, as a compensatory response to certain deficiencies in ETC enzymatic activity. For example, this has been observed in PD patient fibroblasts carrying a PINK1 mutation (Abramov et al., 2011) and in neurons carrying a severe mtDNA mutation, leading to defective complex I of the ETC (Abramov et al., 2010). In our study, mitochondria from Aβ-expressing flies display hyperpolarized mitochondrial membranes, likely in response to Aβ toxicity and reduced mtDNA, which encodes the subunits of the ETC components. Surprisingly,
dPGC-1 over-expression increased brain tissue TMRM fluorescent intensity even more than in flies expressing Aβ alone (Figure 3.10), suggesting that dPGC-1 acts to rescue Aβ toxicity by increasing the hyperpolarising compensatory response.

![Image of TMRM intensity comparison](image)

**Figure 3.10** Both Aβ and dPGC-1 over-expression increased the mitochondrial membrane potential in the fly brain

Aβ-expressing fly brains displayed increased mitochondrial membrane potential at 30-day of age. dPGC-1 over-expression in Aβ co-expressing flies lead to further hyperpolarization of the mitochondrial membrane. The ΔΨm is represented as arbitrary units of TMRM intensities. Data are shown as the mean ± SEM (n=11 for Aβ RU-, n=10 for Aβ RU+ and n=11 for Aβ; dPCG-I RU+; **p<0.01, ***p<0.001 by ANOVA Newman-Keuls test). In collaboration with Dr. Plamena R. Angelova.

dPGC-1 is required for the proper expression of many mitochondrial ETC genes, including *cytochrome c proximal* (*cyt-c-p*), which encodes one of the enzymes in
cytochrome c that participates in the creation of the mitochondrial membrane potential (Tiehenböck et al., 2010). Aβ has been shown to directly inhibit cytochrome c activity \textit{in vitro} (Crouch et al., 2005, Casley et al., 2002), suggesting that elevating \textit{dPGC-1} in neurons might rescue toxicity by increasing expression of \textit{cyt-c-p}. Therefore, I assessed the mRNA transcript levels of \textit{cyt-c-p} in Aβ-expressing fly heads by qRT-PCR, and found that as predicted, mRNA levels of \textit{cyt-c-p} were reduced in response to Aβ-expression, while co-expression with \textit{dPGC-1} significantly rescued this effect (Figure 3.1). These findings suggest that the induction of \textit{dPGC-1} in fly neurons might lead to the elevation of ETC enzymes in association with mitochondrial membrane hyperpolarization.

\textbf{Figure 3.11} Aβ and \textit{dPGC-1} had opposing effects on \textit{cyt-c-p} transcription.

qRT-PCR demonstrated that \textit{cyt-c-p} mRNA levels (relative to \textit{RP49}) in the heads of 30-day-old flies expressing Aβ (RU+) were lower compared to controls (RU-). Co-expression of \textit{dPGC-1} in adult neurons had the opposite effect, increasing \textit{cyt-c-p} mRNA levels. Bars represent means ± SEM (n=3, *p<0.05 by ANOVA Newman-Keuls test). The genotypes used were: \textit{elavGS>UAS-Aβ; elavGS>UAS-Aβ/UAS-dPGC-1}.

To determine whether these changes in \textit{cyt-c-p} had functional consequences, I measured ATP concentrations in fly heads and observed a significant increase in ATP levels in \textit{dPGC-1} flies (Figure 3.12). Although I observed a trend towards a reduction in ATP in response to Aβ, this did not reach statistical significance. Therefore, activation of \textit{dPGC-1} in neurons elevated ATP production by increasing the
membrane potential, through up-regulation of the genes responsible for ETC activity in Aβ-expressing flies.

Taken together my results demonstrated that Aβ reduces mitochondrial DNA copy number and cytochrome c activity, in association with a compensatory increase in the mitochondrial membrane potential. In addition, dPGC-1 may partially ameliorate the Aβ–phenotypes through the compensatory increase in mitochondrial membrane potential, an increase in ATP production, as well as the up-regulation of ETC enzymes.

![Figure 3.12 dPGC-1 over-expression increased ATP levels in Aβ-expressing fly heads](image)

Figure 3.12 dPGC-1 over-expression increased ATP levels in Aβ-expressing fly heads
dPGC-1 over-expression in Aβ-expressing flies leads to an increase in ATP levels in adult neurons at 30 days of age (genotypes: Aβ RU-; Aβ RU+; and Aβ; dPGC-1 RU+). Bars represent the means ± SEM (n=5, *p<0.05 by ANOVA Newman-Keuls test).

### 3.3.4 dPGC-1 over-expression regulated the mitochondrial stress response to Aβ toxicity and protected Aβ flies against oxidative stress

Mitochondrial DNA depletion and increases in mitochondrial membrane potential can lead to excessive production of ROS and deleterious mitochondrial stress. This in turn is detrimental to the maintenance of the mitochondrial proteome and can lead to apoptosis (Haynes and Ron, 2010, Kinghorn et al., 2015). UPR\textsuperscript{mt} is a stress-response pathway that adapts and activates the folding capacity of the mitochondrial matrix in response to mitochondrial dysfunction. It also leads to up-regulation of nuclear-encoded mitochondrial-localized chaperones to promote both protein folding and
degradation, to either restore mitochondrial proteostasis or enter apoptosis (Neupert and Herrmann, 2007, Haynes and Ron, 2010).

I next asked whether the amelioration of the neurotoxic phenotypes associated with Aβ by dPGC-1 over-expression is accompanied by improved mitochondrial proteostasis. Immunoblot analyses against mitochondrial chaperones HSP60 and Grp75 showed a significant reduction in response to Aβ (Figure 3.13 and 3.14), suggesting an impaired UPR\textsuperscript{mt} and proteostastic failure. Interestingly, flies co-expressing both dPGC-1 and Aβ in neurons returned the levels of HSP60 and Grp75 to control levels seen in their un-induced counterparts. Since there was significantly different basal levels of mitochondrial chaperones in the corresponding transgenic control lines, we confirmed our findings by statistical analysis using a two-way ANOVA. This demonstrated a significant interaction between the induction of RU (indicative of gene expression) and the genotypes, suggesting that over-expression of neuronal dPGC-1 reverses the reduction in mitochondrial chaperones seen in Aβ-expressing flies (Figure 3.13 and 3.14). These results suggest that over-expression of dPGC-1 leads to an increase in UPR\textsuperscript{mt} activity, ameliorating Aβ toxicity.

![Figure 3.13](image)

Figure 3.13 Aβ-expressing flies displayed reduced levels of the HSP60 protein

Western blot analysis on 22-day-old Aβ-expressing fly heads (Aβ RU+) showed reduced HSP60 protein levels compared to controls (Aβ RU-). Co-expression of Aβ + dPGC-1 in adult neurons (Aβ;
dPGC-1 RU+) did not show significant difference in HSP60 protein levels compared to controls (Aβ; dPGC-1 RU). The HSP60 levels are shown normalized to β-actin. Bars represent means ± SEM (n=4). The image is a representative gel from one biological repeat (***p<0.01, ***p<0.001 by ANOVA Newman-Keuls test. two-way ANOVA analysis shows **p<0.01 when comparing Aβ response to RU relative to Aβ; dPGC-1 response to RU). The genotypes used were: elavGS>UAS-Aβ; elavGS>UAS-Aβ/UAS-dPGC-1.

Figure 3.14 Aβ-expressing flies displayed reduced levels of the Grp75 protein

Aβ-expressing fly heads display reduced levels of the Grp75 protein as assessed by Western blot analysis on the heads of 22-day-old flies. The Grp75 protein levels were normalized to β-actin. Bars represent means ± SEM (n=3-4). The image is a representative gel from one biological repeat (***p<0.01 by ANOVA Newman-Keuls test. two-way analysis shows *p<0.05 when comparing Aβ response to RU relative to Aβ; dPGC-1 response to RU).

Aβ-induced mitochondrial stress is similar to that caused by exposure to the potent oxidizer paraquat (Cocheme and Murphy, 2008). I therefore determined the effect of dPGC-1 over-expression on the sensitivity of Aβ-expressing flies to paraquat. A single injection of the redox-cycler paraquat, which produces superoxide in part through the mitochondria (Cocheme and Murphy, 2008), caused a significant reduction in the survival of Aβ-expressing flies compared to controls (Figure 3.15). Interestingly, co-expression of Aβ with dPGC-1 restored survival to control levels (Figure 3.15), suggesting that activation of dPGC-1 in neurons protects Aβ-
expressing flies from oxidative stress, likely through an improved mitochondrial stress response.

![Cumulative probability of survival](image)

**Figure 3.15 Over-expression of dPGC-1 rescued the survival of Aβ-expressing female flies injected with paraquat**

The survival curves of flies expressing Aβ or Aβ + dPGC-1 in adult neurons (RU+) and un-induced controls (RU-) demonstrated a rescuing effect of dPGC-1 on the survival of Aβ-expressing flies when injected with paraquat (p<0.0001 when comparing Aβ RU+ and Aβ; dPGC-1 RU+ by log-rank test). This work was performed in collaboration with Dr. Anna Tillmann.

Activation of dPGC-1 in neurons further increases the mitochondrial membrane potential in Aβ flies, which is assumed to increase the ETC leakage, the ROS production and the free radical damage (Green et al., 2011). However, the free radial damage and lifespan can be uncoupled (Zhang et al., 2009, Van Raamsdonk and Hekimi, 2009). Rather than a direct contributor to disease pathology, ROS is more likely a response modulator for mitochondrial stress-elicited survival signal (Sena and Chandel, 2012). Our findings suggest a model in which over-expression of Aβ leads to mtDNA deletion, increased oxidative damage, and impaired mitochondrial stress response and proteostatic failure. However, over-expression of dPGC-1 in neurons switches on a compensatory mechanism involving the further increase in mitochondrial membrane potential to drive ATP synthase. In addition, dPGC-1 protects Aβ flies from mitochondrial stress likely through improvement in the UPRmt.
3.3.5 *dPGC-1* regulated the UPR\textsuperscript{ER} in flies expressing Aβ

PGC-1 activates the UPR\textsuperscript{ER} in mammalian skeletal muscle as an adaptive response to exercise, partially though co-activation of ATF6α (Wu et al., 2011). I therefore assessed whether *dPGC-1* activation in neurons can protect Aβ flies from ER stress. The Grp78 protein is a negative master regulator of the UPR and binds and maintains the downstream cascades in an inactive state (Carrara et al., 2013, Bertolotti et al., 2000, Gorbatyuk et al., 2012, Li et al., 2008), and increases in response to Aβ (Niccoli et al., 2016). Immunoblot analyses confirmed that the levels of the Grp78 protein were increased in Aβ-expressing flies, and that co-expression of *dPGC-1* restored Grp78 expression back to control levels (Figure 3.16). In keeping with this we recently showed that genetic down-regulation of Grp78 protects flies from Aβ toxicity (Niccoli et al., 2016), suggesting that *dPGC-1* could ameliorate Aβ toxicity by modulating the UPR\textsuperscript{ER}.

![Western blot analysis of Grp78 levels](image)

**Figure 3.16** The levels of the Grp78 protein were elevated in Aβ-expressing fly heads but returned towards control levels with co-expression of *dPGC-1*

Western blot analysis of the protein levels of the ER chaperone Grp78 in the heads of 22-day-old flies expressing Aβ or Aβ + *dPGC-1* in neurons (RU+) and un-induced controls (RU-). Protein levels were normalized to β-actin and the bars represent the means ± SEM (n=4, *p<0.05, **p<0.01 by ANOVA Newman-Keuls test). The image is a representative gel of two biological repeats.
Low levels of Grp78 would be predicted to be associated with increased activation of the UPR\textsuperscript{ER}. To confirm this, I analysed the levels of \textit{Xbp1} splicing, a marker of IRE1 activation. Over-expression of A\textbeta in the fly brain resulted in a trend towards an increase in \textit{Xbp1} splicing, but this did not reach significance. Other studies have however shown an increase in \textit{Xbp1} splicing in response to A\textbeta (Casas-Tinto et al., 2011, Niccoli et al., 2016). As predicted, co-expression of A\textbeta with \textit{dPGC-1} increased the levels of spliced \textit{Xbp1} three-fold, suggesting over-expression of \textit{dPGC-1} in neurons increases IRE1 activity (Figure 3.17).

![Figure 3.17](image)

**Figure 3.17** The mRNA levels of \textit{Xbp1} splicing were increased in the heads of A\textbeta-expressing flies co-expressing \textit{dPGC-1}

Spliced \textit{Xbp1} mRNA levels in heads of 15-day-old flies expressing A\textbeta or A\textbeta + \textit{dPGC-1} in neurons (RU+) and un-induced controls (RU-), as measured by qRT-PCR (relative to \textit{RP49}). The bars represent the means ± SEM (n=3, ** P<0.01, ***p<0.001 by ANOVA Newman-Keuls test).

Similarly, \textit{Grp78} mRNA levels, a marker of ATF6\textalpha activation, increased in response to A\textbeta over-expression (Figure 3.18), and co-over-expression of \textit{dPGC-1} resulted in an even greater increase than that seen with A\textbeta alone (Figure 3.18). Grp78 protein levels are tightly controlled at the levels of translation (Gulow et al., 2002), and an increase in the mRNA, therefore, does not necessarily indicate higher protein levels, suggesting that \textit{dPGC-1} reduced either Grp78 translation or stability. Taken together, these findings suggest that A\textbeta activates the UPR\textsuperscript{ER}, and that \textit{dPGC-1} further activates it, by reducing the protein levels of Grp78 and hence activating the IRE1 and ATF6\textalpha pathways, resulting in the amelioration of A\textbeta toxicity.
Figure 3.18 The mRNA levels of Grp78 were increased in Aβ-expressing fly heads and further increased by the co-expression of dPGC-1

Grp78 mRNA levels in the heads of 15-day-old flies expressing Aβ or Aβ + dPGC-1 in neurons (RU+) and un-induced controls (RU-), measured by qRT-PCR (relative to RP49). The bars represent the means ± SEM (n=3, *p<0.05, **p<0.01, ***p<0.001 by ANOVA Newman-Keuls test). The genotypes used were: elavGS>UAS-Aβ; elavGS>UAS-Aβ /UAS-dPGC-1.

As described above, I demonstrated that over-expression of dPGC-1 in neurons modulates the levels of chaperones in both the UPR<sub>ER</sub> and the UPR<sub>mt</sub> in response to Aβ toxicity. I therefore addressed the question of whether dPGC-1 regulates the basal levels of these chaperones in the absence of Aβ. Interestingly, activation of dPGC-1 in neurons did not change the basal levels of Grp75 and Grp78, and even down-regulated the level of HSP60 compared to non-RU treated young control flies (Figure 3.19). This therefore demonstrated that the effect of dPGC-1 on the chaperones involved in the UPR<sub>ER</sub> and the UPR<sub>mt</sub> is specific to Aβ-mediated pathogenesis. It also highlights the potential therapeutic benefits of modulating these chaperones in the treatment of AD.
Figure 3.19 Over-expression of *dPGC-1* in neurons did not change the basal levels of chaperones in young flies

Western blot analysis of the protein levels of the mitochondrial chaperones HSP60, Grp75, and the ER chaperone Grp78 in the heads of 15-day-old flies over-expressing *dPGC-1* (RU+) and un-induced controls (RU-). Protein levels were normalized to β-actin. The bars are plotted below as the means ± SEM (n=4, *p*<0.05 by t-test). The image is a representative gel from one biological repeat.

### 3.3.6 Rifampicin ameliorated Aβ neurotoxicity via activation of the UPR<sup>ER</sup>

Since *dPGC-1* ameliorates Aβ toxicity through activation of UPR<sup>ER</sup>, pharmacological targeting of the UPR<sup>ER</sup> may represent a potential therapeutic avenue for the treatment of AD. Rifampicin, a well-known antibiotic used to treat tuberculosis and leprosy, is known to cross the blood brain barrier following oral administration (Umeda et al., 2016). Interestingly it has been shown that leprosy patients treated with rifampicin show a lower incidence of dementia and a reduction in senile plaques compared to the general population (Mcgeer et al., 1992, Chui et al., 1994, Namba et al., 1992). Rifampicin has also been shown to have neuroprotective effects in a number of animal models of neurodegenerative diseases (Tomiyama et al., 1994, Umeda et al., 2016, Oida et al., 2006, Kilic et al., 2004), but the mechanisms involved have not...
been fully elucidated. Rifampicin reduced Aβ oligomerization and improved memory in an APP mice model through improved autophagy-lysosomal function (Umeda et al., 2016). It was also shown to activate the UPR and offer protection against rotenone-induced cytotoxicity in vitro (Jing et al., 2014).

I therefore treated our Arctic Aβ over-expressing flies with rifampicin to determine whether it can protect against Aβ neurotoxicity in vivo. Indeed, feeding Aβ flies with 200 μM of rifampicin statistically extended lifespan (Figure 3.20, p=0.002 by log-rank test), and improved climbing ability (Figure 3.22) without altering Aβ levels (Figure 3.21). 200 μM of rifampicin did not improve either the lifespan (Figure 3.20, p=0.06 by log-rank test) or the climbing ability in non-induced control flies, suggesting that the effects of rifampicin are specific to AD pathology. Although 500 μM rifampicin increased the lifespan of both Aβ-expressing flies and non-induced controls (Figure 3.20, p=0.0009 and p=0.0006 respectively), their climbing ability was not affected (Figure 3.22), suggesting that the effects on climbing ability and lifespan can be uncoupled. Cox proportional hazard analysis confirmed that there is no significant interaction between the response to RU and the response to rifampicin (500 μM) treatment (Table 1, p=0.89), suggesting that high dose (500 μM) rifampicin extends lifespan regardless of whether Aβ is present or not. Rifampicin may therefore have broad-spectrum effects on lifespan when administrated at high concentrations, but a specific protective effect against Aβ-induced neurotoxicity when administrated at lower concentrations. Rifampicin treatment, similar to dPGC-1 over-expression, also reversed the increase in Grp78 levels associated with Aβ-expression (Figure 3.23), suggesting activation of the UPRER. Rifampicin is therefore a promising ready-to-use medicine for the treatment of AD pathology, through its activation of the UPRER.
Figure 3.20 Rifampicin increased the lifespan of Aβ-expressing female flies

Survival curves of flies expressing Aβ in adult neurons (RU+) and un-induced controls (RU-) using inducible elavGS driver with or without rifampicin treatment at 200 μM (P=0.002 when comparing Aβ RU+ Rifampicin 0 μM and Aβ RU+ Rifampicin 200 μM by log-rank test; p=0.06 when comparing Aβ RU- Rifampicin 0 μM and Aβ RU- Rifampicin 200 μM by log-rank test) and 500 μM (P=0.0009 when comparing Aβ RU+ 0 μM and Aβ RU+ Rifampicin 500 μM by log-rank test; p=0.0006 when comparing Aβ RU- Rifampicin 0 μM and Aβ RU- Rifampicin 500 μM by log-rank test. N= 150 flies per conditions. This work was performed in collaboration with Dr. Jorge Iván Castillo-Quan.

```
coph(formula = Surv(time = dataC$DAY, event = dataC$CENSOR, type = ct("right"), origin = 0) ~ dataC$RU + dataC$RIFAMPICIN)
n = 607, number of events= 601
```

|            | coef | exp(coef) | se(coef) | z     | Pr(>|z|) |
|------------|------|-----------|----------|-------|----------|
| dataC$RU  | 6.54502 | 695.76983 | 0.61510 | 10.641 | <2e-16 *** |
| dataC$RIFAMPICIN | -0.33977 | 0.71193 | 0.11702 | -2.904 | 0.00369 ** |
| dataC$RU:dataC$RIFAMPICIN | -0.02155 | 0.97868 | 0.16472 | -0.131 | 0.89591 |

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table 1 CPH analysis of the survival of Aβ-expressing flies on treatment with 500 μM rifampicin

CPH analysis showed that there was no interaction between the response to RU and the response to rifampicin 500μM (p=0.89). This suggests that treatment with 500μM rifampicin extends lifespan regardless of whether Aβ is present or not.
Figure 3.21 Rifampicin treatment had no effect on Aβ protein levels.

Aβ protein levels, measured by ELISA, in the heads of 15- or 30-day old Aβ-expressing flies (+RU) and un-induced controls (-RU) treated with or without 200uM rifampicin. Bars represent the means ±SEM (n=3, **p<0.01 by ANOVA Newman-Keuls test).

Figure 3.22 Rifampicin rescued the locomotor ability of Aβ-expressing flies

The climbing assay Performance Index (PI) of Aβ-expressing flies in the absence and presence of rifampicin. P<0.001 when comparing the Aβ response to RU relative to the Aβ response to RU + rifampicin 200µM by ordinal logistic regression. There is no significant difference between Aβ RU+ rifampicin 0 µM and Aβ RU+ rifampicin 500 µM.
PGC-1α in neurodegeneration and ageing

Figure 3.23 Rifampicin reduced the increased levels of the Grp78 protein in Aβ-expressing fly heads

Western blot of Grp78 in the heads of 15-day-old flies expressing Aβ in neurons (RU+) and un-induced controls (RU-) with or without 200μM rifampicin treatment. The bars are plotted below as the means ± SEM (n=3, **p<0.01, ***p<0.001 ANOVA Newman-Keuls test). The image is a representative gel of one biological repeat.

3.3.7 Over-expression of dGABPα or dYY1 did not rescue Aβ toxicity

PGC-1α is a transcriptional coactivator involved in mitochondrial biogenesis (Cunningham et al., 2007, Mootha et al., 2004) and the regulation of the circadian clock (Liu et al., 2007). To further explore the downstream effectors of dPGC-1 involved in the amelioration of Aβ neurotoxicity, I performed a small-scale screen of transcription factors previously reported to be required for the co-activation of dPGC-1. I tested the transcription factor PDP1ε, which is involved in the regulation of the circadian clock (Cyran et al., 2003), and two other transcription factors, YY1 and GABPα, known to play a role in mitochondrial biogenesis.

Mammalian PGC-1α has been shown to regulate mitochondrial oxidative phosphorylation by co-activating the transcription factor YY1 (Cunningham et al., 2007). I therefore investigated whether dPGC-1 over-expression ameliorates Aβ toxicity through YY1. Surprisingly, up-regulation of neuronal dYY1 (pho, CG17743) in neurons shortened the lifespan of the Aβ-expressing flies (Figure 3.24).
Figure 3.24 The co-expression of \textit{dYY1} in the neurons of A\textbeta -expressing flies lead to a decrease in lifespan

The survival curves of female flies expressing A\textbeta or A\textbeta + \textit{dYY1} in adult neurons (RU+) and un-induced controls (RU-). The genotypes used were: \textit{elavGS}\textgreater \text{UAS- A\textbeta}; \textit{elavGS}\textgreater \text{UAS- A\textbeta/UAS-dYY1}.

Mammalian PGC-1\textalpha has been also shown to regulate mitochondrial biogenesis through the transcription factor GABP\textalpha (also known as Nuclear respiratory factor 2a, NRF2a) (Mootha et al., 2004, Virbasius et al., 1993). Animal models lacking GABP\textalpha exhibit mitochondrial dysfunction (Yang et al., 2014, Baltzer et al., 2009). I therefore considered whether \textit{dPGC-1} over-expression reduced A\textbeta-mediated toxicity by acting through \textit{dGABP\textalpha}. Up-regulation of \textit{dGABP\textalpha} (\textit{delg}, CG6338) in neurons was not sufficient to the extend lifespan of A\textbeta-expressing flies (Figure 3.25), and therefore it does not appear to be an important downstream mediator of PGC-1\textalpha in extending lifespan.
Figure 3.25 Co-expression of dGABPa in neurons did not rescue the shortened lifespan of Aβ-expressing flies

The survival curves of female flies expressing Aβ or Aβ + dGABPa in adult neurons (RU+) and un-induced controls (RU-). The genotypes used were: elavGS>UAS- Aβ; elavGS>UAS- Aβ/UAS-dGABPa.

3.3.8 PDP1c ameliorated Aβ neurotoxicity via activation of the UPRER

We recently demonstrated that Aβ-expressing flies display an arrhythmic sleep pattern (Niccoli et al., 2016). I confirmed the finding that over-expression of Aβ can disrupt the circadian clock via increasing the period tau. In agreement with previous human and animal studies, our results suggest that Aβ-induced neurotoxicity might directly contribute to circadian rhythm disruption (Harper et al., 2005, Blake et al., 2015, Long et al., 2014). Moreover, metabolic defects and disturbance in redox state can lead to an alteration of circadian rhythm. This in turn has been found not only to occur as a consequence of AD pathology, but may also contribute to APP processing and Aβ accumulation (Kang et al., 2009, Xie et al., 2013, Musiek et al., 2013). Therefore, Aβ-induced defects in circadian rhythm might also lead to a further increase in disease progression.

Circadian clocks coordinate physiological, neurological, and behavioral functions into approximately 24-hour rhythms, and the molecular mechanisms underlying circadian clock oscillation are conserved from Drosophila to humans. Drosophila have a well
studied circadian clock, consisting of two interlocked transcriptional feedback loops (Figure 3.26). In the first feedback loop, dCLOCK/CYCLE activates *period* expression, and the PERIOD protein then inhibits dCLOCK/CYCLE activity. In the second feedback loop, dCLOCK/CYCLE activates *Pdp1ε* and *vri* expression, and then PDP1ε activates *dClock* while VRI inhibits *dClock* expression (Cyran et al., 2003). Flies lacking *Pdp1ε* have impaired circadian clocks, demonstrating that it is important for the rhythmic expression of dCLOCK. Moreover, flies lacking *Pdp1ε* also exhibit increased sensitivity to oxidative damage (Beaver et al., 2010), suggesting its role in maintaining the redox state and circadian rhythm.

**Figure 3.26 The circadian loop model for the Drosophila clock**

Two interconnected transcription feedback loops lie at the core of the *Drosophila* molecular clock. In one loop, dCLOCK and CYCLE directly activate the transcription of *per* and *tim* by binding their promoters. Inhibition of dCLOCK/CYCLE activity is mediated by TIM transporting PER into the nucleus. dCLOCK/CYCLE also directly activate *vri* and *Pdp1ε* transcription. *dClock* transcription is first repressed by VRI, and then activated by PDP1ε. Repression and activation of *dClock* are separated by the different phases of VRI and PDP1ε proteins.

Mammalian PGC-1α has been found to co-activate the transcription factor RORα to activate *Bmal/CLOCK* gene expression. It is therefore involved in the second feedback loop of the circadian clock (Liu et al., 2007). Whether its *Drosophila* counterpart also controls circadian clock awaits investigation. Work in our research group has however shown that *dPGC-1* is rhythmically expressed (unpublished data). Therefore, I hypothesised that *dPGC-1* is a clock gene, regulating the circadian clock in *Drosophila*. As described above I have shown that over-expression of *dPGC-1* in neurons ameliorates Aβ-induced circadian clock disruption, namely the increased period *tau*, suggesting that *dPGC-1* might directly participate in the feedback loop.
Using FRET (Fluorescence Resonance Energy Transfer), a sensitive method to study protein interactions in vivo, I sought to determine whether dPGC-1 and PDP1ε physically interact in the fly brain. Briefly, FRET involves the non-radioactive transfer of energy from an excited state donor fluorophore to a nearby acceptor, which can be detected when two labelled molecules are separated by a distance of 2-10 nm. FRET acceptor photo bleaching is a method that measures the donor “de-quenching” in the presence of an acceptor by comparing donor fluorescence intensity in the same sample/region before and after destroying the acceptor by photobleaching. If FRET was initially present, a resultant increase in donor fluorescence will occur on photobleaching of the acceptor. FRET efficiency is considered positive when the fluorescence intensity of the donor after acceptor photobleaching (D_post) > fluorescence intensity of the donor before acceptor photobleaching (D_pre). I generated transgenic flies carrying both GFP-tagged PDP1ε and HA-tagged dPGC-1 as a donor-acceptor pair for FRET analysis (Figure 3.27). HA-tagged dPGC-1 protein were immune-stained and labelled with RFP as acceptor fluorophore. After acceptor photobleaching in region of interest (ROI), I observed an increase in D_post compared to D_pre (Figure 3.28), suggesting the FRET efficiency was positive. It should be noted that this was a single experiment and more repeats are required to confirm this interaction. Therefore, I can conclude that dPGC-1 and PDP1ε may physically interact in the fly brain.

![Figure 3.27 A model for the FRET donor-acceptor pair](image)

Double mutant flies carried both GFP-tagged PDP1ε and HA-tagged dPGC-1. GFP labeled protein was excited at 488nm as the donor, and HA-labeled protein was stained and labeled by RFP and excited at
568nm as the acceptor. When two molecules are very close to each other (<10nm), then energy will transfer from the excited donor to the acceptor. The emission from the acceptor will include the part transferred from the donor. Acceptor photobleaching is used to stop the energy transfer from the excited donor and involves bleaching the acceptor. The emission of the donor will be increased after photobleaching as energy will no longer be transferred.

**Figure 3.28 Acceptor photobleaching within the regions of interest (ROI)**

FRET photobleaching was conducted using confocal microscopy. The mean fluorescence intensities in the same ROI (see boxes) in the brains before (36.7) and after (10.6) acceptor photobleaching with the 555-nm laser line are shown. The mean fluorescence intensity of the donor after acceptor photobleaching (30.578) is higher than that before acceptor photobleaching (25.481), suggesting a positive FRET efficiency. Fluorescence intensity was measured using ImageJ. This demonstrates that *dPGC-1* and PDP1ɛ may physically interact in the fly brain.
I then explored whether genetic manipulation of \textit{Pdp1\epsilon} may prevent A\beta neurotoxicity. Neuronal over-expression of \textit{Pdp1\epsilon} in adult flies using the inducible \textit{elavGS} driver significantly extended the lifespan of A\beta-expressing flies (Figure 3.29), although the effects were much less than those seen with \textit{dPGC-1} over-expression. However, over-expression of \textit{Pdp1\epsilon} in neurons was unable to rescue the climbing defects (Figure 3.30).

\textbf{Figure 3.29 Over-expression of \textit{Pdp1\epsilon} in neurons extended the lifespan of A\beta flies}

The survival curves of female flies expressing A\beta or A\beta + \textit{Pdp1\epsilon} in adult neurons (RU+) and un-induced controls (RU-). P<0.01 when comparing the A\beta response to RU relative to the A\beta + \textit{Pdp1\epsilon} response to RU by Cox proportional hazard model using JMP and software R. The genotypes used were: \textit{elavGS}\textgreater UAS-A\beta; \textit{elavGS}\textgreater UAS-A\beta/UAS-Pdp1\epsilon. This work was performed in collaboration with Miss. Nikoleta Vavouraki.
Figure 3.30 Co-expression of Pdp1ε did not rescue the locomotor ability of Aβ-expressing flies

The climbing ability shown as the PI of flies of the same genotypes. \( P > 0.05 \) when comparing the Aβ response to RU relative to the Aβ; Pdp1ε response to RU, by ordinal logistics regression. This work was also performed in collaboration with Miss. Nikoleta Vavouraki.

I confirmed that Pdp1ε over-expression was not modulating Aβ toxicity via a direct effect on Aβ levels, by demonstrating that total Aβ protein levels were not significantly different in flies co-expressing Aβ and Pdp1ε compared to those expressing Aβ alone (Figure 3.31). There was however a slight increase in Aβ mRNA levels in response to Pdp1ε over-expression (Figure 3.32), but since this was not correlated with increased protein expression, the significance of this is not clear. Therefore, I demonstrated that over-expression of Pdp1ε reduced the toxicity of Aβ, independently of Aβ protein levels.
Figure 3.31 Co-expression of Pdp1ε in Aβ-expressing flies did not alter Aβ protein levels

Aβ protein levels were measured by ELISA in the heads of 30-day-old flies expressing Aβ or Aβ + Pdp1ε in neurons (RU+) and un-induced controls (RU-). The bars are plotted as the means ±SEM (n=3, ***P<0.001 by ANOVA Newman-Keuls test). This work was performed in collaboration with Miss. Nikoleta Vavouraki.

Figure 3.32 The mRNA levels of Aβ were increased by the co-expression of Pdp1ε in Aβ-expressing flies

Aβ mRNA levels (relative to the internal control gene RP49) in heads of 15-day-old flies were measured by qRT-PCR. The bars are plotted as the means ±SEM (n=4, ***p<0.001, ****p<0.0001 by ANOVA Newman-Keuls test). The genotypes used were: elavGS>UAS-Aβ; elavGS>UAS-Aβ/UAS-Pdp1ε. This work was performed in collaboration with Miss. Nikoleta Vavouraki.

Previous studies demonstrated that Pdp1ε could regulate the expression of genes involved in the detoxification pathway (Beaver et al., 2010). Loss of Pdp1ε leads to reduced levels of cyp6a2 and DHR96, suggesting that over-expression of Pdp1ε might increase the expression of these genes. I therefore measured the transcript levels of
cyp6a2 and DHR96 in flies co-expressing Aβ and Pdp1ɛ, and found that over-expression of Pdp1ɛ increased the mRNA levels of DHR96 compared to the non-induced controls or flies expressing Aβ alone, confirming an increase in the activity of Pdp1ɛ (Figure 3.33). In addition, over-expression of Pdp1ɛ did not change the mRNA levels of cyp6a2. However, cyp6a2 transcript levels were significantly reduced in response to Aβ, suggesting that Aβ may impair the detoxification pathway (Figure 3.34). Our results confirm that Pdp1ɛ over-expression in flies activates the appropriate downstream molecular pathways.

Figure 3.33 Over-expression of Pdp1ɛ in Aβ-expressing flies lead to increased DHR96 levels in fly heads

DHR96 mRNA levels in the heads of 15-day-old flies expressing Aβ or Aβ + Pdp1ɛ in neurons (RU+) and un-induced controls (RU−), measured by qRT-PCR (relative to the internal control gene RP49). The bars represent the means ± SEM (n=4, *p<0.05 by ANOVA Newman-Keuls test). The genotypes used were: elavGS>UAS-Aβ; elavGS>UAS-Aβ/UAS-Pdp1ɛ. This work was performed in collaboration with Miss. Nikoleta Vavouraki.
PGC-1α in neurodegeneration and ageing

Figure 3.34 Over-expression of Aβ, but not Pdp1ε, reduced cyp6a2 mRNA levels in fly heads

cyp6a2 mRNA levels in the heads of 15-days-old flies expressing Aβ or Aβ + Pdp1ε in neurons (RU+) and un-induced controls (RU-), measured by qRT-PCR (relative to the internal control gene RP49). The bars represent the means ± SEM (n=4, *p<0.05, **p<0.01 by ANOVA Newman-Keuls test). The genotypes used were: elavGS>UAS-Aβ; elavGS>UAS-Aβ/UAS- Pdp1ε. This study was performed in collaboration with Miss. Nikoleta Vavouraki.

Since dPGC-1 ameliorates Aβ toxicity through activation of the UPR\textsuperscript{ER}, I assessed whether Pdp1ε activation in neurons can protect Aβ flies against ER stress. I first analysed the levels of Xbp1 splicing, a marker of IRE1 activation. Expression of Aβ significantly increased Xbp1 splicing in agreement with previous studies (Casas-Tinto et al., 2011, Niccoli et al., 2016). Co-expression with Pdp1ε lead to a trend towards an increase in Xbp1 splicing, but this did not reach statistical significance, suggesting that over-expression of Pdp1ε in neurons is not sufficient to increase IRE1 activity (Figure 3.35).

I next assessed Grp78 mRNA levels, a marker of ATF6α activation, and found that in addition to their increase in response to Aβ, there was an even further increase in Pdp1ε-co-expressing flies (Figure 3.36), suggesting activation of the ATF6α branch of the UPR\textsuperscript{ER}. Taken together, over-expression of Pdp1ε activates the UPR\textsuperscript{ER} through activation of the ATF6α branch, in a similar manner to dPGC-1, therefore leading to re-establishment of ER homeostasis in Aβ-expressing flies.
Figure 3.35 mRNA levels of Xbp1 splicing were increased by the expression of Aβ and Pdp1ε in fly heads

Spliced Xbp1 mRNA levels in heads of 15-day-old flies expressing Aβ or Aβ + Pdp1ε in neurons (RU+) and un-induced controls (RU-), measured by qRT-PCR (relative to RP49). Bars are plotted as the means ± SEM (n=3, *p<0.05, **p<0.01, ***p<0.001 by ANOVA Newman-Keuls test). This experiment was performed in collaboration with Miss. Nikoleta Vavouraki.

Figure 3.36 mRNA levels of Grp78 were increased in flies expressing Aβ and Pdp1ε in their heads

Grp78 mRNA levels in the heads of 15-day-old flies expressing Aβ or Aβ + Pdp1ε in neurons (RU+) and un-induced controls (R-U), measured by qRT-PCR (relative to the internal control gene RP49). The bars are plotted below as the means ± SEM (n=3, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by ANOVA Newman-Keuls test). The genotypes used were: elavGS>UAS-Aβ; elavGS>UAS-Aβ/UAS-Pdp1ε. This work was performed in collaboration with Miss. Nikoleta Vavouraki.
3.4 Discussion

AD, triggered by the accumulation of Aβ oligomers or aggregates, causes a neurotoxic cascade including oxidative stress, mitochondrial dysfunction, proteostatic failure, and eventual cell death (Hardy and Higgins, 1992, Musiek and Holtzman, 2015). Ageing-related disturbances in protein homeostasis, neuronal stress response pathways, and circadian rhythm also contribute and exacerbate disease progression (Musiek et al., 2013, Taylor and Dillin, 2011, Hipp et al., 2014). Therefore, genetic or pharmacological interventions targeting these modulators may provide excellent therapeutic avenues.

PGC-1α has been implicated in AD pathology in both human and animal studies (Qin et al., 2009, Helisalmi et al., 2008). However, the effects of elevating PGC-1α in animal models of AD are controversial (Sheng et al., 2012, Katsouri et al., 2011, Dumont et al., 2014). Acting as a transcriptional co-activator, PGC-1α has been broadly implicated in many cellular processes and it is therefore regarded as the master regulator of energy metabolism (Mootha et al., 2004, Scarpulla, 2008, Puigserver and Spiegelman, 2003, Li et al., 2007b). Here I demonstrated that neuronal activation of dPGC-1 can extend lifespan and ameliorate Aβ induced neurotoxicity, including climbing defects and circadian rhythm disruption. This improvement was associated with a compensatory mitochondrial response, namely increased ETC activity and ATP production, and improved mitochondrial proteostasis. Mitochondrial dysfunction plays a central role in disease progression, since it is the energy factory for cells. Damaged mitochondria lead to metabolic defects, including reduced glucose metabolism and ATP production in AD brains (Mosconi et al., 2008, Willette et al., 2015, Winkler et al., 2015, Kapogiannis and Mattson, 2011). In our flies, Aβ over-expression in neurons lead to a trend in ATP reduction. Conversely, dPGC-1 activation in neurons increased the ATP levels in Aβ-expressing flies, suggesting an increase in steady-state homeostasis.

In AD patients, mitochondrial dysfunction has been reported in association with mutations or deletions in mtDNA (Bender et al., 2008, Coskun et al., 2004). I showed that Aβ accumulation in the brain leads to the loss of mtDNA, and a compensatory response involving increased mitochondrial membrane potential. This is consistent
with the hyperpolarization of mitochondrial membranes seen in PD patient fibroblasts carrying PINK1 mutations (Abramov et al., 2011), and in neurons carrying a severe mtDNA mutation (Abramov et al., 2010). I demonstrated that dPGC-1 up-regulation potentiates this compensatory response via up-regulation of the cytochrome c, which leads to a further increase in mitochondrial membrane potential and ATP synthase.

The ETC is the main site of premature electron leakage to oxygen, generating superoxide and reactive oxygen species (ROS). Inefficient electron transport chain activity of mitochondria also produces excessive ROS and mitochondrial-driven apoptosis, which both exacerbate AD pathology (Head et al., 2001, Siegel et al., 2007). In keeping with this I demonstrated that flies over-expressing Aβ in neurons exhibit increased sensitivity to paraquat-induced oxidative stress. Over-expression of dPGC-1 in neurons protected Aβ–expressing flies against paraquat-induced oxidative stress. Our results are consistence with a previous study (St-Pierre et al., 2006), suggesting that PGC-1α suppresses ROS to prevent neurodegeneration.

The mechanisms by which PGC-1α suppresses ROS and protects against oxidative damage have not been fully elucidated. However, it is already known that increased mitochondrial ROS and lifespan can be uncoupled (Zhang et al., 2009). Indeed, in the context of age-related pathology, ROS is likely a response modulator of stress-elicited survival signals (Sena and Chandel, 2012) and therefore has beneficial roles. Several studies have shown that a moderate increase in ROS or mitochondrial stress may be beneficial and extend lifespan in S. cerevisiae and C. elegans via modulation of stress response pathways, including up-regulation of mitochondrial chaperones and chromatin remodelling (Doonan et al., 2008, Mesquita et al., 2010, Van Raamsdonk and Hekimi, 2009, Merkwirth et al., 2016, Tian et al., 2016).

Mitochondrial stress, similar to paraquat-induced oxidative stress (Cocheme and Murphy, 2008), activates the UPR\textsuperscript{mt}. This is a stress signalling pathway designed to ensure mitochondrial homeostasis through up-regulation of mitochondrial chaperones (Merkwirth et al., 2016, Haynes et al., 2007). Recent studies demonstrated that early activation of the UPR\textsuperscript{mt}, prior to mitochondrial dysfunction, can be beneficial for the organism and may extend lifespan in C. elegans (Merkwirth et al., 2016). This
suggests that a moderate increase in the UPR\textsuperscript{mt} is essential for the maintenance of mitochondrial proteostasis. In our model, Aβ over-expression leads to a reduction in mitochondrial chaperones and therefore down-regulation of the UPR\textsuperscript{mt}, leading to an imbalance in mitochondrial homeostasis. Co-expression of \textit{dPGC-1} blocked the down-regulation of mitochondrial chaperones, therefore improving mitochondrial proteostasis and neuronal health. This observation therefore explains why the mitochondrial hyperpolarisation associated with \textit{dPGC-1} activation resulted in overall beneficial effects in Aβ-over-expressing flies. Indeed it has been shown in fly muscle that over-expression of \textit{dPGC-1} increases the levels of the mitochondrial chaperone HSP60 and mitochondrial function (Rera et al., 2011). My work therefore supports the idea that neuronal up-regulation of \textit{dPGC-1} leads to an improved mitochondrial matrix environment, which can reverse the negative effects of Aβ toxicity. A previous study demonstrated that over-expression of \textit{dPGC-1} in the fly gut increased the basal levels of HSP60 (Rera et al., 2011). Conversely, I observed a reduction in the basal levels in HSP60 in young adult brains expressing \textit{dPGC-1} in the absence of Aβ, while the mechanisms are not fully understood. It would be interesting to know whether \textit{dPGC-1} modulates the basal levels of mitochondrial chaperones in fly brains during ageing. My work demonstrated that over-expression of \textit{dPGC-1} in neurons up-regulated mitochondrial chaperones in response to Aβ-induced mitochondrial stress, re-establishing homeostasis and protecting against mitochondrial stress.

Ageing-dependent failure in ER-related proteostasis also contributes to disease progression in AD (Taylor and Dillin, 2011, Hipp et al., 2014). The UPR\textsuperscript{ER} is activated early in AD disease pathogenesis (van der Harg et al., 2014), and both AD patients and animal model studies have shown increased UPR\textsuperscript{ER} components, including Grp78 and Xbp1 (Niccoli et al., 2016, Kondo et al., 2013). Interestingly, I demonstrated that the rescue of Aβ toxicity by \textit{dPGC-1} was associated with a reduction in Grp78 protein levels, leading to a further increase in UPR\textsuperscript{ER} and activation of the IRE1 and ATF6a branches. Indeed, it has been shown that down-regulation of Grp78, the master negative regulator of the UPR\textsuperscript{ER}, leads to activation of the downstream cascades of the UPR\textsuperscript{ER}, to ameliorate Aβ toxicity (Niccoli et al., 2016). Grp78 protein levels are tightly controlled at the levels of translation (Gulow et al., 2002), suggesting that \textit{dPGC-1} reduced either Grp78 translation or stability.
Moreover, over-expression of Xbp1 alone can ameliorate Aβ toxicity (Casas-Tinto et al., 2011), supporting the idea that up-regulation of UPR\textsuperscript{ER} components is beneficial in AD models. Grp78 is becoming an increasingly important therapeutic target for AD (Niccoli et al., 2016) and cancer, where its inhibition increases the cell’s susceptibility to stress (Lee et al., 2014). Although up-regulation of Grp78 showed detrimental effects in an animal model of Parkinson’s disease (Gorbatyuk et al., 2012), its function in other neurodegenerative disorders may depend on the precise experimental conditions. For example, a mild up-regulation of the UPR\textsuperscript{ER} in neurons may increase ER proteostasis in response to the increased burden of mis-folded proteins, whereas, a strong induction of the UPR\textsuperscript{ER} may lead to apoptosis and be detrimental to neurons (Hetz and Mollereau, 2014).

Studies have shown that PGC-1α can modulate Grp78 levels. In skeletal muscle, in response to exercise, PGC-1α can increase the transcription of Grp78 and Xbp1 by co-activating ATF6a (Wu et al., 2011). Already it has been shown that ablation of PGC-1β, the homologue of PGC-1α, increases basal Grp78 levels and modulates the neuronal ER response in the mouse brain (Camacho et al., 2012), suggesting that PGC-1 might directly regulate Grp78 levels. I showed that activation of dPGC-1 in adult neurons does not change basal levels of Grp78 in young flies, but reduces Grp78 levels and activates UPR\textsuperscript{ER} in the presence of Aβ. Since it has been shown that the UPR\textsuperscript{ER} and proteostasis decline with age in C. elegans (Ben-Zvi et al., 2009, Taylor and Dillin, 2013, Taylor and Dillin, 2011), it would therefore be interesting to know whether dPGC-1 affects the basal level of Grp78 levels to maintain proteostasis in old age.

My work suggests a model where Aβ accumulation in the brain induces the UPR\textsuperscript{ER}, and activation of dPGC-1 in neurons blocks the negative feedback loop linked to Grp78 up-regulation, resulting in even further increased UPR\textsuperscript{ER} activity. In turn this allows neurons to re-establish the protein-folding environment and to restore protein homeostasis. This mechanism of action may be responsible for the benefits of PGC-1α observed in models of other neurodegenerative disorders. PGC-1α displayed neuroprotective effects in a mouse model of HD with mechanisms including
attenuation of oxidative stress, activation of the lysosomal autophagy pathway and proteostasis (Tsunemi et al., 2012).

Rifampicin, a well-known antibiotic widely in the treatment of tuberculosis and leprosy, has been proposed to be neuro-protective in PD and AD animal models (Jing et al., 2014, Umeda et al., 2016). Here I showed that treatment with rifampicin increased lifespan and improved locomotor ability in Aβ-expressing flies, in agreement with a previous APP mice model (Umeda et al., 2016). In accordance with previous observations in humans that rifampicin treatment reduced the risk of developing AD (Mcgeer et al., 1992), I also found that rifampicin treatment increased lifespan in wild type Drosophila, suggesting broad spectrum anti-ageing effects. A recent study suggested that rifampicin could reduce Aβ oligomerization and improve the memory defects in an APP mouse model via improved autophagy-lysosomal function (Umeda et al., 2016). Here I confirmed that the amelioration of Aβ-induced neurotoxic phenotypes by rifampicin are not due to a reduction in the level of Aβ peptides, and therefore exclude an effect of rifampicin on APP processing. I found that rifampicin reduced Grp78 levels, suggesting that the rescue of Aβ toxicity is likely due to an increase in the UPRER. Our study points to a novel and unexplored role of rifampicin as a modulator of the UPRER in neurodegeneration downstream of Aβ accumulation. This may provide a useful therapeutic avenue in AD patients already displaying accumulation of Aβ peptides in the brain.

Metabolic defects and disturbances in redox state can lead to alterations in circadian rhythm. In agreement with previous human and animal studies, I showed that Aβ disrupted the circadian rhythm (Harper et al., 2005, Niccoli et al., 2016, Blake et al., 2015), leading to an increase in the period tau. dPGC-1 activation has been shown to restore the period change in Aβ –expressing flies, which likely protects neurons from further deterioration (Kang et al., 2009). Moreover, a previous study found that knockout of the clock gene period did not further affect lifespan, climbing ability or neurodegeneration in an Aβ-expressing fly model that already exhibited circadian rhythm disruption (Long et al., 2014). Therefore, it would be interesting to know whether directly targeting the other clock gene could ameliorate Aβ neurotoxicity. Mammalian PGC-1α regulates the clock gene CLOCK expression by coactivating the
transcription factor RORα and is therefore involved in the second feedback loop (Liu et al., 2007). In the Drosophila second feedback loop, the transcription factor that induces the expression of clock gene dCLOCK is PDP1 (Cyran et al., 2003). I first assessed the interplay between dPGC-1 and PDP1 by FRET, and found these two molecules might physically interact in the fly brain. This suggests that the amelioration of circadian rhythm disruption by dPGC-1 over-expression in Aβ flies may be due to its direct interaction and co-activation with the transcription factor PDP1 in the circadian clock. To evaluate this hypothesis, I over-expressed Pdp1 in the neurons of Aβ-expressing flies, and found that this lead to a small but significant extension in lifespan without affecting Aβ levels. Over-expression of Pdp1 did not rescue the climbing defects and therefore did not prevent Aβ-induced neurodegeneration. Similar to dPGC-1 gain-of-function, over-expression of Pdp1 lead to a further increase in Grp78 transcription, and therefore activation of the UPRER. In keeping with this previous work has shown that flies lacking Pdp1 are sensitive to oxidative damage, confirming its role in the maintenance of the redox state (Beaver et al., 2010). Here I also demonstrated that over-expression of Pdp1 in neurons leads to increased expression of DHR96, suggesting a role in the detoxification pathway. Taken together, my results demonstrate that either genetic activation of dPGC-1 or Pdp1, or pharmacological treatment with rifampicin may provide novel therapeutic strategies in the prevention of AD.

3.5 Materials and Methods

3.5.1 Fly Husbandry and stocks

All flies were reared at 25°C on a 12:12 light: dark cycle at constant humidity and on standard sugar-yeast medium (15g/L agar, 50 g/L sugar, 100 g/L autolyzed yeast). Adult-onset, neuron–specific expression of UAS constructs was achieved as previously described (Sofola et al., 2010). Briefly, 24-28 hours after eclosion female flies carrying a heterozygous copy of elavGS and at least one UAS-construct were fed SY medium supplemented with 200µM mifepristone (RU486) to induce transgene expression. Rifampicin was added to the SY medium food at a concentration of 200µM or 500µM. The elavGS driver line was derived from the original elavGS 301.2 line (Osterwalder et al., 2001) and obtained as generous gift from Dr H. Tricoire.
(CNRS, France). The *UAS-Aβ42Arc* stock was a gift from Dr D. Crowther (University of Cambridge, UK). The *UAS-spargel (UAS-dPGC-1)* line was a gift from Dr. Christian Frei, and the *UAS-Pdp1e* was obtained from FlyORF. All the lines were backcrossed 6 generations into the *w^{1118}* background. The *UAS- Pdp1e* lines were backcrossed over 6 generations into the *w^{Dah}* background. All experiments were carried out on mated females, unless otherwise stated.

### 3.5.2 Lifespan analyses

Flies were raised at a standard density in 200mL bottles. After eclosion, flies were allowed to mate for 24-48 hours in the bottles. Mated females were then split into vials containing SYA medium with or without drugs, with 15 flies in each vial. Approximately 150 females flies were used for each condition in the lifespan experiments. Deaths were scored throughout the lifespan and flies tipped onto fresh food 3 times a week. Data were presented as cumulative survival curves, and survival rates were compared using log-rank tests. All procedures for lifespan experiments were performed at 25°C unless otherwise stated.

### 3.5.3 Climbing assays

The climbing assay was performed as previously described (Kinghorn et al., 2015). Briefly, 15 flies were placed in a 25cm pipette, tapped to the bottom and allowed to climb for 45 seconds. The number of flies in the top 5cm, middle, and bottom 3cm was scored. A performance index was calculated for each time-point and plotted. Statistical analysis was performed in R using ordinal logistics regression, using the individual heights for each fly as data points.

### 3.5.4 Circadian rhythm

Individual, 21-day-old male flies were placed in 65 x 5 mm glass tubes containing standard 1xSYA, and their activity was recorded using the DAM system (*Drosophila* Activity Monitoring System, TriKinetics, Waltham, MA) as described previously (Veleri et al., 2007). Flies were entrained to a 12:12 hour LD cycle at 25°C and 65% humidity 24-36 hours before recording. 5 days of 12:12 hours LD were recorded followed by 5 days of 12:12 hours DD. Analysis of locomotor activity was performed using the fly toolbox and MATLAB software (MathWorks, Natick, MA) as described
previously (Levine et al., 2002). The circadian period was calculated using autocorrelation as described previously (Levine et al., 2002), and was presented by the mean values with their SEM.

3.5.5 The DR protocol
The specific preparation of the DR media has been described in Chapter 2. Work in this chapter used a DR-tent with 0.1SY, 1.0SY, and 2.0SY. RU486 was supplemented into the food to give a final concentration of 200µM.

3.5.6 Paraquat injection
Paraquat (Sigma 856177) was delivered at a dose of 50ng/mg as previously described (Bjedov et al., 2010). To deliver the compound, injection pipettes were prepared from 10mm glass capillaries using the Flaming-Brown micropipette puller (Programme 2). A home-built microinjection machine was used for injections. In parallel flies were also injected with control Ringer’s solution. Both injected solutions contained blue dye for visualization of the injection (FD&C Blue No.1).

3.5.7 Rifampicin treatment
After eclosion flies were split into vials containing standard SY food supplemented with and without 200µM mifepristone (RU486), and with and without 200µM or 500µM Rifampicin (Sigma R3501). Deaths were scored throughout the lifespan and flies tipped onto fresh food 3 times a week. Data were presented as the cumulative survival curves, and survival rates were compared using log-rank tests.

3.5.8 Fly brain imaging using FRET acceptor photobleaching
The following primary antibodies were used: the anti-HA (Abcam, #ab18181, 1:1000) and the anti-GFP (Invitrogen, A21311, 1µg/mL) antibodies. The goat anti-mouse IgG antibody (H+L) (Life technologies, A11019, 1µg/mL) was used as the secondary antibody. Brains were dissected in ice cold PBS and immediately fixed in 4% formaldehyde for 20 mins. Brains were then washed in 0.2% Triton-X/PBS, blocked in 5% bovine serum albumin/PBS, incubated in primary antibody overnight at 4°C and in secondary for 2 hours at RT. Brains were mounted in mounting medium containing DAPI (Vectastain). The method for FRET photobleaching was as
described previously (Broussard et al., 2013). Briefly, the system was set up to use the 488 nm and the 555 nm lines of the laser as an excitation source for the GFP-based donor and RFP acceptor fluorophores. 10% of the laser power was sufficient for the 488 nm and 3% for the 555 nm. The image size was set to 512*512 pixel, with a line averaging of 2. GFP emission was measured from 454 to 505 nm, and the RFP emission from 530 to 590 nm. Photobleaching of the RFP was achieved within the ROI using the 555nm laser line. 100% power was sufficient. Images were captured with a Zeiss (UK) LSM 700 confocal laser-scanning microscope using a 60x oil-immersion objective.

3.5.9 Live cell imaging of the mitochondrial membrane potential

For measurements of mitochondrial membrane potential, the dissected brains were loaded for 40 mins at room temperature with 25 nM tetramethylrhodamine methylester (TMRM) in HBSS (156 mM NaCl, 3 mM KCl, 2 mM MgSO4, 1.25 mM KH2PO4, 2 mM CaCl2 10 mM glucose and 10 mM HEPES at pH 7.35). TMRM was present in the imaging media throughout the experiment and was used in the quench mode to assess Δψm. Hence a reduction in TMRM intensity represents mitochondrial depolarization. Confocal images were obtained using a Zeiss 710 CLSM equipped with a ×40 oil immersion objective. Illumination intensity was kept to a minimum (at 0.1–0.2% of laser output) to avoid phototoxicity. Fluorescence was quantified in individual mitochondria by exciting TMRM using the 561 nm laser line of a confocal microscope. Excitation and emission were measured above 580 nm. 4–5 fields per brain were acquired, and the mean maximal fluorescence intensity was measured for each group. The differences in Δψm were expressed as intensity units, arb.U, of TMRM.

3.5.10 mtDNA measurement

Genomic DNA and mitochondrial DNA from 20 fly heads were isolated using the Qiagen blood and tissue kit according to the manufacturer’s instruction. Quantitative PCR was performed suing the PRISM 7000 sequence-detection system (Applied Biosystem) and SYBR® Green (Molecular Probes) by following the manufacturer’s instructions. mtDNA in each sample were presented as a ratio of mitochondrial DNA/genomic DNA. Each sample was analysed in duplicate and values were
expressed as the mean of the 4 independent biological repeats +/- SEM. Primers used for genomic DNA were as follows:

\textit{GAPDH}:
For: GACGAAATCAAGGCTAAGGTCG
Rev: AATGGGTGTCGCTGAAGAAGTC

For mitochondrial DNA the primers were as follows:

\textit{COXI}:
For: GAATTAGGACATCCTGGAGC
Rev: GCACTAATCAATTCCAAATCC

\section*{3.5.11 Quantitative PCR}
Total RNA was extracted from 20-25 fly heads per sample using TRIzol® (GIBCO) according to the manufacturer’s instructions. The concentration of total RNA purified for each sample was measured using the Nano Drop. 5µg of total RNA was then subjected to DNA digestion using DNase I (Ambion), immediately followed by reverse transcription using the SuperScript® II system (Invitrogen) with oligo(dT) primers. Quantitative PCR was performed using the PRISM 7000 sequence-detection system (Applied Biosystem) and SYBR® Green (Molecular Probes) as per the manufacturer’s instructions. Each sample was analysed in duplicate and the values were presented as the means of 3-4 independent biological repeats +/- SEM. The primers used were:

\textit{dPGC-1}:
For: TCGGCGAGGATTTTTTGATC
Rev: CGATTCGCCGCTCTTTCA

\textit{Aβ42}:
For: CGATCCTTCTCTGCTAACC
Rev: CACCATCAAGCCAATAATCG

\textit{Grp78}:
For: TCTTGTACACACCAACGCAG
Rev: CAAGGAGCTGGGCACAGTGA

\textit{Spliced Xbp1}:
For: CCGAACTGAAGCAGCAACAGC
Rev: GTATACCCCTGCGGAGATCC
3.5.12 ATP assay

ATP concentrations were determined using Roche ATP Bioluminescence Assay Kit HS II (Roche, West Sussex, UK). Briefly, 2 whole flies or 8 fly heads were decapitated and homogenized in 100 µl ice-cold lysis buffer using a Kontes pellet pestle. The lysate was then boiled for 5 min and centrifuged at 20,000g for 1 min. Cleared lysate was then diluted 1 in 200 in dilution buffer and ready to load with 10 µL luciferase. The luminescence was immediately measured using a Tecan Infinite M2000 microplate reader and Magellan V6.5 software. Total proteins were measured using the Thermo Scientific BCA assay. ATP levels in each sample were expressed as a ratio of the total protein content. Data were expressed as the mean+/−SEM and obtained from 5-6 biological repeats for each genotype.

3.5.13 Western blot analysis

15 fly heads in each sample were prepared by homogenising in 2xSDS Laemmli sample buffer (4% SDS, 20% glycerol, 120 mM Tris-HCl pH6.8, 200 mM DTT with bromophenol blue) and boiled at 95°C for 5 minutes. Samples were separated on pre-cast 4-12% Invitrogen Bis-Tris gels (NP0322) and blotted onto nitrocellulose paper in Tris-glycine buffer supplemented with 10% ethanol. Membranes were blocked in 5% milk in TBST (TBS with 0.05% Tween-20) for 1 hour at RT and then incubated with the primary antibody in blocking solution. The primary antibody dilutions used were
anti-Grp78 (novusbio NBP1-06274, 1:1000) in 1% milk, 5% BSA in TBST for 1 hour at RT, anti-β actin (Abcam, #ab8224, 1:5000), anti-HSP60 (Cayman chemical, 10011429, 1:1000) and anti-Grp75 (Abcam, #ab82591, 1:1000). The membranes were incubated in primary antibody in 5% BSA in TBS-T at 4°C overnight unless otherwise stated. Secondary antibodies used were anti-rabbit and anti-mouse (Abcam #ab6721 and Abcam #ab6789 respectively) at 1:5000 dilutions for 1 hour at RT. Bands were visualized with Luminata Forte (Millipore) and imaged with ImageQuant LAS4000 (GE Healthcare Life Science). Quantification was carried out with ImageJ.

3.5.14 Quantification of Aβ42

Five fly heads were homogenized in 50 ml GnHCl extraction buffer (5 M Guanidinium HCl, 50 mM Hepes pH 7.3, protease inhibitor cocktail (Sigma, P8340) and 5mM EDTA), centrifuged at 21,000g for 5 min at 4°C, and cleared supernatant was retained. Aβ42 was measured with an ELISA kit (Millipore, EZHS42), according to the manufacturer’s instructions. The protein concentrations of the samples were measured using a Bradford assay (Bio-Rad protein assay reagent; Bio-Rad laboratories Ltd (UK) and the amount of Aβ42 in each sample expressed as a ratio of the total protein content (pg/µg total protein). Data are expressed as the means +/- SEM and were obtained from three biological repeats for each genotype.

3.5.15 Statistics

Survival experiments were analyzed using a log rank test. The interactions between interventions in lifespan were analyzed using the Cox Proportional Hazard in a survival package developed from Terry M Therneau and Thomas Lumley (https://cran.r-project.org/web/packages/survival/survival.pdf) in R. Climbing assays were analyzed using ordinal logistics regression in R. Circadian rhythm data were analyzed using METLAB. Other data are presented as the means ± SEM obtained from at least 3 independent experiments, and single comparisons were analysed using the Student’s t-test. Other data were tested by either one-way analyses of variance (Casali et al.) for three conditions or two-way ANOVA for four conditions with induction and genotype as main effects, and planned comparisons of means were made using Newman-Keuls test. Statistical analyses were performed using GraphPad Prism.
Chapter 4 The role of PGC-1α in a
Drosophila model of Parkinson’s disease

4.1 Summary
Mitochondrial dysfunction has been implicated in the pathogenesis of PD. PGC-1α is the key regulator of mitochondrial biogenesis, and the possible link to PD arose from studies demonstrating decreased PGC-1α levels in human postmortem brain tissue, as well as in PD mouse models. Moreover, a genome-wide expression meta-analysis demonstrated that a number of genes downstream of PGC-1α are down-regulated in sporadic PD, suggesting that elevation of PGC-1α may offer a potential therapeutic strategy in PD. Here I found that over-expression of dPGC-1 did not rescue the toxic phenotypes of flies lacking PINK1 or parkin. Moreover, activation of dPGC-1 further increased the sensitivity to oxidative stress in PINK1 and parkin null mutants. This work demonstrates that the neuroprotective effects of dPGC-1 seen in the AD fly model are just not effective in these PD models. Further studies using different PD fly models and alternative organisms will be required to fully assess the role of dPGC-1 in PD.

4.2 Introduction
Parkinson’s disease (PD) is the most common movement disorder. Studies on PD related genes have demonstrated that mitochondrial dysfunction plays a central role in the pathogenesis of PD. One of the most significant contributions of Drosophila models to the understanding of PD came from the elegant experiments in which the biology of PINK1 and parkin were elucidated (Greene et al., 2003, Whitworth and Pallanck, 2009, Whitworth et al., 2005, Park et al., 2009, Clark et al., 2006, Yang et al., 2006). Two genes related to autosomal recessive parkinsonism are PINK1 and PARK2. The Drosophila homologue of the PINK1 gene (also called CG4523) encodes a polypeptide of 721 amino acids with a molecular mass of about 80kDa. Similar to human PINK1, the structural analysis of the Drosophila PINK1 (dPINK1) protein also revealed 2 characteristic motifs: a mitochondrial targeting motif and a
PGC-1α in neurodegeneration and ageing

serine/threonine kinase domain, which exhibited 60% similarity (42% identity) to that of human PINK1. Consistent with the localization of human PINK1, *Drosophila* PINK1 was also found localized to mitochondria (Park et al., 2006). Loss of *dPINK1* leads to a number of toxic phenotypes, including reduced longevity, indirect flight muscle and dopaminergic neuronal degeneration, accompanied by locomotor defects, increased sensitivity to oxidative stress, wing and thorax abnormalities and defective mitochondrial morphology and function (Park et al., 2006).

Parkin functions as an E3 ligase in the ubiquitin protein degradation pathway. The *Drosophila* homologue of *parkin* (CG10523) encodes a polypeptide of 482 amino acid exhibiting 42% identity and 59% similarity overall with that of human *parkin*. Loss of the *Drosophila* homologue of *parkin* led to similar phenotypes to those of *PINK1* knockdown, including reduced lifespan, locomotor defects and male sterility (Greene et al 2003). The locomotor defects derive from apoptotic cell death of muscle fibres, whereas the male sterile phenotype derives from a spermatid individualization defect at a late stage of spermatogenesis. Mitochondrial pathology is the earliest manifestation of muscle degeneration and a prominent characteristic of individualizing spermatids in *parkin* mutants (Greene et al., 2003). This study supported the concept that the tissue-specific phenotypes observed in *Drosophila parkin* mutants result from mitochondrial impairment, which is likely a main trigger to neurodegeneration in autosomal recessive juvenile Parkinsonism (ARJP). Further studies have shown that *Drosophila parkin* mutants display degeneration of a subset of dopaminergic neurons in the brain, mimicking the dopaminergic neuronal loss observed in sporadic PD (Whitworth et al., 2005). Moreover, these *parkin* mutant flies have increased oxidative stress and an impaired immune response, suggesting that these processes play a role in the pathogenesis of ARJP.

Interestingly, it has also been demonstrated that over-expression of parkin ameliorates the loss-of-function phenotypes of PINK1, suggesting that parkin acts downstream of PINK1 (Park et al., 2006, Clark et al., 2006). Moreover, PINK1 kinase activity is required for its function in mitochondria, where PINK1 recruits parkin to localize to the mitochondria in a kinase-activity-dependent manner. This work in *Drosophila* has provided significant insights into the essential role of the PINK1-parkin pathway in regulating mitochondrial function, and highlights the importance of mitochondrial
dysfunction in the pathogenesis of PD. Furthermore, it has stimulated considerable research into the precise mechanisms linking loss-of-function of these two genes with mitochondrial dysfunction and neurotoxicity (Whitworth and Pallanck, 2009). In the normal state, PINK1 recruits parkin to translocate from the cytosol to the mitochondria in response to a fall in mitochondrial membrane potential, when mitochondria are under stress or become damaged (Zhang and Ney, 2010, Poole et al., 2008, Narendra et al., 2008, Vives-Bauza et al., 2010, Kim et al., 2008). This physiological process terminates with a specialized form of autophagy, known as mitophagy, in which damaged mitochondria are engulfed in a double membrane and targeted to the lysosome for degradation (Deas et al., 2011, Youle and Narendra, 2011). When either PINK1 or parkin function is lost (i.e. if the \textit{PINK1} or \textit{PARK2} genes are mutated), this process cannot proceed and damaged mitochondria are retained within the cytoplasm. In turn, damaged mitochondria act as a source of oxidative stress that leads to DNA and cellular damage, ultimately causing neuronal death or muscle degeneration (Deas et al., 2011, Youle and Narendra, 2011, Green et al., 2011).

As previously described, most of the physiological functions of PGC-1α have been studies in more details in metabolic tissues such as adipose tissue, liver and muscle. However, there have been only a few studies on the importance of PGC-1α in the brain. PGC-1α null mice show hyperactivity and other movement disorders that represent ganglia pathology, such as stimulus-induced myoclonus, dystonic posturing and frequent limb claspimg. They also develop spongiform lesions in the striatum with axonal loss (Lin et al., 2004). In addition, PGC-1α has been demonstrated to play a role in the stress defense system. PGC-1α null mice display a severe loss of dopaminergic neurons compared to controls when challenged with MPTP (1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine), an oxidative stressor that induces PD in human and animal models (St-Pierre et al., 2006). Flies over-expressing \textit{dPGC-1} show increased resistance to paraquat, a potent oxidizer, highlighting its protective role in response to oxidative stress. In the previous Chapter, I demonstrated the neuroprotective effects of \textit{dPGC-1} in a \textit{Drosophila} model of Aβ toxicity. Over-expression of \textit{dPGC-1} ameliorated the shortened lifespan and neuronal phenotypes, including climbing defects and circadian rhythm disruption. This amelioration was associated with improved mitochondrial function and up-regulation of the UPR.
As described above, mitochondrial dysfunction is a predominant pathogenic feature in both familial and sporadic PD. A recent study demonstrated that parkin plays an important role in regulating mitochondrial biogenesis (Shin et al., 2011). It was shown that parkin physiologically ubiquitinates and thus regulates the proteasomal degradation of the genetic repressor PARIS. In turn, PARIS functions as a major genetic repressor of PGC-1α expression. Therefore, parkin loss-of-function leads to increased PARIS and reduced PGC-1α levels. The conditional knockout of parkin in adult mice leads to the progressive loss of dopaminergic neurons in a PARIS-dependent manner, and this is reversed by over-expression of PGC-1α. This work therefore highlights the importance of PGC-1α in PD, and the potential therapeutic benefits of augmenting PGC-1α, especially in parkin-associated familial PD (Figure 4.1).

Figure 4.1 The PINK1-parkin pathway regulates mitochondrial function via PARIS and PGC-1α.

Under physiological conditions PINK1 recruits parkin to damaged mitochondria to induce mitophagy. Parkin ubiquitinates PARIS, targeting it to degradation by the ubiquitin proteosome system. Reduced levels of PARIS lead to accumulation of PGC-1α, which in turn leads to increased mitochondrial biogenesis. If the parkin gene is mutated and there is loss-of-function of parkin, then it cannot be recruited to the damaged mitochondria to promote mitophagy. This therefore leads to increased production of ROS and mitochondrial damage. As a result of parkin loss-of-function PARIS accumulates and suppresses PGC-1α activity, leading to a reduction in mitochondrial biogenesis. As a result there is an increase in the number of damaged mitochondria and reduced mitochondrial biogenesis, leading to increased oxidative stress and neuronal death.
Moreover, the role of PGC-1α is not confined to patients with parkin mutations, but is also implicated in those with sporadic PD. Human postmortem brain tissue analysis revealed that sporadic PD patients displayed increased levels of PARIS and reduced expression of PGC-1α (Shin et al., 2011). In addition, a genome-wide expression meta-analysis independently demonstrated that 10 genes, which are known downstream targets of PGC-1α, were down-regulated in the SN of PD patients (Zheng et al., 2010). These genes mostly function in mitochondrial the ETC activity, glucose utilization, and glucose sensing, indicating that defects in energy metabolism mediated by PGC-1α occur early in the disease pathogenesis. PD pathology thus causes a reduction in PGC-1α and its downstream factors, leading to worsening of the disease pathology. Interestingly, over-expression of PGC-1α rescues dopaminergic neuronal loss in primary neuronal cultures expressing mutant α-synuclein, in association with up-regulation of genes in the mitochondrial ETC (Zheng et al., 2010). Taken together, this work suggests that elevating PGC-1α may be a useful therapeutic target for treating PD.

The question of whether reduced PGC-1α is a causal factor in the pathogenesis of PD is yet to be resolved. The reduction in PGC-1α seen in PD brains may contribute to the disease progression. Indeed it has been shown that a reduction in PGC-1α leads to increased sensitivity to stressors by elevating ROS, thereby impairing mitochondrial proteostasis, eventually leading to neuronal death (St-Pierre et al., 2006, Lu et al., 2014, Haynes and Ron, 2010). I therefore studied the role of PGC-1α in PD pathogenesis, using a model of PINK1 and parkin loss-of-function in Drosophila melanogaster, which display shortened lifespans, behavioural defects and neurodegeneration (Clark et al., 2006, Greene et al., 2003).

In order to determine whether PGC-1α contributes to the pathogenic cascades downstream of PINK1 and parkin loss-of-function, I genetically over-expressed dPGC-1 in PINK1 and parkin null mutant flies. I first demonstrated that the transcript levels of dPGC-1 were up-regulated in both PINK1 null mutants and parkin null mutants. dPGC-1 over-expression did not rescue the toxic phenotypes in either parkin or PINK1 null mutants in either males or females. Interestingly, PINK1 or parkin null mutants over-expressing of dPGC-1 were more sensitive to H₂O₂ or paraquat-induced
oxidative stress. Taken together, our results showed that elevation of \(dPGC-1\) does not provide a beneficial effect in PD fly models lacking \(PINK1\) and \(parkin\).

4.3 Results:

4.3.1 Over-expression of \(dPGC-1\) did not rescue the toxic phenotypes of \(PINK1\) null mutants

To investigate the role of \(dPGC-1\) in PINK1 loss-of-function, I over-expressed \(dPGC-1\) in \(PINK1^{B9}\) null mutants, which have been previously characterized (Clark et al., 2006). \(PINK1\) is on the X chromosome and null mutant \(PINK1\) males are sterile. I therefore only used male progeny for experiments. qRT-PCR analysis demonstrated that the transcript levels of \(dPGC-1\) were increased in response to PINK1 loss-of-function (Figure 4.2). I then assessed whether over-expression of \(dPGC-1\) can ameliorate the neurotoxic phenotypes induced by PINK1 loss-of-function. I confirmed the over-expression of \(dPGC-1\) using the \(UAS-dPGC-1\) construct driven ubiquitously by the \(ActGS\) driver using qRT-PCR (Figure 4.3). Ubiquitous over-expression of \(dPGC-1\) throughout development using the \(DaGAL4\) driver neither extended lifespan nor improved the climbing defects in \(PINK1^{B9}\) null mutants (Figure 4.4 and 4.5), suggesting that elevation of \(dPGC-1\) does not rescue the toxic phenotypes induced by \(PINK1\) loss-of-function.

![Figure 4.2 The mRNA expression levels of \(dPGC-1\) were increased in PINK1 loss-of-function flies](image)
PGC-1α in neurodegeneration and ageing

The dPGC-1 mRNA levels (relative to actin) in male PINK1<sup>89</sup> null mutant flies at 15-day-old were increased compared to control flies. This was measured using qRT-PCR and the bars represent the means ± SEM (n=4, ***p<0.001 by t test). The genotypes used were: w<sup>1118</sup> and PINK<sup>89</sup>.

![Figure 4.3](image)

Figure 4.3 The mRNA expression levels of dPGC-1 were raised in dPGC-1-over-expressing flies

The dPGC-1 mRNA levels (relative to RP49) were increased in 15-day-old flies ubiquitously over-expressing dPGC-1, as measured by qRT-PCR. The bars represent the means ± SEM (n=4, ***p<0.001 by t test). The genotypes used were: ActGS> UAS-dPGC-1.

![Figure 4.4](image)

Figure 4.4 Ubiquitous over-expression of dPGC-1 did not extend the lifespan of PINK1 null mutant male flies

Male flies lacking PINK1 show reduced survival compared to wild type flies (p=4.5 x 10<sup>-19</sup> when comparing DaGAL4/+ with PINK1<sup>89</sup>/DaGAL4/+). The survival curves demonstrated that ubiquitous over-expression of dPGC-1 did not rescue the shortened lifespan of PINK1<sup>89</sup> males. P=0.04 when comparing PINK1<sup>89</sup> > Da-GAL4/+ with PINK1<sup>89</sup> > Da-GAL4/UAS-dPGC-1. P=0.93 when comparing
PGC-1α in neurodegeneration and ageing

$PINK1^{B9}> UAS-dPGC-1/+ \text{ with } PINK1^{B9}> Da-GAL4/UAS-dPGC-1$ by log-rank test. The genotypes used were: $Da-GAL4/++; UAS-dPGC-1/+$; $Da-GAL4/UAS-dPGC-1$; $PINK1^{B9}> Da-GAL4/+$; $PINK1^{B9}> UAS-dPGC-1/+$; $PINK1^{B9}> Da-GAL4/UAS-dPGC-1$.

![Bar graph showing performance index over age](image)

**Figure 4.5** The over-expression of $dPGC-1$ in $PINK1^{B9}$ null mutant male flies did not rescue the climbing defects

The ubiquitous over-expression of $dPGC-1$ using the $Da-GAL4$ driver did not rescue the locomotor ability of $PINK1^{B9}$ null mutant male flies using the negative geotaxis climbing assay. The bars are plotted as the means ± SEM, n=3, 15 flies per condition. **P<0.01, *p<0.05 by ANOVA analysis.

### 4.3.2 Over-expression of $dPGC-1$ did not rescue the toxic phenotypes of $parkin$ null mutants

To investigate the role of $dPGC-1$ in neurotoxicity mediated by the loss of $parkin$, I first measured the levels of $dPGC-1$ in $park^{25}$ null mutants, which have been previously characterized (Greene et al., 2003). qRT-PCR showed that the mRNA levels of $dPGC-1$ were increased in response to loss of parkin (Figure 4.6). I then assessed whether over-expression of $dPGC-1$ could ameliorate the neurotoxic phenotypes induced by parkin loss-of-function. Ubiquitous over-expression of $dPGC-1$ throughout development using the $ActGAL4$ driver extended lifespan in both male
and female parkin null mutants (Figure 4.7 and 4.8), and improved the climbing defects in female parkin null mutants (Figure 4.9). However, the lifespan extension was not consistent and repeatable (Figure 4.10 and 4.11). These results therefore suggest that over-expression of dPGC-1 does not rescue the toxic phenotypes induced by parkin loss-of-function.

**Figure 4.6** The expression of dPGC-1 was increased in park25 null mutant flies

dPGC-1 mRNA levels (relative to actin) in female park25 null flies at 15-day-old, measured by qRT-PCR. The bars were plotted as the means ± SEM (n=4, *p<0.05 by t test). The genotypes used were: w1118 and park25.

**Figure 4.7** Ubiquitous over-expression of dPGC-1 extended the lifespan of park25 null mutant female flies
PGC-1α in neurodegeneration and ageing

The survival curves of park25 female flies ubiquitously over-expressing dPGC-1. P=3.3 x 10^-8 when comparing ActGAL4>+; park25 with ActGAL4>UAS-dPGC-1; park25. P=0.01 when comparing UAS-dPGC-1>++; park25 with ActGAL4>UAS-dPGC-1; park25 by log-rank test. The genotypes used were: ActGAL4>+; park25, ActGAL4>UAS-dPGC-1; park25, UAS-dPGC-1>++; park25.

Figure 4.8 Ubiquitous over-expression of dPGC-1 extended the lifespan of park25 null mutant male flies

Ubiquitous over-expression of dPGC-1 in park25 null mutant male flies (ActGAL4>UAS-dPGC-1; park25 (red line)) extended lifespan. P=0.03 when comparing ActGAL4>+; park25 with ActGAL4>UAS-dPGC-1; park25, P=1.7 x 10^-6 when comparing UAS-dPGC-1>++; park25 with ActGAL4>UAS-dPGC-1; park25 by log-rank test. The genotypes used were: ActGAL4>+; park25, UAS-dPGC-1>++; park25, ActGAL4>UAS-dPGC-1; park25.
Ubiquitous over-expression of dPGC-1 in park25 null mutant female flies partially ameliorated the climbing defects. The bars represent the means ± SEM, n=3, 15 flies per condition. **P<0.01, *p<0.05 by ANOVA.

Female flies lacking parkin show reduced survival compared to wild type flies (p=4.3 x 10^-42 when comparing DaGAL4/+ with PINKI>DaGAL4/+). Ubiquitous over-expression of dPGC-1 in park25
null mutant female flies did not rescue the lifespan phenotype. \( P=1.6 \times 10^{-6} \) when comparing ActGAL4>+: park25 with ActGAL4>UAS-dPGC-1; park25. \( P=0.03 \) when comparing UAS-dPGC-1>+: park25 with ActGAL4>UAS-dPGC-1; park25 by log-rank test. The genotypes used were: \( w^{1118}, \) ActGAL4>+: park25, UAS-dPGC-1>+: park25, ActGAL4>UAS-dPGC-1; park25.

Figure 4.11 Ubiquitous over-expression of dPGC-1 did not extend the lifespan of park25 null mutant male flies

The survival curves of park25 male flies ubiquitously over-expresses dPGC-1. \( P=0.97 \) when comparing ActGAL4>+: park25 with ActGAL4>UAS-dPGC-1; park25, \( P=0.005 \) when comparing UAS-dPGC-1>+: park25 with ActGAL4>UAS-dPGC-1; park25 by log-rank test. The genotypes used were: ActGAL4>+: park25, UAS-dPGC-1>+: park25, ActGAL4>UAS-dPGC-1; park25.

4.3.3 Over-expression of dPGC-1 increased the sensitivity to oxidative stress of park25 null mutant male flies

To assess whether flies over-expressing dPGC-1 are resistant to oxidative stress I assessed the survival of park25 null mutant flies treated with \( \mathrm{H}_2\mathrm{O}_2 \), a potent oxidizer, and with paraquat, a redox cycler. I demonstrated that both male and female flies lacking parkin had significantly reduced survival upon exposure to \( \mathrm{H}_2\mathrm{O}_2 \) (Figures 4.12 and 4.13) and paraquat (Figures 4.14 and 4.15). Surprisingly ubiquitous over-expression of dPGC-1 (ActGAL4>dPGC-1; park25) even further increased the
sensitivity of park^{25} null mutant male flies to oxidative stress, while further increased the sensitivity of park^{25} null mutant female flies to oxidative stress compared to one the control lines (UAS-dPGC-1>+; park^{25}) but not the others (ActGAL4>+; park^{25}).

Figure 4.12 Ubiquitous over-expression of dPGC-1 did not alter the sensitivity to H_{2}O_{2} of park^{25} null mutant female flies

Female flies lacking parkin (ActGAL4>+; park^{25}) show increased sensitivity to 2.5% H_{2}O_{2} compared to wild type flies (p=3.1 x 10^{-41}). Ubiquitous over-expression of dPGC-1 using the ActGAL4 driver did not alter the sensitivity of park^{25} null mutant female flies on exposure to 2.5% H_{2}O_{2} at 15-days of age. P=0.24 when comparing ActGAL4>+; park^{25} with ActGAL4>UAS-dPGC-1; park^{25}. P=0.01 when comparing UAS-dPGC-1>+; park^{25} with ActGAL4>UAS-dPGC-1; park^{25} by log-rank test. The genotypes used were: w^{1118}, ActGAL4>+; park^{25}, UAS-dPGC-1>+; park^{25}, ActGAL4>UAS-dPGC-1; park^{25}.
Figure 4.13 Ubiquitous over-expression of dPGC-1 increased the sensitivity of park25 null mutant male flies to oxidative stress with H2O2

Male flies lacking parkin (ActGAL4> +; park25) show increased sensitivity to 2.5% H2O2 compared to wild type flies (p=7.1 x 10^-9). Ubiquitous over-expression of dPGC-1 in park25 null mutant flies (ActGAL4>UAS-dPGC-1; park25) reduced the survival on exposure to 2.5% H2O2 at 15-days of age. P=0.005 when comparing ActGAL4> +; park25 with ActGAL4>UAS-dPGC-1; park25, P=0.0003 when comparing UAS-dPGC-1> +; park25 with ActGAL4>UAS-dPGC-1; park25 by log-rank test. The genotype used were: w^1118, ActGAL4> +; park25, UAS-dPGC-1> +; park25, ActGAL4>UAS-dPGC-1; park25.

Figure 4.14 Ubiquitous over-expression of dPGC-1 did not alter the sensitivity of park25 female flies to paraquat

Female flies lacking parkin (UAS-dPGC-1> +; park25) show increased sensitivity to 2.5% H2O2 compared to wild type flies (p=5.5 x 10^-6). Ubiquitous over-expression of dPGC-1 in park25 null mutant flies did not alter the sensitivity to treatment with 10mM paraquat at 15-days of age. P=0.5 when comparing ActGAL4> +; park25 with ActGAL4>UAS-dPGC-1; park25. P=3.2 x 10^-5 when
PGC-1α in neurodegeneration and ageing

comparing $\text{UAS-dPGC-1}^{+} \text{; park}^{25}$ with $\text{ActGAL4}>\text{UAS-dPGC-1}; \text{park}^{25}$ by log-rank test. The genotypes used were: $w^{118}$, $\text{ActGAL4}^{+}$, $\text{UAS-dPGC-1}^{+} \text{; park}^{25}$, $\text{ActGAL4}>\text{UAS-dPGC-1}; \text{park}^{25}$.

![Figure 4.15 Ubiquitous over-expression of dPGC-1 increased the sensitivity to oxidative stress on exposure to paraquat in park25 null mutant male flies](image)

Male flies lacking parkin ($\text{UAS-dPGC-1}^{+} \text{; park}^{25}$) show increased sensitivity to 2.5% H$2$O$2$ compared to wild type flies ($p=3.0 \times 10^{-17}$). The survival curves of $\text{park}^{25}$ null mutant male flies over-expressing $\text{dPGC-1}$ ($\text{ActGAL4}>\text{UAS-dPGC-1}; \text{park}^{25}$) compared to flies lacking parkin only ($\text{ActGAL4}^{+} \text{; park}^{25}$ and $\text{UAS-dPGC-1}^{+} \text{; park}^{25}$) on exposure to 10mM paraquat at 15-days of age. $P=2.2 \times 10^{-5}$ when comparing $\text{ActGAL4}^{+} \text{; park}^{25}$ with $\text{ActGAL4}>\text{UAS-dPGC-1}; \text{park}^{25}$. $P=3.1 \times 10^{-5}$ when comparing $\text{UAS-dPGC-1}^{+} \text{; park}^{25}$ with $\text{ActGAL4}>\text{UAS-dPGC-1}; \text{park}^{25}$ by log-rank test. The genotypes used were: $w^{118}$, $\text{ActGAL4}^{+} \text{; park}^{25}$, $\text{UAS-dPGC-1}^{+} \text{; park}^{25}$, $\text{ActGAL4}>\text{UAS-dPGC-1}; \text{park}^{25}$.

4.4 Discussion

I demonstrated that the gene expression levels of $\text{dPGC-1}$ were up-regulated in $\text{PINK1}$ or parkin null mutant flies. This is opposite to what has been shown in human and mouse models, displaying reduced levels of PGC-1α in familial and sporadic PD (Shin et al., 2011, Zheng et al., 2010). The up-regulation of $\text{dPGC-1}$ that I see in the fly models of familial PD may represent a compensatory response to the mitochondrial dysfunction induced by $\text{PINK1}$ and parkin loss-of-function. Since flies do not have a homologue of PARIS, the transcriptional repressor of PGC-1α, this transcriptional regulation may not be conserved in $\text{Drosophila}$. To dissect the role of $\text{dPGC-1}$ in $\text{PINK1}$ and parkin loss-of-function, I further assessed the lifespan and
behavioral phenotypes in the corresponding null mutants over-expressing dPGC-1. I found that over-expression of dPGC-1 did not extend the lifespan of either PINK1 or parkin null mutant flies. Moreover, dPGC-1 over-expression did not improve the climbing phenotypes of PINK1 or parkin null mutants. These results suggest that elevation of dPGC-1 is not neuroprotective in PD fly models.

4.5 Experimental procedures
4.5.1 Fly strains and backcrossing
The PINK1B9 line (w* PINK1B9/FM7i) was obtained from the Bloomington Drosophila Stock Centre (Park et al., 2006, Park et al., 2009) and contains a deletion of PINK1 on chromosome X. The park\textsuperscript{25} line (w; park\textsuperscript{25}/TM6B. GFP) and the PINK1 genomic rescue line were kind gifts from Dr. Alex Whitworth (University of Cambridge). All of the lines were backcrossed into the w\textsuperscript{1118} strain for at least 6 generations before they were used in experiments. The PINK\textsuperscript{B9} and park\textsuperscript{25} lines have no visible phenotypes to follow for backcrossing. Therefore, the progeny from every generation of backcrossing were identified by PCR analysis with primers designed to detect the presence of the transgenic construct. This was performed as follows: a single cross was set up in an individual vial (one virgin female was crossed with one wild type male). They were then left for several days to mate and lay eggs. Female flies were then individually taken from each vial and used for PCR analysis. Only the progeny from female flies that were PCR-positive were selected for the next backcrossing. The PCR primers were designed using the Primer3 software. The PCR primers for PINK1\textsuperscript{B9} were: FOR: CCAAATGATTCGAGTCCACA+ REV: TCCAGTTCATCCTCCTTGCT. The size of the PCR product for PINK1\textsuperscript{B9} null flies was 721bp, while the size of the full PINK1 construct was 1291bp. For park\textsuperscript{25} the primers used were: FOR: CAGGAGTTTGTTTGTGGACTCCACA+ REV: GCCATTACCCTACCGAAACTTTACA. The PCR product size of the mutant flies was 500bp, while wild type flies had a 1.8kb PCR product size. The PINK1\textsuperscript{B9} null males are sterile, and females are balanced with FM7 on chromosome X. In order to pass the cytoplasmic content from w\textsuperscript{1118} virgin females to the progeny, I made double transgenic lines carrying both PINK1\textsuperscript{B9} and the PINK1 genomic rescue construct, so
that males were fertile and able to mate with \( w^{1118} \) virgin females. Once the initial cross was complete, the PINK1 genomic construct was removed by excluding orange eye color in the following generation. After 6 back-crosses the \( park^{25} \) null mutant lines were balanced twice with \( w^{1118} \); \( Cyo/Sp; TM6B/MKRS \) to create a stable stock. The PINK1\(^{B9} \) lines were balanced with \( FM6 \) to create a stable stock.

### 4.5.2 qRT-PCR

RNA extraction and cDNA synthesis have been previously described. The primers for qRT-PCR were:

- **Actin**
  - For: TTGTCTGGGCAAGAGGATCAG
  - REV: ACCACTCGCACTTGCACCTTC
Chapter 5  PGC-1α-GABPα modulates ageing in Drosophila

5.1 Summary
Dietary restriction is an evolutionarily conserved intervention that extends healthy lifespan across different species. Our understanding of the molecular mechanisms downstream of the nutrient-sensing network has improved significantly over the last 30 years, but is still not fully understood. PGC-1α is a key transcriptional co-activator in energy metabolism, and has recently been identified as a novel modulator of fly ageing, however the mechanism is not completely understood. Here I identified the transcription factor GA-binding protein (dGABPα), downstream of PGC-1α, as a key modulator of fly ageing. dGABPα gain-of-function extended female lifespan beyond dietary restriction (DR), with an associated reduction in IIS/mTOR signalling. Over-expression of dGABPα improved gut homeostasis by delaying age-related over-proliferation in intestinal stem cells. Additionally, dGABPα gain-of-function flies displayed resistance to xenobiotic stress. Finally, I demonstrated that dGABPα up-regulates ImpL2, encoding the insulin ligand binding protein, which down-regulates IIS and extends lifespan. Given the evolutionarily conserved effects of reduced IIS/mTOR, the findings suggest that GABPα may be an effective modulator of mammalian ageing.

5.2 Introduction
An increasing ageing population coupled with the rising prevalence of age-related diseases, highlights the need for effective strategies to improve healthy ageing. Ageing is a complex process that includes genetic and environmental factors. The most successful insights into ageing came from the discovery that ageing could be ameliorated by dietary, genetic, or pharmacological interventions. DR, an intervention that chronically reduces food intake without malnourishment, is the most robust environmental method to extend longevity in a wide range of evolutionarily diverse organisms, from yeast to mammals (Fontana et al., 2010, Colman et al., 2009, Katewa and Kapahi, 2010). DR can also improve healthy lifespan and reduce the prevalence
of age-related diseases in humans and mammalian models (Colman et al., 2014, Mattison et al., 2012, Ikeno et al., 2006, Maeda et al., 1985, Cava and Fontana, 2013, Heilbronn et al., 2006, Masoro, 2003). The mechanisms by which DR improves healthy lifespan have not been fully elucidated. However, the wide use of shorter-lived organisms including, *C. elegans* and *Drosophila*, has facilitated the experimental discovery of genetic and pharmacological interventions that extend healthy lifespan. The molecular mediators of the effects of DR on longevity include the mTOR (Kaeberlein et al., 2005, Chen et al., 2009b, Honjoh et al., 2009, Jia et al., 2004, Hansen et al., 2008), insulin/IGF-1-like signalling (IIS) (Broughton et al., 2010, Laplante and Sabatini, 2012, Takano et al., 2001), the energy sensor 5’ adenosine monophosphate-activated protein kinase (AMPK) (Burkewitz et al., 2014, Greer et al., 2007, Slack et al., 2012), sirtuins (Guarente, 2013), NF-E2-related factor (NRF2) (Castillo-Quan et al., 2016) and heat shock factors (HSFs) (Heydari et al., 1996).

In addition to reducing food intake, the timing of the meal has also emerged as an important determinant of ageing. Intermittent fasting (IF) or chronic starvation extends lifespan in *C. elegans* through mechanisms that include reduced IIS/mTOR signalling (Honjoh et al., 2009, Kaeberlein et al., 2006, Lee et al., 2006, Uno et al., 2013). Moreover, short term fasting in human or mammalian models results in an improved healthy lifespan and the reduced prevalence of age-related chronic disorders. This involves mechanisms such as induction of the chaperone HSP70 and inhibition of the IIS/mTOR signalling network (Brown-Borg and Rakoczty, 2013, Mattson et al., 2014, Cheng et al., 2014). However, the effect of fasting or starvation on cachexia, such as in cancer, may be detrimental, leading to organ wasting and loss of body mass. Fasting in rats increased the production of TGF-beta1, leading to the initiation and promotion of liver carcinogenesis (Tessitore and Bollito, 2006). In a *Drosophila* tumour model, resulting from activation of the oncogene *Yorkie* in intestinal stem cells, there was organ-wasting associated with a sustained and dramatic reduction in systemic IIS signalling, via increased levels of the secreted insulin antagonist ImpL2 (Kwon et al., 2015, Figueroa-Clarevega and Bilder, 2015). Interestingly, over-expression of *ImpL2* extends lifespan in *Drosophila* via mechanisms consistent with reduced IIS signalling, suggesting a possible involvement in mammalian ageing (Alic et al., 2011b). Therefore, wasting, starvation, IF, and DR
might share similar molecular mechanisms, including reduced IIS signalling, in response to nutritional limitation. However, the outcomes are varied depending on multiple factors, including the levels of signalling reduction, nutrient availability and the sensitivity of the host.

The functions of the insulin/IGF-1-like signalling (IIS) pathway include regulation of growth, development, reproduction, stress resistance and metabolic homeostasis. Insulin-like ligands mediate cell-to-cell signalling by activating an insulin-like receptor, leading to the activation of PI3K (Phosphatidylinositide 3-kinase), AKT, mTOR, and ERK (extracellular signal-regulated kinases) intracellular signalling pathways (Tatar et al., 2003, Piper et al., 2008, Slack et al., 2015). Reduced IIS increases longevity in nematodes, fruit flies and mice (Fontana et al., 2010, Kenyon, 2010), highlighting the relevance of IIS in human ageing. Lifespan extension and stress resistance resulting from down-regulation of IIS are mediated by the FOXO family of transcription factors through PI3K and AKT kinase signalling (Laplante and Sabatini, 2012). Inhibition of AKT activates FOXO, which in turn up-regulates several “longevity genes” controlling DNA repair, autophagy, antioxidant activity, stress resistance, as well as cell proliferation (Wang et al., 2014, Webb and Brunet, 2014).

The evolutionarily conserved mTOR signalling pathway responds to growth factors and amino acids to regulate the growth and metabolism of all eukaryotic cells. mTOR functions via two distinct multiprotein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Johnson et al., 2013). mTORC1 regulates growth through phosphorylation of the downstream effectors ribosomal protein S6 kinases (S6Ks). Under favourable conditions, such as an amino acid-rich diet, phosphorylated S6K promotes cellular growth and alters metabolism (Um et al., 2006). mTORC1-dependent phosphorylation of the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) disrupts its association with the translation initiation factor 4E (eIF4E), allowing the latter to promote cap-dependent translation (Sonenberg and Hinnebusch, 2009). Rapamycin, a natural macrolide compound isolated from bacteria, inhibits mTORC1 activity, extending the lifespan across species from yeast to mammals. Rapamycin acts by reducing mRNA translation and by inducing autophagy.
through at least two mTORC1 substrates, S6K and 4E-BP1 (Bjedov et al., 2010, Powers et al., 2006, Robida-Stubbs et al., 2012, Harrison et al., 2009, Medvedik et al., 2007, Miller et al., 2011). Moreover, inhibition of mTORC1 also improves proteostasis and enhances stem cell function (Kapahi et al., 2010, Johnson et al., 2013). mTORC2 is not sensitive to inhibition by rapamycin, and is usually considered not relevant to nutrient sensing. However it can positively regulate the IIS pathway by activating phosphorylation of AKT, the main kinase in the IIS pathway (Guertin and Sabatini, 2009). In addition, AKT kinase can then phosphorylate and inactivate mTORC1, while S6K negatively regulates the insulin receptor substrate 1 (Laplante and Sabatini, 2012, Takano et al., 2001, Um et al., 2006). It has also been shown that the mTORC1 substrate 4E-BP is a transcriptional target of FOXO in fruit flies (Tettweiler et al., 2005).

PGC-1α is the master regulator of many biological processes, including adaptive thermogenesis (Puigserver et al., 1998), mitochondrial biogenesis (Wu et al., 1999), glucose metabolism (Miura et al., 2003), hepatic gluconeogenesis, fatty acid oxidation (Li et al., 2007b, Puigserver et al., 2003), circadian rhythmicity (Liu et al., 2007) as well as stress response systems (St-Pierre et al., 2006). PGC-1α is directly regulated by insulin-stimulated AKT phosphorylation in hepatic metabolism (Li et al., 2007b). Phosphorylation and inhibition prevent the recruitment of PGC-1α to its cognate promoters, impairing its ability to bind and co-activate FOXO1 or HNF4 (hepatocyte nuclear factor) to promote gluconeogenesis and fatty acid oxidation (Puigserver et al., 2003, Li et al., 2007b). Moreover, PGC-1α can also be regulated by FOXO1 in the control of mitochondrial biogenesis in response to insulin signalling (Cheng et al., 2009). *Drosophila* PGC-1 (*dPGC-1*), the only homologue of the mammalian PGC-1 family, regulates mitochondrial activity, cellular growth and metabolism in response to insulin signalling in parallel with FOXO, the main IIS downstream effector responsible for cellular growth (Mukherjee and Duttaroy, 2013, Tiefenböck et al., 2010). Interestingly, *dPGC-1* can also negatively regulate insulin signalling, although the precise mechanisms involved are not known (Tiefenböck et al., 2010).

*dPGC-1* is also a terminal target of the mTOR signalling pathway in regulating cellular growth and metabolism (Mukherjee and Duttaroy, 2013, Tiefenböck et al.,
2010). It may be regulated in a number of different ways. Inhibition of mTOR1 by rapamycin reduces the transcript levels of PGC-1α (Cunningham et al., 2007), and inhibition of mTORC1 induces Mitf, the *Drosophila* homologue of the transcription factor EB (TFEB). This translocates to the nucleus and directly up-regulates the transcript levels of *dPGC-1* (Bouche et al., 2016). Moreover, the mTOR signalling pathway is associated with human longevity (Passtoors et al., 2013), and over-expressing *dPGC-1* in the intestinal system of the fruit fly extends lifespan with improved gut homeostasis and increased mitochondrial biogenesis, suggesting PGC-1α might be a modulator for mammalian ageing. However, the direct contribution of mitochondrial function in regulating longevity has not been investigated (Rera et al., 2011). Therefore, whether mitochondrial function is the major determinant of PGC-1α-mediated lifespan extension in response to reduced mTOR signalling is yet to be proven. Some studies have however demonstrated that inhibition of mTORC1, or up-regulation of 4E-BP, results in a metabolic shift towards greater mitochondrial respiration and increased lifespan across species from yeast to mice (Bonawitz et al., 2007, Polak et al., 2008, Morita et al., 2013, Zid et al., 2009). In addition, mild inhibition of mitochondrial respiration extends lifespan of many organisms including worms, flies and mice (Feng et al., 2001, Dillin et al., 2002, Lee et al., 2003, Lee et al., 2010, Liu et al., 2005, Dell'agnello et al., 2007, Copeland et al., 2009). I therefore hypothesised that PGC-1α, being-downstream of IIS/mTOR signalling, may regulate longevity partially through a mitochondrial-independent manner.

In order to address this hypothesis, I first investigated whether genetic activation of the transcription factors required by PGC-1α in regulating mitochondrial biogenesis and respiration can affect lifespan. PGC-1α co-activates a number of different transcription factors, including NRF1, GABPα, YY1 and ERRα (Puigserver and Spiegelman, 2003, Scarpulla, 2008, Cunningham et al., 2007). GABPα is the alpha subunit of the GA binding protein complex, which is a mammalian E26 transformation-specific (ETS) transcription factor required for mitochondrial biogenesis (Yang et al., 2014). In addition, GABPα can be induced and co-activated by PGC-1α in skeletal muscle, forming a double-positive-feedback loop that drives the expression of many genes in OXPHOS and during neuromuscular junction (NMJ) programming (Handschin et al., 2007, Mootha et al., 2004, Wu et al., 1999). In
PGC-1α in neurodegeneration and ageing

_Drosophila_, PGC-1α and GABPα work by overlapping mechanisms required for mitochondrial abundance and gene expression for OXPHOS (Baltzer et al., 2009), suggesting a conserved function with their mammalian counterparts (Mootha et al., 2004, Yang et al., 2014). I hypothesised that if PGC-1α extends lifespan partially through GABPα, then over-expression of GABPα might be enough to extend lifespan.

As described in this Chapter, I first re-examined the effect of _dPGC-1_ over-expression on the lifespan of _Drosophila_, and found that both ubiquitous and CNS specific over-expression of _dPGC-1_ extends lifespan. In keeping with its human counterpart, _dPGC-1_ over-expression also up-regulated the transcription of _dGABPα_. Either ubiquitous or tissue-specific (brain or gut and fat body) over-expression of _dGABPα_ was sufficient to extend lifespan via mechanisms beyond DR. It also improved measures of health, including increased resistance to xenobiotic stress and improved gut homeostasis. Surprisingly I found that _dGABPα_ up-regulated the levels of _ImpL2_, the secreted insulin antagonist and an important negative regulator of IIS. This suggests that up-regulation of _dGABPα_ may therefore modulate insulin sensitivity. To assess this further I performed both lifespan and bioinformatics analyses, and confirmed that over-expression of _dGABPα_ extends lifespan through overlapping mechanisms with reduced IIS. In addition, over-expression of _dGABPα_ also reduced mTORC1 activity, therefore extending lifespan in a similar manner to rapamycin. My results point to the possibility that GABPα, or a related protein, may be an effective modulator of mammalian ageing and age-related diseases. Further work is now required to study the effects of up-regulating GABPα in specific disease models, such as the AD and PD fly models.

5.3 Results

5.3.1 Over-expression of _dPGC-1_ extended the lifespan of female _Drosophila_

Previous studies demonstrated that over-expression of _dPGC-1_ specifically in the intestine extends lifespan (Rera et al., 2011). I then asked whether over-expression of _dPGC-1_ throughout the fly is also sufficient to extend lifespan. Ubiquitous over-expression of _dPGC-1_, either throughout development using the ActGAL4 driver, or
in adulthood using the Actin-GeneSwitch (ActGS) driver, significantly increased the median lifespan of female flies by 9% (Figure 5.1, p=3.28 x 10^{-18}; Figure 5.3, p=3.54 x 10^{-6}) but not in males (Figure 5.2, p=0.1). Additionally, over-expression of dPGC-1 delayed the age-related decline in climbing ability (Figure 5.4), a behavioural measurement of neuromuscular health. My results therefore confirm that dPGC-1 is indeed a modulator for healthy ageing.

**Figure 5.1 Ubiquitous over-expression of dPGC-1 extended the lifespan of female flies**

Ubiquitous over-expression of dPGC-1 throughout development and adulthood using the ActGAL4 driver (ActGAL4/UAS-dPGC-1, green line) extended the lifespan of female flies (the median lifespan was extended by 9%, p=3.28 x 10^{-18} when comparing ActGAL4/UAS-dPGC-1 and +/UAS-dPGC-1; p=2.2 x 10^{-8} when comparing ActGAL4/UAS-dPGC-1 and ActGAL4/+ by log-rank test). The genotypes used were: ActGAL4/UAS-dPGC-1, ActGAL4+/, +/UAS-dPGC-1.
Figure 5.2 Ubiquitous over-expression of \textit{dPGC-1} during development and in adulthood did not extend the lifespan of male flies

Ubiquitous expression of \textit{dPGC-1} throughout development and in adulthood using the \textit{ActGAL4} driver (\textit{ActGAL4}\textgreater\textit{UAS-dPGC-1}, green line) did not extend the lifespan of male flies. \(P=0.1\) when comparing \textit{ActGAL4}\textgreater\textit{UAS-dPGC-1} and \textit{ActGAL4}++ by log-rank test. The genotypes used were: \textit{ActGAL4}\textgreater\textit{UAS-dPGC-1}, \textit{ActGAL4}++, +\textgreater\textit{UAS-dPGC-1}.

Figure 5.3 Ubiquitous over-expression of \textit{dPGC-1} only in adulthood extended the lifespan of female flies

Ubiquitous over-expression of \textit{dPGC-1} in adulthood only using the \textit{ActGS} driver in the presence of RU (\textit{ActGS}\textgreater\textit{UAS-dPGC-1}, RU+, red line) extended the lifespan of flies compared to un-induced (RU-) controls (the median lifespan was extended by 8.7\%, \(p=3.54 \times 10^{-6}\) by log-rank test). The genotypes used were: \textit{ActGS}\textgreater\textit{UAS-dPGC-1}.
Ubiquitous over-expression of dPGC-1 using the ActGAL4 driver (ActGAL4>UAS-dPGC-1, black bars) partially rescued the age-dependent climbing defects of control flies. ***p<0.001 when comparing the dPGC-1 over-expressing flies to age-matched control flies response by two-way ANOVA analysis. For example, ****p<0.0001 when comparing ActGAL4>UAS-dPGC-1 over-expressing flies and +>UAS-dPGC-1 flies at day 22 by Tukey’s multiple comparisons test. The genotypes used were: ActGAL4>UAS-dPGC-1, ActGAL4>+, +>UAS-dPGC-1.

I previously demonstrated that over-expression of dPGC-1 extends lifespan in AD flies expressing Aβ (Chapter 3). I hence asked whether longevity would be affected if dPGC-1 is over-expressed in neurons in the absence of Aβ. Interestingly, over-expression of dPGC-1 in adult neurons using the elavGS driver was sufficient to extend lifespan (Figure 5.5, the median lifespan was extended by 6%, p<0.0001), suggesting a broad-spectrum effect of neuronal dPGC-1 on lifespan regulation.
Over-expression of dPGC-1 in adult neurons using the elavGS driver induced in the presence of RU (elavGS>UAS-dPGC-1, RU+, red line) extended the lifespan of flies compared to the un-induced control (RU-, black line). The median lifespan was increased by 6%, P<7 x 10^-5 by log-rank test. The genotypes used were: elavGS>UAS-dPGC-1.

Interventions that extend the lifespan of flies are often associated with resistance to various stressors (Castillo-Quan et al., 2016, Broughton et al., 2005, Afschar et al., 2016). Ablation of PGC-1α in cultured cells down-regulates antioxidants including SOD1 and 2, resulting in hypersensitivity to hydrogen peroxide (H$_2$O$_2$)-induced oxidative stress. Conversely, over-expression of PGC-1α in muscles up-regulates the same genes, suggesting that PGC-1α regulates the antioxidant defence system as well as mitochondrial ROS (St-Pierre et al., 2006). Tissue-specific over-expression of dPGC-1 has been reported to suppress ROS in the gut of flies (Rera et al., 2011), and even when dPGC-1 is ubiquitously over-expressed there is resistance to paraquat-induced oxidative stress. However the levels of SOD1 and SOD2 are not changed in response to dPGC-1 (Mukherjee et al., 2014). I therefore investigated the survival of flies over-expressing dPGC-1 under exposure to H$_2$O$_2$, a potent oxidizer, or paraquat, a redox cycler, which produces superoxide in part through the mitochondria (Cocheme and Murphy, 2008). I found that ubiquitous over-expression of dPGC-1 increased the sensitivity of flies to H$_2$O$_2$-induced oxidative stress (Figure 5.6, p=3 x 10^{-21}). Moreover, dPGC-1 activation did not change the survival of flies exposed to paraquat (Figure 5.7, p=8.5). These results therefore do not support previous findings.
PGC-1α in neurodegeneration and ageing

(Mukherjee et al., 2014), and suggest an increased sensitivity to oxidative stress in response to dPGC-1 over-expression.

Figure 5.6 Ubiquitous over-expression of dPGC-1 increased the sensitivity of female flies to H₂O₂

Ubiquitous over-expression of dPGC-1 throughout development and in adulthood using the ActGAL4 driver (ActGAL4>UAS-dPGC-1, red line) decreased the survival of females flies on food containing 2.5% H₂O₂. P=1.61x 10⁻¹⁸ when comparing ActGAL4>UAS-dPGC-1 and +>UAS-dPGC-1. P=3 x 10⁻²¹ when comparing ActGAL4>UAS-dPGC-1 and ActGAL4>+ by log-rank test. The genotypes used were: ActGAL4>UAS-dPGC-1, ActGAL4>+, +>UAS-dPGC-1.

Figure 5.7 Ubiquitous over-expression of dPGC-1 did not significantly affect the lifespan of flies exposed to paraquat.
Ubiquitous over-expression of dPGC-1 using the ActGAL4 driver (ActGAL4>UAS-dPGC-1; red line, RU+) did not significantly alter the survival of female flies exposed to 10mM paraquat. $P=0.85$ when comparing ActGAL4>UAS-dPGC-1 and ActGAL4>+.

The genotypes used were: ActGAL4>UAS-dPGC-1, ActGAL4>+, +>UAS-dPGC-1.

### 5.3.2 Over-expression of dYY1 extended lifespan in female flies

To investigate whether downstream TFs mediate the lifespan extension of dPGC-1 over-expression, the effects of YY1 (Cunningham et al., 2007), PDP1ε (Cyran et al., 2003), and GABPα (Mootha et al., 2004, Baltzer et al., 2009) on survival were tested. Mammalian YY1 regulates mitochondrial biogenesis by co-activation with PGC-1α in response to mTOR signalling. However, the effect of the Drosophila homologue of YY1 (pho, CG17743) on mitochondrial function and lifespan is not yet known. Here I show that ubiquitous over-expression of dYY1 extended the median lifespan by 6% and maximum lifespan by 12% in female flies (Figure 5.8).

---

**Figure 5.8** Ubiquitous over-expression of dYY1 in adulthood extended the lifespan of female flies

Ubiquitous over-expression of dYY1 in adulthood using the daGS driver and inducing expression with RU (daGS>UAS-dYY1, red line, RU+) increased the lifespan of female flies compared with un-induced controls (black line, RU-). The median lifespan extension was 6%, the maximum lifespan extension was 12%, $P=0.003$ by log-rank test. The genotypes used were: daGS>UAS-dYY1.
PDP1ε is a transcription factor involved in the second feedback loop of the circadian clock and positively regulates the transcription levels of *dCLOCK*. Conversely VRI is the transcription factor that negatively regulates *dCLOCK* (Cyran et al., 2003). Flies lacking *Pdp1ε* display hypersensitivity to oxidative stress (Beaver et al., 2010), whereas over-expression of *Pdp1ε* increases the transcription levels of genes involved in the stress-response pathway such as *DHR96, Xbp1* and *Grp78* (Figure 3.34, 3.36, and 3.37). Disturbances in circadian rhythm and the stress response pathway contribute to ageing (Harper et al., 2005, Taylor and Dillin, 2013, Hipp et al., 2014, Musiek et al., 2013), suggesting that PDP1ε and VRI may be significant modulators of fly ageing. I previously showed that *dPGC-1* and PDP1ε may physically interact in the fly brain. I therefore investigated whether over-expression of *Pdp1ε* modulates longevity. I found that ubiquitous over-expression of *Pdp1ε* did not extend the lifespan of flies (Figure 5.9, p=0.9). Similarly, ubiquitous over-expression of *vri* shortened the lifespan of flies (Figure 5.10, p=0.008). These results therefore suggest that activation of the circadian genes involved in the second feedback loop in *Drosophila* does not extend lifespan.

![Image](image-url)

**Figure 5.9** Ubiquitous over-expression of *Pdp1ε* in adulthood does not extend lifespan in female flies

Ubiquitous over-expression of *Pdp1ε* in adulthood using the daGS driver induced by the presence of RU (*daGS>UAS-Pdp1ε*, red line, RU+) did not affect the lifespan compared to non-induced controls (black line, RU-). P=0.9 by log-rank test. The genotypes used were: *daGS>UAS- Pdp1ε*. 
Ubiquitous over-expression of vri in adulthood using the daGS driver induced by RU (daGS>UAS-vri, blue unbroken line, RU+) shortened the lifespan compared to un-induced female flies (blue broken line, RU-). \( P=0.008 \) by log-rank test. The genotypes used were: daGS>UAS-vri.

**5.3.3 Adult-onset over-expression of \textit{dGABP\textalpha} extended lifespan.**

I then assessed whether direct activation of \textit{dGABP\textalpha} was sufficient to extend lifespan. Adult ubiquitous over-expression of \textit{dGABP\textalpha} resulted in a modest but significant lifespan extension in female flies (Figure 5.11), with an increase in median survival of 8.9\% (\( p=1.93 \times 10^{-8} \)). qRT-PCR confirmed the increase in transcript level of \textit{dGABP\textalpha} following induction with RU486 administration (Figure 5.13). In addition, ubiquitous knockdown of \textit{dGABP\textalpha} using RNAi significantly shortened lifespan in female flies (Figure 5.12, \( p=3.6 \times 10^{-20} \)), and qRT-PCR confirmed the reduced transcript levels of \textit{dGABP\textalpha} by \(~20\%\) in these flies (Figure 5.13). Interventions that extend fly lifespan are often associated with reduced fecundity (Bjedov et al., 2010, Alic et al., 2011b, Broughton et al., 2010, Slack et al., 2015). I therefore assessed whether \textit{dGABP\textalpha} over-expression affects reproductive ability. Although there was a trend towards a reduction in egg laying in response to \textit{dGABP\textalpha} over-expression, this did not reach statistical significance (Figure 5.14). Therefore, our results show that \textit{dGABP\textalpha} is a modulator of longevity.
Over-expression of $dGABP\alpha$ in adulthood extended the lifespan in female flies.

Ubiquitous over-expression of $dGABP\alpha$ in adulthood using the $daGS$ driver induced by RU ($daGS>UAS-dGABP\alpha$, red line, RU+) extended lifespan compared with non-induced controls (black line, RU-). The median lifespan was extended by 9% $p=1.93 \times 10^{-8}$ by log-rank test. The genotypes used were: $daGS>UAS-dGABP\alpha$.

Knockdown of $dGABP\alpha$ in adulthood using RNAi shortened the lifespan of female flies.

Knockdown of $dGABP\alpha$ in adulthood using RNAi ($daGS>UAS-dGABP\alpha^{RNAi}$, red line, RU+) resulted in a decrease in lifespan compared with the un-induced control flies (black line, RU-). The median
PGC-1α in neurodegeneration and ageing

Lifespan was shortened by 13% p=3.6 x 10^{-20} by log-rank test. The genotypes used were: daGS>UAS-dGABPαRNAi.

Figure 5.13 dGABPα expression levels in flies either over-expressing dGABPα or expressing the RNAi construct against dGABPα

(A, B) The mRNA levels of dGABPα, GstD2, and cyp6a8 were determined by qRT-PCR and normalized to a control gene (RP49). The bars are plotted as the means ± SEM, n=4. ***p<0.001, **p<0.01, *p<0.05 by t-test. The genotypes used were: (A) daGS>UAS-dGABPα and (B) daGS>UAS-dGABPαRNAi.

Figure 5.14 Ubiquitous over-expression of dGABPα did not affect fecundity

Ubiquitous over-expression of dGABPα in adulthood using the daGS driver (daGS>UAS-dGABPα, RU+) did not alter the average number of eggs laid per female over 24 hours compared to that of un-induced control flies (-RU) at 12-days of age. The bars represent the means ± SEM, p>0.05 by t-test. The genotypes used were: daGS>UAS-dGABPα.

I then asked whether dGABPα, similar to its mammalian counterpart, is itself downstream of dPGC-1 (Mootha et al., 2004). Indeed I showed that over-expression of dPGC-1 significantly increased the transcript levels of dGABPα (Figure 5.15, p<0.01), suggesting that dGABPα is activated downstream of dPGC-1.
Ubiquitous over-expression of \(dPGC-1\) in adulthood using the \(ActGS\) driver by induction with RU (\(ActGS>UAS-dPGC-1\), red bar, \(RU^+\)) resulted in an up-regulation of \(dGABPa\) gene expression. The mRNA levels of \(dGABPa\) were determined by qRT-PCR and normalized to the control gene (\(RP49\)). The bars are representative of the means ± SEM, \(n=4\). **\(P<0.01\), by t-test. The genotypes used were: \(ActGS>UAS-dPGC-1\).

5.3.4 The tissue-specific over-expression of \(dGABPa\) extended lifespan

The \(Drosophila\) brain, gut, and fat body, the latter functionally being equivalent to the mammalian liver and adipose tissue, have an evolutionarily conserved function in ageing (Fontana et al., 2010). I therefore examined whether confining \(dGABPa\) over-expression to these organs is sufficient to extend lifespan. Using the inducible, pan-neuronal \(elavGS\) driver or the gut- and fat body-specific \(S_{106}\) driver, I over-expressed \(dGABPa\) specifically within these tissues. In each case, induction of expression with RU486 resulted in a significant extension of lifespan (Figures 5.16 and 5.17, median lifespan extensions were 6% and 3%, \(p=4.8 \times 10^{-8}\), \(p=0.002\), respectively). Taken together, these data clearly show that over-expression of \(dGABPa\) in the brain as well as in the gut and fat body is sufficient to extend lifespan.
Figure 5.16 Over-expression of \(dGABP\alpha\) in adult neurons extended the lifespan in female flies

Neuronal expression of \(dGABP\alpha\) in adulthood using the \(elavGS\) driver induced by RU (\(elavGS>UAS-dGABP\alpha\), red line, RU+) extended the lifespan compared to non-induced control flies (black line, RU-). The median lifespan was extended by 6%, \(p=4.8 \times 10^{-8}\) by log-rank test. The genotypes used were: \(elavGS>UAS-dGABP\alpha\).

Figure 5.17 Over-expression of \(dGABP\alpha\) in the adult gut and fat body extended the lifespan in female flies

Over-expression of \(dGABP\alpha\) in the adult gut and fat body using the \(S_{106GS}\) driver induced by RU (\(S_{106GS}>UAS-dGABP\alpha\), red line, RU+) extended the lifespan compared with un-induced female flies (black line, RU-). The median lifespan was extended by 3% \(p=0.003\) by log-rank test. The genotype used was: \(S_{106GS}>UAS-dGABP\alpha\).
5.3.5 Energy production was uncoupled from energy consumption in dGABPα-over-expressing flies

Long-lived invertebrate mutants often display a reduction in the rate of cellular functions, with a slowing in energy metabolism, and a lower accumulation of age-related stress. This manifests in resistance to various stressors, including oxidative stress and xenobiotic stress (Castillo-Quan et al., 2016, Broughton et al., 2005, Afschar et al., 2016, Braeckman et al., 1999, Lakowski and Hekimi, 1996). Animal models lacking dGABPα exhibit mitochondrial dysfunction (Baltzer et al., 2009, Yang et al., 2014). It is therefore interesting to assess whether dGABPα gain-of-function leads to an improvement in mitochondrial function. To test this hypothesis, I determined whether up-regulation of dGABPα increased mitochondrial activity by measuring four independent mitochondrial markers: ATP production, mitochondrial mass, mitochondrial protein synthesis and oxygen consumption. Firstly, I found that over-expression of dGABPα significantly increased ATP production by approximately 50% (Figure 5.18), suggesting an increased steady state of mitochondrial homeostasis. I then measured the amount of mtDNA, relative to the amount of the nuclear DNA (nDNA) amplicon, in heads and thoraxes of young and old flies expressing dGABPα. Interestingly, there was no significant difference in mitochondrial mass between dGABPα-over-expressing flies and wild type controls at either young or old age. There was however a significant increase in aged dGABPα-over-expressing flies compared to their younger counterparts (Figure 5.19). In addition, immunoblots with antibodies against NDUFS3, one of the complex I subunits of the ETC, did not show any significant difference between flies over-expressing dGABPα and un-induced controls (Figure 5.20).

Figure 5.18 ATP levels were increased in dGABPα-over-expressing flies.
Ubiquitous over-expression of \(dGABP\alpha\) using the \(daGS\) driver induced with RU (\(daGS>UAS-dGABP\alpha\), blue bar, RU+) resulted in increased ATP levels compared to un-induced control female flies (red bar, RU-). ATP levels were measured in 40-day-old whole fly lysates with the ovaries removed. The bars represent the means ± SEM (n=6), *p<0.05 by t-test. The genotype used was: \(daGS>UAS-dGABP\alpha\).

**Figure 5.19 Over-expression of \(dGABP\alpha\) did not alter the mitochondrial mass in young or older flies**

Mitochondrial DNA (mtDNA), relative to the nuclear DNA (nDNA) amplicon, was measured by qRT-PCR in the heads and thoraxes of flies over-expressing \(dGABP\alpha\) (+RU) and un-induced controls (-RU) at 12 or 40 days of age. A significant increase was observed in old flies over-expressing \(dGABP\alpha\) (\(daGS>UAS-dGABP\alpha\), blue bar, RU+) compared to young flies over-expressing \(dGABP\alpha\) (red bar). The bars represent the means ± SEM (n=4, *p<0.05 by ANOVA). The genotype used was: \(daGS>UAS-dGABP\alpha\).

**Figure 5.20 Over-expression of \(dGABP\alpha\) did not alter NDUFS3 protein levels**
Western blot analysis of NDUFS3 in 14-day-old flies demonstrated that ubiquitous over-expression of dGABPα using the daGS driver induced by RU486 (daGS>UAS-dGABPα, red bar, RU+) did not alter the protein levels of NDUFS3 compared to un-induced controls (black bar RU-). The protein levels were normalized to β-actin. The bars represent the means ± SEM (n=3). The image is a representative gel from one biological repeat. The genotype used was: daGS>UAS-dGABPα.

Finally, I sought to analyse the effect of elevated dGABPα expression on mitochondrial respiratory chain activity. The rate of oxygen consumption was measured using the Oroboros Oxygraph-2k on fly heads rather than whole fly lysates, enabling a more defined and tissue-specific signal. The steps in respiration were compared in mitochondria separated from dGABPα-over-expressing fly head homogenates and controls by using substrates, uncouplers, and inhibitors specific to individual respiratory complexes. In doing so, I observed there was no significant difference in the oxygen consumption rate (OCR) for Complex I-, and II-dependent respiration (Figure 5.21). Moreover, there was no significant difference in OCR in state 2, state 3, or in the maximum uncoupled state between flies over-expressing dGABPα and controls (Figure 5.21). This is consistent with the observations of mitochondrial mass and mitochondrial-related protein levels, suggesting that over-expression of dGABPα does not change energy consumption. Taken together, these data suggest that over-expression of dGABPα increases ATP production, but this is uncoupled from mitochondrial respiration rates.
Oxygen consumption rate (OCR) were measured in the heads of flies over-expressing dGABPα (RU+) and un-induced controls (RU-) at day 14. There was no significant difference in OCR in state 2, state 3, and in the maximum uncoupled state, or for Complex I- and II-dependent respiration. The values are plotted as the means ± SEM, n=5, p>0.05 by t-test. The genotype used was: elavGS>UAS-dGABPα. These experiments were performed in collaboration with Dr. Vassilios N Kotiadis.

5.3.6 Over-expression of dGABPα increased the resistance to phenobarbital stress

I then assessed whether the long-lived flies over-expressing dGABPα were resistant to different stressors. I tested the survival of dGABPα-over-expressing flies under starvation conditions and on exposure to oxidative stress with H2O2 and paraquat, and phenobarbital. Here we show that dGABPα over-expression did not significantly affect survival compared to controls under starvation conditions or when challenged with H2O2 (Figure 5.22, p=0.3, p=0.7 respectively). However, flies over-expressing dGABPα exhibited a small but significant increase in sensitivity to paraquat (Figure 5.22, p<0.05). In addition, flies over-expressing dGABPα were significantly resistant...
to phenobarbital stress (Figure 5.22, p=1.75 x 10^{-11}). Taken together these results suggest that dGABPa may extend lifespan through increased detoxification of endobiotic and xenobiotic compounds.

Figure 5.22 The effect of ubiquitous over-expression of dGABPa in adulthood on exposure to various stressors in female flies

The survival curves of flies over-expressing dGABPa in adulthood (RU+) or un-induced control flies (RU-) using the inducible daGS driver. Flies were pre-treated with RU for 14 days and then transferred to either agar-only food for starvation assays (p=0.7), or to sugar/agar food supplemented with paraquat (p=0.04), H₂O₂ (p=0.33) or phenobarbital (p=1.75 x 10^{-11} by log-rank test). The genotype was: daGS>UAS-dGABPa.

Increased resistance to phenobarbital stress in dGABPa over-expressing flies may rely on the UPS (Ubiquitin-Proteasome System), the lysosomal-autophagy pathway, and the chaperone network, acting to maintain cellular protein homeostasis by removing unwanted or damaged proteins that could become toxic to cells (Jung et al., 2009, Glickman and Ciechanover, 2002, Ross et al., 2015, Lopez-Otin et al., 2013). I therefore investigated whether over-expression of dGABPa affects proteasomal activity and autophagy. Here I show that the 26S proteasomal activity was significantly increased in flies over-expressing dGABPa compared to controls (Figure 5.23). Immunoblots with antibodies against the alpha subunit of the 26S proteasome complexes demonstrated that levels of the 26S were not altered in flies over-expressing dGABPa compared to controls (Figure 5.24). This suggests that over-
expression of \( dGABP\alpha \) may increase the assembly of the proteasome and therefore its activity, rather than the total load of the proteasomal machinery. In addition, western blot analysis with an antibody against the fly LC-3 orthologue, Atg8, demonstrated no significant change in Atg8-I levels or Atg8-II levels in response to \( dGABP\alpha \) over-expression (Figure 5.25). Even the ratio of Atg8-II/Atg8-I was not changed, indicating no change in autophagosome formation (Figure 5.25). Taken together, over-expression of \( dGABP\alpha \) increased proteasome activity but not autophagy.

**Figure 5.23 Over-expression of \( dGABP\alpha \) increased proteasome activity**

Proteasome activity, as measured using the fluorogenic peptide substrate LLVY-AMC, was increased in flies over-expressing \( dGABP\alpha \) (\( daGS>UAS-dGABP\alpha \), red bar, +RU) compared to non-RU induced controls (blue bar, RU-). The bars are plotted as the means of activity (pmol/min/mg protein) ± SEM. *\( p<0.05 \), n=6 by t-test. The genotype used was: \( daGS>UAS-dGABP\alpha \).
PGC-1α in neurodegeneration and ageing

Figure 5.24 Ubiquitous over-expression of dGABPα in adulthood did not alter the protein levels of the 26S proteasome

Western blot analysis with an antibody against the alpha subunit of the 26S proteasome demonstrated no significant change in the amount of the alpha subunit protein in dGABPα-over-expressing flies (daGS>UAS-dGABPα, red bar, +RU) compared with age-matched non-RU induced control flies (blue bar, RU-). The bars represent the means ± SEM, n=3 by t-test. The genotype used was: daGS>UAS-dGABPα.

Figure 5.25 Flies over-expressing dGABPα did not show any change in autophagy

Western blot analysis with an antibody against the fly LC-3 orthologue, Atg8, demonstrated no significant change in Atg8-I levels (normalized to β-actin levels), Atg8-II levels or the Atg8-II/Atg8-I
ratio in flies over-expressing \( dGABP\alpha \) (\( daGS>UAS-dGABP\alpha \), red bars, RU+) compared with age-matched non-RU induced control flies (green bars, RU-) at day 14. The bars represent the means ± SEM, n=3 by t-test. The genotype used was: \( daGS>UAS-dGABP\alpha \).

5.3.7 Over-expression of \( dGABP\alpha \) extended the lifespan via mechanisms beyond those of DR

To explore the mechanisms of lifespan extension by \( dGABP\alpha \) activation, I first investigated whether over-expression of \( dGABP\alpha \) was acting as a DR mimetic. DR is a well-established anti-ageing intervention that extends healthy lifespan in diverse species (Fontana and Partridge, 2015, de Cabo et al., 2014), and some genetic interventions that extend lifespan have features of DR mimetics (Ingram and Roth, 2015, de Cabo et al., 2014). To determine whether \( dGABP\alpha \) and DR extend lifespan by similar mechanisms, I assessed whether \( dGABP\alpha \) can extend lifespan beyond the maximum achievable by DR. To maximize lifespan under DR, the yeast concentrations were varied in the food, while maintaining a constant concentration of sucrose (Bass et al., 2007), resulting in a typical tent-shaped response, with a peak median lifespan with food containing a 1.0 yeast concentration (Figure 5.26 A and B). If \( dGABP\alpha \) activation and DR share overlapping pathways, then activation of \( dGABP\alpha \) would not be able to further extend lifespan already maximized by DR (Castillo-Quan et al., 2016, Gems et al., 2002, Broughton et al., 2010, Bjedov et al., 2010). Activation of \( dGABP\alpha \) significantly extended median lifespan in the yeast condition that maximized lifespan (1.0 yeast; \( p=1.93 \times 10^{-8} \)), in full feeding (2.0 yeast; \( p=3.9 \times 10^{-5} \)), and under reduced yeast concentrations (0.5 yeast; \( p=2.94 \times 10^{-7} \)), with the greatest extension of median lifespan seen using 2.0 SY food (Figures 5.26 A and B). Cox proportional hazards (CPH) analysis confirmed that the response to RU was not significantly different across the different yeast concentrations on the right side of the DR-tent (Table 3, \( p=0.11 \)), suggesting that over-expression of \( dGABP\alpha \) extended the lifespan via mechanisms independent to those of DR and beyond DR. Interestingly, \( dGABP\alpha \) over-expression significantly reduced lifespan (Figure 5.26 A and B, \( p=3.27 \times 10^{-14} \)) on the ‘starvation-inducing’ yeast concentration of 0.1 SY food. CPH analysis confirmed that the response to RU was modulated by food on the ‘starvation side’ of the DR tent (Table 2, \( p=0.02 \)), suggesting that the effect of over-expression of \( dGABP\alpha \) on lifespan is dependent on nutrient-availability.
Figure 5.26 Ubiquitous over-expression of dGABPa extended the lifespan via mechanisms independent of those of DR

(A) The survival curves of female flies ubiquitously over-expressing dGABPa in adulthood using the daGS driver induced by RU (daGS>UAS-dGABPa, RU+, full lines) compared to non-induced controls (RU-, dash lines). The RU was supplemented into food containing different yeast concentrations (0.1x, 0.5x, 1.0x, and 2.0x yeast concentrations). (B) The median lifespans are plotted at four different yeast concentrations: over-expression of dGABPa extended lifespan under the 0.5x, 1.0x, and 2.0x dietary
conditions tested. Over-expression of dGABPa significantly shortened the lifespan on the 0.1x yeast containing diet. ***P<0.001 by log-rank test; n=145 flies per condition. The genotype used was: daGS>UAS-dGABPa.

Table 2 CPH analysis of the survival of flies over-expressing dGABPa on different yeast concentrations of food

CPH analysis showed that there is an interaction between the response to different diets and the response to over-expression of dGABPa (p=0.02). This means that the response to RU (absence or presence of dGABPa over-expression) is modulated by diet. Flies fed with ‘starvation-inducing’ low yeast food (0.1SY) were shorter lived in response to dGABPa over-expression, while flies fed with any other diets were longer lived in response to dGABPa over-expression. Therefore the responses to RU across all the diets are not in same direction (p=0.15).

Table 3 CPH analysis of the survival of flies over-expressing dGABPa on DR and full-feeding food

CPH analysis showed that there is no interaction between the response to different diets (DR food and full-feeding food) and the response to over-expression of dGABPa (p=0.11). This means that the response to RU is not modulated by food. Therefore over-expression of dGABPa extends lifespan independently of DR and beyond DR.

5.3.8 Next-generation Transcriptome profiling

In order to determine the molecular mechanisms by which dGABPa over-expression mediates its pro-longevity effects, I undertook next generation sequencing to analyse the transcript profile in flies over-expressing dGABPa. RNAseq analysis was
performed at the Glasgow Polyomics Unit, and the bioinformatics analysis was performed in collaboration with Dr. Dobril Ivanov. Given that the brain specific over-expression of dGABPα using the inducible elavGS driver significantly extended lifespan (Figure 5.16), I sought to investigate the brain specific transcriptional response to dGABPα over-expression. Fly heads over-expressing dGABPα for 10 days were subjected to RNAseq analysis and compared to non-RU induced controls, and 4 replicates each condition were used. According to FDR correction for multiple hypothesis testing, which is an acronym for adjusted p-value, there is around 50 differentially expressed genes in response to dGABPα over-expression, including genes involved in memory, learning, synaptic formation and in the perception of sound and pain (Figure 5.27). For example, SIFαR (SIFamide receptor) was up-regulated in response to dGABPα over-expression. This is a G-protein coupled peptide receptor involved in both neuropeptide and the G-protein coupled receptor signalling pathways. It is required for the sensory perception of pain and is involved in sexual behaviour and the determination of lifespan (Figueroa-Clarevega and Bilder, 2015, Paik et al., 2012). In addition, dGABPα regulated several genes involved in insulin signalling pathway, including up-regulated nord, daw, ImpL2, and down-regulated Glt, tobi, and Gnmt, suggesting that over-expression of dGABPα might regulate IIS.
Figure 5.27 Heat-map of the genes differentially expressed in fly heads over-expressing dGABPa

Heat-map showing genes that were significantly up-regulated (red colour) or down-regulated (blue colour) in fly heads over-expressing dGABPa (RU+) compared to non-induced controls (RU-). The intensity of the color represents the statistical significance. N=4 per condition, P<0.05 for statistically
significant changes according to multiple hypothesis testing. Genotypes used were: \textit{elavGS>UAS-dGAPBa}.

I then sought to look at the gene ontology (GO) annotations using Catmap analysis (Breslin et al., 2004). GO categories include three classes: Biological Process ontology, Cellular Component ontology and Molecular Function ontology. The Biological Process ontology includes terms that represent collections of processes as well as terms that represent a specific, entire process that the gene product is involved in. The Cellular Component ontology describes locations, at the levels of subcellular structures and macromolecular complexes. The Molecular Functions of a gene product are the functions that it performs or the "properties" that it has. These may include transporting molecules around, binding to molecules, holding molecules together and changing one molecule into another. This is different from the Biological Processes, which involve more than one activity. Down-regulated GOs mean that the results represent GO categories are significantly enriched in genes that are mainly down-regulated, while up-regulated GO categories are enriched in genes that are predominantly up-regulated. For GO analysis, all genes were used along with p-values (not-adjusted for multiple hypothesis testing), and the statistical significance of the GO categories is calculated using p-value-adjusted FDR correction for multiple hypothesis testing. Here I have listed the top 10 GO categories in each class (Figure 5.28). For example, over-expression of \textit{dGAPBa} up-regulated biological processes including ‘development’, ‘regulation of signalling pathway’, and ‘regulation of response to stimulus’, while down-regulated biological processes including ‘nitrogen compound metabolic process (cellular amino acid metabolic process)’, ‘translation’, and ‘oxidation-reduction process’. In terms of molecular function, over-expression of \textit{dGAPBa} up-regulated ‘transcription factor activity’ and ‘protein kinase activity’, while down-regulated ‘ribosome’, ‘translation activity’, and ‘electron carrier activity’. In addition, over-expression of \textit{dGAPBa} up-regulated cellular components mainly involved in membrane, neurons, synapses and axon, while down-regulated GO terms involved in mitochondrion, respiratory chain complex, lipid particle and ribosome.
Figure 5.28 GO categories of fly heads over-expressing dGAPBa
The graphs show the top 10 GO categories that significantly enriched in genes that are mainly up-regulated (left side) or down-regulated (right side) in response to dGAPBa over-expression in fly heads using inducible elavGS driver (RU+) compared to non-induced control (RU−). GO terms are classified into 3 different groups, including Biological Process (blue colour), Molecular Function (orange colour) and Cellular Components (green colour). GO annotations have been analyzed using Catmap, and calculated using p-value-adjusted FDR correction for multiple hypothesis testing. N=4 per condition, p<0.05 for statistically significance. Genotypes used were: elavGS>UAS-dGAPBa.

5.3.9 Over-expression of dGABPa extended lifespan in overlapped mechanisms with reduced IIS likely through up-regulation of ImpL2
As described above, overexpression of dGAPBa regulates several genes involved in insulin signalling pathway. Among those differentially expressed genes that were up-
regulated in response to \(dGABP\alpha\) over-expression, ImpL2 is particularly interesting. ImpL2 (Imaginal morphogenesis Protein-late 2), the ortholog of the mammalian insulin-like growth factor-binding protein 7 (IFGBP7), resembles IGFBP7 in sequence and acts as a negative regulator of IIS during development, regulating growth cell-non-autonomously (Sloth Andersen et al., 2000, Alic and Partridge, 2008, Honegger et al., 2008). ImpL2 is a secreted protein belonging to the immunoglobulin superfamily that forms a protein complex with the circulating dilps, and inhibits their ability to bind the insulin receptor, thus reducing IIS (Sloth Andersen et al., 2000, Alic et al., 2011b). In addition, a modest over-expression of \(ImpL2\) extends lifespan in association with reduced IIS (Alic et al., 2011b, Paik et al., 2012). Therefore, I hypothesised that over-expression of \(dGABP\alpha\) may mediate lifespan extension through mechanisms that up-regulate \(ImpL2\) to down-regulate IIS. Although the direct evidence showing that ImpL2 mediates the lifespan extension of \(dGABP\alpha\) over-expression is still lacking, it is possible that activation of \(dGABP\alpha\) may reduce insulin signalling and therefore affect IIS-related traits.

It is known that down-regulation of IIS extends lifespan in diverse organisms (Piper et al., 2008). To further assess whether \(dGABP\alpha\) activation extends lifespan through reduced IIS, I investigated whether \(dGABP\alpha\) over-expression can further extend the lifespan of flies that already have reduced IIS. To do this I tested the lifespan effect of \(dGABP\alpha\) over-expression on flies co-expressing the \(INR^{DN}\) (Slack et al., 2011). Adult-onset, ubiquitous over-expression of either \(dGABP\alpha\) or the \(INR^{DN}\) significantly extended lifespan (Figure 5.29, the median lifespans were increased by 7% and 9.8%, \(p = 3.04 \times 10^{-7}\) and \(p = 0.0001\), respectively). In addition, co-expressing \(dGABP\alpha\) and \(INR^{DN}\) also extended lifespan (Figure 5.29, the median lifespan extension was 9.7%, \(p = 3 \times 10^{-8}\)). To compare the extent of lifespan extension between the two interventions, CPH analysis was used. The lifespan extension observed in the \(INR^{DN}\) flies was not significantly different to that in the \(dGABP\alpha\) over-expressing flies in the response to RU486 (Table 4, RU486 by genotype interaction, \(p = 0.76\)). CPH analysis also confirmed that there was no significant difference between the lifespan extension in \(INR^{DN}\) expressing flies and \(dGABP\alpha-INR^{DN}\) co-expressing flies (Table 4, RU486 by genotype interaction, \(p = 0.43\)), suggesting that \(dGABP\alpha\) over-expression and reduced IIS signalling may mediate lifespan extension via overlapping mechanisms.
Figure 5.29 Female flies over-expressing INR<sup>DN</sup> and dGABPα mediated lifespan extension

The survival curves of flies over-expressing either the dominant negative form of the insulin receptor (INR<sup>DN</sup>, black line), or dGABPα (red line), or both INR<sup>DN</sup> and dGABPα (green line, +RU) and non-RU induced controls (broken lines, -RU) are shown. Ubiquitous over-expression of dGABPα extended lifespan (p=3.04 x 10<sup>-7</sup>). Ubiquitous over-expression of a dominant negative form of INR also extended lifespan (p=0.0001), and co-expression of both dGABPα and the INR<sup>DN</sup> also extended lifespan (p= 3 x 10<sup>-8</sup>). The lifespans were analyzed by the log-rank test. N=130 flies per condition. The genotypes used were: daGS>UAS-INR<sup>DN</sup>, daGS>UAS-dGABPα, daGS> UAS-INR<sup>DN</sup>; UAS-dGABPα.

| n = 773, number of events= 755 |

| dataC$RU$ | coef | exp(coef) | se(coef) | z  | Pr(>|z|) |
|-----------|------|-----------|----------|----|---------|
| dataC$S$genotypedaDELG | -0.70476 | 0.49423 | 0.10625 | -4.239 | 2.24e-05 | *** |
| dataC$S$genotypedaINRDDELG | -0.13434 | 0.87429 | 0.16429 | -0.818 | 0.414 |
| dataC$SRU$;dataC$S$genotypedaINRDDELG | -0.71837 | 0.48755 | 0.16581 | -4.333 | 1.47e-05 | *** |
| dataC$SRU$;dataC$S$genotypedaINRDDELG | 0.85181 | 1.86376 | 0.28392 | 0.383 | 0.762 |
| dataC$SRU$;dataC$S$genotypedaInRDNDDELG | 0.16112 | 1.17483 | 0.20340 | 0.792 | 0.428 |

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table 4 CPH analysis of the survival of flies co-expressing dGABPα and INR<sup>DN</sup>

CPH analysis demonstrated that three genotypes (daGS>UAS-INR<sup>DN</sup>, daGS>UAS-dGABPα, daGS> UAS-INR<sup>DN</sup>; UAS-dGABPα) responded to RU in the same way (p=2.24 x 10<sup>-5</sup>). There was no significant difference between the genotypes daGS>UAS-INR<sup>DN</sup> and daGS>UAS-dGABPα (p=0.414). There was a significant difference between the genotypes daGS>UAS-INR<sup>DN</sup> and daGS> UAS-INR<sup>DN</sup>; UAS-dGABPα (p=1.47 x 10<sup>-5</sup>). There was no significant difference between the genotypes daGS>UAS-INR<sup>DN</sup> and daGS> UAS-dGABPα in response to RU (P=0.762). There was also no significant difference between daGS>UAS-INR<sup>DN</sup> and daGS> UAS-INR<sup>DN</sup>; UAS-dGABPα in response to RU (P=0.428). This
means that the three genotypes are responsive to RU in a similar way, suggesting that over-expression of $dGABPa$ and over-expression of $INR^{DN}$ extended lifespan via similar mechanisms.

5.3.10 $dGABPa$ over-expression and reduced IIS in flies shared overlapping transcriptional responses

I next investigated whether the transcriptional response to $dGABPa$ over-expression is similar to that observed when IIS is genetically down-regulated. I used a recently published microarray analysis performed on whole flies over-expressing the dominant negative form of the insulin receptor, $INR^{DN}$, as a means of reducing IIS (Alic et al., 2011a). I compared the overlap of genes elicited by both interventions, and detected statistical significance for down-regulated genes (Figure 5.30 A, p=0.004). By comparing the overlap of GO categories elicited by both interventions, I found the two most significant overlapping enriched GO terms in both interventions are either up-regulated or down-regulated (Figure 5.30 B, p=6.5 x 10^{-87} and p=1.5 x 10^{-12} respectively), suggesting that these two interventions share highly overlapped transcriptional response in same direction. There was also a significant overlap between GOs down-regulated by lowering IIS and those up-regulated by over-expressing $dGABPa$ (Figure 5.30 B, p=8.54 x 10^{-11}), suggesting that they may regulate some of the transcriptional responses in opposite ways.
PGC-1α in neurodegeneration and ageing

Figure 5.30 Comparative analyses of the transcriptional response of IIS down-regulation and dGABPα over-expression.

(A) Venn diagrams showing overlap between genes regulated by IIS down-regulation via the over-expression of a dominant negative version of InR (Blue color), and the transcriptional response to dGABPα over-expression (red color). P<0.05. The heat map shows genes that were overlapped between the two interventions.

(B) Venn diagrams demonstrating overlapping enriched GO categories regulated by IIS down-regulation (yellow color) and dGABPα over-expression (green color). P<0.05. The bioinformatics analysis was performed by Dr. Dobril Ivanov.

5.3.11 dGABPα over-expression mediated the lifespan extension of reduced IIS signalling in flies

The long-term beneficial effect of modest reductions in IIS on lifespan is largely dependent on the fine-tuning of the IIS down-regulation and the potential tissue and systemic adaptation. However, when dGABPα is genetically knocked-down, ImpL2 maybe down-regulated, which would reduce binding to the circulating dilps, leading to increased binding to the insulin receptor. Continuous ligand stimulation of the insulin receptor and the resulting increase in IIS activity can be harmful, because it eventually leads to tissue insensitivy to the ligand, which is referred to insulin resistance. I previous showed that down-regulation of dGABPα increased the early mortality, which mechanisms might be associated with a constant insulin receptor stimulation and resulting “insulin resistance”. Down-regulation of IIS through the co-expression of a dominant negative form of the insulin receptor may further exacerbate
the \( dGABP\alpha \)-mediated insulin resistance, accelerating mortality. In keeping with this, I showed that adult-onset ubiquitous over-expression of the \( INR^{DN} \) significantly extended lifespan (Figure 5.31, \( p=0.002 \)), and RNAi-mediated ubiquitous knockdown of \( dGABP\alpha \) shortened lifespan (Figure 5.31, \( p=0.005 \)). Co-expression of \( dGABP\alpha^{RNAi} \) in flies expressing \( INR^{DN} \) shortened lifespan (Figure 5.31, \( p=4.8 \times 10^{-7} \)) even further than flies expressing \( dGABP\alpha^{RNAi} \) alone (\( p=0.01 \)). This result suggested that \( dGABP\alpha \) is at least partially required for the lifespan extension of reduced IIS, and that reduction of the insulin receptor, and hence IIS signalling, further exacerbates the negative effect of knocking down of \( dGABP\alpha \).

![Figure 5.31 dGABPα mediated the lifespan extension of reduced IIS](image)

The survival curves of flies over-expressing either \( INR^{DN} \) (black line, RU+) or \( dGABP\alpha^{RNAi} \) (green line, RU+), or both \( INR^{DN} \) and \( dGABP\alpha^{RNAi} \) (red line, +RU) and non-RU induced controls (broken lines, RU-). Ubiquitous RNAi-mediated down-regulation of \( dGABP\alpha \) using the \( daGS \) driver shortened the lifespan (\( p=0.005 \)), and abolished the lifespan extension mediated by over-expression of a dominant negative form of \( INR \) (\( p=4.8 \times 10^{-7} \)). Over-expression of the dominant negative form of the \( INR \) extended lifespan (\( p=0.002 \)), but further shortened the lifespan when \( dGABP\alpha \) was knocked-down using RNAi (\( p=0.01 \)). The lifespans were analyzed by using the log-rank test. \( N=150 \) flies per condition. The genotypes used were: \( daGS>UAS-INR^{DN}, daGS>UAS-dGABP\alpha^{RNAi}, daGS>UAS-INR^{DN}; UAS-dGABP\alpha^{RNAi} \).

### 5.3.12 Over-expression of \( dGABP\alpha \) reduced AKT signalling

Reducing IIS inhibits the PI3K-PDK1-AKT signalling pathway and activates FOXO, a transcription factor that up-regulates several “longevity pathways” (Webb and...
Brunet, 2014, Wang et al., 2014). To examine whether over-expression of \(dGABP\alpha\) reduces systemic insulin signalling, I firstly measured the levels of phosphorylated AKT (p-AKT), a downstream target of the insulin receptor, by western blot analysis. Given the lack of an antibody recognizing the PDK1 phospho-site on AKT, I evaluated the phosphorylation status at the S505 site, which is phosphorylated by mTORC2 (Guertin and Sabatini, 2009). Previous studies have used this antibodies as a readout of insulin activity (Palu and Thummel, 2016). This analysis demonstrated that over-expression of \(dGABP\alpha\) caused a modest but significant reduction in the levels of phosphorylated AKT normalized to total AKT (Figure 5.32, \(p=0.03\)), suggesting reduced activity of the IIS pathway.

![Figure 5.32 Over-expression of \(dGABP\alpha\) reduced systemic insulin signalling](image)

5.3.13 \(dGABP\alpha\) over-expression inhibited mTORC1 and mimicked the effect of rapamycin on lifespan

The transcriptional profiling showed that over-expression of \(dGABP\alpha\) significantly down-regulated the biological functions including amino acid metabolism and translation, which both are tightly linked with reduced mTOR signalling pathway.
The mTOR signalling pathway regulates ageing by mechanisms that overlap with IIS (Johnson et al., 2013). mTORC1 is activated by insulin through PI(Powers et al.)K and AKT kinase signalling (Laplante and Sabatini, 2012). I demonstrated above that over-expression of dGABPα inhibited AKT kinase activity. I therefore assessed whether mTORC1 activity is altered by dGABPα activation. To do this I measured the phosphorylation levels of S6K, a well-described downstream target of TORC1, using western blot analysis with the phospho-Thr398-dependent S6K antibody. I observed a significant reduction in phospho-T398-S6K levels in response to dGABPα over-expression (Figure 5.33), confirming that dGABPα inhibits mTORC1 activity in vivo.

**Figure 5.33** The ubiquitous over-expression of dGABPα reduced the phosphorylation levels of S6K

Whole fly lysates from flies over-expressing dGABPα ubiquitously in adulthood (daGS>UAS-dGABPα, red bar, +RU) demonstrated reduced levels of S6K phosphorylation compared to non-induced controls (blue bar, RU-). Western blots were performed using antibodies directed against phosphorylated S6K (p-S6K), total S6K and β-actin as a loading control. Animals were collected at two weeks of age. The same samples were loaded onto separate gels for blotting either p-S6K or total S6K. The levels of p-S6K and t-S6K were measured using ImageJ and normalized to same-gel loading control. The ratio of p-S6K levels to total S6K levels was then calculated. Bars represent the means ± SEM, n=3. **P=0.004 using the t-test. A representative gel is shown for the loading control β-actin, which was derived from the p-S6K gel. The genotype used was: daGS>UAS-dGABPα.
I next investigated whether the lifespan extension of \( dGABP\alpha \) is mediated by mTORC1 signalling. Rapamycin inhibits mTORC1 activity and extends lifespan by mechanisms that include reduced mRNA translation and autophagy induction (Bjedov et al., 2010). I therefore assessed whether the lifespan extension mediated by \( dGABP\alpha \) over-expression mimics the effects of reduced mTORC1 activity. To do this I observed whether \( dGABP\alpha \) over-expression can further extend the lifespan extension mediated by rapamycin treatment. I first confirmed that induction of \( dGABP\alpha \) with RU extended the lifespan compared to un-induced controls (Figure 5.34, the median lifespan was extended by 6%, \( p=0.03 \)). I then used two concentrations of rapamycin, 50 and 200\( \mu M \), both of which have been shown to mediate lifespan extension through a dose-dependent reduction of S6K activity, the most effective dose being 200\( \mu M \) (Bjedov et al., 2010). I showed that both 50 and 200\( \mu M \) of rapamycin extended lifespan in a dose-dependent manner (Figure 5.34, the median lifespan was extended by 16%, and 25%, \( p= 2.29 \times 10^{-14} \), \( p= 1.52 \times 10^{-28} \) respectively by log-rank test). However no further extension occurred when the rapamycin-flies over-expressed \( dGABP\alpha \) (for rapamycin 50\( \mu M \): \( p=0.24 \) and for rapamycin 200\( \mu M \): \( p=0.67 \) by log-rank test), suggesting that \( dGABP\alpha \) acts in a similar manner to rapamycin. Indeed, 200\( \mu M \) rapamycin-treated flies over-expressing \( dGABP\alpha \) showed reduced survival compared to 200\( \mu M \) rapamycin-treated controls (the median lifespan was shortened by 5%, \( p=0.046 \) by Wilcoxon test), suggesting a toxic effect likely due to the excessive inhibition of mTOR that is detrimental to the lifespan of the flies. Surprisingly, the CPH analysis failed to detect any significant interaction between the response to RU and the response to rapamycin 50\( \mu M \) (Table 5, \( p=0.47 \)), likely due to the limitation of the test. Taken together, these results demonstrated that over-expression of \( dGABP\alpha \) may act via similar mechanisms of action with rapamycin/mTORC1 in regulating of lifespan, likely through inhibition of S6K.
Ubiquitous over-expression of dGABPa extended lifespan (p=0.03 by log-rank test). Rapamycin treatment (50µM) also extended lifespan compared to non-treated controls (p=2.29 x 10^{-14}), and no further extension occurred with the over-expression of dGABPa (p=0.24 by log-rank test). Treatment with a higher dose of rapamycin (200µM) extended lifespan, to a greater extent than 50µM, compared to non-treated controls (p= 1.52 x 10^{-25}), but the lifespan was shortened when dGABPa was over-expressed in the rapamycin-treated flies (p=0.67 by log-rank test and p=0.046 by Wilcoxon test). N=140 flies per condition. The lifespans were analyzed using the log-rank test or Wilcoxon test. The genotype used was: daGS>UAS-dGABPa.

### Table 5 The CPH analysis of the survival of rapamycin-treated flies co-expressing dGABPa

|        | coef | exp(coef) | se(coef) | z     | Pr(>|z|) |
|--------|------|-----------|----------|-------|----------|
| dataC$RU | -0.2410 | 0.7859      | 0.1226   | -1.966 | 0.0493   *  |
| dataC$RAPA | -0.8878 | 0.4124      | 0.1249   | -7.910 | 2.5e-15 *** |
| dataC$RU:dataC$RAPA | 0.1234 | 1.1314      | 0.1737   | 0.711  | 0.4773   |

Signif. codes:  0 '***'  0.001 '**'  0.01 '*'  0.05 '.'  0.1 ' ' 1

**5.3.14 dGABPa modulated intestinal homeostasis**

The *Drosophila* intestine is an accessible model system to study tissue homeostasis in ageing (Biteau et al., 2010). Intestinal stem cells (ISCs) in the gut drive cell division
in response to environmental stress (Choi et al., 2008, Biteau et al., 2008, Jiang et al., 2009, Cronin et al., 2009, Buchon et al., 2009, Amcheslavsky et al., 2009). However, age-related over-proliferation of ISCs has deleterious consequences with accumulation of mis-differentiated cells ultimately contributing to the disruption of epithelial integrity and to the increased mortality of ageing flies (Choi et al., 2008, Biteau et al., 2008). Previous studies have shown that limiting cell proliferation rates by moderately reducing IIS/mTOR signalling in stem cells, is sufficient to delay intestinal dysplasia, improving metabolic health and extending lifespan (Biteau et al., 2010, Regan et al., 2016). Since over-expression of dGABPa extends female lifespan in the mechanisms associated with a reduction in IIS/mTOR signalling, I hypothesised that improvements in gut homeostasis may be responsible for some of the beneficial effects of dGABPa over-expression. Therefore, I assessed whether over-expression of dGABPa improves the proliferative homeostasis in the ageing fly gut. I therefore over-expressed dGABPa using the gut-specific driver TIGs-Geneswitch-GAL4 (TIGs-GS), to direct intestinal-specific expression in adulthood. Female flies over-expressing dGABPa showed a moderate, but significant, reduction in the number of actively dividing (pH3+) cells at 45-days of age (Figure 5.35, p=0.02). This suggests that over-expression of dGABPa modulates intestinal homeostasis by preventing over-proliferation of stem cells with age, likely through a reduction in IIS/mTOR signalling.

Figure 5.35 Over-expression of dGABPa in gut prevented the over-proliferation of stem cells
Quantification of pH3+ cells per gut demonstrated that over-expression of dGABPa specifically in the fly gut, using the TIGs-GS driver (TIGs-GS>UAS-dGABPa, red bar, RU+) showed a reduced number of mitoses compared with non-induced controls (black bar, RU-) at 45 days. N>16 per condition; p=0.02 using the t-test. The genotype used was: TIGs-GS>UAS-dGABPa. The gut dissection was performed in collaboration with Dr. Mobina Khericha and Dr. Jennifer C Regan.
5.3.15 Over-expression of dGABPa extended lifespan via mechanisms independent of those of lithium treatment

Over-expression of dGABPa increases resistance to phenobarbital stress (Figure 5.22). The responses to xenobiotics in Drosophila are not only regulated by the transcription factors dFOXO and DHR96 (Salih and Brunet, 2008, Sykiotis and Bohmann, 2010, Hoffmann and Partridge, 2015), which mediate the xenobiotic stress response in insulin mutants (Afschar et al., 2016), but also by the transcription factor CncC (Sykiotis and Bohmann, 2010). We recently showed that CncC-induced xenobiotic resistance and longevity can be achieved through the administration of lithium (Castillo-Quan et al., 2016). I therefore assessed whether the lifespan extension mediated by dGABPa over-expression mimics the effect of lithium treatment. To test this, I investigated whether dGABPa over-expression could further extend the lifespan of lithium-treated flies. Flies treated with 10mM lithium displayed a significant lifespan extension (Figure 5.36, p= 5.3 x 10^{-5}), consistent with previously reported observations (Castillo-Quan et al., 2016), and further extension occurred when the lithium-flies over-expressed dGABPa (p=0.001). Induction of dGABPa by RU486 extended the lifespan of wild type flies (Figure 5.36, p=0.007), and further extension occurred when dGABPa-over-expressing flies were treated with 10mM lithium (p=0.01). CPH analysis also confirmed that there was no significant interaction between the response to RU and the response to lithium treatment (Table 6, p=0.7), suggesting that over-expression of dGABPa extends lifespan via mechanisms independent of those of lithium treatment.
Ubiquitous over-expression of \(dGABP\alpha\) extended the lifespan of lithium-treated flies

The survival curves of flies over-expressing \(dGABP\alpha\) with the \(daGS\) driver (RU+) and non-RU induced controls (RU+) with and without lithium treatment. Ubiquitous over-expression of \(dGABP\alpha\) extended lifespan in wild types flies \((p=0.007)\), and a further extension occurred in \(dGABP\alpha\)-over-expressing flies treated with 10mM lithium \((p=0.01)\). 10mM lithium extended lifespan in wild type flies \((p= 5.3 \times 10^{-5})\), and a further extension occurred when the lithium-flies over-expressed \(dGABP\alpha\) \((p=0.001)\). The lifespans were analyzed using the log-rank test. \(N=150\) flies per condition. The genotype used was: \(daGS>UAS-dGABP\alpha\).

The CPH showed that there is no significant interaction between the response to RU and the response to lithium treatment \((p=0.7)\). This means that lithium extended lifespan regardless of whether \(dGABP\alpha\) is over-expressed. This suggests that over-expression of \(dGABP\alpha\) extends lifespan via mechanisms independent of those of lithium treatment.

I then assessed whether the transcriptional response mediated by \(dGABP\alpha\) over-expression was similar to that observed in lithium treatment. I used our recently published microarray data for whole flies treated with 10mM lithium (Castillo-Quan et al., 2016). By comparing the overlap of GO categories elicited by both
interventions, I detected statistical significance in GO terms of those that are up-regulated or down-regulated in both interventions (Figure 5.37), suggesting that the transcriptional response changed in the same directions.

Lithium protects flies from xenobiotic stress by up-regulating the genes involved in the detoxification pathway (Castillo-Quan et al., 2016). The RNAseq data suggested that over-expression of dGABPa does not change the transcript levels of GstD2, and down-regulates the levels of cyp6a8, two genes involved in the detoxification pathway. I further confirmed these findings by qRT-PCR analysis of whole flies over-expressing or lacking dGABPa (Figure 5.13). Since the direct contribution of individual detoxification proteins to the xenobiotic stress and lifespan regulation is as yet unexplored, it will be interesting to further study whether any of these proteins mediate the effects of dGABPa on xenobiotic stress resistance and lifespan extension.

![Venn diagrams showing the overlap between the GO terms enriched by 10mM lithium treatment (yellow color) and those enriched by dGABPa over-expression (green color). P<0.05. Bioinformatics analysis was performed by Dr.Dobril Ivanov.](image)

**Figure 5.37 Comparative analyses of the transcriptional response of lithium and dGABPa over-expression**
5.3.16 Ubiquitous knockdown of AKT increased ER stress resistance

We previously demonstrated the importance of nutrient sensing pathway in regulation of the lifespan, although the molecular mechanisms of the improvement of healthy ageing are not fully elucidated. Age-related proteostatic failure contributes to ageing and age-related diseases (Taylor and Dillin, 2011, Hetz, 2012). Direct up-regulation of the components of the UPR\textsuperscript{ER} delays ageing in \textit{C. elegans} (Taylor and Dillin, 2013). Whether the UPR\textsuperscript{ER} plays an important role in fly ageing has not yet been determined. I therefore asked whether long-lived mutants mediate their effects via modulation of the UPR\textsuperscript{ER}. To test this, I genetically knocked down AKT, the main kinase in the nutrient-sensing pathway that has been previously reported to extend lifespan (Biteau et al., 2010). RNAi knockdown of AKT in flies dramatically increased the resistance to tunicamycin, a well-known ER stress inducer (Figure 5.38), suggesting up-regulation of the UPR\textsuperscript{ER}. Given the central position of AKT in the nutrient-sensing pathway, this result suggests that the UPR\textsuperscript{ER}, being downstream of the nutrient-sensing pathway, may play an important role in lifespan regulation.

Figure 5.38 Flies lacking AKT are more resistant to the ER stressor tunicamycin

The survival curves of flies over-expressing \textit{AKT}\textsuperscript{RNAi} in adulthood using the inducible \textit{daGS} driver (\textit{daGS}>\textit{UAS-\textit{AKT}\textsuperscript{RNAi}}, dark blue line, RU+) and un-induced controls (red line, RU-). Flies were pretreated with RU486 for 14 days and then transferred to sugar/agar food supplemented with tunicamycin. \(P=1.52 \times 10^{-11}\) using the log-rank test. The genotype used was: \textit{daGS}>\textit{UAS-\textit{AKT}\textsuperscript{RNAi}}.
5.4 Discussion

PGC-1α is a key transcriptional co-activator in energy metabolism, regulating growth and mitochondrial biogenesis in response to IIS/mTOR signalling (Mukherjee and Duttaroy, 2013, Tiefenböck et al., 2010). Recent studies highlight its importance for longevity in Drosophila melanogaster (Rera et al., 2011), suggesting an evolutionary conserved function in lifespan regulation. Here I showed that ubiquitous over-expression of dPGC-1 extends lifespan and improves behavioural phenotypes in Drosophila, confirming it is indeed a modulator for healthy longevity. Interestingly, over-expression of dPGC-1 only extended female lifespan, and not the lifespan of males, suggesting that sex difference may mediate the effects of dPGC-1 on lifespan regulation. Indeed, interventions that down-regulate the nutrient-sensing pathway extend fly lifespan mostly in female flies, and furthermore the sex differences in the ageing-gut have been identified as mediators of this response (Regan et al., 2016). This highlights the intricate link between gut homeostasis, the nutrient sensing pathway and female lifespan. dPGC-1 has been shown to improve gut homeostasis (Rera et al., 2011) and therefore improving gut health and homeostasis during the ageing process may be an important determinant of healthy ageing. The link between dPGC-1 and reduced nutrient-sensing pathway signalling in ageing has been highlighted in a developmental study, showing that dPGC-1 forms a negative feedback loop to fine-tune the insulin signalling. However, the mechanisms mediating this are yet to be fully elucidated (Mukherjee and Duttaroy, 2013, Tiefenböck et al., 2010).

To identify the mechanisms involved in the regulation of lifespan by dPGC-1, I assessed the effect of downstream TFs on longevity. I identified that ubiquitous over-expression of the Drosophila homologue of human GA-binding protein α subunit (also known as nuclear respiratory factor 2α) extended lifespan. Moreover, tissue specific dGABPα gain-of-function in the central nervous system or in the gut and fat body was sufficient to extend lifespan, suggesting its broad beneficial effects in different tissues. In addition, dGABPα was up-regulated followed by dPGC-1 over-expression, consistent with human data suggesting that GABPα is induced by PGC-1α (Mootha et al., 2004).
Consistent with their mammalian counterparts, dPGC-1 and dGABPα are required for the appropriate expression of genes in mitochondrial OXPHOS (Tiefenböck et al., 2010, Mootha et al., 2004). Previous work has already shown that dPGC-1 gain-of-function correlates with increased mitochondrial oxygen consumption, mitochondrial mass, and ATP production (Rera et al., 2011). However, the transcriptional profiling suggested that over-expression of dGABPα in CNS down-regulated the cellular components involved in respiratory chain complex and mitochondrial part, suggesting mitochondrial-related functions might be repressed in response to dGABPα over-expression. I found that over-expression of dGABPα increased ATP production, but not oxygen consumption and mitochondrial mass, suggesting that energy production and consumption are uncoupled. Indeed this has been observed in long-lived worms showing elevated ATP but not respiration rate (Braeckman et al., 1999). In addition, the contribution of mitochondrial function to longevity is controversial, and whether direct up-regulation of mitochondrial components extend lifespan is yet to be unequivocally answered. It is already known that mild inhibition of mitochondrial respiration extends lifespan in diverse organisms, including worms, flies and mice (Feng et al., 2001, Dillin et al., 2002, Lee et al., 2003, Lee et al., 2010, Liu et al., 2005, Dell'agnello et al., 2007, Copeland et al., 2009). The role of mitochondrial function in the coordination of the ageing process is therefore very complex and yet to be fully elucidated.

The ETC is the major site of ROS production. PGC-1α, the “captain” of the mitochondrion, has dual effects, not only regulating mitochondrial biogenesis, but also controlling mitochondrial ROS, by regulating the antioxidant defence system (St-Pierre et al., 2006). Although Drosophila PGC-1 gain-of-function results in resistance to paraquat-induced oxidative stress, the studies have failed to detect any changes in the main antioxidant enzymes, including SOD1, and SOD2 (Mukherjee et al., 2014). Here I demonstrated that the long-lived flies over-expressing dPGC-1 does not show increased resistance to paraquat-induced oxidative stress, and shows hypersensitivity to H2O2-induced oxidative stress. The difference in the two studies may be attributable to the background strains used to over-express dPGC-1, possibly with different expression levels of dPGC-1. In addition, the contribution of ROS to the ageing process is still a controversial area, since ROS production and lifespan can be
uncoupled (Copeland et al., 2009). Increasing ROS and oxidative damage in mice does not lead to increased ageing (Zhang et al., 2009), and reducing ROS via activation of the antioxidant defence system in mice does not extend lifespan (Van Raamsdonk and Hekimi, 2009). There is increasing evidence that a moderately inducing ROS may be beneficial and leads to lifespan extension in yeast and worms (Doonan et al., 2008, Mesquita et al., 2010, Van Raamsdonk and Hekimi, 2009). Here I demonstrated that \textit{dGABPa} gain-of-function does not change sensitivity to either H$_2$O$_2$- or paraquat-induced oxidative stress, therefore excluding the possibility that oxidative stress is the driving force behind the lifespan prolonging effects of \textit{dGABPa}.

To investigate the mechanisms by which \textit{dGABPa} over-expression extends lifespan, I first assessed whether \textit{dGABPa} acts like a DR-mimetic. DR is the most established anti-ageing intervention that extends lifespan in diverse species, by shifting metabolism from reproduction and growth to somatic maintenance (Fontana and Partridge, 2015, de Cabo et al., 2014, Holliday, 1989). By comparing the effects of \textit{dGABPa} gain-of-function and DR on lifespan, I found that \textit{dGABPa} gain of function: 1) further extended lifespan beyond that already maximized by DR; 2) extended lifespan via mechanisms independent of DR; and 3) shortened lifespan in starvation-like low yeast food.

To further explore the molecular mechanisms involved in these effects, I performed transcript profiling to assess the transcriptional response to \textit{dGABPa} gain-of-function. This also enabled me to compare the data with the available sequencing data from previous studies. I showed that \textit{dGABPa} regulates several genes involved in insulin signalling, in which over-expression of \textit{dGABPa} up-regulates ImpL2, the secreted antagonist of insulin signalling (Sloth Andersen et al., 2000, Alic and Partridge, 2008, Honegger et al., 2008), thereby reducing insulin signalling. I showed that \textit{dGABPa} gain-of-function shares similar transcriptional response with reduced IIS. In addition, over-expression of \textit{dGABPa} extends lifespan via overlapping mechanism with reduced IIS. This finding supports the negative feedback loop of \textit{dPGC-1} on IIS that has been proposed (Tiefenböck et al., 2010), involving up-regulation of \textit{dGABPa} and \textit{ImpL2}. It will be interesting to determine whether the \textit{dGABPa}-IIS link is conserved across species.
It is known that a modest reduction in insulin signalling extends lifespan in diverse species (Piper et al., 2008), likely due to a beneficial effect of increased insulin sensation in later life. Here I showed that down-regulation of \(dGABP\alpha\) increased the early mortality, which is likely an outcome secondary of reduced \(ImpL2\), leading to increased binding of dilps to the insulin receptor, with a reduction in the insulin sensitivity. Insulin resistance in type-2 diabetes and leptin resistance in obesity are common clinical examples (Chiu et al., 2007, Pan et al., 2014). In both of these conditions, cells fail to respond normally to the hormone. Type-2 diabetes is a chronic metabolic disease that normally develops over several years. Under physiological conditions, insulin is secreted from the pancreas in response to increased blood glucose levels after a meal, and binds to the insulin receptor on the cell surface of tissue-sensitive organs, triggering a series of downstream cascades including glucose uptake and metabolism. However, a long-term high-fat diet/high-calorie diet causes a constant stimulus to the insulin-insulin receptor interaction, which in turn leads to a reduction in sensitivity of the insulin receptor to insulin. This is referred to as insulin resistance. Patients with insulin resistance, or leptin resistance in obesity, normally show increased levels of circulating insulin or leptin, with a lower tissue sensitivity to these hormones. In keeping with this, I demonstrated that down-regulation of \(dGABP\alpha\) shortens lifespan and knockdown of the insulin receptor further accelerates ageing, resulting in down-regulation of IIS beyond beneficial levels. Thus \(dGABP\alpha\) is required for reduced IIS, in order to maintain the insulin sensitivity necessary for lifespan extension.

I further showed that flies over-expressing \(dGABP\alpha\) displayed reduced phosphorylation of AKT, the main enzyme in the IIS/mTOR signalling pathway, suggesting a reduction in IIS/mTOR signalling activity (Webb and Brunet, 2014, Wang et al., 2014, Guertin and Sabatini, 2009, Palu and Thummel, 2016, Laplante and Sabatini, 2012). Interestingly, the transcriptional profiling showed that over-expression of \(dGABP\alpha\) significantly down-regulated the biological functions including amino acid metabolism and translation, which both are tightly linked with reduced mTOR signalling pathway. Indeed, \(dGABP\alpha\) gain-of-function inhibited mTORC1 activity, as evident from the reduction in S6K phosphorylation levels (Um
et al., 2006). In addition, \textit{dGABP\textalpha} gain-of-function extended the lifespan via overlapping mechanisms with rapamycin, the mTORC1 inhibitor, likely through inhibition of S6K (Bjedov et al., 2010). Taken together, \textit{dGABP\textalpha} gain-of-function extends lifespan via overlapping mechanisms with that of reduced IIS/mTOR signalling. \textit{dFOXO} is one of the main transcription factors responsible for many metabolic traits of reduced IIS/mTOR signalling (Alic et al., 2011a, Slack et al., 2011).

I then looked at whether \textit{dGABP\textalpha} gain of function improved healthy lifespan. Here I found that over-expression of \textit{dGABP\textalpha} reduced the age-related over-proliferation of intestinal stem cells, preventing the disruption of epithelial integrity, and reducing the mortality of ageing flies (Choi et al., 2008, Biteau et al., 2008). Our results are consistence with previous findings showing that \textit{dPGC-1} gain-of-function and reduced IIS/mTOR signalling, all contribute to improved gut homeostasis via similar mechanisms (Rera et al., 2011). Since all of the above effectors extend only female lifespan, this indicates that the gut differences between the sexes may mediate the lifespan extension from reduced nutrient sensing pathway.

Interestingly, I observed that, unlike many other genetic and pharmacological interventions (eg. DR, reduced IIS, rapamycin, or trametinib treatment), \textit{dGABP\textalpha} gain-of-function did not reduce fecundity (Slack et al., 2011, Slack et al., 2015, Bjedov et al., 2010). It therefore promotes healthy lifespan in adult \textit{Drosophila} with limited side effects. Indeed, extended longevity is not consistently correlated with reduced fertility (Copeland et al., 2009) and in our recently published work, we pointed out that flies treated with lithium show extended lifespan without a reduction in fecundity (Castillo-Quan et al., 2016). Lithium induces lifespan-extension and protection against xenobiotic via GSK3/NRF2-dependent mechanisms. Here I show that \textit{dGABP\textalpha} gain-of-function shares overlapped transcriptional responses with those of lithium, suggesting they might share similar mechanisms in mediating lifespan regulation. However, over-expression of \textit{dGABP\textalpha} does further extend lifespan in flies treated with lithium, suggesting that over-expression of \textit{dGABP\textalpha} also extends lifespan via mechanisms independent of lithium treatment. In addition, flies over-expressing \textit{dGABP\textalpha} show increased resistance to phenobarbital stress, indicating that it improves
healthy lifespan through up-regulation of the detoxification pathway. Since both lithium and NRF-2 and reduced IIS are involved in the detoxification pathway (Salih and Brunet, 2008, Sykiotis and Bohmann, 2010, Hoffmann and Partridge, 2015, Afschar et al., 2016, Castillo-Quan et al., 2016), it will be interesting to know which mediator is particularly responsible for this effect of \( dGABPa \) over-expression in flies.

My work has thus shown that \( dGABPa \) gain-of-function extends lifespan via similar mechanisms to those of reduced IIS and rapamycin treatment, all of which further extend lifespan beyond the maximum achieved by DR in \textit{Drosophila} (Bjedov et al., 2010, Broughton et al., 2010). This suggests that \( dGABPa \) may be one of the main molecular modulators of lifespan extension beyond DR. In addition, \( dGAPBa \) gain-of-function up-regulates the chaperones HSP70, together with inhibition of IIS/mTOR signalling pathway, which both mediate the beneficial effects in short-term fasting in humans or mammalian models (Brown-Borg and Rakoczy, 2013, Mattson et al., 2014, Cheng et al., 2014, Hine et al., 2015). This therefore highly suggests that GABP\( \alpha \) may be the central mediator of the benefits of short term fasting in mammalian ageing and chronic diseases. Moreover, \( dGABPa \) up-regulates HSP70, which is known to improve proteostasis in IF and short-term fasting (Brown-Borg and Rakoczy, 2013). Whether direct over-expression of \( dGABPa \) improves proteostasis is yet to be determined. Furthermore I demonstrated here that down-regulation of AKT, the key enzyme in the nutrient-sensing pathway, increased the resistance to ER stress in flies, suggesting activation of the UPR\( _{ER} \). These results therefore indicate that up-regulation of UPR\( _{ER} \) may be beneficial for fly ageing.

Finally, the effects of both genetic interventions that down-regulate the IIS/mTOR signalling pathway, and the environmental interventions that alter meal timing, are dependent on the levels of the signal reduction and nutrient availability. Under normal conditions, GABP\( \alpha \) activation may be beneficial for mammalian ageing through the use of mechanisms that mimic IF. However, in cachexic states, such as in cancer and type-2 diabetes, activation of GABP\( \alpha \) maybe harmful. A previous study suggested that under adverse nutritional conditions, \textit{ImpL2} is up-regulated in the fat body and represses systemic IIS activity (Honegger et al., 2008). Indeed, starvation or fasting in cachexic states, as seen in \textit{Drosophila} tumour models (Kwon et al., 2015, Figueroa-
Clarevega and Bilder, 2015), lead to organ wasting and loss of body mass (Tessitore and Bollito, 2006). This is associated with a sustained and dramatic reduction in systemic IIS signalling via increased levels of secreted ImpL2, suggesting that sustained activation of the ImpL2/IIS pathway under malnutrition accelerates ageing. Here I show that under low-yeast food conditions, flies over-expressing dGABPα are more sensitive than non-induced controls to malnutrition, through activation of ImpL2 and repression of IIS. In type-2 diabetes (high-insulin but profound insulin resistance), hepatic gluconeogenesis is highly induced, through both up-regulation of PGC-1α mRNA levels via either the cAMP-CREB pathway (Herzig et al., 2001), or through co-activation with the transcription factor HNF4 (Rhee et al., 2003). This leads to increased blood glucose levels and exacerbates insulin resistance. Therefore in conclusion up-regulation of PGC-1α or GABPα activity may prove to be a useful therapeutic strategy in modulating human ageing and age-related diseases. However, activation of these genes will require careful optimisation, especially in the established disease state.

5.5 Experimental procedures

5.5.1 Fly strains

The UAS-dPGC-1 and UAS-dGABPα lines were gifts from Dr. Christian Frei and Dr. Hugo Stocker (Tiefenböck et al., 2010, Baltzer et al., 2009). The AKTRNAi and dGABPαRNAi lines were obtained from the VDRC. Actin-GAL4 (ActGal4) and UAS-INRDN (K1409A) stocks were obtained from the Bloomington Drosophila Stock Centre. The S106-GeneSwitch (S106GS) driver was a kind gift from the R. Davis lab (Roman et al., 2001). The Daughterless-GeneSwitch (daGS) driver line was kindly provided by Dr. Veronique Monnier (Tricoire et al., 2009). The ActGS driver line was kindly provided by Dr. Laurent Seroude (Poirier et al., 2008). All of the lines were backcrossed over 6 generations into the whiteDahomey (wDah) stock, which was maintained in population cages. wDah was derived by backcrossing the w1118 into the outbred wild-type Dahomey background. Flies were raised and maintained on standard sugar/yeast medium (Bass et al., 2007). Experiments were conducted at 25°C in a 12:12 hours light/dark cycle at constant humidity.
5.5.2 Dietary restriction protocol
The specific preparation of the DR media has been described in Chapter 2. In this chapter, I used 0.1SY, 0.5SY, 1.0SY and 2.0SY. RU486 was supplemented into the SY food to give a final concentration of 200 µM.

5.5.3 Rapamycin preparation and delivery
Rapamycin (LC Laboratories #R-500) was dissolved in 100% ethanol at a concentration of 50mM and then added to the fly medium to give the final concentration.

5.5.4 Lithium preparation and delivery
Lithium chloride (LiCl; Sigma L0505) was dissolved in ddH₂O to give a stock concentration of 5M and then supplemented into 1.0 SY medium to give the final concentration. Food was then dispensed into plastic vials and stored at 4 °C. Cold refrigerated vials were placed at 25 °C for a minimum of 2 hours before use.

5.5.5 Stress experiments
Flies were reared and housed as for lifespan experiments until 14 days of age, and then flies were transferred onto the specific foods containing the various stress reagents. The preparation and delivery of the various stress assay foods has been described in Chapter 2.

5.5.6 Fecundity assays
For fecundity measurement, eggs were collected over a 24-hour period and counted. Data was reported as the mean number of eggs laid per female ± SEM over this 24-hour period.

5.5.7 Oxygen consumption
The mitochondrial Oxygen Consumption Rate (OCR) was analyzed using an Oroboros Oxygraph-2k (O2k). Drosophila heads (30-40 heads per run) were collected and placed in ice-cold homogenization buffer (pH 7.4, Sucrose 320 mM, Tris-HCl 10
mM, K+EDTA 1 mM, BSA essentially fatty acid free 2.5 g/L), homogenized with a Teflon homogenizer over ice, spun down and re-suspended in ice-cold homogenization buffer without BSA. The homogenate was added to the MiR05 buffer (pH 7.1, EGTA 0.5 mM, MgCl2.6H2O 3 mM, Lactobionic Acid 60 mM, Taurine 20 mM, KH2PO4 10 mM, HEPES 20 mM, D-Sucrose 110 mM, BSA essentially fatty acid free 1 g/L) for OCR recordings. The experimental temperature of the O2k was set at 30°C and electrodes were calibrated according to the manufacturer’s protocol. Acquisition and analysis of the OCR was done through the Oroboros Datlab software. Substrates, uncouplers and inhibitors were added in the following order and final concentrations: 2 mM malate, 10 mM glutamate, 2.5 mM ADP, 10 µM cytochrome c, 10 mM succinate, 0.25 µM titrations with the protonophore uncoupler Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) until maximal uncoupled respiration was reached, 0.5 µM rotenone, and finally 2.5 µM antimycin A. State 2 respiration was obtained after the additions of malate and glutamate, State 3 after ADP, and maximal uncoupling after FCCP. The OCR was normalized to total protein levels obtained from the homogenate and units of activity are presented as pmol O2/mg*s. All reagents were from Sigma, unless stated otherwise

### 5.5.8 Immunohistochemistry

The following antibodies were used for cell division analyses: the primary antibody rabbit anti-PH3 (Cell Signaling (Danvers, MA) 9701) 1:500, and the secondary antibody Alexa Fluor 594 donkey anti-rabbit (A21207) Thermo Fisher Scientific, Waltham, MA) 1:1000. Guts were dissected in ice cold PBS and immediately fixed in 4% formaldehyde for 15 mins. They were then serially dehydrated in MeOH, stored at -20°C and subsequently stained. For staining guts were defrosted and washed in 0.2% Triton-X/PBS, blocked in 5% bovine serum albumin/PBS, incubated in primary antibody overnight at 4°C and in secondary for 2 hour at RT. At least 16 guts per condition were mounted in mounting medium containing DAPI (Vectastain). Scoring was performed under the Zeiss (UK) fluorescence microscope using a 40X oil-immersion objective.
**5.5.9 Quantitative RT-PCR**

Total RNA was isolated from 10 whole adult flies using standard TRIZOL (Invitrogen) protocols and converted to cDNA using oligo d(T)s, primers and Superscript II reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed using the Power SYBR Green PCR Master Mix (ABI), and relative quantities of transcripts were determined using the relative standard curve method normalized to the internal control gene *RP49*.

The following primers sequences were used for the qRT-PCR:

- **dGABPa:**
  - **FOR:** GCAACATCTTTCTGGACCCAC
  - **REV:** CCGCATATAATCCGCTGTCAAA

- **Cyp6a8:**
  - **FOR:** CAAGATAAGCTGCTGGCTGA
  - **REV:** TGGTGTACAGTCGCAGGTTC

- **GstD2:**
  - **FOR:** CCGACATTGCCATCTCTGT
  - **REV:** TGCTGAAGCTGCAAGGTCTTTC

- **4EBP:**
  - **FOR:** CACTCCTGGAGGCACCA
  - **REV:** GAGTTCCCCTCACAGCAAGCAA

- **RP49:**
  - **FOR:** ATCGGTTACGGATCGAACAA
  - **REV:** GACGGTCTCCTTGCGCTTCT

- **ImpL2:**
  - **FOR:** CCTCATTAAGTACGGGATAC
  - **REV:** CTTCTGATCTCCGAGATCAAG

**5.5.10 Immunoblot analyses**

Protein extraction and western blot techniques were described in Chapter 2. Primary antibodies used in this work were: pSer505-AKT (Cell Signalling Technologies #4054, 1:1000), total-AKT (Cell Signalling Technologies #9272, 1:1000), pThr398-S6K (Cell Signalling Technologies #9209, 1:1000), custom made total-S6K (kindly provided by Dr. Cathy Slack, 1:1000), β-actin (Abcam #ab8224, 1:5000), tubulin
PGC-1α in neurodegeneration and ageing

(Sigma-aldrich T6199, 1:5000), NDUFS3 (Abcam #ab14711, 1:1000), and custom made Atg8 (kindly provided by Dr. Ivana Bjedov, 1:1000). Secondary antibodies used in this work were: anti-rabbit and anti-mouse (Abcam #ab6721 and Abcam #ab6789) at 1:5000 dilutions.

5.5.11 Proteasome activity
Two whole flies were homogenized in 25mM Tris, pH 7.5 and the protein content was determined using a BCA assay. Chymotrypsin-like peptidase activity of the proteasome was assayed by using the fluorogenic peptide substrate Succinyl-Leu-Leu-Val-Tyr-amidomethylcoumarin (LLVY-AMC), based on a previously published protocol (Bulteau et al., 2002, Rogers et al., 2012). 20 µg of crude fly homogenate total protein was incubated at 37°C with 25uM LLVY-AMC in a final volume of 200 µLS. Enzymatic kinetics were measured in a temperature-controlled microplate fluorimeter (Tecan Infinite M200), at excitation/emission wavelengths of 360/460 nm, measuring fluorescence every 2 min for 1 h. Proteasome activity was determined as the slope of AMC accumulation over time per mg of total protein (pmoles/min/mg).

5.5.12 RNA sequencing
RNA was extracted using Trizol (Invitrogen) from four biological repeats of 25 heads of 10-day-old flies of the following genotypes: elavGS>UAS-dGABPα (+RU) and non-RU treated controls. The RNA was purified with the RNeasy columns (Qiagen) and its quality and concentration were determined using a NanoDrop. The RNA was further processed for RNAseq analysis at the Glasgow Polyomics Unit, University of Glasgow. The differentially expressed genes were measured for multiple hypothesis testing. The raw data were analysed by Dr. Dobril Ivanov, University of Cambridge.

5.5.13 Gene-Ontology (Catmap analysis)
For the functional analysis of all the expressed genes, I used the Wilcoxon rank sum test available in Catmap (Breslin et al., 2004). Ranks of genes were based on the Bayes t-statistic for differential expression and for a given functional category. The significance of the rank sum for all genes in the category was calculated analytically based on a random gene-rank distribution. Catmap analysis was performed by Dr. Dobril Ivanov.
5.5.14 Statistical analysis

Survival experiments were analysed using log-rank test or wilcoxon test. Cox Proportional Hazards analysis was performed in R to compare interactions for survivals. Other statistical analyses were performed using Excel and Prism (GraphPad, La Jolla, CA), have been indicated in the figure legends.
Chapter 6 General Discussion and future plan

PGC-1α is the key transcriptional co-activator in energy metabolism, regulating many biological processes, including growth, mitochondrial biogenesis, stress defence system, circadian rhythm and proteostasis. In my thesis, I focused on dissecting the role of PGC-1α in ageing and age-related neurodegeneration. I have found that genetic activation of PGC-1α in the central nervous system could ameliorate the neurotoxic phenotypes in a *Drosophila* model of Aβ toxicity. The improvements were associated with an improved mitochondrial homeostasis and the activation of UPR^ER^.

In keeping with this, I found that both rifampicin, the antibiotic used in the treatment of tuberculosis and leprosy, and metformin, the drug used in the treatment of type-2 diabetes, activated the UPR^ER^ and rescued the neurotoxicity in Aβ-expressing flies. These findings suggested that the pharmacological interventions that activating UPR^ER^, or elevating PGC-1α may offer significant therapeutic benefits in AD. My findings are significant in the field. Although both mitochondrial dysfunction and ER-related proteostastic failure have been implicated in the disease progression in AD, *in-vivo* studies using animal models targeting above effectors are still limited. It would be interesting to investigate in future studies whether directly targeting components of UPR^mt^ or UPR^ER^ could provide beneficial effects in animal models. This knowledge could eventually be translated into drug discovery for human use.

There is urgent need for effective strategies to improve healthy ageing, with facts that the increasing ageing population coupled with the rising prevalence of age-related neurodegeneration. PGC-1α has also been implicated in fly ageing, however the mechanisms are not fully elucidated. Previous studies suggested that over-expression of PGC-1α in intestine extends lifespan associated with improved stress-defence system. However, I found that the long-lived flies were not resistant to different oxidative stressors, therefore excluding the possibility that oxidative stress is the driving force behind the lifespan prolonging effects of PGC-1α. There are three clues as to the mechanisms underlying the anti-ageing effects of PGC-1α over-expression. Over-expression of PGC-1α: 1) extended lifespan only in females but not males; 2)
formed an un-defined negative feedback loop on IIS/mTOR signalling pathway; 3) improved gut homeostasis. The sex difference in gut pathology in modulating lifespan prolonging effects has previously been observed in interventions involving down-regulation of nutrient-sensing pathways. To investigate the link between PGC-1α and nutrient-sensing pathways, I identified that transcription factor GABPα being downstream of PGC-1α, extended fly lifespan, through mechanisms associated with reduced IIS/mTOR signalling pathway. This finding supports the negative feedback loop of dPGC-1 on IIS that has been proposed (Tiefenböck et al., 2010), involving up-regulation of dGABPα and ImpL2. It will be interesting to determine whether the dGABPα-IIS link is conserved in mammalian systems. Moreover, over-expression of GABPα improved gut homeostasis by delaying age-related over-proliferation in intestinal stem cells. My findings are novel and important in the ageing field. Given the evolutionarily conserved effects of reduced IIS/mTOR, the findings suggest that GABPα may be an effective modulator of mammalian ageing.

Moreover, I have demonstrated that lifespan could be uncoupled from oxidative stress, mitochondrial function, and fecundity. Xenobiotic stress seems to play an important role in the lifespan prolonging effects of GABPα over-expression. It would be interesting in future studies to target this pathway genetically or pharmacologically for healthy ageing.
References:


Dopaminergic midbrain neurons are the prime target for mitochondrial DNA deletions. J Neuro, 255, 1231-5.


CHEN, C., LIU, Y., LIU, Y. & ZHENG, P. 2009a. mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells. Sci Signal, 2, ra75.


PGC-1α in neurodegeneration and ageing


PGC-1α in neurodegeneration and ageing


KAMP, F., EXNER, N., LUTZ, A. K., WENDNER, N., HEGERMANN, J., BRUNNER, B., NUSCHER, B., BARTELS, T., GIESE, A., BEYER, K., EIMER, S., WINKLHOFER,
K. F. & HAASS, C. 2010. Inhibition of mitochondrial fusion by alpha-synuclein is rescued by PINK1, Parkin and DJ-1. EMBO J, 29, 3571-89.


MEDAWAR, P. B. 1957. The Uniqness of the Individual. *(Basic Books, Inc.)*


MERKWIRTH, C., JOVAISAIKE, V., DURIEUX, J., MATILAINEN, O., JORDAN, S. D., QUIROS, P. M., STEFFEN, K. K., WILLIAMS, E. G., MOUCHIROUD, L., TRONNES, S. U., MURILLO, V., WOLFF, S. C., SHAW, R. J., AUWERX, J. &


MOOTHAv, K., LINDGREN, C. M., ERIKKSSON, K. F., SUBRAMANIAN, A., SIHAG, S., LEHAR, J., PUIGSERVER, P., CARLSSON, E., RIDDERSTRALE, M., LAURILA,


Appendix

Appendix 1
Lithium suppresses Aβ pathology by inhibiting translation in an adult Drosophila model of Alzheimer’s disease

Oyinkan Sofola-Adesakin1,2, Jorge I. Castillo-Quan1,2†, Charalampos Rallis1†, Luke S. Tain1†, Ivana Bjedov1,3, Iain Rogers1, Li Li1, Pedro Martinez1,2, Mobina Khericha1,2, Melissa Cabecinha1, Jürg Bähler1 and Linda Partridge1,2,*

†These authors contributed equally to this work.

INTRODUCTION

Alzheimer’s disease (AD) is the most common form of dementia in the ageing population. The proportion of deaths due to heart disease and stroke decreased by 13 and 20% respectively between 2000 and 2008, while those due to AD increased to heart disease and stroke decreased by 13 and 20% respectively between 2000 and 2008, while those due to AD increased in Alzheimer’s disease (AD), is thought to initiate the pathogenesis of AD, eventually leading to neuronal cell death. Previously, we developed an adult-onset Drosophila model of AD. Mutant Aβ42 accumulation led to increased mortality and neuronal dysfunction in the adult flies. Furthermore, we showed that lithium reduced Aβ42 protein, but not mRNA, and was able to rescue Aβ42-induced toxicity. In the current study, we investigated the mechanism/s by which lithium modulates Aβ42 protein levels and Aβ42 induced toxicity in the fly model. We found that lithium caused a reduction in protein synthesis in Drosophila and hence the level of Aβ42. At both the low and high doses tested, lithium rescued the locomotory defects induced by Aβ42, but it rescued lifespan only at lower doses, suggesting that long-term, high-dose lithium treatment may have induced toxicity. Lithium also down-regulated translation in the fission yeast Schizosaccharomyces pombe associated with increased chronological lifespan. Our data highlight a role for lithium and reduced protein synthesis as potential therapeutic targets for AD pathogenesis.

The greatest risk factor for Alzheimer’s disease (AD) is age, and changes in the ageing nervous system are likely contributors to AD pathology. Amyloid beta (Aβ) accumulation, which occurs as a result of the amyloidogenic processing of amyloid precursor protein (APP), is thought to initiate the pathogenesis of AD, eventually leading to neuronal cell death. Previously, we developed an adult-onset Drosophila model of AD. Mutant Aβ42 accumulation led to increased mortality and neuronal dysfunction in the adult flies. Furthermore, we showed that lithium reduced Aβ42 protein, but not mRNA, and was able to rescue Aβ42-induced toxicity. In the current study, we investigated the mechanism/s by which lithium modulates Aβ42 protein levels and Aβ42 induced toxicity in the fly model. We found that lithium caused a reduction in protein synthesis in Drosophila and hence the level of Aβ42. At both the low and high doses tested, lithium rescued the locomotory defects induced by Aβ42, but it rescued lifespan only at lower doses, suggesting that long-term, high-dose lithium treatment may have induced toxicity. Lithium also down-regulated translation in the fission yeast Schizosaccharomyces pombe associated with increased chronological lifespan. Our data highlight a role for lithium and reduced protein synthesis as potential therapeutic targets for AD pathogenesis.

Lithium has been used to treat psychiatric conditions such as bipolar disorder, and it also has interesting neuroprotective effects (Rybakowski, 2011). Lithium is able to promote neurogenesis, and increase the levels of neurotrophins such as brain-derived neurotrophic factor (BDNF), and to inhibit glycogen synthase kinase-3 (GSK-3), which is involved in AD (Machado-Vieira et al., 2009; Rybakowski, 2011). Lithium also reduces amyloid production by affecting APP processing/cleavage in cells and mice, presumably by down regulating the levels of phosphorylated APP (Phiel et al., 2003; Rockenstein et al., 2007). Lithium can also influence various ageing-regulated processes that could interfere with protein turnover and consequently affect neurological function. For instance, lithium has
been shown to induce autophagy (Sarkar et al., 2005), promote proteasome-mediated degradation (Jing et al., 2013), and influence components of the translational machinery (Bosetti et al., 2002; Karyo et al., 2010). Also, inhibiting GSK-3 in HCC1806 cells by a GSK-3 inhibitor or knockdown of GSK-3β has been reported to significantly decrease polysome assembly, and thus affect translation (Shin et al., 2014). GSK-3 was shown in these cells to partially exert its effects on translation via eIF4E-binding protein 1 (4E-BP1). Knocking down 4E-BP1 only partially restored the cap-dependent translation suppressed by GSK-3 inhibition, suggesting that GSK-3β may regulate other components involved in protein synthesis (Shin et al., 2014).

In this study, we investigated underlying mechanism/s by which lithium can reduce Aβ protein levels and thus pathology in the adult-onset, Drosophila model of AD. Aβ peptide is directly expressed in this model, and therefore, any effect of lithium on Aβ levels is not due to its ability to alter APP processing, but rather a consequence of its role in protein synthesis or degradation.

MATERIALS AND METHODS

FLY STOCKS AND MAINTENANCE

All fly stocks were maintained at 25°C on a 12:12-h light:dark cycle at constant humidity on a standard sugar-yeast (SY) medium (15 g l⁻¹ agar, 50 g l⁻¹ sugar, 100 g l⁻¹ autolyzed yeast, 100 g l⁻¹ nipagin and 3 ml l⁻¹ propionic acid). Adult-onset, neuronal-specific expression of Arctic mutant Aβ42 peptide was achieved by using the elav GeneSwitch (elavGS)-UAS system [GAL4-dependent upstream activator sequence; (Osterwalder et al., 2001)]. ElavGS was derived from the original elavGS 301.2 line (Osterwalder et al., 2001) and obtained as a generous gift from Dr H. Tricoire (CNRS, France). UAS-ArcAβ42 were obtained from Crowther et al. (2005), elavGS and UAS-lines used in all experiments were backcrossed six times into the w¹¹¹B genetic background. For the fly AD model, flies carrying homozygous UAS-ArcAβ42;elavGS constructs were out-crossed to either w¹¹¹B flies, or flies expressing EGFP in a w¹¹¹B background; and adult-onset neuronal expression was induced in the female progeny by treatment with mifepristone (RU486; 200 µM) added to the standard SY medium.

LITHIUM TREATMENT PROTOCOL

Lithium chloride solution was made at 10 M concentration and added to 200 µM RU486 standard SY medium for final concentrations of lithium.

LIFESPAN ANALYSES

For all experiments, flies were raised at a standard density on standard SY medium in 200 ml bottles. Two days after eclosion once-mated females were transferred to experimental vials containing SY medium with or without RU486 (200 µM) at a density of 15 flies per vial. Deaths were scored almost every other day and flies were transferred to fresh food. Data are presented as survival curves and statistical analysis was performed using log-rank tests to compare survival of groups.

NEGATIVE GEOTAXIS ASSAYS

Climbing assays were performed at 25°C according to previously published methods (Sofola et al., 2010). Climbing was analyzed every 2–3 days post-RU486 treatment. Fifteen adult flies were placed in a vertical column (25 cm long, 1.5 cm diameter) with a conic bottom end, tapped to the bottom of the column, then their climb to the top of the column was analyzed. Flies reaching the top and flies remaining at the bottom of the column after a 45 s period were counted separately, and three trials were performed at 1 min intervals for each experiment. Scores recorded were the mean number of flies at the top (n_top), the mean number of flies at the bottom (n_bottom) and the total number of flies assessed (n_tot). A performance index (PI) defined as \( \frac{n_{\text{top}} + n_{\text{bottom}}}{n_{\text{tot}}} \) was calculated. Data are presented as the mean PI ± s.e.m. obtained in three independent experiments for each group, and analyses of variances (ANOVA) were performed using JMP 10.0 software.

QUANTIFICATION OF Aβ42 PEPTIDE

To extract total Aβ42, five Drosophila heads were homogenized in 50 µl GnHCl extraction buffer (5 M Guanidine HCl, 50 mM Heps pH 7.3, protease inhibitor cocktail (Sigma, P8340) and 5 mM EDTA), centrifuged at 21,000 g for 5 min at 4°C, and cleared supernatant retained as the total fly Aβ42 sample. Aβ42 content was measured in Arctic mutant Aβ42 flies and controls using the hAmyloid β42 ELISA kit (HS) (Millipore). Samples were diluted in sample/standard dilution buffer and the ELISA performed according to the manufacturers’ instructions. Protein extracts were quantified using the Bradford protein assay (Bio-Rad protein assay reagent; Bio-Rad laboratories (UK) Ltd) and the amount of Aβ42 in each sample expressed as a ratio of the total protein content (pmol/g total protein). Data are expressed as the mean ± s.e.m. obtained in three independent experiments for each genotype. ANOVAs and Tukey’s-HSD post-hoc analyses were performed using JMP 7.0 software.

WESTERN BLOTTING

The same number of Drosophila heads for each sample were homogenized in Laemmli sample buffer containing β-mercaptoethanol and boiled for 10 min. Proteins were separated on SDS polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were incubated in a blocking solution containing 5% milk proteins in TBST for 1 h at room temperature, then probed with primary antibodies diluted in TBST + 5% BSA overnight at 4°C. Antibodies were from Cell Signaling unless specified. GFP antibody was used at 1 in 1000 dilution (2955), phospho eIF2B and total eIF2B at 1 in 1000 (Ab4775 and Ab32713), phospho and total eEF2 at 1 in 500 (2331 and 2332), and phospho S6K (9206), total S6K at 1 in 1000 [made from previously published sequence (Pearson et al., 1995)]. Appropriate secondary antibodies were used at a dilution of 1 in 10,000.

PROTEASOME ACTIVITY

Fly heads were homogenized in 25 mM Tris, pH 7.5 and protein content determined by Bradford assay. Chymotrypsin-like peptidase activity of the proteasome was assayed the...
using fluorogenic peptide substrate Succinyl-Leu-Leu-Val-Tyr-amidomethylcoumarin (LLVY-AMC), based on a previously published protocol (Butteau et al., 2002; Rogers et al., 2012). 20 µg of crude fly head homogenate total protein was incubated at 37°C with 25 µM LLVY-AMC in a final volume of 200 µLs. Enzymatic kinetics were measured in a temperature-controlled microplate fluorimeter (Tecan Infinite M200), at excitation/emission wavelengths of 360/460 nm, measuring fluorescence every 2 min for 1 h. Proteasome activity was determined as the slope of AMC accumulation over time per mg of total protein (pmoles/min/mg).

35S-METHIONINE INCORPORATION

Protein translation was measured in fly heads using a method adapted from Bjedov et al. (2010). Standard SY medium was first supplemented with 100 µCi 35S methionine/mL of food (American Radiolabeled Chemicals 1mCi/37MBq ARS0104A). 15 flies were transferred to each vial containing 1 mL radioactive SY medium. After three-hours of feeding, flies were transferred to non-radioactive SY for 30 min in order to purge any undigested radioactive food from the intestines. Flies that were in contact with the radioactive food for 1 min were used as a background control. Flies were then decapitated using liquid nitrogen and the heads and bodies homogenized in 1% SDS and heated for 5 min at 95°C. Samples were centrifuged twice for 5 min at 16,000 g. Proteins were precipitated by the addition of the same volume of 20% cold TCA (10% TCA final concentration) and incubated for 15 min on ice. Samples were then centrifuged at 16,000 g for 15 min, the pellet washed twice with acetone and re-suspended in 200 µl of 4 M guanidine-HCl.

Samples (100 µl) were mixed with 3 mL of Fluoran-Safe 2, BDH and radioactivity counted in a liquid scintillation analyzer (TriCarb 2800TR, Perkin Elmer), with appropriate quench corrections. SDS-homogenates, prior to TCA precipitation, were also sampled and analyzed as a measure of the total radioactivity (incorporated and un-incorporated) present. Total protein for each sample was determined by Bradford assay and a translation index was calculated as follows: (TCA protein cpm/total cpm) /µg protein per sample.

CHRONOLOGICAL LIFESPAN ASSAYS IN FISSION YEAST

Standard S. pombe laboratory strain 972 h+ cells were grown in EMM2 as previously described (Rallis et al., 2013). When cultures reached a stable maximal density, cells were harvested, serially diluted and plated on YES plates. The measurement of Colony Forming Units (CFUs) was taken as time-point 0 at the beginning of the CLS curve (i.e., 100% cell survival). Measurements of CFUs were conducted on the following days. Error bars represent standard deviation calculated from three independent cultures, with each culture measured three times at each time-point. Statistical significance (t-test) was determined at the time-point when median lifespan (50% cells dead) was reached for the untreated cells.

POLYSOME PROFILING IN FISSION YEAST

Translational profiles were acquired as previously described (Rallis et al., 2013). Briefly, S. pombe cells were treated with 100 µM Cycloheximide for 5 min. Cells were then collected by centrifugation at 3500 rpm for 5 min and diluted in 20 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM MgCl2 supplemented with protease (PMSF), 100 µM cycloheximide, 1 mM DTT and 200 ng/mL Heparin. Cells were lysed in a Fastprep-24 machine using glass beads. Sucrose gradients (10–50%) were generated using a Biocomp Gradient Master, and protein preparations were loaded and centrifuged at 35,000 rpm for 2 h 40 min. Polysome gradients were then loaded to the fractionator to obtain the translational profiles.

POLYSOME PROFILING IN DROSOPHILA

Polysome profiles were generated as previously described with minor modifications (Dinkova et al., 2005). Heads (300) were homogenized on ice in 1200 µl polysome extraction buffer (300 mM NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 1 mM EGTA, 200 µg heparin/ml, 400 U RNAsin/ml, 1.0 mM phenylmethylsulfonyl fluoride, 0.2 mg cycloheximide/ml, 1% Triton X-100, 0.1% Sodium Deoxycholate). Lysates were mixed gently and placed on ice for 10 min. Debris was removed by spinning at 20,000 g (4°C) for 10 min and the supernatant was layered onto a 10–50% sucrose gradient in high salt resolving buffer (140 mM NaCl, 25 mM Tris-HCl (pH 8.0), 10 mM MgCl2). Using a Beckman SW41Ti rotor (38,000 rpm at 90 min, 4°C) polysomes and ribosomal subunits were separated before the gradients were fractionated. Profiles were continuously monitored (Ab 252 nm) using a Teledyne density gradient fractionator.

STATISTICAL ANALYSES

Data are presented as means ± s.e.m. obtained in at least three independent experiments. JMP (version 10.0) software (SAS Institute, Cary, NC, USA) was used for data analyses.

RESULTS

LITHIUM REDUCED Aβ LOAD IN ARCTIC Aβ42 EXPRESSING FLYES THROUGH PROTEIN CLEARANCE/DEGRADATION-INDEPENDENT MECHANISMS

To understand the mechanism/s by which lithium reduces Aβ42 protein level, we first investigated the speed of Aβ42 accumulation after induction of expression, in the presence and absence of lithium. We measured total Aβ protein in the flies expressing Arctic mutant Aβ42 specifically in adult neurons (elav gene switch system was used to induce Aβ expression with the activator mifepristone, RU486/RU) (Sofola et al., 2010) at a very early time-point, 2 days post Aβ induction. We found by ELISA analysis that even at this early time point, UAS-ArcA42/UAS-gfpelavGS/+ +RU/+Li flies that were fed either 25 or 100 mM lithium showed a major reduction in total Aβ burden in comparison to UAS-ArcA42/UAS-gfpelavGS/+ +RU controls, P < 0.01 and P < 0.001 respectively (Figure 1A). These data suggest that lithium may affect synthesis rather than degradation of Aβ, because a reduction in Aβ levels was already present at such an early time point.

Next, we directly assessed the effects of lithium on degradation of Aβ42. We induced Aβ expression for 6 days in the presence of lithium (UAS-ArcA42/UAS-gfpelavGS/+ +RU +Li), then stopped Aβ induction and divided the flies randomly into two
FIGURE 1 | Lithium reduced the level of Aβ42 peptide in the adult Drosophila nervous system. (A) Lithium reduced amyloid levels of Arctic Aβ42 flies at a very early time-point. Protein levels of UAS-ArcAβ42/UAS-gfp; elavGS/+ flies on +RU+88 SY medium, and UAS-ArcAβ42/UAS-gfp;elavGS/+ flies on +RU +lithium (LiCl), were measured by ELISA at 2 days post-induction (see Materials and Methods). Data were compared using One-Way ANOVA, number of independent tests (n) = 4. *P < 0.01 and *P < 0.001 when comparing UAS-ArcAβ42/UAS-gfp;elavGS/+ RU to UAS-ArcAβ42/UAS-gfp;elavGS/+ RU +LiCl 25 or 100 mM respectively. (B) Protein levels of UAS-ArcAβ42/UAS-gfp;elavGS/+ flies on RU +100 mM LiCl medium at 6 days post induction, and then switched to –RU LiCl for 5 days were not significantly different from ArcAβ42/UAS-gfp;elavGS/+ flies on RU +100 mM LiCl medium for 6 days, P = 0.6, nor were UAS-ArcAβ42/UAS-gfp;elavGS/+ flies on +RU 6 days post induction different in comparison to UAS-ArcAβ42/UAS-gfp;elavGS/+ flies on +RU –LiCl for 6 days switched on to –RU + LiCl for 5 days, P = 0.7. Data were compared using One-Way ANOVA, number of independent tests (n) = 4. (C) Proteasome activity, as measured using the fluorogenic peptide substrate LLVY-AMC, was not changed in flies over-expressing Arctic Aβ42 with or without LiCl treatment. Data are presented as mean activities (pmol/min/mg protein) ± s.e.m., P = 0.72 and 0.82 for 10 and 100 mM LiCl (n = 5).

LITHIUM DOWN-REGULATED TRANSLATION
To determine whether the effect of lithium on Aβ protein level was specific to Aβ, we investigated whether it affected
the level of green fluorescent protein (GFP), a protein that is irrelevant to AD pathology. Surprisingly, we found that 100 mM lithium significantly reduced levels of GFP in the neurons of adult flies (UAS-egfp/+;elavGS/+ +RU), $P = 0.01$ (Figure 2A). These data suggest that lithium affects protein synthesis through a mechanism that is not specific to Aβ. To determine whether overall level of translation was reduced by lithium, we carried out S-methionine radioactive tracer experiments in UAS-egfp/+;elavGS/+ flies. Interestingly, we found lower $^{35}$S-methionine incorporation into protein in the bodies of flies treated with lithium (pooled data of 10 and 100 mM lithium) in comparison to untreated control flies (Figure 2B), $P < 0.05$. Based on this finding, we measured the effect of lithium on polysome profiles of Aβ flies, as an indication of activity of the translation machinery. There was a significant reduction in the ratio of high (polysomes >4) to low (monosome/polysome 1-2-3) fraction in flies treated with lithium (10 mM) (UAS-ArcAβ42/+;elavGS/+ +RU +Li) in comparison to untreated controls (UAS-ArcAβ42/+;elavGS/+ +RU flies), 0.34 vs. 0.40, $P < 0.001$ (Figure 2C), again demonstrating that lithium reduced activity of the translation machinery, possibly through a stall in translation initiation.

We next determined whether lithium exerted its effect on translation through an effect on the activity of the mechanistic target of rapamycin (mTOR) pathway, which is involved in control of translation. S6K is a phosphorylation target of mTOR kinase in the mTORC1 complex (Bjedov and Partridge, 2011); interestingly, both phosphorylated S6K and total S6K were reduced in Aβ flies treated with lithium (UAS-ArcAβ42/+;elavGS/+ +RU +Li) in comparison to untreated flies (UAS-ArcAβ42/+;elavGS/+ +RU) (Figure 3A). However, the ratio of phosphorylated S6K to total S6K was not significantly

**FIGURE 2 | Lithium reduced overall protein synthesis/translation.** (A) Protein levels of UAS-egfp/+;elavGS/+ flies on +RU486 SY medium, and UAS-egfp/+;elavGS/+ flies on +RU +100 mM LiCl were measured by Western blot analyses at 7 days post-induction (see Materials and Methods). GFP levels were significantly reduced when comparing UAS-egfp/+;elavGS/+ +RU to UAS-egfp/+;elavGS/+ +RU +LiCl 100 mM, $P < 0.001$. Data were compared using One-Way ANOVA, number of independent tests ($n = 6$). (B) Protein synthesis was reduced in pooled UAS-egfp/+;elavGS/+ +RU +LiCl 10 mM and Li 100 mM treated flies in comparison to UAS-egfp/+;elavGS/+ +RU flies measured by $^{35}$S-methionine incorporation, $P < 0.05$. Data were analyzed by One-Way ANOVA ($n = 7$ for +RU and $n = 13$ for +RU +LiCl treated flies). (C) Polysome profiles showed a reduction in translation in lithium treated Aβ expressing flies, measured by calculating the area under the different fractions, and their ratios (area under the first 3 peaks for low fraction, and >4 for high, ratio >4/1-2-3) $P < 0.001$. Data were analyzed by paired t-test ($n = 5$). One representative figure is shown.
different between the two groups (Figure 3A). In these blots, because of the possibility that total protein content of the flies could have been altered by lithium, samples derived from a fixed number of fly heads were used. Interestingly, actin levels did not seem to be affected by lithium and were used as an additional loading control. This finding suggests that either the turnover rate of actin is very low, or there is a subset of proteins because of the possibility that total protein content of the flies did not seem to be affected by lithium and were used as an additional loading control. This finding suggests that either the turnover rate of actin is very low, or there is a subset of proteins that is not down-regulated by lithium. Treatment of Aβ-expressing flies with lithium (UAS-ArcAβ42++;elavGS/+ +RU +Li) in comparison to untreated flies (UAS-ArcAβ42++;elavGS/+ +RU), but the ratio of phosphorylated to total eIF2B-epsilon or eEF-2 was not significantly affected (Figures 4A,B). These data again demonstrate that lithium down-regulated protein levels, but they do not point to a specific role for eIF2B-epsilon or eEF-2.

Indeed, a clear-cut explanation of the effects of lithium may be elusive, since translation of many proteins involved in the protein synthesis machinery is presumably inhibited by lithium. Furthermore, we used a high dose of lithium (100 mM) to maximize our chances of identifying these downstream factors that are responsible for the effect of lithium on translation, and were unable to identify a specific role for the proteins we tested; and thus did not test lower concentrations of lithium. Overall, the data suggest that the reduction in Aβ levels we observe upon lithium treatment is as a consequence of reduction in protein synthesis, probably because of reduced expression of multiple proteins involved in initiation of translation, but do not identify the specific targets responsible.

**LITHIUM ALSO INHIBITS PROTEIN SYNTHESIS IN FISSION YEAST**

Reduction in protein synthesis has been frequently linked to increased lifespan, possibly attributable to both a reduction in energy consumption, because translation requires a significant amount of the energy budget, and a reduction in mis-translated polypeptides (Browne and Proud, 2002; Proud, 2002; Hansen et al., 2007; Hipkiss, 2007). Additionally, lithium has been shown to cause significant lifespan-extension in the nematode worm C. elegans and in Drosophila (McCull et al., 2008; Kasuya et al., 2009; Zarse et al., 2011). Therefore, we decided to turn into a simpler system to determine whether lithium has an evolutionarily conserved effect on translation, and whether it has an associated effect on lifespan. Unicellular yeast have been pivotal in the advancement of understanding of mechanisms of ageing (Kaeberlein et al., 2005; Kallis et al., 2013). Fast-growing fission yeast cells in mid-log phase were treated with 0.1 mM lithium chloride for 1 h and their translational profiles were compared to those of untreated (control) cells. Following lithium treatment, translation was reduced: high (polysomes 1–2–3) ratios were lower (0.51) in lithium treated yeast cells in comparison to untreated yeast cells (0.63), \( P < 0.001 \) (Figure 5A). Interestingly, the same dose of lithium extended the chronological lifespan of fission yeast, \( P = 0.001 \) (Figure 5B). Yeast is hence an ideal model organism in which to perform a genetic screen to identify the relevant targets of lithium for reduced protein synthesis and increased lifespan.
Lithium suppresses Aβ pathology by inhibiting translation

**FIGURE 4** | (A,B) Lithium reduced protein levels of phosphorylated and total eIF2β and eEF2. There was no change in the ratio of phosphorylated eIF2β or eEF2 to total protein levels of eIF2β or eEF2 in UAS-ArcAβ42/+;elavGS/++RU+LiCl 100 mM flies in comparison to UAS-ArcAβ42/+;elavGS/++RU controls, \( P = 0.6 \) and 0.7 respectively \( (n = 3) \).

**LITHIUM EXTENDED LIFESPAN OF FLIES EXPRESSING Aβ**

Since we observed an association between reduced global protein synthesis and increased longevity in fission yeast, we determined whether lithium could also extend the survival of Aβ expressing flies. We measured lifespan of flies expressing Arctic Aβ42 (UAS-ArcAβ42/+;elavGS/+ +RU) and treated chronically with different concentrations of lithium from two days post-eclosion. Flies expressing Arctic Aβ42 in adult neurons had a shortened median and maximum lifespan as previously reported \( (\text{Sofola et al., 2010}) \), which could be extended with 10, 25, and 50 mM \( (P < 0.001 \text{ for all doses}) \), but not with 1 or 100 mM lithium \( (\text{Figure 5C}) \). The data suggest that above a certain a threshold, somewhere between 50 and 100 mM, lithium no longer rescued the shortened lifespan of flies expressing Aβ, possibly because it became toxic. However, this toxicity with a high lithium dose was only evident when taken long term and/or in older flies, since both 10 mM \( (\text{Supplemental Figure 1B}) \) and 100 mM lithium previously published \( (\text{Sofola et al., 2010}) \) were able to protect against the Aβ-induced locomotor dysfunction measured after shorter term treatment and earlier in life.

**DISCUSSION**

Human life expectancy continues to increase at a steady rate in most countries worldwide, and has done so by almost 3 months per year in the last 160 years \( (\text{Oeppen and Vaupel, 2002}) \). Therefore, it is of great importance to tackle ageing-related diseases such as AD, because they are becoming increasingly prevalent. Because age is the biggest risk factor for AD, interventions that promote general increases in health during ageing could also be important and beneficial in AD.

Lithium is becoming increasingly implicated as a drug that can ameliorate ageing and neurodegenerative diseases. Several groups have shown that it extends lifespan in model organisms such as the nematode worm *C. elegans* and *Drosophila* \( (\text{McColl et al., 2008; Kasuya et al., 2009; Zarse et al., 2011}) \). Here, we showed that it also extends lifespan in fission yeast *Schizosaccharomyces pombe*, highlighting that this effect is conserved over large evolutionary distances. Fission yeast is an ideal organism for genetic screens, and future work should identify the molecular targets of lithium both for control of protein synthesis and of lifespan. Furthermore, slightly higher levels of lithium present in the drinking water have been reported as associated with reduced mortality in a Japanese human population \( (\text{Zarse et al., 2011}) \).

A substantial body of work has demonstrated that several neurodegenerative diseases and neurological disorders, including but not confined to stroke, schizophrenia, Fragile X syndrome, Huntington’s disease and Parkinson’s disease, benefit from the therapeutic properties of lithium \( (\text{Chiu et al., 2011}) \). In addition, several studies have investigated whether lithium has a beneficial effect in AD pathogenesis. Clinical trials conducted with lithium have yielded conflicting results; some have found benefits, whilst others have not \( (\text{Nunes et al., 2007; Macdonald and Briggs, 2008; Hampel et al., 2009}) \). Interestingly, a correlative study conducted in patients with bipolar disorder, suggested that patients that had been on chronic lithium treatment showed a reduced incidence of AD in comparison to patients that had not been on treatment \( (\text{Nunes et al., 2007}) \). And a more recent small-scale clinical trial on
mild cognitive impaired (MCI) patients found that low doses of lithium slowed cognitive decline (Forlenza et al., 2011). The investigators suggested that a reason for the previous conflicting data on the efficacy of lithium was probably attributable to the pathological states/stages at which the patients were given lithium. It is becoming increasingly evident that drug trials are most likely to yield positive effects when initiated early, at the MCI stage.

Our results add to existing data suggesting that lithium could be beneficial in ameliorating Aβ toxicity, and should be considered for a potential large-scale trial on MCI patients. It has the added advantage of being an already approved drug, used to treat bipolar patients. It does have side-effects, but these are minimal at the low doses used in the recent small-scale clinical study (Forlenza et al., 2011). We also found that there are limits to the beneficial/therapeutic benefits of lithium in fission yeast in chronological lifespan—lithium was unable to increase chronological lifespan at higher doses (data not shown) as well as in the Drosophila AD model. Previously, we showed in Sofola et al. (2010) that administering both 30 and 100 mM lithium into the fly food are effective in modulating Aβ neuronal toxicity as evidenced by the improved locomotor function in young flies (Sofola et al., 2010). These lithium concentrations were initially chosen based on the paper by Dokucu et al. (2005)—they showed that lithium concentrations ranging from 10 to 100 mM lithium in the fly food translates to roughly 0.05–0.4 mM in the fly tissue (Dokucu et al., 2005), so well below the toxic levels in patients and mice (Wood et al., 1986; Schou, 2001; Can et al., 2011). In this paper, we show that both 25 and 100 mM lithium reduce Aβ levels in a dose dependent manner at an early time point. We also find that lower doses of Lithium (10 and 25 mM) rescue the shortened longevity of the Aβ flies, but 100 mM lithium was unable to extend lifespan when given to the flies throughout adulthood. It will be important to determine the therapeutic thresholds for lithium in patients that could offer therapeutic benefits without overt side effects.

Similar to the published data on the role of GSK-3 inhibition in down-regulating translation in HCC1806 cells (Shin et al., 2014), we find that lithium is able to reduce translation in fission yeast and flies, suggesting that perhaps some of the effect of lithium on translation down-regulation is via GSK-3 inhibition. However, this is correlative and future work will involve carrying out epistasis interactions between lithium and GSK-3, and identifying molecular targets of GSK-3 and lithium for control of protein synthesis.

Nonetheless, our study highlights the potential benefits of lithium through down-regulation of translation, associated with extension of lifespan in very distantly related organisms. By reducing protein synthesis, lithium may reduce the increased proteostatic burden in ageing, a recognized hallmark of ageing (López-Otin et al., 2013). Lithium is also of specific benefit in AD, because of its ability to down-regulate translation, and hence levels of proteins involved in promoting the presence of toxic Aβ.

The mutant Arctic Aβ42 protein present in the transgenic flies used in this study has a propensity to aggregate faster than wild type Aβ (Spires and Hyman, 2005). However, we have observed both soluble and insoluble Aβ in the Arctic Aβ42 flies ([Rogers et al., 2012], and data not shown); and the ability of lithium to reduce translation of the Aβ peptide without affecting its clearance may lower the level of soluble Aβ. In a wider context, lithium might be beneficial in ameliorating toxicity of AD by lowering expression of APP and of proteins that are involved in the generation of Aβ from APP. Our model does not express full length APP,
and may therefore not include other potential/additional benefits of lithium on Aβ toxicity. As well as the increased ratio of Aβ42 to Aβ40 peptide observed in familial AD cases with APP mutations (De Jonghe et al., 2001), increased levels of APP could also contribute to AD pathogenesis. Indeed, patients with Down syndrome have a high risk of developing AD possibly due to trisomy of the APP gene which leads to increased APP expression (Wiseman et al., 2009). Also, several mutations in the APP promoter region were found to significantly increase APP expression in SH-SY5Y cells, and were associated with risk for AD (Theuns et al., 2006).

The ability of lithium to down-regulate translation could therefore be beneficial at several stages in AD pathogenesis. Lithium might also have therapeutic benefits for other neurodegenerative disorders that are caused by over-expression of wild type or mutant forms of proteins such as α-synuclein in Parkinson’s disease. Lithium could also reduce the production of mis-translated polypeptides, and free proteases or/and chaperones that can then participate in cellular proteostasis (Proud, 2002; Singleton et al., 2003). Furthermore, diseases where protein turnover is compromised by loss of function of the degradation machinery could also benefit from lowering the burden of protein production hence reducing cellular stress. This could be particularly important in lysosomal storage diseases, where the intrinsic function of the degradative machinery is compromised (Kinghorn, 2011; Sarkar et al., 2013). Moreover, induction of autophagy in some cases increases the load of an already dysfunctional lysosome, worsening the cellular proteostatic stress (Proud et al., 2002). Thus the role of lithium in lowering protein translation could be beneficial and, for example, the Drosophila models of Pink1 and Parkin, which do not include the over-expression of toxic proteins (Whitworth et al., 2006; Castillo-Quan, 2011). Flies lacking either of these proteins accumulate unfolded proteins in mitochondria, resulting in mitochondria impairment (Pimenta de Castro et al., 2012). It would be interesting to study whether lithium could ameliorate mitochondrial stress by reducing the production of the proteins accumulating in the mitochondria of the Pink1 or parkin null flies. Lithium could hence be a useful drug with an overall benefit for health during ageing and protection against AD and other neurodegenerative diseases.

ACKNOWLEDGMENTS

We thank Dr. D. Crowther (University of Cambridge) and Dr. H. Tricoire (CNRS, France) for their kind donation of UAS-Aβ42 and elavGS fly stocks, and Dr. Cathy Slack for total Drosophila antibody. This work was supported by grants from the Max Plank Institute for the Biology of Ageing (Oyinfolka-Adesakin, Jorge I. Castillo-Quan, Luke S. Tain, Iain Rogers, Ivana Bjedov, Mobina Khericha, Linda Partridge), Alzheimer’s Society (Oyinfolka-Adesakin), Eisai London Research Laboratories (UK) (Iain Rogers), Alzheimer’s Research UK (Pedro Martinez), a BBSRC Research Grant (Charalampos Rallis, Jürg Bähler), and the Wellcome Trust (Linda Partridge). This work is dedicated to Pedro Martinez—you’re legacy lives on.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnagi.2014.00190/abstract

Supplemental Figure 1 | (A) Rapamycin did not rescue the climbing phenotype of UAS-ArcA42+/+;elavGS/+ flies on +RU486 +200μM rapamycin, P = 0.83 (B) Lithium suppressed the locomotor dysfunction phenotype, P < 0.05. Climbing ability of UAS-ArcA42/UAS-gfp;elavGS/+ +RU486 and UAS-ArcA42/+/+;elavGS/+ flies on +RU486 +LIC1 10 mM SY medium or +Rapamycin 200 μM was assessed at the indicated time-points (see Materials and Methods). Data are presented as the percentage climbing performance of flies ± s.e.m. [Two-Way ANOVA, number of independent tests n = 3].

REFERENCES


Appendix 2

Loss of \textit{PLA2G6} leads to elevated mitochondrial lipid peroxidation and mitochondrial dysfunction

Kerri J. Kinghorn,\textsuperscript{1,2} Jorge Iván Castillo-Quan,\textsuperscript{1,2} Fernando Bartolome,\textsuperscript{2} Plamena R. Angelova,\textsuperscript{2} Li Li,\textsuperscript{1,2} Simon Pope,\textsuperscript{4} Helena M. Cocheme,\textsuperscript{1,3} Shabana Khan,\textsuperscript{1} Shabnam Asghari,\textsuperscript{5} Kailash P. Bhatia,\textsuperscript{2} John Hardy,\textsuperscript{2} Andrey Y. Abramov\textsuperscript{2} and Linda Partridge\textsuperscript{1,3}

The \textit{PLA2G6} gene encodes a group VIA calcium-independent phospholipase A2 beta enzyme that selectively hydrolyses glycerophospholipids to release free fatty acids. Mutations in \textit{PLA2G6} have been associated with disorders such as infantile neuroaxonal dystrophy, neurodegeneration with brain iron accumulation type II and Karak syndrome. More recently, \textit{PLA2G6} was identified as the causative gene in a subgroup of patients with autosomal recessive early-onset dystonia-parkinsonism. Neuropathological examination revealed widespread Lewy body pathology and the accumulation of hyperphosphorylated tau, supporting a link between \textit{PLA2G6} mutations and parkinsonian disorders. Here we show that knockout of the \textit{Drosophila} homologue of the \textit{PLA2G6} gene, \textit{iPLA2-VIA}, results in reduced survival, locomotor deficits and organisinal hypersensitivity to oxidative stress. Furthermore, we demonstrate that loss of \textit{iPLA2-VIA} function leads to a number of mitochondrial abnormalities, including mitochondrial respiratory chain dysfunction, reduced ATP synthesis and abnormal mitochondrial morphology. Moreover, we show that loss of \textit{iPLA2-VIA} is strongly associated with increased lipid peroxidation levels. We confirmed our findings using cultured fibroblasts taken from two patients with mutations in the \textit{PLA2G6} gene. Similar abnormalities were seen including elevated mitochondrial lipid peroxidation and mitochondrial membrane defects, as well as raised levels of cytoplasmic and mitochondrial reactive oxygen species. Finally, we demonstrated that deuterated polyunsaturated fatty acids, which inhibit lipid peroxidation, were able to partially rescue the locomotor abnormalities seen in aged flies lacking \textit{iPLA2-VIA} gene function, and restore mitochondrial membrane potential in fibroblasts from patients with \textit{PLA2G6} mutations. Taken together, our findings demonstrate that loss of normal \textit{PLA2G6} gene activity leads to lipid peroxidation, mitochondrial dysfunction and subsequent mitochondrial membrane abnormalities. Furthermore we show that the \textit{iPLA2-VIA} knockout fly model provides a useful platform for the further study of \textit{PLA2G6}-associated neurodegeneration.

\textsuperscript{1} Institute of Healthy Ageing and Department of Genetics, Evolution and Environment, University College London, London WC1E 6BT, UK
\textsuperscript{2} Institute of Neurology, University College London, Queen Square, London WC1N 3BG, UK
\textsuperscript{3} Max Planck Institute for Biology of Ageing, Joseph-Stelzmann Str. 9b, D-50931, Cologne, Germany
\textsuperscript{4} Neurometabolic Unit, National Hospital for Neurology and Neurosurgery, London WC1N 3BG, UK
\textsuperscript{5} Department of Family Medicine, Memorial University, St. John’s, NL, Canada

Correspondence to: Kerri Kinghorn,
Institute of Healthy Ageing and Department of Genetics, Evolution and Environment,
University College London,

\textsuperscript{1} Institute of Healthy Ageing and Department of Genetics, Evolution and Environment, University College London, London WC1E 6BT, UK
\textsuperscript{2} Institute of Neurology, University College London, Queen Square, London WC1N 3BG, UK
\textsuperscript{3} Max Planck Institute for Biology of Ageing, Joseph-Stelzmann Str. 9b, D-50931, Cologne, Germany
\textsuperscript{4} Neurometabolic Unit, National Hospital for Neurology and Neurosurgery, London WC1N 3BG, UK
\textsuperscript{5} Department of Family Medicine, Memorial University, St. John’s, NL, Canada

Received November 9, 2014. Revised March 9, 2015. Accepted March 17, 2015. Advance Access publication May 23, 2015
© The Author (2015). Published by Oxford University Press on behalf of the Guarantors of Brain.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.
Introduction

The PLA2G6 gene encodes an 85-kDa group VI calcium-independent phospholipase A2 beta (PLA2G6). This enzyme hydrolyses the sn-2 acyl chain of glycerophospholipids to release free fatty acids and lysophospholipids (Wolf and Gross, 1996; Ma and Turk, 2001). PLA2G6 localizes to the mitochondria, and has proposed roles in the remodelling of membrane phospholipids, signal transduction and calcium signalling, cell proliferation and apoptosis (Gadd et al., 2006; Seleznev et al., 2006; Strokin et al., 2012).

Humans with PLA2G6 mutations can show progressive cognitive and motor skill regression, as displayed in disorders such as infantile neuroaxonal dystrophy, neurodegeneration with brain iron accumulation type II and Karak syndrome (Khateeb et al., 2006; Morgan et al., 2006). Infantile neuroaxonal dystrophy is a neurodegenerative disease with onset in infancy and fatality in the teenage years or in early adulthood. It is characterized neuropathologically by axonal swelling and the presence of spheroid bodies in the central and peripheral nervous systems in addition to hallmark cerebellar atrophy. Neurodegeneration with brain iron accumulation comprises a clinically and genetically heterogeneous group of disorders with a progressive extrapyramidal syndrome and high basal ganglia iron, and includes pantothenate kinase-associated neurodegeneration caused by mutations in PANK2 (neurodegeneration with brain iron accumulation type I). Post-mortem examination of the brain of a patient with neurodegeneration with brain iron accumulation comprised of homozygous PLA2G6 mutations demonstrated both Parkinson’s and Alzheimer’s disease pathology with widespread Lewy bodies, dystrophic neurites and cortical neuronal neurofibrillary tangles (Gregory et al., 2008).

Furthermore, PLA2G6 has also been implicated in a number of other brain diseases including Alzheimer’s disease (Schaeffer and Gattaz, 2008) and bipolar disorder (Xu et al., 2013). More recently, PLA2G6, at the PARK14 locus, has also been characterized as the causative gene in a subgroup of patients with autosomal recessive early-onset dystonia-parkinsonism. Interestingly these patients did not display cerebellar atrophy or basal ganglia iron on MRI (Paisan-Ruiz et al., 2009), and neuropathological examination revealed widespread Lewy body pathology and the accumulation of hyperphosphorylated tau (Paisan-Ruiz et al., 2012). These clinical and neuropathological features further support a link between PLA2G6 mutations and parkinsonian disorders, and demonstrate the clinical heterogeneity in PLA2G6-associated neurodegeneration.

It is not known how mutations in PLA2G6 cause neuro-pathology. However, it has been suggested that the loss of normal PLA2G6 activity in neurodegenerative disease involves structural abnormalities of cell or mitochondrial membranes and disturbances in lipid homeostasis and lipid metabolism. Indeed, a study in a mouse Pla2g6 knockout model demonstrated ultrastructural abnormalities in mitochondrial and synaptic membranes (Beck et al., 2011). As well as possible mitochondrial defects, it is thought that changes in the composition of the plasma membrane and other intracellular membranes may in turn affect the normal processes responsible for the movement of membranes within axons and dendrites, subsequently leading to accumulation of membranes as so-called spheroids.

Support for a loss-of-function of PLA2G6 enzyme activity in causing disease comes from a recent study on recombinant wild-type and mutant human PLA2G6 proteins. This demonstrated that mutations in PLA2G6 associated with infantile neuroaxonal dystrophy or neurodegeneration with brain iron accumulation resulted in encoded proteins that exhibited <20% of control levels of phospholipase and lysophospholipase activities. Conversely, mutations associated with dystonia-parkinsonism did not impair catalytic activity. The differential enzymatic activities associated with PLA2G6 mutations in infantile neuroaxonal dystrophy/neurodegeneration with brain iron accumulation and dystonia-parkinsonism may explain the relatively later onset and milder phenotype seen in the latter. Furthermore, the preserved enzymatic function of dystonia-parkinsonism causing mutations must be interpreted with caution as there may be differences in PLA2G6 activity in vivo that are not detected by these in vitro assays. For example, there may be alternative mechanisms that alter the regulation of PLA2G6 activity, such as changes in the binding to calmodulin or other proteins (Engel et al., 2010). Further support for the loss of enzymatic function hypothesis comes from a recent study of a Chinese population with Parkinson’s disease, which identified novel PLA2G6 mutations occurring in the heterozygous state, associated with an inhibition in the phospholipid-hydrolysing functions of PLA2G6 (Gui et al., 2013). Moreover Gregory et al. (2008) found a genotype–phenotype correlation in patients with infantile neuroaxonal dystrophy and neurodegeneration with brain iron accumulation: mutations that are predicted to lead to an absence of protein were associated with more severe.

Abbreviations: D/H-PUFA = deuterated/hydrogenated polyunsaturated fatty acids; D$_2$-linoleic acid = 11-11-D$_2$-linoleic acid; D$_5$-linoleic acid = 11,11,14,14-D$_5$-linoleic acid; TMRM = tetramethylrhodamine methylester
infantile neuroaxonal dystrophy-type clinical phenotypes, while those with compound heterozygous missense mutations correlated with the less severe phenotype of neurodegeneration with brain iron accumulation and are predicted to result in protein with some residual enzyme function.

Genetic ablation of Pla2g6 in mice is associated with an \textit{in vivo} disturbance of brain phospholipid metabolism (Cheon \textit{et al.}, 2012), mitochondrial membrane degeneration (Beck \textit{et al.}, 2011) and marked cerebellar atrophy (Zhao \textit{et al.}, 2011). Furthermore, \textit{in vitro} experiments demonstrated a disturbance in calcium signalling in astrocytes from PLA2G6-deficient mice (Strokin \textit{et al.}, 2012). However, despite these studies, the precise molecular mechanisms linking loss of PLA2G6 activity to mitochondrial morphological abnormalities and neurodegeneration remain unclear and further studies are required.

The fruit fly \textit{Drosophila melanogaster} has proven to be an excellent model system for studying neurodegenerative diseases, especially the role of mitochondrial activity in Parkinson’s disease (Clark \textit{et al.}, 2006; Park \textit{et al.}, 2006). Once a faithful model is established, the fly has the advantage over mouse models in that it can be used to perform high-throughput screening to identify genetic modifiers and novel therapeutic targets. Here we show that \textit{iPLA2-VIA}, the \textit{Drosophila} homologue of PLA2G6, plays an essential role in maintaining normal mitochondrial function. We demonstrate that knockout of the \textit{iPLA2-VIA} gene activity in the fly results in reduced survival, locomotor deficits and organisal hypersensitivity to oxidative stress. Furthermore, loss of \textit{iPLA2-VIA} function leads to a number of mitochondrial abnormalities, including reduced mitochondrial membrane potential, mitochondrial respiratory chain dysfunction and reduced ATP synthesis. We also show that levels of lipid peroxidation are significantly elevated in the brains of flies lacking \textit{iPLA2-VIA}. In addition, we examined cultured fibroblasts taken from two patients with known \textit{PLA2G6} mutations and found similar abnormalities to those seen in the fly, including mitochondrial membrane abnormalities and raised levels of cytoplasmic and mitochondrial reactive oxygen species. Moreover, lipid peroxidation levels, especially in the mitochondria, were elevated in the \textit{PLA2G6} mutant human fibroblasts compared to controls. Taken together, our findings suggest that loss of normal \textit{PLA2G6} gene activity leads to mitochondrial lipid peroxidation, mitochondrial dysfunction and mitochondrial membrane abnormalities. Finally we demonstrate that reduction of lipid peroxidation, by treatment with deuterated polyunsaturated fatty acids (D-PUFAs), is able to recover the mitochondrial membrane potential of \textit{PLA2G6} mutant human fibroblasts and partially rescue the locomotor deficits in \textit{iPLA2-VIA} knockout flies. We have therefore shown that the \textit{iPLA2-VIA} knockout fly appears to be a useful model organism for studying PLA2G6-associated neurodegeneration.

### Materials and methods

#### Fly stocks and husbandry

The fly stocks \textit{y[1]} \textit{w[67C23]; P[w[ + mC] y[ + mDm2]=EPgy2]} \textit{iPLA2-VIA[EY05103]}. \textit{actin-5C-GAL4, elav-GAL4} were obtained from the Bloomington \textit{Drosophila} Stock Centre. The \textit{iPLA2-VIA} RNAi (HMS01544) line was provided by the TRiP at Harvard Medical School. All fly strains used were backcrossed at least six generations into the \textit{w[1118]} background to obtain isogenic lines. All fly stocks were maintained at 25°C on a 12:12 hour light: dark cycle at constant humidity on a standard sugar-yeast (SY) medium (15 g/l agar, 50 g/l sugar, 100 g/l autolysed yeast, 100 g/l nipagin and 3 ml l/ propionic acid). For all experiments, flies were raised at a standard density on standard SY medium in 200 ml bottles unless otherwise stated. Tissue-specific expression of \textit{iPLA2-VIA} RNAi constructs was achieved by using the \textit{GAL4-UAS} system [GAL4-dependant upstream activator sequence (Brand and Perrimon, 1993)].

#### Lifespan analyses

The survival assays were performed using newly eclosed flies that were allowed to mature and mate for 24 h before the females and males were separated and collected. One hundred and fifty flies were housed in groups of 15 and the flies were transferred every 2 to 3 days onto fresh food and the number of dead flies recorded. Data are presented as cumulative survival curves, and survival rates were compared using log-rank tests.

#### Climbing assays

The climbing assay was performed using a negative geotaxis assay according to previously published methods (Rival \textit{et al.}, 2004). Briefly, 15 adult flies were placed in a vertical column (25 ml tissue culture pipette), and allowed to climb for 45 s before the number of flies at the top and bottom was determined. A performance index (PI) defined as 0.5*(n(top) + n(bottom))/ n(total) was calculated. The experiment was repeated three times for each time point.

#### Fertility tests

For female fecundity tests, female flies were housed with males for 48 h post-eclosion and then separated into vials at a density of 10 females per vial. Eggs were collected over a 24-h period at different time points. The number of eggs laid per vial at each time point was counted.

#### Quantitative RT-PCR

Total RNA was extracted from 15 flies per sample using TRIzol \textsuperscript{®} (GIBCO) according to the manufacturer’s instructions. The concentration of total RNA purified for each sample was measured using an Eppendorf biophotometer. One microgram of total RNA was then subjected to DNA digestion using DNase I (Ambion), immediately followed by reverse transcription using the SuperScript\textsuperscript{®} II system.
(Invitrogen) with oligo(dT) primers. Quantitative PCR was performed using the PRISM 7000 sequence-detection system (Applied Biosystems, SYBR® Green (Molecular Probes), ROX Reference Dye (Invitrogen), and HotStarTaq (Qiagen) by following the manufacturer’s instructions. Each sample was analysed in triplicate with both target gene (iPLA2-VIA) and reference gene (RP49) primers in parallel. The end products of the RT-PCR reactions were also visualized by staining with ethidium bromide following separation with agarose-gel electrophoresis. The primers for the Drosophila iPLA2-VIA used in the RT-PCR of the EY05103 fly line were as follows: forward 5'--TACGGAATTTGCAGGATAA GG-3' and reverse 5'-GATTGGATATTCGACCGAGAG-3'. The primers used for the RT-PCR performed on the iPLA2-VIA RNAi (HMS01544) fly line were as follows: forward 5'-GAtriaxglycerolTAGTGCCCCGATGGTCCAA-3' and reverse 5'-GAACCTGATATCCCTGGCAGG-3'. The reference gene primers were as follows: forward 5'-ATGACCATCGCCCCAGCATCAGG-3' and reverse 5'-ATCTGGGCCAGTAAAACG-3'.

Stress experiments

For all stress assays, flies were reared and housed as for life-span experiments. For oxidative stress assays, 7-day or 15-day old flies were transferred onto 5% sucrose/agar containing 5% hydrogen peroxide (Sigma) or 20 mM paraquat (Sigma). For starvation and osmotic stress experiments, 7-day or 15-day old flies were transferred to 1% agar or 500 mM NaCl respectively. For hypoxia experiments 15-day old flies were transferred to a hypoxia chamber with oxygen levels of 7%. After 15 h the flies were removed from the chamber and the number of dead flies was counted.

Triacylglyceride and ATP assays

Levels of triacylglyceride in adult females were measured using the Triglyceride Infinity Reagent (ThermoScientific) and normalized to total body protein using a BCA assay (Sigma). The ATP concentration of whole female flies was determined using the Roche ATP Bioluminescence Assay Kit HS II (Roche). Briefly, two live whole flies or eight fly heads were homogenized in 100 μl ice-cold lysis buffer (provided in the kit) for 1 min using a Kontes pellet pestle. The lysate was then boiled for 5 min and centrifuged at 20,000 g for 1 min. 2.5 μl of cleared lysate was added to 187.5 μl dilution buffer and 10 μl luciferase and the luminescence was immediately measured using a Tecan Infinite M2000 microplate reader and Magellan V6.5 software. Each reading was converted to the amount of ATP per fly based on the standard curve generated with ATP standards. At least three measurements were made for each genotype and the experiment was repeated three times.

Isolation of Drosophila mitochondria

Fly mitochondria were isolated by differential centrifugation, essentially as described in Miwa et al. (2003). Briefly, adult flies (n = 500 for each time point) were chilled on ice and gently pressed using a pre-chilled pestle and mortar. Importantly, the pestle was moved in a vertical motion (with no horizontal motion) until the shape of the flies was no longer visible. The flies were then washed in STE + BSA buffer [250 mM sucrose, 5 mM Tris, 2 mM EGTA, pH 7.4 (4°C), 1% bovine serum albumin]. The flies were then pressed further in 5 ml of STE + BSA buffer. The squashed flies were then passed through double-layered muslin cloth. The collected pulp was then spun for 3 min at 4°C at 1500 rpm to remove debris. The supernatant was then passed through a single layer of muslin into a clean centrifuge tube and spun for 10 min at 4°C and 10,000 rpm to collect the mitochondria. The mitochondrial pellet was then suspended in 250 μl STE + BSA buffer and stored on ice. A BCA assay (Sigma) was used to determine the protein concentration of the mitochondrial preparations. The isolated mitochondria were kept on ice and used immediately for experiments.

Drosophila mitochondrial respiration measurement

Oxygen consumption was measured using a Clark-type oxygen electrode thermostatically maintained at 25°C. Glutamate (5 mM) and malate (5 mM) or pyruvate (5 mM) were added to measure Complex I-linked respiration, and succinate (5 mM) with rotenone (5 μM) were added to measure Complex II-linked respiration. All data were obtained using an OxycopGraph Plus system with Chart recording software.

Light and electron microscopy of fly brains

Flies were decapitated and the proboscis was removed to allow penetration of the fixative. The heads were perfused with 3% glutaraldehyde in phosphate buffer overnight. Brains were then treated with 1% osmium tetroxide for 3 h at 4°C and embedded in Araldite® CY212 resin. Thin sections were stained with toluidine blue and the brain visualized with the light microscope. Ultrathin sections (70 μm) were stained with lead citrate and uranyl acetate and digital images taken on a Phillips CM10 electron microscope with a Megaview III digital camera (Olympus).

Human fibroblast collection and culture

The p.R747W PLA2G6 fibroblasts were generated from a 4 mm skin punch biopsy taken under local anaesthetic. Biopsies were dissected into ~1 mm pieces and cultured in 5 cm petri dishes in Dulbecco’s modified Eagle’s medium (DMEM), 10% foetal bovine serum, 1% L-glutamine until fibroblasts were seen to grow out from the explants. When fibroblasts reached confluency, they were detached from culture dishes using TrypLe™ (Invitrogen) and transferred to larger culture vessels for further expansion and cryopreservation. This sample was collected with the written consent of the participant and formal ethical approval from the National Hospital for Neurology and Neurosurgery-Institute of Neurology Joint Research Ethics Committee (London, UK). The GM11529 mutant PLA2G6 human fibroblast cell cultures were obtained from the Coriell Cell Repositories. This line was sampled from a patient with infantile neuroaxonal dystrophy.
Fibroblasts were seeded at a density of $4 \times 10^5$ cells/cm$^2$, grown to 75–80% confluence and maintained at 37°C and 5% CO$_2$ in DMEM supplemented with 10% (v/v) foetal bovine serum, 2mM L-glutamine and 1% (v/v) penicillin/ streptomycin.

**Measurement of mitochondrial membrane potential and cytosolic and mitochondrial reactive oxygen species**

For measurements of mitochondrial membrane potential ($\Delta \Psi_{mt}$), dissected fly brains or cells were loaded with 25nM tetramethylrhodamine methylester (TMRM) for 30 min at room temperature and the dye was present for the duration of the experiment. TMRM is used in the redistribution mode to assess $\Delta \Psi_{mt}$, and therefore a reduction in TMRM fluorescence represents depolarization.

**Confocal microscopy of mitochondrial membranes in fly brains and human fibroblasts**

Confocal images were obtained using a Zeiss 710 VIS CLSM equipped with a META detection system and a ×40 oil immersion objective. Illumination intensity was kept to a minimum (at 0.1–0.2% of laser output) to avoid phototoxicity and the pinhole set to give an optical slice of ~2μm. TMRM was excited using the 560 nm laser line and fluorescence measured above 580 nm. All data presented were obtained from at least five coverslips and two to three different cell preparations for cell experiments, and six coverslips and at least six counts for fly brain experiments. The same anatomical area of the fly brain was used each time.

**Measurement of reactive oxygen species levels in human fibroblasts**

For measurement of mitochondrial reactive oxygen species production, cells were preincubated with 5μM MitoSOX™ (Molecular Probes) for 10 min at room temperature. Cytosolic reactive oxygen species generation was measured with 2μM dihydroethidium with the dye present in all solutions throughout the duration of the experiment.

Fluorescence images were obtained on an epifluorescence inverted microscope equipped with a ×20 fluorite objective (Cairn Research). Ratiometric dihydroethidium and MitoSOX fluorescence was recorded with an excitation light at 380 and 530nm and emission at 440 and 630nm, respectively.

**Measurement of lipid peroxidation in fly brains and human fibroblasts**

C11-BODIPY581/591 (Molecular Probes) is a sensitive fluorescent probe for indexing lipid peroxidation in model membrane systems and living cells undergoing a shift from red to green fluorescence emission upon oxidation, even under physiological oxidation (Vaarmann et al., 2010). Fly brains were dissected in phosphate-buffered saline and placed into Hank’s balanced salt solution (HBSS). C11-BODIPY581/591 (5μM) was loaded for 15 min at 20°C. Coverslips were washed once and resuspended in 1 ml of HBSS. Ratiometric measurements of probe oxidation were taken over a 12 min time course. Fluorescence excitation was at 488 nm, green fluorescence was detected at 530 nm and red fluorescence at 670 nm.

**Cardiolipin analysis by mass spectrometry**

Cardiolipin and oxidized cardiolipin were measured by mass spectrometry essentially as described previously (Pope et al., 2008; Heather et al., 2010; Rodriguez-Cuenca et al., 2010) with some minor modifications to make the analysis specific for Drosophila cardiolipin species. In brief, 500 flies of each genotype were homogenized in NaPi buffer (50mM NaPi, 1 mM EDTA, 100mM DTPA, pH 7.5) using a Kontes pestle. The homogenates were then spun for 2 min at 13,000 rpm to remove fly debris. Lipids were extracted using a modified Folch extraction and the combined organic layers were evaporated to dryness under nitrogen. Before analysis, samples were rehydrated in 1 ml of a 1:1 mix of buffers A and B (buffer A, 15% water, 85% methanol containing 0.1 ml of 250 ml/l aqueous ammonia per litre; buffer B, 97% chloroform, 3% methanol containing 0.1 ml of 250 ml/l aqueous ammonia per litre). 1 nmol of (C14:0)$_2$-cardiolipin (Avanti Polar Lipids) was used as an internal standard and was added to Drosophila head homogenates.

The LC/MS/MS system (Waters 2795 separation unit and a Quattro Micro API triple quadrupole mass spectrometer) and parameters were as described previously (Heather et al., 2010; Rodriguez-Cuenca et al., 2010) except that the multiple reaction monitoring (MRM) transitions were specific for Drosophila cardiolipin. Mass spectra from retention times 5.5–6.0 min were combined to generate cardiolipin spectra. Cardiolipin species were identified in MRM mode with the collision gas at 0.25Pa and collision energy at 35 eV. Cardiolipin species were analysed as their dianions [CL–2H]$^{2-}$. In these samples, the dominant form of cardiolipin was (C16:1)$_2$-cardiolipin (m/z 671), which was therefore selected for analysis based on its fragmentation to a C16:1 fatty acid (m/z 253) by monitoring the 671 > 253 transition. Similarly, other cardiolipin species (C18:2) _2_ (C16:1)$_2$-cardiolipin (m/z 684) and (C18:2) _2_ (C16:1)$_2$-cardiolipin (m/z 697) were analysed by assessing transitions of 684 > 253 and 697 > 253, respectively. Oxidized cardiolipin was analysed by the transitions 678 > 253 and 691 > 253 transition. The internal standard (C14:0)$_2$-cardiolipin (m/z 619) was analysed by its fragmentation to a C14:0 fatty acid (m/z 227) using the transition 619 > 227. Data were analysed with QuanLynx software (Waters). Total cardiolipin content was expressed as peak area relative to that of the internal standard. The amounts of each cardiolipin and oxidized cardiolipin were expressed relative to the total amount of cardiolipin in the sample, giving dimensionless ratios.
Deuterated polynsaturated fatty acids

Deuterated polynsaturated fatty acids (D-PUFAs) 11,11-2H-linoleic acid (D2-linoleic acid) and 11,11,14-1806-2H-linoleic acid (D4-linoleic acid) were kindly provided by Dr Mikhail S. Schepinov (Retrototope, Inc), and their synthesis has been previously described (Hill et al., 2012). Hydrogenated polynsaturated fatty acids (H-PUFAs) were obtained from Sigma–Aldrich (99%). Cells were preincubated with 10 μM H- or D-PUFA (D2-linoleic acid) in the culture media for 24 h and then washed with HBSS before experiments. For the fly experiments, D2-linoleic acid was diluted in water and added to the standard fly medium to give a final concentration of 10 or 100 μM.

Statistics

Data were expressed as means ± standard error of the mean (SEM). Significant differences were evaluated by unpaired two-tailed Student’s t-test and significance levels are described in individual figure legends. Climbing assays were analysed using a two-way ANOVA or a one-way ANOVA with Bonferroni correction.

Results

Drosophila lacking iPLA2-VIA activity display age-dependent locomotor deficits and reduced lifespan

A null mutant fly stock completely lacking the Drosophila orthologue of the PLA2G6 gene, iPLA2-VIA, was obtained from the Bloomington Drosophila Stock Centre. We will refer to null mutant flies lacking both iPLA2-VIA genes as iPLA2-VIA−/− henceforth. This fly strain carries a P-element insertion (EY05103) in the iPLA2-VIA gene (Fig. 1A). As previously shown, RT-PCR analysis confirmed that there was complete knockout of iPLA2-VIA gene expression in this strain with no detectable SYBR green signal produced. The end products of the RT-PCR reactions were also visualized on agarose gel electrophoresis, which confirmed the complete absence of iPLA2-VIA gene expression in the iPLA2-VIA−/− flies (Fig. 1B) (Malhotra et al., 2009). Knockout of iPLA2-VIA activity in the fly did not produce any gross changes in body size or obvious differences in morphology (data not shown).

iPLA2-VIA is expressed throughout the adult fly body, including the brain and eye. The iPLA2-VIA gene displays good homology with the human PLA2G6 gene, with a 51% identity and 67% positivity (FlyBase).

The iPLA2-VIA−/− flies displayed progressive locomotor deficits, with the climbing ability of iPLA2-VIA−/− flies in response to light tapping progressively falling below that of w1118 controls from ~20 days of age (Fig. 1C). Accompanying this climbing defect the iPLA2-VIA−/− flies also showed an ~50% reduction in lifespan, in both females (Fig. 1D) and males (Supplementary Fig. 1A). Furthermore, flies heterozygous for the EY05103 P-element insertion (iPLA2-VIA), with presumed 50% gene activity only, did not display reduced survival (Fig. 1D and Supplementary Fig. 1A). To ensure that the reduced lifespan was, indeed, due to the EY05103 P-element insertion, we generated flies with precise excisions of the P-element, by introducing a transposase in an appropriate cross, and found that this restored a normal lifespan (Supplementary Fig. 1B).

To confirm the effect of iPLA2-VIA deficiency in the fly, we also inhibited iPLA2-VIA gene expression by transgenic RNA interference (RNAi) technology, using the GAL4/UAS system (Brand and Perrimon, 1993). Ubiquitous expression of iPLA2-VIA dsRNAi (UAS-iPLA2-VIA-RNAi) with the actin-GAL4 driver resulted in a ~40% reduction in whole body iPLA2-VIA gene expression using RT-PCR analysis (Supplementary Fig. 1C). This reduction was associated with an age-dependent decrease in climbing ability (Fig. 1E) and a shortened lifespan (Fig. 1F). It can therefore be robustly concluded that genetic inactivation of iPLA2-VIA activity results in progressive locomotor deficits and a reduction in lifespan in Drosophila. To determine whether these phenotypes were specific to loss of iPLA2-VIA activity in the brain, we knocked down iPLA2-VIA in fly neural tissue using UAS-iPLA2-VIA-RNAi driven by the elavC118-Gal4 driver. We observed a significant reduction in lifespan in these flies (Fig. 1G), confirming the brain-specific effects of iPLA2-VIA deficiency.

We also assessed the fecundity of iPLA2-VIA−/− female flies. The number of eggs laid per female significantly declined with age compared with age-matched w1118 control flies, and by Day 25 the null mutant flies were almost completely infertile (Supplementary Fig. 1D).

Brains lacking iPLA2-VIA display severe, widespread neurodegeneration and mitochondrial degeneration

Light microscopic examination of thin paraffin sections of iPLA2-VIA−/− fly brain revealed normal brain architecture in young Day 2 flies that was indistinguishable from w1118 controls (Fig. 2A and B). However, by Day 32 there was significant widespread vacuolation in the iPLA2-VIA−/− fly brains that was not present in w1118 control flies (Fig. 2C and D). Ultrastructural examination using electron microscopy revealed that the mitochondria were grossly abnormal and swollen with fragmented cristae in aged Day 32 iPLA2-VIA−/− fly brains as compared to the mitochondria in age-matched w1118 control brains (Fig. 2E–H) or younger Day 2 iPLA2-VIA−/− flies (data not shown), which were both filled with densely packed cristae. Furthermore, examination of the fly eye using electron microscopy revealed abnormal retinal structure in the aged Day 32 iPLA2-VIA−/− flies compared with age-matched
We next isolated mitochondria from older flies, with grossly abnormal ommatidial structure (Supplementary Fig. 2A and B).

Loss of iPLA2-VIA resulted in reduced mitochondrial membrane potential and abnormal mitochondrial respiratory chain activity

Given the abnormal mitochondrial membrane morphology in older iPLA2-VIA−/− flies, we investigated whether loss of iPLA2-VIA affected mitochondrial membrane potential (ΔΨm), an indicator of mitochondrial health and function. Mitochondrial membrane potential was assessed in the brains of 25-day-old flies using TMRM fluorescence and imaging with confocal microscopy. This revealed a significant reduction in mitochondrial membrane potential in iPLA2-VIA−/− fly brains compared to controls (Fig. 3A). We next isolated mitochondria from iPLA2-VIA−/− flies and assessed respiratory chain activity by measuring oxygen consumption using a Clark-type oxygen electrode. The steps in respiration were compared in mitochondria isolated from iPLA2-VIA−/− flies and control w1118 flies with substrates and inhibitors specific to individual respiratory complexes. We found that knockout of iPLA2-VIA activity conferred a decrease in complex I and II-dependent oxygen consumption in state 3 respiration, whether using substrates for complex I (5 mM glutamate and 5 mM malate) or for complex II (5 mM succinate in the presence of rotenone) (Fig. 3B). The respiratory control ratio is a measure of the degree of coupling of mitochondrial respiration to oxidative phosphorylation (Chance and Williams, 1955). The respiratory control ratio was significantly lower in iPLA2-VIA−/− mitochondria compared with age-matched control w1118 flies (Fig. 3C), because of...
Figure 2 Fly brains lacking iPLA2-VIA display severe mitochondrial degeneration at the ultrastructural level. (A) Light microscopic examination of Day 2 w1118 control (scale bar = 20 μm) and (B) iPLA2-VIA−/− fly brains (scale bar = 30 μm). (C) At Day 32 w1118 control fly brains display healthy brain tissue compared with (D) iPLA2-VIA−/− fly brains, which display widespread vacuolation (arrows) (× 60 magnification; scale bars 20 μm). (E and F) Ultrastructural examination of aged Day 32 w1118 control fly brains (× 2500; scale bar = 0.6 μm and × 5300; scale bar = 0.2 μm) revealed normal mitochondrial morphology, which is in contrast to (G and H) Day 32 iPLA2-VIA−/− fly brains, which display significant degeneration of the mitochondrial cristae (× 3100; scale bar = 0.5 μm and × 5300; scale bar = 0.2 μm). Images representative of six fly brains examined for each genotype. No abnormal mitochondria were seen in the w1118 control fly brains.
the inhibition of state 3 respiration. Furthermore, the respiratory control ratio of iPLA2-VIA−/− flies in the presence of glutamate/malate was dependent on the age of the flies: older flies having lower respiratory control ratio values (Fig. 3C). Thus, loss of iPLA2-VIA activity reduces mitochondrial membrane potential as a result of mitochondrial uncoupling, which increases with age in the iPLA2-VIA−/− flies.

Loss of iPLA2-VIA activity results in reduced ATP levels and increased sensitivity to oxidative stress

In keeping with the mitochondrial dysfunction caused by loss of iPLA2-VIA activity, we also demonstrated that ATP levels in aged, Day 25, iPLA2-VIA−/− fly heads were reduced by 40% compared with control flies (Fig. 4A). Levels were also reduced in whole iPLA2-VIA−/− flies compared with controls (Supplementary Fig. 3A).

Mitochondrial dysfunction can lead to decreased resistance to reactive oxygen species, which has been implicated in many neurodegenerative diseases. To explore the role of iPLA2-VIA in resistance to oxidative stress, we examined the survival of iPLA2-VIA−/− flies after exposure to the potent oxidizer hydrogen peroxide and paraquat, a free radical generator. Consistent with the structural mitochondrial abnormalities in aged flies, iPLA2-VIA−/− flies showed an age-dependent, mild sensitivity at Day 15 to hydrogen peroxide and, more so, to paraquat (Fig. 4B and C) that was not consistently present in younger 7-day-old flies (data not shown). Both young Day 7 and older Day 15 iPLA2-VIA−/− flies were also sensitive to osmotic stress and showed reduced survival compared to w1118 control flies when fed 500 mM NaCl (Supplementary Fig. 3B). Day 15 iPLA2-VIA−/− flies also displayed increased sensitivity to hypoxic and xenobiotic (dichlorodiphenyltrichloroethane, DDT) stresses (Supplementary Fig. 3C and D).

Loss of iPLA2-VIA was not associated with changes in cardiolipin composition

Given that mitochondrial membrane morphology appeared to be abnormal in the iPLA2-VIA−/− flies, we performed mass spectroscopic analysis of cardiolipin, one of the major phospholipids in mitochondrial membranes. It has been hypothesized that PLA2G6 is critical for the normal repair and remodelling of damage to cardiolipin caused by mitochondrial reactive oxygen species, and that loss of PLA2G6 activity may lead to cardiolipin oxidation and pathogenic downstream events such as induction of apoptosis. To test this hypothesis, we performed mass spectroscopic analysis on iPLA2-VIA−/− fly heads to compare the cardiolipin composition with that in w1118 control flies. We found no significant differences in the cardiolipin content, monolysocardiolipin content or fatty acid composition between iPLA2-VIA−/− and control w1118 fly brains (Supplementary Fig. 4). Similar results have previously been reported in whole flies lacking iPLA2-VIA (Malhotra et al., 2009). Furthermore, we found that oxidized cardiolipin was not detected in significant amounts in either the iPLA2-VIA−/− or control w1118 fly head homogenates (Supplementary Fig. 4). Oxidized cardiolipin

Figure 3 Flies lacking iPLA2-VIA have reduced mitochondrial membrane potential and decreased mitochondrial respiratory chain activity. (A) The mitochondrial membrane potential measured using TMRM fluorescence in the brains of 25-day-old flies was significantly decreased in iPLA2-VIA−/− brains compared to w1118 controls (***P < 0.001; n = 6 brains for each genotype). (B) The respiratory chain activity was measured by assessing oxygen consumption and demonstrated that mitochondria from young Day 2 and old Day 32 iPLA2-VIA−/− flies have reduced complex I and II-dependent respiration compared with w1118 control flies, manifested as significantly decreased oxygen consumption in state 3 respiration (**P < 0.05, ***P < 0.001). (C) The respiratory control ratio was significantly lower in iPLA2-VIA−/− mitochondria compared with age-matched control w1118 mitochondria. Furthermore, the respiratory control ratio of iPLA2-VIA−/− flies in the presence of glutamate/malate was dependent on the age of the flies: older flies having lower respiratory control ratio values (**P < 0.05, ***P < 0.001).
species would be expected to be present as additions of $+7/8 \text{ m/z}$ on the scan spectra. Our results therefore do not support a role for oxidized cardiolipin in causing mitochondrial dysfunction and neurodegeneration in flies lacking iPLA2-VIA activity.

Loss of iPLA2-VIA promotes brain lipid peroxidation and reduces whole body triacylglycerol levels

Despite the absence of changes in oxidized cardiolipin, we hypothesized that loss of PLA2G6 may lead to abnormal and excessive oxidation of other mitochondrial membrane lipids, which in turn would lead to the observed mitochondrial dysfunction. We therefore studied the effect of knocking out iPLA2-VIA on lipid peroxidation in Drosophila. Rates of lipid peroxidation were measured by live imaging of Day 25 iPLA2-VIA$^{-/-}$ and $w^{1118}$ control dissected fly brains, using the fluorescent ratiometric oxidation-sensitive dye C11 BODIPY581/591, which shifts its fluorescence from red to green in a time-dependent manner, the rate being proportional to levels of lipid peroxidation. The changes in colour shift were detected by confocal microscopy. This demonstrated that levels of lipid peroxidation in flies lacking the iPLA2-VIA gene were 3.5-fold higher than in age-matched control fly brains (Fig. 5A).

There is evidence that PLA2G6 may play a role in the regulation of triacylglycerol synthesis by providing endogenous fatty acids (Gubern et al., 2009). Triacylglycerol is the main form of lipid storage in the fly and therefore reflects the ability of flies to respond to starvation conditions (Ballard et al., 2008). Measurement of whole-body triacylglycerol levels in female flies revealed a significant age-dependent reduction in both control $w^{1118}$ and iPLA2-VIA$^{-/-}$ flies. However, the reduction was significantly greater by Day 20 in the flies lacking iPLA2-VIA activity (Fig. 5A). In keeping with an age-dependent decrease in triacylglycerol levels, iPLA2-VIA$^{-/-}$ flies were not sensitive to starvation conditions at Day 7 but became sensitive at Day 15 compared with control $w^{1118}$ flies (Fig. 5C).

Human mutant PLA2G6 fibroblasts display abnormal mitochondrial physiology

Given that loss of iPLA2-VIA led to increased lipid peroxidation and mitochondrial dysfunction in Drosophila, we examined fibroblasts taken from a patient with a known homozygous p.R747W mutation in PLA2G6 causing dystonia-parkinsonism (Paisan-Ruiz et al., 2009). Using TMRM fluorescence to probe for mitochondrial membrane potential, we found that there was a significant decrease in basal mitochondrial membrane potential in the mutant human fibroblasts (Fig. 6A and B), similar to that seen in fly brains lacking iPLA2-VIA.

Abnormal respiration and low mitochondrial membrane potential can induce excessive reactive oxygen species production in the matrix of mitochondria. Indeed the rate of mitochondrial reactive oxygen species production, as assessed using MitoSOX fluorescence, was increased by 28% relative to control human fibroblasts (Fig. 6C and E). Cytosolic reactive oxygen species were also significantly higher in the fibroblasts with a PLA2G6 mutation (Fig. 6D and E).

Mutations in PLA2G6 are associated with elevated mitochondrial lipid peroxidation levels in human fibroblasts and are reversed by treatment with deuterated polyunsaturated fatty acids

Following the demonstration that there is an increase in lipid peroxidation in the brains of flies lacking iPLA2-VIA compared with controls, we also assessed lipid peroxidation in two human PLA2G6 fibroblast lines; one taken
from a patient with dystonia-parkinsonism with a p.R747W mutation (Paisan-Ruiz et al., 2009) and the other from a child with infantile neuroaxonal dystrophy. We assessed lipid peroxidation levels using the fluorescent ratiometric oxidation-sensitive C11 BODIPY581/591 dye. This demonstrated that PLA2G6 mutant fibroblasts had an increased level of lipid peroxidation compared to controls (Fig. 7A), suggesting therefore that lipid peroxides may be a potential therapeutic target.

Natural polyunsaturated fatty acids (PUFAs) such as linoleic acid undergo autoxidation by reactive oxygen species, resulting in the production of toxic reactive carbonyl compounds that can mediate DNA damage, carcinogenesis and inflammation. However, replacement of the bis-allylic hydrogen atoms with deuterium atoms (termed site-specific isotope-reinforcement) arrests PUFA autoxidation due to the isotope effect. Indeed, even small amounts of isotope-reinforced D-PUFA are able to protect yeast from lipid autoxidation-mediated cell killing (Hill et al., 2011, 2012). These benefits have also been confirmed in vivo with the demonstration that D-PUFAs reduce nigrostriatal degeneration in a mouse model of Parkinson’s disease (Shchepinov et al., 2011). We therefore pretreated human PLA2G6 mutant fibroblasts for 24 h with D-PUFA (D2-linoleic acid) and found that this decreased lipid peroxidation in the cells to below control levels (Fig. 7A). This was also associated with a rescue in the reduced mitochondrial membrane potential (Fig 7B). Moreover, assessment specifically of lipid peroxidation levels in digitonin-permeabilized cells, which destroys all cell membranes except mitochondrial ones, also demonstrated that lipid peroxidation was substantially increased. In addition, when rotenone was added to the fibroblasts there was no further significant increase in the lipid peroxidation level, indicating that the lipid peroxidation was predominantly produced by mitochondria in the cell (Fig. 7C).

Deuterated linoleic acid partially rescued the locomotor abnormalities of flies lacking iPLA2-VIA

Finally, we tested the effect of inhibiting lipid peroxidation with D-PUFAs on the neurodegenerative locomotor phenotype seen in flies lacking iPLA2-VIA. Crosses for iPLA2-VIA−/− and w1118 control flies were set up on food containing vehicle (H2O), 10 or 100 μM D2-linoleic acid. Enclosed offspring were also kept on standard food containing either vehicle or D2-linoleic acid and aged while their climbing ability was assessed. iPLA2-VIA−/− flies treated with 10 μM D2-linoleic acid had significantly improved motor performance in old age compared to flies treated with control food (Fig. 7D). There was a trend towards improved climbing ability at older ages when iPLA2-VIA−/− flies were treated with 100 μM D2-linoleic acid and aged while their climbing ability was assessed. iPLA2-VIA−/− flies treated with 10 μM D2-linoleic acid had significantly improved motor performance in old age compared to flies treated with control food (Fig. 7D). There was a trend towards improved climbing ability at older ages when iPLA2-VIA−/− flies were treated with 100 μM D2-linoleic acid, but this was not statistically significant. The rescuing effect of D2-linoleic acid on climbing ability was specific to iPLA2-VIA−/− flies, as the climbing ability of control flies treated with 10 μM D2-linoleic acid was indistinguishable from controls (Supplementary Fig. 5B). The effect of D2-linoleic acid on the survival of iPLA2-VIA−/− flies was also assessed. There was no significant effect of 10 μM D2-linoleic acid on the lifespan of flies lacking iPLA2-VIA. Indeed, at the higher concentration of 100 μM D2-linoleic, the lifespan of the iPLA2-VIA−/− flies was reduced compared to control w1118 flies demonstrating that D2-linoleic acid is toxic to flies at higher concentrations (Supplementary Fig. 5B). Despite the lack of lifespan-prolonging effects, the fact that deuterated linoleic acid significantly improved the locomotor abnormalities of iPLA2-VIA flies suggests that low concentrations of D-PUFAs, or similar compounds, may act as potential therapeutic compounds for the treatment of PLA2G6-associated neurodegeneration.
Discussion

As the energy factories of cells, mitochondria play an essential role in neurons, in which oxidative phosphorylation is the main source of ATP. A previous study in a mouse model demonstrated that knockout of \textit{Pla2g6} results in abnormal mitochondrial membrane morphology (Beck \textit{et al.}, 2011). These findings lead to the question as to how loss of normal \textit{PLA2G6} gene function leads to abnormal mitochondrial morphology, and whether mitochondrial dysfunction is an early feature of \textit{PLA2G6}-associated neurodegeneration.

\textbf{Figure 6} Human mutant PLA2G6 fibroblasts have reduced mitochondrial membrane potential and increased levels of mitochondrial and cytosolic reactive oxygen species levels. (A) The mitochondrial membrane potential of human p.R747W mutant PLA2G6 fibroblasts is reduced compared with control human fibroblasts (\(* P < 0.05\) control 1 and \(* * P < 0.002\) control 2) as measured by TMRM fluorescence. (B) This is clearly seen as a reduction in red TMRM staining in the p.R747W mutant PLA2G6 human fibroblasts compared with control fibroblasts (Scale bars = 44 µm). (C) Mitochondrial reactive oxygen species production is increased 28% in human p.R747W mutant PLA2G6 fibroblasts compared to control fibroblasts (\(P < 0.001; n = 6\)). (D) Cytosolic reactive oxygen species production is also increased in human p.R747W mutant PLA2G6 fibroblasts compared with control fibroblasts (1.47-fold increase; \(P < 0.001; n = 6\)). (E) Rates of reactive oxygen species production in \(C\) and \(D\) are shown as a percentage. Het = dihydroethidium.
Elevated lipid peroxidation is a possible therapeutic target in PLA2G6-associated disease. (A) Lipid peroxidation levels in intact human fibroblasts taken from two patients with PLA2G6 mutations (mutant 1 fibroblasts were taken from a patient with a p.R747W PLA2G6 mutation and mutant 2 fibroblasts were from a patient with a PLA2G6 mutation and infantile neuroaxonal dystrophy) are increased compared with fibroblasts from healthy controls. In addition, pretreatment with D-PUFAs (D4-linoleic acid) for 24 h significantly rescues the elevated lipid peroxidation rates in the human PLA2G6 mutant fibroblasts (**P < 0.0001; n.s = non-significant). (B) The mitochondrial membrane potential of human mutant PLA2G6 fibroblasts is considerably reduced compared with control fibroblasts and is significantly recovered below control levels following 24 h pre-treatment with D4-linoleic acid (**P < 0.0001). (C) Lipid peroxidation levels are also increased in permeabilized mutant p.R747W PLA2G6 fibroblasts compared with controls in the absence (**P < 0.05 for control 1 and control 2) and presence of rotenone (****P < 0.05 for controls 1 and 2), suggesting that it is generated from a mitochondrial source. (D) Treatment of iPLA2-VIA+/−/− flies with 10 μM D4-linoleic acid partially rescues the climbing deficits in an age-dependent manner compared with untreated controls (n = 45 flies per group, three repeats per group; one-way ANOVA with Bonferroni correction ***P < 0.003). There is a trend for 100 μM D4-linoleic acid to rescue the climbing deficits of iPLA2-VIA+/−/− flies but this is not statistically significant. (E) Schematic diagram showing how loss of normal PLA2G6 activity might lead to mitochondrial dysfunction. A mutation in PLA2G6 results in loss of PLA2G6 activity. In turn this results in elevated lipid peroxidation of mitochondria, with the resultant decrease in mitochondrial membrane potential, decrease in ATP synthesis and mitochondrial reactive oxygen species production. The mitochondria eventually degenerate and become swollen in appearance. All of these abnormalities result in mitochondrial dysfunction and subsequent cellular dysfunction with consequent neuronal death. NBI = neurodegeneration with brain iron accumulation; ROS = reactive oxygen species.
Loss of normal phospholipase A2 activity leads to mitochondrial dysfunction, which precedes mitochondrial membrane defects

In this study, we demonstrated that loss of normal phospholipase A2 activity in the fruit fly and in fibroblasts taken from patients harbouring mutations in PLA2G6, results in striking abnormalities in both mitochondrial function and morphology. Furthermore, we demonstrate that mitochondrial dysfunction precedes the mitochondrial morphological abnormalities that are seen at the ultrastructural level. Deficits in mitochondrial oxidative respiration are seen in iPLA2-VIA knockout flies as young as 2 days of age, when there are no associated mitochondrial membrane abnormalities present on electron microscopy.

Moreover, our findings that mitochondrial dysfunction occurs early in PLA2G6-associated neurodegeneration, is consistent with the clinical features seen in infantile neuroaxonal dystrophy, neurodegeneration with brain iron accumulation and dystonia-parkinsonism, all of which display clear early-onset phenotypes, that have previously been associated with mitochondrial disease.

Loss of normal PLA2G6 activity leads to reactive oxygen species production

In addition to mitochondrial dysfunction, we also demonstrate that loss of PLA2G6 activity is associated with raised levels of reactive oxygen species. These species play important roles in cell signalling, but at abnormally high levels are detrimental to cells, ultimately leading to cell death (Martindale and Holbrook, 2002). Lipids are important targets of reactive oxygen species and therefore elevated reactive oxygen species levels will lead to increases in mitochondrial lipid peroxidation. Furthermore, it may also be that elevated lipid peroxidation arises in the absence of phospholipase A2 activity because some phospholipases preferentially cleave oxidized lipids to maintain redox homeostasis (Domijan et al., 2014). In turn, lipid peroxides are known to trigger a cascade of events that include a fall in mitochondrial membrane potential, mitochondrial swelling and loss of mitochondrial matrix components (Castilho et al., 1994). Furthermore, it has been shown that complex I, the largest complex of the respiratory chain, is susceptible to oxidative stress (Lin and Beal, 2006). Indeed we saw a decrease in oxidative respiration in flies lacking iPLA2-VIA when we used substrates specific for complex I of the respiratory chain. Furthermore, Lewy body disease has been shown to be associated with reduced levels of complex I activity (Gu et al., 1998), further implicating mitochondrial dysfunction in neurodegeneration, and more specifically in PLA2G6-associated neurodegeneration, which has been linked to parkinsonism and Lewy body pathology.

In addition, lipid peroxidation is strongly catalysed through the Fenton reaction by iron and iron complexes (Koster et al., 1980) and it is iron that is often deposited in the basal ganglia of the brains of patients affected with PLA2G6 mutations (Morgan et al., 2006). Thus, iron-mediated oxidative insults seem to have a crucial role in the function of PLA2G6 and in the progression of diseases associated with mutations in this gene.

PLA2G6-associated neurodegeneration is not likely mediated by oxidization of mitochondrial cardiolipin

Given the increase in levels of lipid peroxidation in flies lacking iPLA2-VIA, we looked for elevated levels of oxidized cardiolipin, one of the most common lipids in the inner mitochondrial membrane. It has previously been hypothesized that PLA2G6 is involved in the remodelling of cardiolipin’s fatty acid side chains (Malhotra et al., 2009) and in removing oxidized fatty acid side chains. Oxidation of mitochondrial phospholipids such as cardiolipin leads to cytochrome c release and apoptosis (Kagan et al., 2005). To determine whether cardiolipin is oxidized in PLA2G6 loss-of-function, we analysed the cardiolipin composition of iPLA2-VIA−/− fly heads using mass spectrometry. We demonstrated that mitochondrial membrane disintegration and mitochondrial dysfunction occur in the absence of any significant differences in cardiolipin amount or side chain composition in fly brains lacking iPLA2-VIA. Furthermore, oxidized cardiolipin was not detected in significant amounts. This may be related to the types of fatty acids present in Drosophila cardiolipin. Palmitic acid is the major component of cardiolipin side chains in Drosophila and is more resistant to oxidation than linoleic acid, the main cardiolipin side chain in most mammalian tissues. However, it could also point towards redundancy in the cardiolipin remodelling enzymes. There are a number of enzymes involved in cardiolipin remodelling and it may be the case that fatty acid remodelling of the side chains, in particular removal of oxidized fatty acids, can be performed by other enzymes involved in cardiolipin remodelling such as tafazzin (Schlame, 2013).

The abnormal lipid peroxidation seen in PLA2G6 knockout leads to a toxic cascade of downstream events

In this work we report that loss of normal PLA2G6 function is strongly associated with elevated mitochondrial lipid peroxidation. Furthermore lipid peroxidation is known to induce mitochondrial injury, leading to a decrease in oxidative respiratory chain function, reduced mitochondrial...
membrane potential, reduced cellular ATP levels and raised reactive oxygen species levels, all features we have demonstrated in flies and human fibroblasts lacking normal PLA2G6 gene activity (Fig. 7D). This in turn would be predicted to lead to cell death and neurodegeneration. As lipid peroxides are harmful mediators of oxidative stress, strategies focused on quenching, recycling or protecting such products could be highly effective in the treatment of the disease.

This work suggests that lipid peroxidation-lowering strategies may be beneficial in PLA2G6-associated neurodegeneration

In addition to showing that lipid peroxidation is increased in both fly brains lacking iPLA2-VIA and human PLA2G6 mutant fibroblasts, we have demonstrated that D-PUFAs reduce the rates of lipid peroxidation in two lines of PLA2G6 mutant fibroblasts and restore mitochondrial membrane potential. This suggests that D-PUFAs may have potential therapeutic roles in PLA2G6-associated neurodegeneration and its associated mitochondrial dysfunction.

Our data promote Drosophila as a useful model for studying PLA2G6-associated neurodegeneration, and for manipulating pathways and therapeutic targets in order to reverse or prevent the deleterious effects of lipid peroxides. Loss of iPLA2-VIA gene function results in reduced lifespan and locomotor ability, increased sensitivity to oxidative insults as well as hypoxia, osmotic and starvation stressors. Thus flies lacking iPLA2-VIA display phenotypes that can be used as markers in genetic modifying screens or in the screening of potential therapeutic compounds. To test the validity of the fly model for drug screening and to confirm the benefits of D-PUFAs in vivo in an organism lacking iPLA2-VIA, we examined the effects of D-PUFAs on the locomotor deficits of iPLA2-VIA−/− flies. This demonstrated that treatment with D2-linoleic acid partially rescues the climbing abnormalities in aged iPLA2-VIA knockout flies. Therefore this approach of using compounds, which act to reduce the autoxidation of lipids, may represent a future therapeutic strategy for patients with PLA2G6-associated neurodegeneration and warrants further investigation.

Acknowledgements

We would like to thank Drs Fiona Kerr, Teresa Niccoli, Nazif Alic and Ekin Bolukbasi for technical advice and useful discussions, and Filiz Senbabaoglu and Helena Cantwell for technical assistance. We also thank Kerrie Venner for help with electron microscopy and Dr Una Sheerin for help with patient fibroblast sample collection. We also thank the TRIP at Harvard Medical School (NIH/ NIGMS R01-GM084947) for providing a transgenic RNAi fly stock used in this study, and Dr Mikhail S. Shchepinov (Retrotepe, Inc) for providing D-PUFAs.

Funding

K.J.K, L.P. and J.H. are funded by the Wellcome Trust. F.B. is supported by a fellowship from the Spanish Ministerio de Educacion through the FECYT and partially supported by an ALS Association Initiated award (ID 2109) and Motor Neuron Disease Association Small Grants (Hardy/Oct12/6603). L.P. is also supported by the Max Planck Society. This work was also supported by the Welcome Trust MRC strategic neurodegenerative disease initiative award (Ref. number WT089698). K.J.K. was the recipient of a post-doctoral Wellcome Trust fellowship (Ref. number 090541/Z/09/Z). Part of this work was undertaken at the University College of London Hospitals who received a proportion of their funding from the Department of Health’s NIHR Biomedical Research Centres funding scheme. K.B.B. was also funded by a Parkinson’s UK Grant (Ref. number G-1009). L.L. is also the recipient of a Parkinson’s UK PhD studentship (Ref. number H-1105).

Supplementary material

Supplementary material is available at Brain online.

References


Appendix 3
Lithium Promotes Longevity through GSK3/NRF2-Dependent Hormesis

Jorge Iván Castillo-Quan,1,2,3 Li Li,1,3 Kerri J. Kinghorn,1,3 Dobril K. Ivanov,4 Luke S. Tain,2 Cathy Slack,1,2 Fiona Kerr,1 Tobias Nespital,5 Janet Thornton,4 John Hardy,3 Ivana Bjedov,1,5 and Linda Partridge1,2
1Institute of Healthy Ageing and Department of Genetics, Evolution and Environment, University College London, Darwin Building, Gower Street, London WC1E 6BT, UK
2Max Planck Institute for Biology of Ageing, Joseph-Stelzmann Strasse 9-b, 50931 Köln, Germany
3Department of Molecular Neuroscience, Institute of Neurology, University College London, Queen Square, London WC1N 3BG, UK
4European Molecular Biology Laboratory, European Bioinformatics Institute (EMBL-EBI), Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK
5UCL Cancer Institute, Paul O’Gorman Building, 72 Huntley Street, London WC1E 6BT, UK
*Correspondence: l.partridge@ucl.ac.uk
http://dx.doi.org/10.1016/j.celrep.2016.03.041

SUMMARY
The quest to extend healthspan via pharmacological means is becoming increasingly urgent, both from a health and economic perspective. Here we show that lithium, a drug approved for human use, promotes longevity and healthspan. We demonstrate that lithium extends lifespan in female and male Drosophila, when administered throughout adulthood or only later in life. The life-extending mechanism involves the inhibition of glycogen synthase kinase-3 (GSK-3) and activation of the transcription factor nuclear factor erythroid 2-related factor (NRF-2). Combining genetic loss of the NRF-2 repressor Kelch-like ECH-associated protein 1 (Keap1) with lithium treatment revealed that high levels of NRF-2 activation confer stress resistance, while low levels additionally promoted longevity. The discovery of GSK-3 as a therapeutic target for aging will likely lead to more effective treatments that can modulate mammalian aging and further improve health in later life.

INTRODUCTION
Lithium is the most commonly prescribed drug for the treatment of bipolar disorder. It also improves disease phenotypes in animal models of many clinical conditions including Alzheimer disease, depression, and stroke (Chiu and Chuang, 2010). The effects of lithium on aging have been documented in yeast and Caenorhabditis elegans, with lithium extending lifespan (McColl et al., 2008; Zarse et al., 2011; Tam et al., 2014; Sofola-Adesakin et al., 2014). The effects of lithium on Drosophila aging have previously been inconclusive, with demonstration of both positive and negative effects on survival (Matsagas et al., 2009; Zhu et al., 2015). Moreover, lithium concentration in the drinking water of a large Japanese population has been associated with reduced all-cause mortality (Zarse et al., 2011), suggesting that lithium may be a bona fide anti-aging drug. However, the mechanisms by which lithium acts in humans remain poorly understood.

In vitro studies have reported that lithium can protect against several forms of oxidative and xenobiotic stressors (Lai et al., 2006; Schäfer et al., 2004), but in vivo evidence for such protective effects of lithium is lacking. Longevity has been extensively correlated with resistance to stress (Minois, 2000; Rattan, 2008; Calabrese et al., 2011; Epel and Lithgow, 2014). Transcriptomic analysis of interventions known to extend lifespan have identified particular genes likely to be involved in stress resistance (McElwee et al., 2007; Steinauher et al., 2012). Up-regulation of the transcription factor cap’n’collar C (CcnC, an NRF-2 homolog) has been shown not only to confer resistance to toxic compounds, but also to promote longevity in C. elegans and flies (Tullet et al., 2008; Sykiotis and Bohmann, 2008; Ewald et al., 2015). In flies and mammals, NRF-2/CcnC is negatively inhibited through cytosolic sequestration and proteasomal degradation by the canonical Keap1 (Hayes and Dinkova-Kostova, 2014; Ritiuniak and Bohmann, 2015). However, a second emerging upstream regulator of NRF-2/CcnC is GSK-3, a well-documented target of lithium (Jope, 2003; Hayes and Dinkova-Kostova, 2014; Cuadrado, 2015; Hayes et al., 2015; Blackwell et al., 2015). GSK-3 regulates NRF-2 by phosphorylation and nuclear exclusion, an effect that is evolutionarily conserved from invertebrates to mammals (Salazar et al., 2006; An et al., 2005). Interestingly, GSK-3 inhibition has been shown to phenocopy the effects of lithium for protection against xenobiotic stress in vitro (Lai et al., 2006; Schäfer et al., 2004).

Activation of NRF-2/CcnC produces hormetic effects on lifespan, such that at low level NRF-2/CcnC activity extends lifespan while higher levels of activation limit it (Mattson, 2008; Maher and Yamamoto, 2010). Interestingly a hormetic signature was recently reported for the survival of a mammalian cell line treated with lithium (Suganthi et al., 2012), suggesting that lithium and GSK-3 inhibition could influence...
animal lifespan and stress resistance through activation of NRF-2.

Here we show that lithium supplementation in the diet can modulate longevity, stress resistance, and metabolism in Drosophila through the inhibition of GSK-3. Correspondingly, genetic downregulation of GSK-3 and lithium treatment are epistatic, suggesting a common molecular pathway. We also show that lithium and the genetic inhibition of GSK-3 promote xenobiotic stress resistance and lifespan extension through the activation of a transcriptional response mediated by CncC/NRF-2. Furthermore, lithium protects against a high-sucrose diet and acts through mechanisms that only partially overlap with those mediating lifespan extension by dietary restriction (DR). These findings demonstrate an alternative genetic and pharmacological target for the promotion of longevity and stress resistance, and emphasize the potential of pharmacological inhibitors of GSK-3 as viable anti-aging treatments.

RESULTS

Lithium Extends Healthy Lifespan in Drosophila

To assess the role of lithium in Drosophila aging, we treated adult female flies with lithium chloride (LiCl) by supplementation in their food. Lithium treatment in the range of 1 to 25 mM resulted in lifespan extension, whereas higher doses (50–100 mM) shortened lifespan (Figure 1A). These effects of lithium treatment on lifespan extension were also observed in an independent genetic background (Figure S1A) and in males (Figure S1B). Thus, lithium treatment extended Drosophila lifespan independently of genetic background and sex.

Figure 1. Lithium Regulated Longevity and Metabolism in Drosophila

(A) Lithium extended lifespan of w^Dah Drosophila females (n = 160 flies per condition) at concentrations between 1 and 25 mM (+16% and +18% median and maximum lifespan extension; p < 0.001), but resulted in a dose-dependent reduction in lifespan at concentrations between 50 and 100 mM (p < 0.001).

(B) Lithium treated female w^1118 flies showed a significant improvement and protection against age-related locomotor decline (p < 0.01, two-way ANOVA for 10 mM).

(C) Lithium extended lifespan of aged, 32-day-old female w^1118 flies at concentrations from 1 to 25 mM (30 days later than in Figure 1A); 1 mM extended median lifespan by 5% (4 days) and maximum lifespan by 13% (8 days; p < 0.05); 10 and 25 mM lithium increased median lifespan by 9% (8 days); 10 mM increased maximum lifespan by 4.5% (3.5 days); whereas 25 mM lengthened it by 8% or 6 days (p < 0.01); and 50 and 75 mM significantly shortened lifespan (p < 0.01). n = 150 flies per condition.

(D) Brief treatment with lithium for 15 days early in adulthood extended lifespan of female w^Dah flies (p < 0.05 for 1 mM and p < 0.01 for 10 mM; n = 150 flies per condition).

(E) Lithium induced a dose-dependent reduction in triglyceride levels. Bars represent means of six replicas of five flies per condition ± SEM. *p < 0.01, **p < 0.001.

(F) Female w^1118 flies pre-treated with lithium for 15 days were subsequently sensitive to starvation in a dose-dependent manner (n = 90 flies per condition).

(G) Lithium treatment significantly extended the lifespan of w^1118 female flies exposed to a four times higher sucrose concentration (2g/L; p < 0.001; n = 120 flies per condition).

(H) The increase of triglycerides observed on a high-sucrose diet was completely blocked after 15 days of treatment with 1 mM lithium. Bars represent means of six replicas of five w^1118 female flies per condition ± SEM. *p < 0.01.
Interestingly, we observed that, unlike with many other genetic and pharmacological interventions (e.g., DR, insulin/IGF down-regulation, rapamycin, or trametinib treatment), lithium did not reduce fecundity at life-extending doses or compromise feeding behavior (Figures S1E and S1F). Moreover, it delayed locomotor decline at two concentrations that extend lifespan (Figure 1B). Thus, lithium promotes healthspan in adult Drosophila with limited side effects.

**Lithium Extends Lifespan in Mid-life or with Short-Term Treatment in Young Flies**

To limit the side effects of long-term use, a drug that improves lifespan and healthspan will ideally do so with late-onset administration (Castillo-Quan et al., 2015; London et al., 2015). We therefore assessed the effect of commencing lithium treatment at older ages. Flies were switched onto food containing a range of lithium concentrations (1–75 mM) at 32 days of age (Figure 1C). Lower doses (1–25 mM) of lithium extended lifespan, whereas higher doses (50 and 75 mM) significantly reduced lifespan, similar to the dose-dependent effects we observed in younger flies.

We also tested whether transient lithium treatment early in life could increase lifespan. We therefore exposed young flies to 1 or 10 mM lithium for 15 days and then switched them to control food for the remainder of their lifespans. Early treatment with these doses of lithium extended lifespan (Figure 1D). Lithium treatment early in life, and for a transient period, can therefore increase survival later in life.

**Lithium Alters Lipid Metabolism and Promotes Survival under a High-Sugar Diet**

Genetic and environmental interventions that extend lifespan often induce abnormalities in carbohydrate and lipid metabolism (Barzilai et al., 2012; Wang et al., 2014; Lamming et al., 2013). We therefore examined the effects of lithium on whole body trehalose, glycogen, and triglyceride levels. Following 15 days of lithium treatment, and over a wide range of lithium concentrations, we were unable to detect a significant change in the levels of either trehalose or glycogen (Figures S1G and S1H). However, we observed a dose-dependent reduction in whole body triglycerides, the main lipid storage in flies (Ballard et al., 2008; Skorupa et al., 2008) (Figures 1E and S1I). In keeping with the lowered triglyceride levels (Ballard et al., 2008; Ulgherait et al., 2014), lithium treatment reduced survival under starvation conditions in a dose-dependent manner (Figures 1F and S1J). Moreover, lithium also extended lifespan under dietary conditions that promote triglyceride accumulation (Skorupa et al., 2008). Flies fed a high-sucrose diet were short lived and lithium was able to partially rescue this defect (Figure 1G) while completely blocking the increase in triglycerides observed with a sucrose-rich diet (Figure 1H). Therefore, lithium can extend lifespan under obesogenic dietary conditions.

**Lithium and DR Extend Lifespan via Partially Overlapping Mechanisms**

We next investigated whether lithium treatment was acting as a DR mimetic. DR is a well-established anti-aging intervention that extends healthy lifespan in diverse species (de Cabo et al., 2014; Fontana and Partridge, 2015), and some pharmacological and genetic interventions that extend lifespan have features of DR mimetics (Ingram and Roth, 2015; de Cabo et al., 2014). To determine whether lithium and DR extend lifespan by similar mechanisms, we assessed whether lithium could extend lifespan beyond the maximum achievable by DR. To maximize lifespan under DR, we varied the yeast concentration in the food while maintaining a constant concentration of sucrose (Bass et al., 2007), resulting in a typical tent-shaped response, with peak lifespan at food containing a 1.0 yeast concentration (Figures 2A and S2A–S2D). If lithium treatment and DR share overlapping pathways, then lithium would not be able to further extend lifespan already maximized by DR (Gems et al., 2002; Castillo-Quan et al., 2015). All lithium doses tested significantly extended median lifespan in both the yeast condition that maximized lifespan (1.0 yeast; Figures 2A and S2C) and under full feeding (2.0 yeast; Figures 2A and S2D), with greatest extension of median lifespan with 10 mM lithium under full feeding. However, under reduced yeast concentrations that shorten lifespan (0.2 and 0.5 yeast), 10 mM lithium either significantly reduced lifespan (Figure S2B) or did not confer a significant lifespan benefit (Figure S2A). Cox proportional hazards analysis showed a significant interaction between lithium and yeast concentrations for lifespan (interaction term p < 0.0001). The extension of lifespan from lithium increased with the level of yeast in the fly diet, suggesting partially overlapping mechanisms to those of DR.

**Lithium Extends Lifespan through Inhibition of GSK-3**

A well-known target of lithium is GSK-3 (Phiel and Klein, 2001; Jope, 2003; Eldar-Finkelman and Martinez, 2011). We therefore evaluated the phosphorylation status of the fly ortholog of GSK-3, Shaggy (Sgg), in response to lithium treatment. Lithium addition to the fly medium resulted in a dose-dependent increase in the inhibitory phosphorylation (Serine 9 or S9) of Sgg (Figure 2B). To evaluate the role of Sgg in lithium-mediated lifespan extension, we directly manipulated its activity in adult flies. Ubiquitous overexpression of wild-type or constitutively active Sgg (SggS9A) significantly reduced lifespan by ~30% and 50%, respectively (Figures 2C and S2E). This reduction in lifespan was almost completely reversed by lithium treatment. Furthermore, RNAi-mediated reduction in sgg expression using two independent dsRNA-expressing transgenes significantly increased lifespan (Figures 2D and S2F). Importantly, lithium was unable to further increase the lifespan of these sgg RNAi knockdown mutants flies (Figure 2D). Taken together, these findings suggest that Sgg/GSK-3 inhibition and lithium treatment increase lifespan by acting on the same downstream targets.

**Lithium Activates the Cap’n’Collar C/NRF-2 Transcription Factor**

To identify downstream mediators of lifespan extension by lithium and of GSK-3 inhibition, we analyzed the genome-wide transcriptional response could underlie the translational repression following lithium treatment that has been previously observed.
Figure 2. Lithium Extended Lifespan beyond Dietary Restriction by Inhibiting Sgg/GSK-3

(A) Median lifespans at different lithium concentrations (0, 1, 2.5, 5, or 10 mM) are plotted for four different yeast concentrations (0.2, 0.5, 1.0, and 2.0 yeast): 1–5 mM lithium extended lifespan under all dietary conditions tested. Although 10 mM lithium prolonged life at 1.0 and 2.0 yeast, it showed no effect at 0.2 yeast and significantly shortened lifespan at 0.5 yeast. *p < 0.05, **p < 0.01, ***p < 0.001, from 0 lithium; n = 160 flies per condition. Complete survival curves are shown in Figures S2A–S2D.

(B) Lithium treatment for 15 days significantly increased the inhibitory phosphorylation of Sgg/GSK-3 in a dose-dependent manner. Bars represent means of triplicates of ten flies per biological repeat ± SEM, *p < 0.05, **p < 0.01.

(C) Ubiquitous overexpression of wild-type sgg significantly shortened lifespan (p < 0.001) and this was partially rescued by lithium treatment at two concentrations (10 and 25 mM; p < 0.001). See Figure S2E for the interaction of sgg(S9A) and lithium treatment on lifespan.

(D) Ubiquitous RNAi-mediated downregulation of sgg extended lifespan (p < 0.001) and no further extension occurred when the flies were treated with 1 or 5 mM lithium (p > 0.05), whereas 10 mM lithium treatment restored the lifespan to control levels (p > 0.05), and 25 mM was significantly toxic (p > 0.05). See Figure S2F for lifespan extension obtained with an independent RNAi line.
in fission yeast and Drosophila heads (Sofola-Adesakin et al., 2014). In addition, five GO terms for genes encoding enzymes in the detoxification pathway were also in the ten most upregulated categories (Figure 3A).

The responses to xenobiotics and oxidative stress in Drosophila are regulated by the transcription factors dFOXO, CncC, and DHR96 (Salih and Brunet, 2008; Sykiotis and Bohmann, 2010; Tullet, 2015; Hoffmann and Partridge, 2015; Blackwell et al., 2015). We therefore assessed whether transcriptional responses to activation of these transcription factors overlapped with that of lithium treatment. The transcriptomic response to lithium did not overlap with that of dFOXO-dependent or -independent transcriptional regulation downstream of IIS (Figure S3A) (Alcântara et al., 2011). Further, although we detected a significant overlap in the transcriptional signatures of lithium and DHR96 (King-Jones et al., 2008), they did not share the same directionality (Figure S4). However, we found a significant overlap (Figure 3B) between the genes that were upregulated by lithium and cncC overexpression (Misra et al., 2011), but not between genes downregulated by both treatments (Figure S5A), suggesting that lithium might activate a CncC transcriptional response downstream of GSK-3. The barbiturate phenobarbital activates CncC and induces a similar transcriptional response to that of cncC overexpression (Misra et al., 2011). We therefore analyzed the overlap between the transcriptional profiles induced by lithium and phenobarbital treatment, and again found a significant overlap (Figure 3B) between upregulated, but not downregulated, genes (Figure S5B). The genes upregulated in common between lithium treatment, phenobarbital treatment and cncC overexpression (Figures 3B and S5C) encoded enzymes that participate in all three phases of xenobiotic metabolism (Figure 3C). To further confirm the activation of CncC by lithium, we used a previously generated CncC reporter that responds to both chemical and genetic inducers of CncC (Sykiotis and Bohmann, 2008). Flies carrying the GsDGFP CncC reporter showed a dose-dependent increase in GFP expression with increasing concentrations of lithium (Figure 3D). Taken together, our results suggest that lithium activates CncC to upregulate the expression of genes in the detoxification pathway.

Lithium Induces Lifespan-Extension, Hormesis, and Protection against Xenobiotics via CncC-Dependent Mechanisms

We next assessed whether CncC activity is required for the pro-longevity effects of lithium. Ubiquitous, RNAi-mediated knockdown of cncC expression blocked the lifespan extension of 1 to 10 mM lithium, but was detrimental to survival when flies treated with 25 mM lithium, the highest dose that extends lifespan under basal conditions, albeit to a lesser extent (Figure 4A). Thus, lithium treatment requires CncC activity to confer its longevity benefits.

Because CncC/NRF-2 can induce hormesis (Mattson, 2008; Maher and Yamamoto, 2010), we assessed whether lithium can also do this. To test for a hormetic effect of lithium at low doses, we pre-treated flies with a range of concentrations of lithium and then challenged them with a toxic dose of 500 mM. Most pre-treatment doses of lithium induced subsequent resistance to the toxic dose (Figure 4B). To assess whether the hormetic response of lithium was mediated by CncC, we knocked down expression of cncC using RNAi, and treated the flies with 1–25 mM lithium. Reduction in cncC expression completely blocked the hormetic response induced by 10 mM lithium pre-treatment, and significantly reduced the effect of 25 mM lithium (Figure 4C).

We next assessed the ability of lithium pre-treatment to protect against other xenobiotics. Flies pre-treated with increasing concentrations of lithium ranging from 1 to 100 mM were significantly resistant to a toxic concentration of phenobarbital, with lithium doses between 1 and 75 mM almost doubling survival (Figure 4D). Lower doses of lithium also protected against a toxic concentration of the anti-malarial drug, chloroquine (Figure S5D; 1–10 mM), and the pesticide paraquat (Figure 4E). Thus, low to intermediate concentrations of lithium protect against xenobiotic toxicity. To determine the role of CncC activity in lithium-mediated protection against phenobarbital, we used RNAi to knock down expression of cncC, which sensitized the flies to phenobarbital and completely abrogated the protection against phenobarbital afforded by lithium supplementation (Figure 4F). Thus, CncC is at least partly responsible for the hormetic effect induced by low-level treatment with lithium.

To confirm that Sgg, upstream of CncC, is also necessary for the resistance to xenobiotic stress (Blackwell et al., 2015; Cuadrado, 2015; Hayes et al., 2015), we assessed the effect of ubiquitous overexpression of wild-type sgg or the constitutively active Sgg(S9A) on xenobiotic resistance. Both significantly sensitized flies to phenobarbital (Figures 5A and S5E). We confirmed that sgg or sgg(S9A) overexpression regulated CncC by showing significantly lower levels of MRP and keap1 (Figures 5B and S5F), both CncC target genes. Correspondingly, RNAi-mediated knockdown of sgg resulted in resistance to phenobarbital (Figure 5C), and paraquat (Figure S5G). An increase of mRNA levels of cncC, keap1, and gsto2 confirmed that CncC was active in sgg knockdown flies (Figure 5D). Thus, increased Sgg activity sensitizes against xenobiotic stressors, whereas its inhibition protects against them.

Lifespan and Stress Resistance Depend on the Degree of Activation of CncC by Keap1 and Lithium Treatment

In addition to activating CncC by repressing Sgg/GSK-3, lithium could potentially increase CncC activity by inhibiting its canonical repressor Keap1 (Cuadrado, 2015; Pitio and Bohmann, 2015). Hence, we analyzed the interaction between lithium treatment and Keap1. Overexpression of Keap1, which inhibits CncC activity in vivo (Sykiotis and Bohmann, 2008), was unable to prevent the lifespan-extending properties of lithium (Figure 6A), suggesting that the longevity effect of lithium treatment is independent of Keap1. Next, we analyzed the interaction of loss of Keap1 and lithium treatment. We generated a deletion of the keap1 coding sequence by P-element-mediated male recombination using a previously described P-element insertion line (Sykiotis and Bohmann, 2008) (Figure 6B). The keap1 deletion (keap1D1) was homozygous lethal, but activated CncC 4-fold in the heterozygous state, as measured by the CncC reporter (Figure 6C). Lithium treatment of the keap1D1 flies further activated CncC (Figure 6C). We next tested whether this effect on CncC
activation protected against paraquat and lithium toxicity. \textit{keap}^{Dox} flies were significantly resistant to both paraquat and lithium (Figures 6D and 6E), and pre-treatment with lithium further protected them. We confirmed these findings using a previously described heterozygous loss-of-function mutation in the \textit{keap1} gene (\textit{keap}^{EY5} \text{Sykiotis and Bohmann, 2008}) (Figures

**Figure 3. Lithium Activated a Transcriptional Response Similar to that of CncC/NRF-2**
(A) Ten most significantly upregulated GO categories induced by lithium treatment of \textit{w}^{1118} female flies. See Figure S3A for downregulated GO categories.
(B) Lithium treatment of \textit{w}^{1118} females flies induced a transcriptional response that significantly overlapped with that induced by \textit{cncC} overexpression (p = 7.83 \times 10^{-14}) or phenobarbital treatment (p = 3.85 \times 10^{-14}) (Misra et al., 2011). Heatmap showing the 57 genes most significantly changed by lithium or phenobarbital treatment and overexpression of \textit{cncC}.
(C) Genes upregulated by lithium treatment mapped to the three phases of the xenobiotic detoxification pathway in flies.
(D) Lithium treatment of \textit{w}^{1118} females pre-treated with lithium 1 to 100 mM protected against a 6% phenobarbital (p < 0.001 for all doses).
(E) Lithium pre-treatment (for 15 days) protected against the herbicide paraquat in a dose-dependent manner (p < 0.001 for all doses, with maximal protection at 50 mM).
(F) RNAi-mediated downregulation of \textit{cncC} completely blocked the protective effect of lithium against phenobarbital.

**Figure 4. Lithium-Induced Xenobiotic Resistance and Longevity Were Mediated by CncC**
(A) Ubiquitous knockdown of \textit{cncC} blocked lifespan extension by lithium.
(B) Pre-treatment with increasing concentrations of lithium protected against a subsequent toxic dose of lithium (500 mM; p < 0.05 for 1 mM; p < 0.005 for 100 mM).
(C) Ubiquitous downregulation of \textit{cncC} blocked the protective effect of 10 mM lithium pre-treatment against a subsequent toxic dose and partially blocked the protective effect of 25 mM lithium pre-treatment.
(D) 1 to 100 mM lithium pre-treatment protected against a 6% phenobarbital (p < 0.001 for all doses).
(E) Lithium pre-treatment (for 15 days) protected against the herbicide paraquat in a dose-dependent manner (p < 0.001 for all doses, with maximal protection at 50 mM).
(F) RNAi-mediated downregulation of \textit{cncC} completely blocked the protective effect of lithium against phenobarbital.
produces autophagy in a tissue-specific manner. Inactivation of autophagy occurs in CncC/NRF-2 (Sarkar et al., 2005) through the inhibition of GSK-3 and the subsequent activation of mTOR. Treatment further protected against toxic doses of lithium and paraquat. The span is considerably lower than that which maximizes protection against these xenobiotics.

We subsequently evaluated the interaction between loss of keap1 and lithium treatment for longevity. Survival analysis showed that the lifespan of keap1D allele mutant flies was indistinguishable from controls, but that addition of 1 mM lithium marginally, yet significantly, extended lifespan (Figure 6A). Increasing the dose of lithium to 10 mM restored longevity to control levels. The keap1/− mutant flies showed a significant lifespan extension (Figure 6G). However, supplementation of either 1 or 10 mM lithium to the keap1/− mutant shortened lifespan in a dose-dependent manner. These results suggest that the level of activation of CncC that maximizes extension of lifespan is considerably lower than that which maximizes protection against toxic doses of lithium and paraquat.

**Lithium Does Not Induce or Require Autophagy to Promote Longevity**

Activation of autophagy has been proposed as a mechanism for the beneficial effects of lithium (Sarkar et al., 2005). We therefore analyzed the induction of autophagy by LC3-I/LC3-II (Atg8 in Drosophila) levels without detecting statistically significant changes. Indeed, there was a tendency for lower LC3-I that did not reach statistical significance (Figure S7A). Moreover, lithium treatment was able to extend the lifespan of flies with autophagy defects due to heterozygous loss of atg1 (Figure S7B) (Lee et al., 2007). Thus, taken together our results do not immediately support a role for autophagy in the pro-longevity effects of lithium treatment, and strengthen our conclusion that they are mediated through the inhibition of GSK-3 and the subsequent activation of CncC/NRF-2 (Figure 7). However, it remains possible that induction of autophagy occurs in atg1-deficient flies, or that lithium induces autophagy in a tissue-specific manner.

**DISCUSSION**

**Lithium Acts as a Pro-longevity Drug**

Drug repositioning is the most promising approach for developing pharmacological agents to improve healthy aging. So far, two medically approved drugs, metformin and rapamycin, have been reported to promote longevity and provide health benefits across species from invertebrates to mammals (McColl et al., 2008; Matsagas et al., 2009; Sofola-Adesakin et al., 2014). We also showed that this effect was common between two different laboratory strains and, unlike other interventions that seem to be more effective in females (Austad and Bartke, 2015), lithium similarly extended lifespan in both sexes.

Lifespan-extending drugs can often act like DR mimetics (Madeo et al., 2014; Ingram and Roth, 2015); hence, it was important to determine whether lithium was acting in a similar manner. While low doses of lithium were able to extend lifespan at all dietary levels tested, median lifespan extension was greatest under full feeding conditions. Our data thus suggest that lithium and DR act via partially overlapping mechanisms and confirms the observation made in C. elegans that lithium extends lifespan of eat-2 mutants (McColl et al., 2008), a genetic model of DR in worms. Lithium also extended the lifespan of flies fed a diet enriched with sucrose, possibly by modulating lipid metabolism (Sykiotis et al., 2011; Pang et al., 2014; Karim et al., 2015; Steinbaugh et al., 2015). However, the role of CncC in modulating the triglyceride phenotype of lithium remains to be explored. Overall, our observations strongly suggest that lithium is a pro-longevity drug capable of extending lifespan at low doses independent of sex and genetic background, and under a variety of dietary conditions.
Figure 6. Higher Activation Levels of CncC Promote Xenobiotic Resistance but Not Lifespan

(A) Overexpression of keap1 did not prevent the lifespan-modulatory effects of lithium treatment. n = 150 flies per condition.
(B) Schematic of the keap1 gene showing the portion deleted in the keap1mutant (top) and agarose gel showing start and end of P-element disrupting keap1coding sequence in the keap1mutant (bottom).
(C) Combination of heterozygous deletion of keap1 and lithium treatment showed a greater activation of CncC than on their own. Bars represent means of four replicas of five flies per repeat ± SEM. **p < 0.01.

(legend continued on next page)
In humans, the therapeutic window for lithium treatment of bipolar disorder lies between 0.5 and 1 mM in serum, whereas concentrations of 1.5 mM and above severely increase the risk of tissue damage (Malhi and Tanious, 2011). We found that lithium extends lifespan when first administered in mid-late life. In humans, long-term treatment with lithium for psychiatric disorders is associated with progressive and permanent renal damage (Malhi and Tanious, 2011). We showed that short treatment periods in Drosophila, 15 days during early adulthood, are sufficient to prolong life. Taken together, our data suggest that when testing lithium as a pro-longevity drug in mammals, lower doses than those used in psychiatric disorders are likely to be sufficient, and other strategies such as alternate-day dosing or transient treatment periods (either early or late in life), may be sufficient to reduce undesirable side effects and maximize the potential health benefits.

Interestingly, doses of lithium that shortened lifespan were protective against certain forms of xenobiotic stress. In vitro studies in mammalian cells have shown that lithium, and other GSK-3 inhibitors, protect against cell death caused by rotenone-induced oxidative stress (Lai et al., 2006), glutamate excitotoxicity, and H$_2$O$_2$ (Schäfer et al., 2004). This is likely mediated through a hormetic response (Suganthi et al., 2012), in this case orchestrated by NRF-2 activation. We observed that while simultaneous activation of CncC by loss of Keap1 and lithium treatment is additive and confers greater stress resistance to xenobiotics, the threshold for lifespan extension is perhaps considerably lower. A similar situation has been observed in C. elegans in which strong activation of the endoplasmatic reticulum unfolded protein response conferred stress resistance benefits, while shortening lifespan (Taylor and Dillin, 2013). Our findings thus suggest that while NRF-2 activation either by loss of Keap1 or inhibition of GSK-3 is beneficial for longevity and stress resistance, at low levels of activation, stronger induction is detrimental for lifespan. This suggests that the hormetic benefits of lithium are more likely to occur at low levels under basal non-stress conditions (Calabrese, 2013). Hence, when testing for GSK-3 inhibitors or NRF-2 activators in modulating animal (and especially mammalian) aging, the degree of NRF-2 activation within the hormetic curve will determine positive or negative longevity outcomes. Future work studying the convergence of the salutary and damaging effects of lithium will aid in understanding to what extent the molecular mechanisms are shared (Calabrese and Mattson, 2011; Calabrese et al., 2013; Epel and Lithgow, 2014). Additionally, our microarray analysis was performed in heads and thoraces; therefore, it remains to be explored to what extent systemic or localized activation of NRF-2 modulates longevity, stress resistance, and lipid metabolism at the tissue level (Douglas et al., 2015).

Figure 7. Lithium Regulates Longevity, Metabolism, and Stress Resistance by Inhibiting GSK-3 and Activating NRF-2

(A) Summary of findings with lithium for longevity, stress resistance, starvation, and triglyceride levels.

(B) Proposed model showing the mechanism by which lithium, Sgg/GSK-3, and CncC/NRF-2 act in the same pathway to modulate longevity and xenobiotic resistance.

**Lithium Toxicity, Hormesis, and Stress Resistance**

Drug interventions to promote healthy lifespan are less likely to have side effects if started late in life (Castillo-Quan et al., 2015). Only a handful of drugs approved by the US Food and Drug Administration, namely rapamycin, metformin, and the Ras inhibitor trametinib, induce lifespan extension when commenced at later ages in model organisms (Harrison et al., 2009; Cabrero et al., 2013; Martin-Montalvo et al., 2013; Slack et al., 2015). We found that lithium extends lifespan when first administered in mid-late life. In humans, long-term treatment with lithium for psychiatric disorders is associated with progressive and permanent renal damage (Malhi and Tanious, 2011). We showed that short treatment periods in Drosophila, 15 days during early adulthood, are sufficient to prolong life. Taken together, our data suggest that when testing lithium as a pro-longevity drug in mammals, lower doses than those used in psychiatric disorders are likely to be sufficient, and other strategies such as alternate-day dosing or transient treatment periods (either early or late in life), may be sufficient to reduce undesirable side effects and maximize the potential health benefits.

Interestingly, doses of lithium that shortened lifespan were protective against certain forms of xenobiotic stress. In vitro studies in mammalian cells have shown that lithium, and other GSK-3 inhibitors, protect against cell death caused by rotenone-induced oxidative stress (Lai et al., 2006), glutamate excitotoxicity, and H$_2$O$_2$ (Schäfer et al., 2004). This is likely mediated through a hormetic response (Suganthi et al., 2012), in this case orchestrated by NRF-2 activation. We observed that while simultaneous activation of CncC by loss of Keap1 and lithium treatment is additive and confers greater stress resistance to xenobiotics, the threshold for lifespan extension is perhaps considerably lower. A similar situation has been observed in C. elegans in which strong activation of the endoplasmatic reticulum unfolded protein response conferred stress resistance benefits, while shortening lifespan (Taylor and Dillin, 2013). Our findings thus suggest that while NRF-2 activation either by loss of Keap1 or inhibition of GSK-3 is beneficial for longevity and stress resistance, at low levels of activation, stronger induction is detrimental for lifespan. This suggests that the hormetic benefits of lithium are more likely to occur at low levels under basal non-stress conditions (Calabrese, 2013). Hence, when testing for GSK-3 inhibitors or NRF-2 activators in modulating animal (and especially mammalian) aging, the degree of NRF-2 activation within the hormetic curve will determine positive or negative longevity outcomes. Future work studying the convergence of the salutary and damaging effects of lithium will aid in understanding to what extent the molecular mechanisms are shared (Calabrese and Mattson, 2011; Calabrese et al., 2013; Epel and Lithgow, 2014). Additionally, our microarray analysis was performed in heads and thoraces; therefore, it remains to be explored to what extent systemic or localized activation of NRF-2 modulates longevity, stress resistance, and lipid metabolism at the tissue level (Douglas et al., 2015).
NRF-2 modulates longevity in et al., 2014; Rizak et al., 2014 for the lifespan extension conferred by lithium. In keeping with lithium acts via CncC/NRF-2. CncC activity was indispensable to identify a transcriptional signature that indeed suggested that induced a CncC/NRF-2-dependent protective response (Jones et al., 2015; Blackwell et al., 2015). GO enrichment analysis identified a transcriptional signature that indeed suggested that lithium acts via CncC/NRF-2. CncC activity was indispensable for the lifespan extension conferred by lithium. In keeping with our results, work in rodents and mammalian cell lines has shown that lithium treatment and GS3 kinase inhibition activate NRF-2 (Lee et al., 2014; Rizak et al., 2014). Because activation of CncC/NRF-2 modulates longevity in C. elegans and Drosophila (Tullet et al., 2008; Sykiotis and Bohmann, 2008; Ewald et al., 2015), our results provide evidence that GS3 kinase and NRF-2 is a viable therapeutic target to promote longevity via activation of NRF-2.

To date, the only GS3 kinase inhibitor approved for human use is lithium (Williams and Harwood, 2000; Meijer et al., 2004; Martinez et al., 2011). However, researchers and pharmaceutical companies have developed more selective GS3 kinase inhibitors, some of which have already entered the early stages of clinical trials for obesity, Alzheimer disease, and progressive supranuclear palsy (Eldar-Finkelman and Martinez, 2011). Our results call for a reassessment of the potential use of GS3 kinase inhibitors and NRF-2 activators as potential anti-aging compounds.

**EXPERIMENTAL PROCEDURES**

**Fly Stocks and Husbandry**

The w^{118} stock was obtained from Bloomington Drosophila Stock Center. The control white Dahomey (w^{DAH}) stock has been maintained in large population cages with overlapping generations since 1970. The w^{DAH} stock was initially derived by incorporation of the w^{118} mutation into the outbred Dahomey background by backcrossing (Bass et al., 2007). Further details concerning fly mutants can be found in the Supplemental Experimental Procedures.

**Lithium Treatment**

LiCl (Sigma) or NaCl (Sigma) were dissolved in ddH2O at a concentration of 5 M before supplementing to the medium. Equivalent volumes of vehicle were supplemented to the medium to compensate for dilution.

**Dietary Restriction Protocol**

The DR protocol was performed as described previously (Bass et al., 2007).

**Statistical Analyses**

Statistical analyses were performed using Excel, GraphPad Prism, or JMP software version 9 (SAS Institute). Survival experiments were analyzed using log rank test. Other data were tested by one-way analyses of variance (ANOVA) and planned comparisons of means were made using Tukey-Kramer HSD test. Cox proportional hazards analysis was performed to compare interactions for survival.

**ACCESSION NUMBERS**

The accession number reported for the microarray data in this paper is ArrayExpress: E-MTAB-3809.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cearep.2016.03.041.

**AUTHOR CONTRIBUTIONS**


**ACKNOWLEDGMENTS**

We thank Profs. D. Gems, M. Murphy, and D. Rubinsztain, and Drs. H. Cochemé, F. Cabreiro, S. Enman, Y. de la Guardia, M. Piper, N. Wooding, A. Gilliat, and O. Sofia-Adesakin for insightful advice and comments; Dr. N. Aic for advice and assistance with statistical testing; and Dr. D. Wieser for initial analysis of microarray data. We thank the Max Planck-Genome-centre Cologne (http://mpgc.mpipz.mpg.de/home/) for microarray analysis. We acknowledge funding from UCL Scholarships (to J.I.C.-Q.), the Max Planck Society (to J.I.C.-Q., L.S.T., C.S., T.N., and L.P.), European Research Council (to I.B. and L.P.), Research Into Ageing (to I.B. and L.P.), Wellcome Trust (to D.K.I., K.J.K., J.T., J.H., and L.P.), Parkinson’s UK (to L.L. and L.P.), and Alzheimer’s Research UK (to F.K. and L.P.).

Received: November 9, 2015
Revised: January 31, 2016
Accepted: March 10, 2016
Published: April 7, 2016

**REFERENCES**


Appendix 4

Appendix 5

Increased Glucose Transport into Neurons
Rescues Aβ Toxicity in Drosophila

Teresa Niccoli,1 Melissa Cabecinha,1 Anna Tillmann,1 Fiona Kerr,1 Chi T. Wong,1 Dalia Cardenes,1 Alec J. Vincent,1 Lucia Bettedi,1 Li Li,1 Sebastian Grönke,2 Jacqueline Dols,2 and Linda Partridge1,2,*

1Institute of Healthy Ageing, Department of Genetics, Evolution and Environment (GEE), University College London, Darwin Building, Gower Street, London WC1E 6BT, UK
2Max Planck Institute for Biology of Ageing, Joseph-Stelzmann-Strasse 9b, 50931 Cologne, Germany
*Correspondence: partridge@age.mpg.de
http://dx.doi.org/10.1016/j.cub.2016.07.017

SUMMARY

Glucose hypometabolism is a prominent feature of the brains of patients with Alzheimer’s disease (AD). Disease progression is associated with a reduction in glucose transporters in both neurons and endothelial cells of the blood-brain barrier. However, whether increasing glucose transport into either of these cell types offers therapeutic potential remains unknown. Using an adult-onset Drosophila model of Aβ (amyloid beta) toxicity, we show that genetic overexpression of a glucose transporter, specifically in neurons, rescues lifespan, behavioral phenotypes, and neuronal morphology. This amelioration of Aβ toxicity is associated with a reduction in the protein levels of the unfolded protein response (UPR) negative master regulator Grp78 and an increase in the UPR. We further demonstrate that genetic downregulation of Grp78 activity also protects against Aβ toxicity, confirming a causal effect of its alteration on AD-related pathology. Metformin, a drug that stimulates glucose uptake in cells, mimicked these effects, with a concomitant reduction in Grp78 levels and rescue of the shortened lifespan and climbing defects of Aβ-expressing flies. Our findings demonstrate a protective effect of increased neuronal uptake of glucose against Aβ toxicity and highlight Grp78 as a novel therapeutic target for the treatment of AD.

A prominent feature of AD progression is a substantial reduction in glucose metabolism [4]. This drop precedes the onset of clinical symptoms [4], worsens with disease progression [4], and is a more accurate marker of neuronal atrophy than is Aβ accumulation itself [5]. Patients with type 2 diabetes, who are at higher risk of AD, display increased insulin resistance, which has been linked both to reduced glucose uptake in the brain and to memory impairments [6]. Mouse models of AD also show a decrease in glucose metabolism, suggesting that it may be part of the disease process [7]. However, the exact role of lowered glucose metabolism in disease progression is unknown.

Glucose does not freely cross cell membranes and is, instead, actively shuttled by transporters. In humans, there are 12 glucose transporters, with different expression patterns and affinities. In the brain, Glut1 is expressed mainly in glia and endothelial cells, whereas Glut3 is expressed in neurons [7]. A reduction in expression of a number of glucose transporters has been observed in the brains of mouse AD models [8] and of human patients [7]. The timing of this decrease correlates with increases in Tau phosphorylation and neurofibrillary tangles (NFTs) [7]. In a mouse model of AD pathogenesis, a reduction in neuronal Glut3 expression coincided with a reduction in glucose metabolism [8], while a drop in Glut1 in endothelial cells exacerbated pathology in another mouse AD model [9].

Whether impaired neuronal glucose metabolism plays a causal role in neurodegeneration in AD awaits investigation. The drop in glucose metabolism could contribute to disease progression in several ways. It could lead to a reduction in ATP in neurons, since glucose is the main source of energy. Downregulation of the hexosamine pathway, which relies on glucose for GlcNAc production, would lead to a reduction in Tau GlcNAcylation, which, in turn, could drive up toxic Tau phosphorylation, since the two are negatively correlated [10]. Hypometabolism and glucose deprivation have been shown to induce the unfolded protein response (UPR) [11]; this, too, could drive Tau phosphorylation [11]. Any or all of these mechanisms could contribute to neurodegeneration.

To begin to experimentally test the role of glucose transport and metabolism in AD pathogenesis, we used a model of Aβ toxicity in the fruit fly Drosophila melanogaster [12]. Drosophila has proved to be an excellent model system in which to study neurodegenerative diseases. The fly has a distinct brain structure with cell types analogous to the human brain, as well as a blood-brain barrier (BBB), and is, therefore, ideal for studying...
the neurodegenerative process in a complex tissue. The metabolic coupling between glia and neurons observed in mammalian brains is also conserved in flies [13]. The fly AD model that we used expresses pathogenic Arctic Aβ42 tagged with an endoplasmic reticulum (ER) export signal peptide [14] exclusively in the neurons of the adult fly, thereby removing any confounding developmental effects. These flies have shortened lifespans, behavioral defects, and neurodegeneration [12].

If lowered glucose metabolism in neurons is part of the pathogenic cascade from toxic Aβ, then experimentally increasing glucose metabolism in neurons should ameliorate the pathogenesis in the AD model. Therefore, we assessed the effect of overexpressing a glucose transporter, Glut1, in the neurons of the AD flies. While sleep patterns are an obvious candidate, we focused on brain systems that are independent of each other, and we could thus monitor the morphology of a sub-population of neurons in the presence of Aβ and of overexpression of Glut1. When Aβ was expressed, a number of filamentous structures clearly visible in the wild-type brain were lost, reflecting the degeneration of axons or dendrites. Strikingly, when Glut1 was overexpressed, the neuronal morphology was completely restored (Figure 1D).

Surprisingly, Glut1 overexpression did not affect Aβ-42 protein or mRNA levels in heads (Figures 1E and 1F), suggesting that Glut1 reduced the toxicity, rather than the total load, of Aβ.

In humans and mouse models of AD, disease progression has been linked to a decrease in glucose transporters [8], and in mouse AD models, a reduction of glucose transporters in endothelial cells exacerbates disease development. Similarly, in our Drosophila model, RNAi of Glut1 in neurons (Figure S1E) reduced the lifespan of Aβ-expressing flies, indicating that reduction of glucose import into neurons worsens Aβ toxicity, similar to the mechanism suggested in humans.

In both mammalian and fly brains under normal physiological conditions, glycolysis occurs primarily in glia [13]. Therefore, we assessed whether increasing glucose uptake in glia could also affect pathology. For this, we generated a fly model concomitantly expressing Aβ in neurons and Glut1 in glia. Aβ was driven in neurons by the nSyb-QF2 driver [17], which was induced starting from eclosion, whereas Glut1 was induced by the constitutive glial driver repo-Gal4. Overexpression of Glut1 in glia did not rescue the toxicity of neuronal Aβ, as assessed by lifespan (Figure S1F). Hence, either the Aβ toxicity in neurons was too great for the induction of Glut1 that we achieved to rescue it, or Glut1 in glia cannot rescue Aβ toxicity.

Glucose uptake can influence many cellular processes and could, therefore, rescue Aβ toxicity in several ways. The most obvious candidate is energy metabolism, since glucose is the source of most cellular energy. However, we did not observe an obvious energy deficit in the brains of our Aβ-expressing flies, and the ADP/ATP ratio in brains was unchanged when Glut1 was overexpressed (Figure S1G).

Next, we considered whether Glut1 overexpression reduced Aβ toxicity by acting through the hexosamine biosynthetic pathway, by increasing protein GlcNAcylation. However, upregulation in neurons of GFTA2, the first and rate-limiting enzyme in the hexosamine biosynthetic pathway, shortened the lifespan of the Aβ-expressing flies (Figure S1H), contrary to what would be predicted from this hypothesis.
Glucose uptake can affect the UPR [18], which is becoming increasingly recognized as important in neurodegenerative diseases, including AD [19], although whether it plays a protective or detrimental role remains unclear [19]. The UPR signaling cascade is activated in response to ER stress, allowing the cell either to restore protein homeostasis or to enter apoptosis [19], and is mediated by three trans-membrane proteins: pancreatic ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). Grp78, also known as BiP, binds and keeps these three proteins in an inactive state. Upon ER stress, Grp78 targets misfolded proteins to act as a chaperone, and thus releases PERK, IRE1, and ATF6 to activate a series of downstream cascades, leading to the phosphorylation of eIF2alpha and reduction of protein translation, activation of downstream transcription factors such as ATF4 and Xbp1, and increases in chaperones such as Grp78 itself (Figure S2A) [19].

The main marker of PERK activation, eIF2alpha phosphorylation, was not affected by either Aβ or Glut1 expression in neurons (Figure 2A); however, we did confirm that the antibody we used was able to detect eIF2alpha phosphorylation in response to a strong UPR inducer (Figure S2B). However, Grp78 mRNA expression, a marker of ATF6 activation, was increased in flies expressing Aβ (Figure 2B). Xbp1 splicing, a marker of IRE1 activation, increases in response to Aβ in flies [20]. In our model, we noticed a trend toward an increase in the fluorescence of a GFP reporter for Xbp1 splicing, but this did not reach significance (Figure 2C). However, when we measured the spliced Xbp1 isoform by qPCR, it was significantly increased in response to Aβ (Figure 2D), in agreement to what has previously been described [20]. Aβ thus induces the ATF6 and IRE1, but not the PERK, branches of the UPR. Unexpectedly, co-expression of Glut1 increased Grp78 mRNA (Figure 2B) and Xbp1 splicing (Figure 2C) even further, suggesting an additional increase of the UPR in...
response to glucose uptake. Grp78 protein levels are tightly controlled at the level of translation [21], and an increase in the mRNA, therefore, does not necessarily indicate higher protein levels. Therefore, we measured Grp78 protein and found that it also increased in the presence of Aβ, albeit to a smaller extent (Figure 2E). However, surprisingly, overexpression of Glut1 resulted in reduced expression of Grp78 protein (Figure 2E), suggesting that Glut1 and the increased glucose uptake that it produced reduced either Grp78 translation or stability.

Grp78 is broadly considered a negative regulator of the UPR, since it binds and maintains ATF6 and IRE1 in an inactive state. Overexpression of Grp78 can attenuate UPR signaling both in non-neuronal cells [22] and in neurons [19], and its knockdown could also increase resistance to tunicamycin. Indeed, Glut1 overexpression protected flies from tunicamycin stress (Figure 3A), accompanied by a block in Grp78 induction (Figure 3B) and a trend toward a further increase in UPR markers upon Glut1 expression (Figures 3C and 3D), similar to what was observed in the Aβ-expressing brains. Glut1 could, therefore, protect against UPR stress, attributable to a reduction in Grp78 levels.

If Glut1 overexpression protects against Aβ-induced UPR stress by reducing Grp78 levels, then a reduction in Grp78 activity should also rescue Aβ toxicity. We tested this by overexpressing a dominant-negative version of Grp78, Grp78K97S, which carries a point mutation affecting the coupling of ATP binding to substrate release [25]. Expression of Grp78K97S in neurons increased both the lifespan and the climbing ability of Aβ-expressing flies (Figures 4A and 4B), suggesting that, indeed, reduced Grp78 activity is causal in the amelioration of Aβ toxicity by Glut1.

AD is characterized by a deregulation of protein homeostasis [26], which could contribute to disease. Therefore, we hypothesized that increased glucose metabolism in neurons allows the UPR to increase even further and, thus, to restore protein homeostasis. To test this idea, we measured insoluble ubiquitinated protein levels in heads and found that Aβ expression led to the accumulation of insoluble ubiquitinated proteins, which was abrogated by Glut1 overexpression (Figure S5). Therefore, allows neurons to
re-establish protein homeostasis, presumably via upregulation of the UPR.

Metformin is a drug used to treat type 2 diabetes, and it increases glucose uptake in several tissues [27, 28] by increasing the translocation of glucose transporters to the plasma membrane [27]. Therefore, we treated our Aβ-overexpressing flies with metformin to determine whether we could recapitulate the rescue observed by overexpressing Glut1. Indeed, feeding Aβ-overexpressing flies with a range of metformin concentrations resulted in a significant lifespan extension (Figure 6A) and increase in climbing ability (Figure 6B) without altering Aβ levels (Figure 6C). Interestingly, 80 mM metformin reduced the lifespan of flies that did not express Aβ and was also less effective than lower doses at extending the lifespan of Aβ-expressing flies, but it gave the strongest rescue of climbing ability. These results suggest that this high concentration of metformin shows a beneficial effect on neuronal-related health before 25 days but that continuous exposure to a high dose of metformin leads, at later ages, to systemic toxicity and reduced lifespan. The rescue was dependent on Glut1 expression, since RNAi of Glut1 blocked the lifespan extension from metformin treatment (Figure 6D). Metformin treatment, like Glut1 overexpression, also blocked the increase in Grp78 levels associated with Aβ expression (Figure 6E). Metformin could, therefore, be a potential therapeutic modulator of Aβ pathology by blocking toxic Grp78 induction.

**DISCUSSION**

Glucose metabolism has been strongly implicated in the pathogenesis of AD [5, 7]. Patients display a marked reduction in glucose metabolism in brain areas vulnerable to degeneration, and this precedes the onset of clinical symptoms and mirrors disease progression more closely than does Aβ deposition [4]. Expression of neuronal glucose transporters also drops in AD patients...
and in AD mouse models [7]. Recently, it was shown that reduc-
tion of glucose transport across the BBB in mouse AD models
exacerbates Aβ toxicity [9]. However, whether impaired glucosemetabolism in neurons plays a causal role in AD pathogenesis
has not been addressed directly.

Our study has shown that experimentally increasing glucose
uptake in neurons can protect against Aβ toxicity. In our
Drosophila AD model, Glut1 overexpression led to a lifespans
increase, an amelioration of phenotypes linked to neuronal
health—namely, climbing and sleep—and a restoration of normal
neuronal morphology. This improvement was associated with
a reduction in Grp78 protein levels and an upregulation of
the UPR.

In AD patients, the UPR is activated early in disease pathogen-
esis [11]. Similarly, Aβ expression in the neurons of adult flies led
to the induction of the UPR. Grp78 levels were increased, simi-
larly to those of mice models of AD [29], of AD patients early in
disease development [30], and in neuronal cells derived from
AD patients’ induced pluripotent stem cells (iPSCs) [31].

Intriguingly, the rescue of Aβ toxicity by Glut1 was associated
with a reduction in Grp78 expression. Grp78 is becoming an
increasingly important therapeutic target, especially in cancer
biology, where its inhibition increases cells’ susceptibility to
chemotherapy agents [32]. Its role in neurodegeneration is less
well defined. In rat models of Parkinson’s disease, activation
of Grp78 is protective [33], and its downregulation is detrimental
[34]. However, its role in AD models has not been tested. Our
studies suggest that Grp78 downregulation can ameliorate Aβ
toxicity by allowing upregulation of the UPR. Already, it has
been shown in flies that overexpression of Xbp1 can ameliorate
Aβ42 toxicity [20], supporting the idea that upregulation of UPR
components could be beneficial in AD models. The role of
Grp78 has not been tested in other models of neurodegenera-
tion, and a complex picture is emerging regarding the role of
downstream UPR effectors, where, depending on the precise
experimental conditions, upregulation of the UPR appears to
be protective or detrimental [19]. For example, downregulation
of PERK is protective in some ALS (amyotrophic lateral sclerosis)
or prion disease models, whereas increased Xbp1 can be pro-
tective in Parkinson’s and Huntington’s disease models [19].
This could be because the UPR is a complex pathway with
extensive crosstalk between the different signaling cascades,
so it would be difficult to predict reliably the outcome of an in-
tervention. Alternatively, a mild upregulation of the UPR could
allow the induction of an ER-hormetic response, increasing ER proteo-
stasis to allow a neuron to deal with an increase in misfolded
proteins, whereas, in other conditions, a strong induction of
the UPR could lead to apoptosis, and, therefore, blocking this
response could also increase neuronal survival [19].

How glucose regulates Grp78 expression is unclear. It is
well established that glucose starvation induces expression of
Grp78 [35], and, in one study, increased glucose reduced
Grp78 expression in cultured neurons [36]. However, little is
known about how physiological changes in glucose metabolism
modulate Grp78, and it will be important to identify the mecha-
nisms at work.

Our study suggests a model where Aβ accumulation in the
brain induces the UPR, and increased glucose uptake in neurons
blocks the negative feedback loop linked to Grp78 upregulation,
resulting in even further increased UPR, which allows neurons
to clear insoluble ubiquitinated proteins and restore protein
homeostasis, resulting in the rescue of neurodegeneration and
increased lifespan. This mechanism of action could be relevant
to the benefits observed by administration of insulin nasal
spray in AD patients [37]. Insulin is thought to act via PI3K/
MAPK (phosphatidylinositol 3-kinase/mitogen-activated protein
kinase) to influence Aβ trafficking and decrease Tau phosphory-
lization by inhibiting Gsk3 [38]. However, insulin can upregulate
glucose transporters in neurons [39] and increase glucose meta-
bolism [39]. It would be interesting to determine whether insulin
administration also upregulates the UPR.

Glucose metabolism has also been implicated in the increased
risk of AD associated with type 2 diabetes. The link is well estab-
lished, but the mechanism is less so [40]: possibilities include
increased vascular risk factors associated with metabolic syn-
drome in type 2 diabetes, as well as hyperglycemia-linked dysregu-
lation of cellular signaling pathways, leading to advanced
glycation products and increased reactive oxygen species.
Also, the rise in brain insulin resistance, which could, in part,
lead to increased production of Aβ [40], could also result in
decreased glucose transporters and glucose metabolism within
neurons, leading to an upregulation of Grp78.

Metformin, a type 2 diabetes therapy, is a drug that has been
shown to increase glucose uptake in cells. Interestingly, treat-
ment with a metformin dose known not to affect lifespan in
wild-type Drosophila [41] increased the lifespan of Aβ-express-
ing flies. In accordance with previous studies [42], we also found
that metformin decreased expression of Grp78, suggesting that
the rescue of Aβ toxicity could be due to increased UPR. Metfor-
im’s effect in AD is controversial, with some studies reporting
patient benefits [43] but others finding that it worsened cognitive
performance [44], possibly related to metformin’s ability to
increase APP levels and processing to increase Aβ production [45]. Our study points to a novel and unexplored role of metformin as a modulator of the UPR in neurodegeneration downstream of Aβ accumulation, which could provide a useful therapeutic avenue in a clinical context where AD patients present quite late in disease development and have already accumulated Aβ peptide in their brain.

EXPERIMENTAL PROCEDURES

Fly Husbandry and Stocks
All flies were reared at 25°C on a 12-hr:12-hr light:dark (LD) cycle at constant humidity and on standard sugar-yeast-agar (SYA) medium (agar, 15 g/l; sugar, 50 g/l; autolyzed yeast, 100 g/l; nipagin, 100 g/l; and propionic acid, 2 ml/l). Adult-onset, neuron-specific expression of UAS constructs was achieved as described elsewhere [12]. Briefly, 24–48 hr after eclosion, female flies carrying a heterozygous copy of elavGS and at least one UAS construct were fed SYA medium supplemented with 200 μM mifepristone (RU486) to induce transgene expression. For induction with quinic acid (QA), flies were put on food containing 7.5 g of QA per liter. Metformin was added to the food at the stated concentrations. ElavGS was derived from the original elavGS 301.2 line [46] and obtained as a generous gift from Dr. H. Tricoire (CNRS); the UAS-Aβ stock was a gift from Dr. D. Crowther (University of Cambridge). The nSyb-QF2 stock was a gift from Dr. C. Potter (Johns Hopkins School of Medicine) [17], W1118, tubulin-QS (tub-QS), UAS-Gpr78/k97S, UAS-Xbp1GFP, daughterless-Gal4 (daGal4), Glut1 RNAi line (TRP.H00241532) and Repo-Gal4 were obtained from the Bloomington Drosophila Stock Center. All transgenes were backcrossed into the w1118 background to ensure a homogeneous genetic background between transgenic lines. All experiments were carried out on mated females, unless otherwise stated.

Lifespan Analysis
Flies were raised at standard density in 200-ml bottles. After eclosion, flies were allowed to mate for 24–48 hr. At least 110–150 females of the appropriate genotype were split into groups of 15 and housed in vials containing SYA medium with or without drugs. Deaths were scored, and flies tipped onto fresh food three times a week. Data are presented as cumulative survival curves, and survival rates were compared using log-rank tests or Cox proportional hazards performed in JMP (version 9.0) software (SAS Institute). All lifespans were performed at 25°C unless otherwise stated.

Figure 5. Glut1 Reduces Accumulation of Insoluble Ubiquitinated Proteins
Western blots of SDS-soluble protein fraction from the heads of day-17 flies expressing Aβi or Aβi+Glut1 in neurons (+RU) and uninduced controls (-RU), probed for ubiquitinated proteins (FK2) and for actin, plotted below as means ± SEM (n = 4). The two blots shown are from different experiments. *p < 0.05; **p < 0.01, by ANOVA. Genotypes: UAS Aβi; elavGS; UAS Aβi/UAS Glut1; elavGS.

Climbing Assay
The climbing assay in Figure 1 was performed as previously described [47]. Briefly, 15 flies were placed in a 25-cm pipette, tapped to the bottom, and allowed to climb for 45 s. The number of flies in the top 5 cm, center, and bottom 3 cm was scored. A performance index was calculated for each time point and plotted. Statistical analysis was performed in R using ordinal logistic regression, using the individual heights for each fly as data points. For the climbing assay in Figures 4 and 6, the assay was performed with the following modifications: 45 flies were housed in a glass-walled chamber 25 cm tall, and flies were tapped to the bottom as described earlier and allowed to climb for 20 s before scoring. The analysis was the same as described earlier.

Western Blotting
Protein samples were prepared by homogenizing in 2x SDS Laemmli sample (4% SDS, 20% glycerol, 120 mM Tris-HCl [pH 6.8], 200 mM DTT with bromophenol blue) and boiled at 95°C for 5 min. Samples were separated on pre-cast 4%–12% Invitrogen Bis-Tris gels (NP0322) and blotted onto PVDF (polyvinylidene fluoride) or nitrocellulose membrane (for Gpr78) in Tris-glycine buffer supplemented with 10% ethanol. Membranes were blocked in 5% milk and 1% BSA in TBS-T (Tris-buffered saline with 0.05% Tween-20) for 1 hr at room temperature (RT) and then incubated with primary antibodies in block. Ubiquitin westerns were blocked in 1% BSA. Primary antibody dilutions used were as follows: anti-Gpr78, 1:1,000 (Novus Biologicals, NBP1-08274); anti-actin, 1:1,000 (Abcam, ab1801); anti-Ubiquitin, 1:1,000 (Millipore, FK2); and anti-eIF2A-phospho, 1:1,000 (Cell Signaling, 3597). Secondary antibodies were anti-rabbit and anti-mouse (Abcam, ab6789 and ab6721) at 1:10,000 dilutions for 1 hr at RT. Bands were visualized with Luminata Forte (Millipore) and imaged with ImageQuant LAS4000 (GE Healthcare Life Sciences). Quantification was carried out with ImageQuant software or ImageJ.

Preparation of Detergent-Soluble Fractions for Insoluble Ubiquitinated Protein Gels
The method was adapted from [48]. Briefly, 10–20 heads were extracted in 75 μl Triton X extraction buffer and spun at 13,000 x g for 10 min at 4°C, and the supernatant was collected as the Triton X soluble fraction. The pellet was re-suspended in 50 μl SDS extraction buffer and spun again, and the supernatant was collected as the SDS soluble fraction. Samples were stored at ~8°C and run as described earlier.

gPCR
Total RNA was extracted from heads or whole flies (for Figure 3) and converted to cDNA (see Supplemental Experimental Procedures for details). gPCR was performed using the PRISM 7000 sequence detection system (Applied Biosystems). Each sample was analyzed in duplicate, and values are the mean of three or four independent biological repeats ±SEM.

Quantification of Aβ42
Five fly heads were homogenized in 50 ml G×HCl extraction buffer (5 M guanidinium HCl, 50 mM HEPES [pH 7.3], 1:10 dilution of protease inhibitor cocktail...
(Sigma, P8340), and 5 mM EDTA) and centrifuged at 21,000 g for 5 min at 4°C, and cleared supernatant was retained as the total fly Aβ42 sample. Aβ42 was measured with an ELISA kit (Millipore, EZHS42), according to the manufacturer’s instructions, and protein was measured with a Bradford assay (Bio-Rad protein assay reagent), and the amount of Aβ42 in each sample was expressed as a ratio of the total protein content (picograms per microgram of total protein). Data are expressed as the mean ± SEM obtained from three biological repeats for each genotype.

Figure 6. Metformin Extends the Lifespan of Aβ-Expressing Flies
(A) Lifespan survival curves of flies expressing Aβ (+RU) and controls (-RU) in the presence of different metformin (Met) concentrations (all the metformin-treated +RU flies were significantly longer lived than the +RU-alone control: p < E-4 for 5 mM, p < E-6 for 10 mM, p < E-8 for 20 mM, and p < 0.01 for 80 mM by log-rank test).
(B) Climbing assay performance index (PI) for the same flies (p < 1E-5 when comparing the +RU control relative to the +RU flies treated with 20 mM or 80 mM metformin by ordinal logistics regression).
(C) Aβ42 protein levels, measured by ELISA, in the heads of 15-day-old flies expressing Aβ (+RU) and controls (-RU) in the presence or absence of 10 mM metformin, plotted as means ± SEM (n = 3). *p < 0.0001, by ANOVA; ns, not significant.
(D) Lifespan survival curves of flies expressing Aβ Glut1 RNAi (+RU) and controls (-RU) in the presence or absence of 10 mM metformin (no difference between +RU-treated flies by log-rank test). Genotype: wv; UAS Aβ; elavGS/Glut1RNAi (C).
(E) Western blots for Grp78 and actin control in the heads of flies treated with 10 mM metformin, plotted as means ± SEM (n = 4). *p < 0.01, by ANOVA; ns, not significant.
Genotype for (A)–(C) and (E): UAS Aβ; elavGS.
Microscopy
Brains were dissected in 4% paraformaldehyde in PBS with Tween 20 (PBST), incubated for 20 min, rinsed in PBST, mounted invectashield with DAPI, and imaged on a Zeiss LSM510 inverted confocal microscope. Images were taken on the 20x or 40x objective as stacks and are shown as maximum intensity projections of the complete stack. The same size stacks were taken for experimental and control samples. All images for one experiment were taken at the same settings. For Xbp1 fluorescence (in Figure 2C), total fluorescence intensity of a given area of the brain was measured with ImageJ. Values shown are the averages for 6–13 brains ± SEM. Samples were compared by ANOVA. For neuronal GFP, images were blind scored and given a score from 1 to 5 based on fluorescent intensity; average scores are presented with their SEM (n = 3–8).

Analysis of Activity and Sleep
Individual, 21-day-old, mated female flies were placed in glass tubes (65 mm x 5 mm) containing standard 1x SYA, and activity was recorded using the DAM System (Drosophila Activity Monitoring System; TriKinetics) as described previously [49]. Flies were entrained to a 12:12-hr light:dark (LD) cycle at 25°C and 65% humidity 24–36 hr before recording. 5 days of the 12:12 hr LD cycle were recorded, followed by 5 days of a 12-hr:12-hr dark:light (DD) cycle. Analysis of locomotor activity was performed using the fly toolbox and MATLAB software (MathWorks), as described previously [9]. Sleep was defined as a bout of inactivity lasting 5 min or more, and sleep analysis was performed with pySOLO [81]. All behavioral data (activity and sleep duration) are represented by mean values with their SEM.

Tunicamycin Stress Assay
Flies were tipped into vials containing 1% agar, 1.5% sucrose, and 10 mg/l of tunicamycin. Dead flies were scored at regular intervals, and lifespan curves were compared with log-rank test.

SUPPLEMENTAL INFORMATION
Supplemental Information includes two figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.07.017.

AUTHOR CONTRIBUTIONS

ACKNOWLEDGMENTS
We thank Nazif Alic for help with statistical analysis and Nathan Woodling for comments on the manuscript. We also thank the neuro group for helpful discussion and Alexander Hassabis for help with scoring. This work was supported by the Wellcome Trust (grant number WT086555/Z/12/Z to L.P.), the APUK (grant number ART/P200984 to L.P.), and the Max Planck Society (to L.P.).

Received: November 29, 2015
Revised: June 9, 2016
Accepted: July 11, 2016
Published: August 11, 2016

REFERENCES
Appendix 6
Neurobiology of Disease

A Drosophila Model of Neuronopathic Gaucher Disease Demonstrates Lysosomal-Autophagic Defects and Altered mTOR Signalling and Is Functionally Rescued by Rapamycin

Kerri J. Kinghorn,1,2 Sebastian Grönke,3 Jorge Iván Castillo-Quan,1,2,3 Nathan S. Woodling,1 Li Li,1,2 Ernesteas Sirka,4 Matthew Gegg,5 Kevin Mills,4 John Hardy,7 Ivana Bjedov,6 and Linda Partridge1,3

1Institute of Healthy Ageing and Department of Genetics, Evolution and Environment, University College London, London WC1E 6BT, United Kingdom, 2Institute of Neurology, University College London, London WC1N 3BG, United Kingdom, 3Max Planck Institute for Biology of Ageing, D-50931 Köln, Germany, 4Centre for Translational Omics, Institute of Child Health, University College London, London WC1N 1EH, United Kingdom, 5Clinical Neuroscience, Institute of Neurology, University College London, London NW3 2PF, United Kingdom, and 6University College London Cancer Institute, London WC1E 6DD, United Kingdom

Glucocerebrosidase (GBA1) mutations are associated with Gaucher disease (GD), an autosomal recessive disorder caused by functional deficiency of glucocerebrosidase (GBA), a lysosomal enzyme that hydrolyzes glucosylceramide to ceramide and glucose. Neuronopathic forms of GD can be associated with rapid neurological decline (Type II) or manifest as a chronic form (Type III) with a wide spectrum of neurological signs. Furthermore, there is now a well-established link between GBA1 mutations and Parkinson’s disease (PD), with heterozygote mutations in GBA1 considered the commonest genetic defect in PD. Here we describe a novel Drosophila model of GD that lacks the two fly GBA1 orthologs. This knock-out model recapitulates the main features of GD at the cellular level with severe lysosomal defects and accumulation of glucosylceramide in the fly brain. We also demonstrate a block in autophagy flux in association with reduced lifespan, age-dependent locomotor deficits and accumulation of autophagy substrates in dGBA-deficient fly brains. Furthermore, mechanistic target of rapamycin (mTOR) signaling is downregulated in dGBA knock-out flies, with a concomitant upregulation of Mitf gene expression, the fly ortholog of mammalian TFEB, likely as a compensatory response to the autophagy block. Moreover, the mTOR inhibitor rapamycin is able to partially ameliorate the lifespan, locomotor, and oxidative stress phenotypes. Together, our results demonstrate that this dGBA1-deficient fly model is a useful platform for the further study of the role of lysosomal-autophagic impairment and the potential therapeutic benefits of rapamycin in neuronopathic GD. These results also have important implications for the role of autophagy and mTOR signaling in GBA1-associated PD.

Key words: autophagy; Drosophila; Gaucher disease; glucocerebrosidase; mTOR; rapamycin

Significance Statement

We developed a Drosophila model of neuronopathic GD by knocking-out the fly orthologs of the GBA1 gene, demonstrating abnormal lysosomal pathology in the fly brain. Functioning lysosomes are required for autophagosome-lysosomal fusion in the autophagy pathway. We show in vivo that autophagy is impaired in dGBA-deficient fly brains. In response, mechanistic target of rapamycin (mTOR) activity is downregulated in dGBA-deficient flies and rapamycin ameliorates the lifespan, locomotor, and oxidative stress phenotypes. dGBA knock-out flies also display an upregulation of the Drosophila ortholog of mammalian TFEB, Mitf, a response that is unable to overcome the autophagy block. Together, our results suggest that rapamycin may have potential benefits in the treatment of GD, as well as PD linked to GBA1 mutations.

Introduction

Homozygous mutations in the glucocerebrosidase gene (GBA1) can cause Gaucher disease (GD), the commonest lysosomal storage disorder. GBA1 encodes glucocerebrosidase (GBA, also known as glucosylceramidase [GCase]), which mediates the lysosomal hydrolysis of glucosylceramide to form ceramide and glucose. Loss of GBA activity leads to lysosomal accumulation of glucosylceramide and its nonacetylated analog glucosylsphingosine in a number of cell types,
including macrophages and neurons, giving rise to the visceral and neurological manifestations of GD, respectively (Cox, 2010). GD is classified into three clinical subtypes based on the absence (Type I) or presence (Types II and III) of neurological involvement. Type II, or acute neuronopathic GD, is rare but results in rapidly progressive neurological decline, with a wide spectrum of clinical signs and death in infancy or early childhood. Type III, or chronic neuronopathic GD, is a more slowly progressive disorder with neurological features, including eye movement abnormalities, myoclonic epilepsy, ataxia, and dementia (Sidransky, 2004). There are currently no therapies available that target the neuronal pathology associated with GD because existing treatments, such as recombinant enzyme replacement therapy, fail to permeate the blood–brain barrier (Cox, 2010).

As well as causing GD, heterozygous GBA1 mutations are now well-established genetic risk factors for PD and other systemicinopathies, characterized by the presence of intraneuronal aggregates called Lewy bodies (LBs), predominantly containing α-synuclein as well as ubiquitin (Shults, 2006). Both loss-of-function and gain-of-function hypotheses have been put forward to explain how mutations in GBA1 lead to lysosomal dysfunction and neurodegeneration, and there is mounting evidence in support of the loss of GBA function hypothesis (Kinghorn, 2011). Lysosomal dysfunction in GD has far-reaching implications, not only for the degradation of α-synuclein, but also for the autophagic degradation of other superfluous or damaged cellular material and organelles, as the fusion of lysosomes with autophagosomes is a critical step in the autophagic pathway (Ravikumar et al., 2010). The process of macroautophagy can be divided into key stages, including initiation, elongation, and maturation of autophagosomes, followed by the fusion with lysosomes.

In keeping with the importance of lysosomes in the autophagic pathway, it was recently shown in macrophages and induced pluripotent stem cell (iPSC)-derived neuronal cells from GD patients that autophagy is impaired (Awad et al., 2015; Afalaki et al., 2016). Furthermore, this defect in autophagy was shown to trigger inflammasome activation and the production of inflammatory cytokines (Afalaki et al., 2016). Moreover, Osellame et al. (2013) demonstrated that there is accumulation of dysfunctional and fragmented mitochondria in a neuronopathic mouse model of GD, thought to be secondary to autophagic and proteasomal defects.

To further understand the mechanisms linking loss of GBA activity to neurodegeneration in neuronopathic GD, and also to determine whether autophagy plays a role in neurons in vivo, we developed a novel Drosophila model that lacks the two GBA1 orthologs, dGBA1a and dGBA1b. The fruit fly has proven to be a useful model system for studying neurodegenerative diseases (Kinghorn et al., 2015) and lysosomal storage disorders, including those affecting the nervous system, such as neuronal ceroid lipofuscinoses (Myllykangas et al., 2005; Hindle et al., 2011). It is therefore a useful model for studying the mechanisms of pathogenesis of GD as well as the role of GBA1 in neurodegeneration.

The dGBA knock-out flies proved to be faithful models of GD, with significant lysosomal dysfunction and buildup of the dGBA substrate, glucosylceramide, in the fly brain. Furthermore, there was significant neuronal autophagy impairment, which was associated with reduced survival and age-dependent locomotor abnormalities. At the cellular level, we demonstrated accumulation of p62 and polyubiquitinated proteins, both of which accumulate to form LBs in PD (Zatloukal et al., 2002), in addition to mitochondrial abnormalities. Moreover, dGBA-deficient flies displayed decreased mechanistic target of rapamycin (mTOR) signaling with a reduction in p70 S6 kinase (S6K) phosphorylation, and the mTOR inhibitor rapamycin was able to partially ameliorate the neurodegenerative phenotypes. Our results thus show an important role of autophagy and mTOR signaling in GD, and provide a useful model for the further study of pathogenic mechanisms downstream of lysosomal dysfunction.

Materials and Methods

Fly stocks and husbandry. All fly strains used were backcrossed at least 6 generations into the w^1118 background to obtain isogenic lines. All fly stocks were maintained at 25°C on a 12:12 h light:dark cycle at constant humidity on a standard sugar-yeast medium (15 g/L-1 agar, 50 g/L-1 sugar, 100 gl-1 autolyzed yeast, 3g/L nipagin, and 3 ml/L propionic acid). For all experiments, flies were raised at a controlled density on standard sugar-yeast medium in 200 ml bottles.

Generation of dGBA1a and dGBA1b mutants by ends-out homologous recombination. The genes encoding dGBA1a (CG31148) and dGBA1b (CG31414) are located in close proximity on Drosophila chromosome 3, separated by the CG31413 gene (see Fig. 1A). We generated specific mutants for dGBA1a, dGBA1b, and a dGBA1a and dGBA1b double-mutant, without affecting the coding sequence of CG31413 by ends-out homologous recombination (Gong and Golic, 2004). dGBA1a was mutated by replacing the whole ORF with a white" marker gene. dGBA1b was mutated by introducing a stop codon and a subsequent frame shift mutation 12 bp downstream of the predicted ATG start codon. In addition, an EcoRV restriction site was introduced to allow PCR screening of the introduced mutations. To clone the donor construct for homologous recombination, ~4 kb of 5' and 3' flanking sequences of the dGBA1a gene were cloned into the pBluescript II SK+ vector (Stratagene) by ET recombination using BAC clone CH321-50J5 (BACPAC Resource Center, Oakland, California) as template and primers SOL474/475 and SOL476/477 for the 5' and 3' arms, respectively. Mutations in the 3' arm were introduced using the QuikChange site-directed mutagenesis kit (Agilent Technologies) and primers SOL482/483. Both 5' and 3' homologous arms were subcloned into the pW25 vector (Gong and Golic, 2004) and full-length sequenced. pW25 was obtained from the Drosophila Genomics Resource Centre. The DGBA donor construct was transformed into the germline of Drosophila melanogaster by P-element-mediated germ line transformation using the Best Gene Drosophila Embryo Injection Service. Crosses for ends-out homologous recombination were performed according to the rapid targeting scheme (Gong and Golic, 2004). Subsequently, the white" marker gene was genetically mapped and homologous recombination events were identified by PCR. Point mutations introduced into dGBA1b were identified by PCR with primers SOL484 and SOL485 and subsequent digestion with EcoRV, which results in two specific DNA fragments of 120 and 193 bp from the unc131 bp wild-type DNA fragment. Oligonucleotide primers used for cloning and genotyping are listed in Table 1.

qRT-PCR. Total RNA was extracted from 25 heads or 25 thoraces and abdomens of 6–d-old adult flies per sample using Trizol (Invitrogen) and further purified over an RNeasy Mini column (QIAGEN) with on-column DNase digestion. cDNA was prepared from 2.5 μg RNA using the SuperScript III cDNA synthesis kit (Invitrogen). qRT-PCR data were performed with TaqMan primers (Applied Biosystems) in a 7900HT
real-time PCR system (Applied Biosystems). The TaqMan primers used were as follows: Dm02150538_s1 for dGBA1b, Dm02143806_s1 for dGBA1a, Dm02150538_s1 for CG31413, and a custom-made probe against Rpl32. For each sample, 4 biological replicates and 4 technical replicates were used. Relative expression was calculated using the ΔΔCT method and Rpl32 as normalization control.

For assessment of Mitf and Atg8a gene expression levels, total RNA was extracted from 20–25 fly heads per sample using TRIzol (Invitrogen) by following the manufacturer’s instructions. Each sample was subjected to DNA digestion using DNase I (Ambion), immediately followed by reverse transcription using the SuperScript II system (Invitrogen). The reaction was stopped with 0.25 M 4-methyllumbelliferyl-D-glucopyranoside substrate. Both enzyme activity and expressed as pmol/h/mg protein. Approximately 50% of the total dGBA activity was abolished by incubation with conduritol-β-epoxide in w1118 control flies.

Measurement of glucosylceramide levels. Fly heads (n = 70) were analyzed for glucosylceramide accumulation. The substrate was extracted in 500 μl of chloroform/methanol (2:1 v/v) containing 80 μg/ml of d4-C16:0-glucosylceramide internal standard, which was synthesized in house (Mills et al., 2005). The samples were shaken for 30 min at room temperature before the addition of 100 μl of PBS for phase separation. After a 10 min centrifugation at 16,000 × g, upper and lower phases were collected and 5 μl of each was injected into the ultra-performance liquid chromatography–tandem mass spectrometry system. Synthetic glucosylceramide standard (Matreya) was also analyzed to confirm analyte identity.

The samples were injected onto Waters ACQUITY UPLC system operated in partial loop mode and separated on Waters ACQUITY UPLC BEH C18 column (130A, 1.7 μm, 2.1 mm × 50 mm) under the following gradient conditions: 0.00–0.20 min 80% A; 9.01–11.00 min 80% A; 11.01–20.00 min 0.1% A; 20.01–50.00 min 0.1% A; 50.00–99.00 min 0.1% A; 99.01–100.00 min 0.1% A.

Measurement of lysosomal enzymes. Twenty flies were homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% (w/v) NP-40, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, protease inhibitor mixture) with a micro-pestle. Debris was pelleted at 17,000 × g and protein concentration of the supernatant was determined using the protein BCA kit (Pierce). β-Hexosaminidase (10 μg protein) was measured in McIlvaine citrate buffer, pH 4.2, with 2 mM 4-methylumbelliferyl-N-acetyl-glucosamide substrate. β-Galactosidase activity (10 μg protein) was measured in McIlvaine citrate buffer, pH 4.1, with 0.25 mM 4-methylumbelliferyl-β-D-galactopyranoside substrate. Both enzyme assays were incubated at 37°C for 30 min and the reaction stopped by addition of 0.25 mM glycine, pH 10.4. Fluorescent product was measured using a plate reader (excitation, 380 nm; emission, 460 nm). dGBA activity was also measured in the presence of the inhibitor conduritol-β-epoxide (100 μM) and dGBA activity calculated as total GBA activity minus CBE-sensitive GBA activity and expressed as pmol/h/mg protein. Approximately 50% of the total dGBA activity was abolished by incubation with conduritol-β-epoxide in w1118 control flies.

Measurement of triacylglyceride and ATP assays. Levels of triacylglyceride (TAG) in adult females were measured using the Triglyceride Infinity Reagent (Thermo Scientific) and normalized to total body protein using a BCA assay (Sigma). The ATP concentration of whole female flies was determined using the ATP Bioluminescence Assay Kit HS II (Roche) as previously described (Kinghorn et al., 2015).
**Antibodies and Western blotting.** For Atg8 Western blotting, 10 to 15 fly heads or 5 headless bodies were homogenized in 2 × Laemmli loading buffer (100 mM Tris 6.8, 20% glycerol, 4% SDS) containing 5% β-mercaptoethanol and then boiled for 5 min. Approximately 40 μg of protein extract was loaded per lane. Proteins were separated on SDS polyacrylamide gels and transferred to a nitrocellulose membrane. For ubiquitin, Western blots, 12 fly heads per sample were homogenized in Triton-X buffer (1% Triton-X, 10 mM NEM, 50 μM MG132, Complete Mini protease inhibitors, Roche, in PBS). After centrifugation, the insoluble pellet was resuspended in SDS buffer (2% SDS, 10 mM NEM, 50 μM MG132, complete mini protease inhibitors, Roche, 50 mM Tris, pH 7.4), centrifuged, and the supernatant collected as the insoluble sample for Western blot. Proteins were separated on 4%–12% NuPage Bis-Tris gels (Invitrogen) and transferred to a PVDF membrane. The membranes were then blocked in 5% BSA in TBST (TBS with 0.05% Tween 20) for 1 h at room temperature, after which they were probed with primary antibodies diluted in 5% BSA in TBST overnight at 4°C. Blots were developed using the ECL detection system. The following primary antibodies were used: β-actin (Abcam, #ab1801; 1:5000), mouse anti-poly-ubiquitinated proteins (clone FK2, Millipore, 1:1000) and anti-Atg8 (1:1000) (Castillo-Quan et al., 2016).

For detection of St6K and Ts6K protein levels, 15 fly heads in each sample were prepared by homogenization in 2 × SDS Laemmli sample buffer (4% SDS, 20% glycerol, 120 mM Tris-HCl, pH 6.8, 200 mM DTT with bromophenol blue), and boiled at 95°C for 5 min. Samples were separated on precast 4%–12% Invitrogen Bis-Tris gels (NP0322) and blotted onto nitrocellulose paper in Tris-glycine buffer supplemented with 10% ethanol. They were then blocked as above, and the primary antibodies used were anti-phosphorylated St6K anti-phospho-Thr398-St6K (Cell Signaling Technology, #9209, 1:1000), anti-total-S6K (1:1000) (Sofola-Adesakin et al., 2014). The appropriate secondary antibodies (Abcam) were diluted 1:10,000 in 5% BSA in TBST for 1 h at room temperature. Bands were visualized with Luminata Forte (Millipore).

All blots were imaged with ImageQuant LAS4000 (GE Healthcare Life Science). Quantification was performed using the ImageJ program (National Institutes of Health).

**Immunohistochemistry, LysoTracker, confocal imaging, and quantification.** For immunohistochemistry, adult brains were dissected in PBS and immediately fixed in cold 4% PFA (v/v) for 20 min at room temperature. After fixation, the samples were washed in PBT (PBS containing 0.3% Triton X-100, v/v) four times for 20 min then blocked in PBT containing 10% BSA (PBT-BSA) for 2 h at 20°C-30°C. For primary antibody treatment, samples were incubated in PBT-BSA containing the primary antibody for 3 d at 4°C. After primary antibody incubation, brains were washed in PBT, four times for 20 min at 20°C-30°C, then incubated in PBT-BSA containing the secondary antibody for 24 h at 4°C. Brains were washed four times with PBT for 20 min at 20°C-30°C. Brains were finally mounted in Vectashield containing DAPI overnight (Vector Laboratories). Antibodies were used at the following dilutions: rabbit anti-Ref(2)P (Abcam) 1:200; mouse anti-polyubiquitinated proteins (FK2, Millipore) 1:200; mouse anti-Bruchpilot (DshB antibody nc82), 1:100; secondary goat anti-rabbit AlexaFluor-568 1:250, and secondary goat-anti-mouse AlexaFluor-488 1:250.

For Ref(2)P and ubiquitin staining, image stacks of specimens were obtained on a Zeiss LSM700 confocal microscope using a 40 × objective for whole-brain imaging. Stacks of 7.25 μm Z distance and 14 images per stack were taken. Images were quantified using ImageJ software. Briefly, confocal stacks were merged into a single plane by using the average projection function. A region of each central brain was manually selected (200 μm high, 100 μm wide) from the dorsal to the ventral aspect of the brain. Thresholds were then set for p62/Ref(2)P and ubiquitin, and the area above threshold within this region was measured.

For Bruchpilot staining, image stacks were obtained using a 40 × objective in the region encompassing the ventral edge of the antennal lobe and dorsal edge of the subesophageal ganglion. Stacks of 1.00 μm Z distance and 11 images per stack were taken. Images were quantified using ImageJ software. Briefly, confocal stacks were merged into a single plane by using the average projection function. Two regions of each image were manually selected (10 μm high, 10 μm wide), one in the center of the antennal lobe region and one in the center of the subesophageal ganglion region, by an observer blinded to genotype. The mean intensity of Bruchpilot staining was then measured for each region of interest.

For LysoTracker staining, adult brains were dissected in PBS and immediately transferred to a solution of PBS with 1 μM LysoTracker Red DND-99 (Invitrogen) and 1 μM DAPI. Brains were mounted on slides in this solution and imaged within 15 min of dissection on a Zeiss LSM700 confocal microscope using a 40 × objective. For each replicate within the experiment, overt control and one mutant brain were imaged side by side, with identical microscope settings and images taken from the same region of the subesophageal ganglion for each brain. Images were quantified using ImageJ software, by setting a threshold for LysoTracker and measuring the area above threshold.

**Electron microscopy of fly brains and image analysis.** The flies were decapitated and the proboscis removed to allow penetration of the fixative. They were then processed and visualized using previously described methods (Kinghorn et al., 2015). Cross-sectional mitochondrial and rhabdomere areas were measured manually using ImageJ software.

**Statistical analyses.** Survival experiments were analyzed using log rank test. Single comparisons were analyzed using the Student’s t-test. Other data were tested by ANOVA, and planned comparisons of means were made using Tukey-Kramer HSD test. Statistical analyses were performed using Excel, GraphPad Prism, or JMP software version 9 (SAS Institute).

**Results**

**Knock-out of the Drosophila orthologs of GBA1 using homologous recombination.**

Drosophila melanogaster has two orthologs of the human GBA1 gene, CG31148 and CG31414, which we refer to as dGBA1a and dGBA1b, respectively. These two genes are found on the same chromosome, separated by the CG31413 gene (Fig. 1A), and show differential tissue expression. dGBA1b is expressed in the adult brain, albeit at low levels, as well as moderately expressed in the adult fat body. dGBA1a is predominantly expressed in the adult fly digestive system and shows no expression in the adult brain (Flyatlas) (Robinson et al., 2013). To study the function of the dGBA1 genes, we produced loss-of-function mutants lacking dGBA1a (dGBA1a<sup>−/−</sup>), dGBA1b (dGBA1b<sup>−/−</sup>), or double-mutants lacking both genes (dGBA1a,b<sup>−/−</sup>) using ends-out homologous recombination. The complete open reading frame of dGBA1a was knocked out by homologous recombination, whereas dGBA1b expression was disrupted by the introduction of a stop codon and a frame shift 12b downstream of the putative ATG start codon (Fig. 1A; for more details, see Materials and Methods). Deletion of dGBA1a and integrity of the CG31413 gene were confirmed by PCR on genomic DNA (Fig. 1B). Furthermore, introduction of specific point mutations within the dGBA1b gene were verified by PCR (Fig. 1C) and sequencing. To test whether these mutations affected expression of the dGBA genes and the intergenic CG31413 gene, we measured mRNA transcript levels by qRT-PCR data in female heads and bodies for dGBA1b (Fig. 1D), in female bodies for dGBA1a (Fig. 1E), and in male bodies for CG31413 (Fig. 1F). The latter gene is specifically expressed in male accessory glands (Flyatlas) (Robinson et al., 2013). dGBA1b transcript levels were barely detected in dGBA1b<sup>−/−</sup> single-mutant and dGBA1a,b<sup>−/−</sup> double-mutant flies (Fig. 1D), suggesting that the introduced point mutations affect the stability of the dGBA1b transcript. dGBA1a expression was absent from dGBA1a<sup>−/−</sup> single-mutant and dGBA1a,b<sup>−/−</sup> double-mutant flies (Fig. 1E) and CG31413 expression was slightly reduced in dGBA1a,b<sup>−/−</sup> double-mutants (Fig. 1F). Thus, the qRT-PCR data suggest that both dGBA1a<sup>−/−</sup> and dGBA1b<sup>−/−</sup> are loss-of-function alleles for the respective dGBA genes, with the single mutations not affecting expression of the CG31413 gene, and the double-mutation very slightly reducing it. Knock-out of dGBA1a, dGBA1b, or both genes was not associated with any gross changes in body size or obvious differences in morphology (data not shown). In keeping
with the expression of dGBA1b, and not dGBA1a, in the fly brain, dGBA activity levels were decreased by 95% in dGBA1b−/− fly heads and were undetectable in the heads of dGBA1a,b−/− flies. Conversely, the levels of dGBA activity in dGBA1a−/− fly heads were not statistically different from that of controls, consistent with its predominant expression within the fly midgut (Fig. 1G).

Flies lacking dGBA displayed reduced lifespan and progressive age-dependent locomotor deficits
The lifespan of flies lacking dGBA1a (dGBA1a−/−), dGBA1b (dGBA1b−/−) or both dGBA1 genes (dGBA1a,b−/−) was assessed. dGBA1b−/− flies displayed reduced survival compared with control flies. However, dGBA1a−/− flies, which lack the dGBA isoform ex-
pressed mainly in the digestive system, showed a significantly increased survival compared with control flies (Fig. 2A), and this trend was seen using 4 additional dGBA1a null mutant fly lines (Fig. 2B). Interestingly, double-mutant dGBA1a,b/−/− flies displayed a lifespan that was similar to that of the single dGBA1b knock-out line (Fig. 2A), suggesting that the detrimental effects of dGBA1b deficiency on lifespan cannot be reversed by the prolongevity effects of dGBA1a knock-out. Given that GBA1 mutations are usually found in the heterozygous state in PD and other synucleinopathies, we also assessed dGBA1b and dGBA1a heterozygote knock-out flies (dGBA1b+/− and dGBA1a+/−) and found that the lifespans of these flies were not significantly different from those of controls (Fig. 2C).

In keeping with the lifespan phenotypes, dGBA1b+/− and dGBA1a,b+/− flies, which lack the dGBA isoform expressed in the brain, also displayed progressive age-dependent locomotor deficits, with the climbing ability of dGBA1b+/− and dGBA1a,b+/− flies in response to light tapping falling below that of controls at older ages (Fig. 2D). Knock-out of dGBA1a, despite its prolongevity effects, did not have any significant effect on climbing ability over time compared with control flies (Fig. 2E). Therefore, because loss of dGBA1a was not associated with any lifespan defect or change in locomotor ability, and has no expression in the adult brain, we concentrated on studying the dGBA1b+/− and dGBA1a,b+/− flies in the subsequent experiments.

We also assessed the fecundity of dGBA1b+/− and dGBA1a,b+/− female flies as a further measure of healthspan. The number of eggs laid per female significantly declined with age compared with age-matched control flies, and day 30 null mutant

Figure 2. Lack of dGBA activity led to reduced survival and locomotor deficits. A, dGBA1b−/− and dGBA1a,b−/− female flies had significantly reduced lifespan compared with w1118 control flies (p = 3 × 10−20 and p = 7 × 10−23). Furthermore, dGBA1a−/− flies displayed a significant lifespan extension compared with control flies (p = 8 × 10−25). B, This lifespan extension was consistent using a further 4 independent dGBA1a−/− lines (all p < 6 × 10−15, n = 150 flies per genotype). C, Flies heterozygous for dGBA1b or both dGBA1 genes (dGBA1b+/− and dGBA1a,b+/−) did not display a lifespan phenotype (p = 0.69 and p = 0.33). D, dGBA1b+/− and dGBA1a,b+/− flies showed an age-dependent reduction in climbing ability compared with w1118 control flies (p = 0.0001 and p = 0.0004; n = 45 flies per genotype, 3 repeats each). E, The climbing ability of dGBA1a−/− flies was indistinguishable from that of controls (p = 0.38). F, The fecundity of dGBA1b+/− and dGBA1a,b+/− flies was significantly reduced at day 20 compared with age-matched control flies. *** p = 3 × 10−8. *** p = 2 × 10−5. They were almost completely infertile at day 30, whereas >20% of the control flies were still able to lay eggs.
Figure 3. Loss of dGBA activity resulted in lipid defects and substrate accumulation in fly brains. **A**, Mass spectroscopic analysis revealed a ~7-fold increase in accumulation of the dGBA substrate, glucosylceramide, in the heads (n = 70) of 25-d-old dGBA1b−/− compared with age-matched w1118 control flies. **p = 0.01. The isoforms that were predominantly elevated were C16:0, C18:0, and C20:0. **B**, Similar results were seen in dGBA1a,b−/− fly heads (n = 70 heads per sample, 3 technical repeats). ***p = 0.0008. **C**, There was no age-dependent decrease in hexosaminidase (HEX) activity or (D) β-galactosidase (β-GAL) activity in dGBA1b−/− fly heads. There was a significant increase in β-GAL activity in older 25-d-old dGBA1b−/− fly heads compared with age-matched w1118 control flies. ***p = 2 × 10−3. Data are the mean ± SEM of three independent experiments. **E**, The 25-d-old dGBA1b−/− and dGBA1a,b−/− flies had reduced whole-body levels of TAG compared with age-matched controls. *p = 0.046 (paired t test). ***p = 0.00082 (paired t test). **F**, Day 15 dGBA1b−/− and dGBA1a,b−/− flies were more sensitive to starvation conditions than age-matched w1118 control flies (p = 3 × 10−20 and p = 5 × 10−11; n = 150 flies per genotype). **G**, Day 15 dGBA1b−/− flies had reduced feeding activity as assessed by the proboscis-extension assay (n = 55 flies per genotype). *p = 0.040.
dGBA1b and the double-mutant dGBA1a,b−/− flies were almost completely infertile (Fig. 2F).

Knock-out of dGBA resulted in changes in lipid metabolism and accumulation of glucosylceramide

We next assessed whether knock-out of dGBA activity in the fly is associated with accumulation of its substrate, glucosylceramide. Indeed, using lipid mass spectrometry, we found that dGBA1b−/− and dGBA1a,b−/− flies displayed significant accumulation of glucosylceramide in their heads, whereas the heads of w1118 control flies contained very low glucosylceramide (Fig. 3A, B). Furthermore, the predominant glucosylceramide isomers produced were C:16, C:18, and C:20 fatty chains (Fig. 3A, B). To determine whether loss of dGBA caused any change in the activity of other lysosomal enzymes, we also assessed hexosaminidase (Fig. 3C) and β-galactosidase (Fig. 3D). There was no age-dependent decrease in the activity of these two enzymes in the heads of dGBA1b−/− flies compared with controls. Indeed, the activity of β-galactosidase in day 25 dGBA1b−/− fly heads was significantly higher than in the controls (Fig. 3D).

In addition to specific lipid defects caused by the loss of dGBA we also looked at more general lipid abnormalities. As mentioned above, dGBA1 is expressed in both the midgut and the fat body of the fly, an organ analog of vertebrate adipose tissue and liver that acts as a major organ of energy metabolism and nutrient storage (Tatar et al., 2014). We therefore assessed whether loss of dGBA in these tissues might result in general defects in lipid metabolism. TAG is the main form of lipid storage in the fly and therefore reflects the ability of flies to respond to starvation conditions (Ballard et al., 2008). Measurement of whole-body TAG levels in day 25 female flies revealed a significant reduction in dGBA knock-out flies compared with age-matched controls (Fig. 3E). In particular, there was a greater reduction in TAG levels in double-mutant dGBA1a,b−/− flies compared with the single-mutant dGBA1b−/− flies (Fig. 3E). The physiological relevance of the decrease in TAG levels was confirmed in both dGBA1b−/− and, to a greater extent, in dGBA1a,b−/− flies, which were more sensitive to starvation conditions compared with controls (Fig. 3F). Furthermore, assessment of feeding behavior by measuring proboscis-extension (Wong et al., 2009) demonstrated that dGBA1b−/− flies had significantly reduced feeding activity compared with controls (Fig. 3G). This suggests that reduced food intake may contribute to the reduced TAG levels and hypersensitivity to starvation.

Loss of dGBA activity led to lysosomal-autophagic deficits

To determine whether the accumulation of the dGBA substrate corresponds with abnormal lysosomal pathology, which is characteristic of GD, we stained the brains of dGBA1b−/− and dGBA1a,b−/− flies with LysoTracker and visualized the lysosomes with confocal microscopy. Aged dGBA1b−/− and dGBA1a,b−/− flies displayed numerous enlarged and abnormal lysosomes in their brains, which were not seen in age-matched control brains (Fig. 4A, B). Given that normal lysosomes are required to fuse with autophagosomes to form autophagolysosomes in the process of autophagy (Rubinsztein et al., 2012), we next studied autophagy. During the elongation phase, the cytosolic form of protein microtubule-associated protein 1 light chain 3 (LC3), LC3-I, is conjugated to phosphatidylethanolamine to form LC3-II, which is specifically targeted to the autophagosomal membrane, where it remains until fusion with lysosomes. LC3 is therefore considered a good marker for studying autophagy (Ravikumar et al., 2010). We therefore measured the accumulation of Atg8, the fly ortholog of LC3, in both the heads of dGBA1b−/− and headless bodies of dGBA1a,b−/− flies, respectively (Fig. 5A, B). Western blot analysis demonstrated that Atg8-I and Atg8-II accumulated in the heads of flies lacking dGBA1b−/− (Fig. 5A). Atg8-II also accumulated in the headless bodies of flies lacking both dGBA1 genes (dGBA1a,b−/−) (Fig. 5B) compared with controls. Increased steady-state levels of Atg8-II suggest increased autophagosome number, but the marked increase in Atg8-I also could indicate a stall in flux activity in the brains of dGBA1b−/− flies (Fig. 5A). Analysis of the Atg8-II/Atg8-I ratio also confirmed increased autophagosome formation and autophagy block in the dGBA knock-out fly heads and bodies (Fig. 5A, B). Furthermore, Atg8 levels in homogenates of heads from control and dGBA1b−/− mutant flies demonstrated that this tissue was unresponsive to nutrient deprivation (Fig. 5A), unlike headless bodies, in which a brief 48 h period of prior starvation slightly increased Atg8-II levels, suggestive of autophagy induction (Fig. 5B).

p62 and polyubiquitinated proteins accumulated in dGBA-deficient fly brains

To study the downstream effects of autophagic dysfunction, we next performed immunostaining on the brains of dGBA-deficient flies with antibodies against ubiquitin and p62, markers of lysosomal-autophagic degradation (Bartlett et al., 2011). Under normal physiological conditions, p62 can link ubiquitinated proteins to the autophagy machinery (e.g., Atg8-II), targeting them for degradation (Rogov et al., 2014). Moreover, both ubiquitin and p62 accumulate when autophagy is inhibited (Bartlett et al., 2011) and are abundant constituents of protein inclusions associated with neurological diseases, including LBs in PD (Zatiloukal et al., 2002; Bartlett et al., 2011). We first dissected Drosophila brains and visualized these proteins using antibodies against polyubiquitinated proteins and the fly ortholog of p62, Ref(2)P. Aged dGBA1b−/− brains displayed elevated levels of both ubiquitinated proteins and p62/Ref(2)P-containing aggregates under the confocal microscope, which were not seen in age-matched control fly brains (Fig. 6A). We also confirmed elevated levels of polyubiquitinated proteins in dGBA1b−/− fly heads using Western blot analysis (Fig. 6B). Thus, dGBA knock-out led to lysosomal-autophagic defects and the accumulation of ubiquitin and p62/Ref(2)P-containing deposits in the fly brain.

Knock-out of dGBA in the fly brain led to synaptic loss and neurodegeneration

To study the effect of dGBA deficiency on synaptic viability, we performed immunostaining for Bruchpilot, an essential component of synaptic active zones in Drosophila (Wagh et al., 2006). This revealed significantly reduced Bruchpilot in the brains of dGBA1b−/− flies compared with controls, suggesting synaptic loss (Fig. 7A).

We also performed an ultrastructural examination of dGBA1b−/− fly brains using transmission electron microscopy. This demonstrated grossly abnormal morphology of the ommatidia, the units of the compound eye, in dGBA1b−/− flies compared with controls (Fig. 7B). Quantitative analysis showed that the average rhabdomere area within each ommatidium was significantly reduced in the eyes of dGBA1b−/− flies (Fig. 7B).
Knock-out of dGBA in the brain was associated with mitochondrial abnormalities and hypersensitivity to oxidative stress

Ultrastructural examination of the brains of flies lacking dGBA using electron microscopy revealed giant mitochondria in day 25 dGBA1b−/− fly brains compared with age-matched controls (Fig. 8A) or younger day 5 dGBA1b−/− brains (data not shown), with an ~25% increase in the average mitochondrial area (Fig. 8B). The presence of giant mitochondria, in addition to the increase in autophagy substrates and increased Atg8-II, further supported the conclusion that there was impaired autophagic flux.

In keeping with our ultrastructural data, mitochondrial dysfunction has been shown in a mouse model of GBA deficiency (Osellame et al., 2013) and GD human fibroblasts (de la Mata et al., 2015). To assess mitochondrial function, we measured ATP levels and found that they were reduced by ~40% in the brains of dGBA1b−/− flies as well as in the whole bodies of dGBA1b−/− and dGBA1a,b−/− flies compared with controls (Fig. 8C,D). Mitochondrial dysfunction can lead to decreased resistance to reactive oxygen species, which has been implicated in many neurodegenerative diseases (Kinghorn et al., 2015). To explore the role of dGBA in resistance to oxidative stress, we examined the survival of dGBA-deficient flies after exposure to the potent oxidizer hydrogen peroxide (H₂O₂) and paraquat, a free radical generator. Consistent with the mitochondrial abnormalities in aged flies, day 15 dGBA1b−/− and dGBA1a,b−/− flies showed increased sensitivity to H₂O₂ and paraquat stress (Fig. 8E,F).

Targeting downstream autophagy defects with the mTOR inhibitor rapamycin

The nutrient-sensing mTOR pathway regulates lysosomal biosynthesis and autophagy (Ravikumar et al., 2010). We hence assessed the activity of mTOR by measuring the phosphorylation level of S6K, which acts downstream of mTOR complex 1 (mTORC1) (Castillo-Quan et al., 2015). Western blot analysis revealed a decrease in S6K phosphorylation in the heads of dGBA1b−/− flies compared with controls, indicating downregulation of mTORC1 (Fig. 9A), which would be predicted to lead to an increase in autophagy. We also tested whether rapamycin, a known mTORC1 inhibitor (Bjedov et al., 2010), could further downregulate mTOR in dGBA-deficient flies. Treatment of control flies with 200 μM rapamycin resulted in a ~50% decrease in S6K phosphorylation. There was a trend for rapamycin treatment of dGBA1b−/− flies to further decrease S6K phosphorylation, although this was not statistically significant. We therefore tested the effect of rapamycin on the lifespan of dGBA1b−/− flies. Interestingly, we found that rapamycin supplementation to the fly medium (both 200 and 400 μM) from 2 d of age was able to partially ameliorate the lifespan defect in dGBA1b−/− flies (Fig. 9B). Consistent with previous work (Bjedov et al., 2010), rapa-
mycin also increased the lifespan of control flies (Fig. 9B). In keeping with the effect on lifespan, 400 μM rapamycin, but not 200 μM (data not shown), was also able to partially rescue the locomotor deficits in aged dGBA1b−/− flies (Fig. 9C). An even greater rescue was seen in flies lacking both dGBA1 genes (dGBA1a,b−/−). This effect of rapamycin appeared to be specific to dGBA knock-out flies, as rapamycin did not have a significant effect on the climbing ability of age-matched control flies (Fig. 9C). We also assessed the effect of rapamycin on the response to oxidative stress. Treatment with 200 μM rapamycin from 2 to 15 d of age subsequently prolonged the survival of dGBA1b−/− flies on food containing H2O2 (Fig. 9D). This effect was also specific to dGBA-deficient flies, as surprisingly rapamycin-treated control flies had reduced lifespan on exposure to H2O2 (Fig. 9D).

Rapamycin treatment is also associated with an increased resistance to starvation in wild-type flies (Bjedov et al., 2010). We therefore treated dGBA1b−/− flies with rapamycin for the

**Figure 5.** Flies lacking dGBA displayed a block in autophagy. A, Western blot analysis with an antibody against the fly LC3 ortholog, Atg8, demonstrated increased levels in dGBA1b−/− fly heads compared with age-matched w1118 control flies. Both Atg8-I and Atg8-II were increased compared with controls, and the ratio of Atg8-II/Atg8-I in dGBA1b−/− flies indicated increased autophagosome formation (n = 3 experiments). *p < 0.05. B, Western blot analysis of Atg8 levels in the headless bodies of dGBA1a,b−/− flies demonstrated no significant change in Atg8-I levels, with an increase in Atg8-II levels and an increased Atg8-II/Atg8-I ratio. This effect was more pronounced after starvation in dGBA1a,b−/− flies (n = 3 experiments). *p < 0.05.
first 15 d of adulthood before exposing them to starvation conditions. The 200 μM rapamycin was able to rescue the survival of dGBA1b−/− flies and control flies under starvation conditions (Fig. 9E). These findings suggest that, despite the autophagy block in flies lacking dGBA, rapamycin is still able to exert protective effects on lifespan, locomotor defects, and oxidative and starvation stress responses, likely by exerting a small but functionally significant increase in mTOR inhibition.

Loss of dGBA function in the brain resulted in an upregulation of Mitf gene expression and its downstream target gene Atg8a

To further study the effects of dGBA deficiency and rapamycin treatment on autophagy, we measured the expression levels of Mitf and its downstream target Atg8a. Mitf is the fly ortholog of the mammalian transcription factor EB (TFEB) gene, the master regulator of lysosomal biogenesis and autophagy (Bouché et al., 2016; Tognon et al., 2016). TFEB is critical for the autophagic cellular clearance of abnormal proteins and organelles, as well as lipid metabolism. TFEB acts by coordinating the expression of a number of genes belonging to the Coordinated Lysosomal Expression and Regulation (CLEAR) network, by directing binding to specific CLEAR-box sequences located near their promoters. TFEB is usually cytosolic where it interacts with mTORC1 at the surface of the lysosome (Rocznik-Ferguson et al., 2012), but during starvation it translocates to the nucleus where it induces target gene transcription. Studies have shown that Mitf has conserved regulatory mechanisms with mammalian TFEB, translocating to the nucleus when Drosophila mTORC1 is inhibited (Bouché et al., 2016).

We first measured Mitf gene expression in the heads of dGBA1b−/− flies. qRT-PCR data analysis showed that, in response to the loss of dGBA activity in the brain, there is a highly significant upregulation of Mitf gene expression (Fig. 10A). We next studied the levels of the Atg8a gene, the fly ortholog of the mammalian GABARAPL1 gene, which acts downstream of Mitf. This gene is involved in the main steps of autophagosome formation and maturation. In keeping with the upregulation of Mitf in dGBA1b−/− fly heads, Atg8a was also upregulated in response to dGBA knockdown in the fly brain (Fig. 10B).

We also studied the effect on Mitf and Atg8a gene expression of treating dGBA-deficient and control flies with 200 μM rapamycin from day 2 (Fig. 10A,B). Interestingly, we did not see any increase in Mitf gene expression in dGBA1b−/− flies treated with rapamycin compared with untreated flies (Fig. 10A). Moreover, the levels of Atg8a in rapamycin-treated dGBA1b−/− flies were significantly downregulated compared with untreated flies (Fig. 10B). Rapamycin significantly increased the expression levels of Mitf in control flies (Fig. 10A); and although there was a trend
toward increasing Atg8a levels, it was not statistically significant (Fig. 10B). Thus, loss of dGBA activity in the brain is associated with increased expression levels of Mitf, and its downstream target gene, Atg8a, but upregulation of Mitf expression is not responsible for the protective effect of rapamycin on lifespan, locomotor, and oxidative stress phenotypes.

Discussion

We generated a Drosophila model of neuronopathic GD. Simple genetics could not be used to precisely excise both Drosophila GBA1 genes, dGBA1a and dGBA1b, due to the presence of the CG31413 gene between them. Therefore, we used serial homologous recombination to knock out both dGBA1 genes separately or together in the fly. Of the two fly GBA orthologs, only dGBA1b is expressed in the fly brain (Flyatlas). dGBA1b−/− fly heads, lacking dGBA1b expression, displayed accumulation of the dGBA substrate glucosylceramide. Furthermore, the glucosylceramide isoforms (C16:0, C18:0, C22:0) were similar to those found in human spleen tissue (with the addition of hydroxylated or longer chain isoforms including C18:0-OH, C20:0-OH, C24:1, and C24:0 in the human tissue (N. Sebire and S. Paine, unpublished data). Moreover, this is consistent with data showing accumulation of glucosylceramide and glucosylsphingosine in Types II and III GD patient brains (Orvisky et al., 2002).

Abnormally engorged lysosomes in dGBA1b−/− fly brains were visualized with LysoTracker, confirming the validity of this neuronopathic GD fly model. Lysosomal integrity is necessary for normal functioning of the autophagy degradation machinery (Xu and Ren, 2015), and accordingly we observed autophagy dysfunction in dGBA1b−/− brains, with accumulation of the autophagy lysosomal protein Atg8/LC3. Thus, we demonstrate autophagy defects at the level of the autophagic machinery in vivo in a GD Drosophila model, following on from in vitro studies in GD macrophages and iPSCs showing impaired autophagy (Awad et al., 2015; Aflaki et al., 2016; Fernandes et al., 2016).

Neuronal-specific autophagy deficits, through loss of the mouse autophagy genes atg7 or atg5, result in accumulation of polyubiquitinated proteins, behavioral defects, and reduced longevity (Hara et al., 2006; Komatsu et al., 2006). Consistent with this, we found that dGBA1b−/− flies displayed reduced survival and locomotor ability and brain accumulation of p62/Ref(2)P and polyubiquitinated proteins. Both p62 and ubiquitin accumulate in neurodegenerative diseases, such as Alzheimer’s disease and PD (Zatloukal et al., 2002; Bartlett et al., 2011), and multiple pathogenic proteins, including p62 and β-amyloid, have been identified in neuronopathic GD mouse brains (Xu et al., 2014).

Surprisingly, knock-out of dGBA1a, which is predominantly expressed in the digestive system, significantly increased survival. This finding parallels recent research in Drosophila and Caenorhabditis elegans demonstrating that the intestine is an important target organ for mediating lifespan extension at the organismal
level, with single-gene manipulations specifically in the gut prolonging lifespan (Rera et al., 2011; Tatar et al., 2014). The role of dGBA in the gut in regulating lifespan was beyond the scope of this study but is an area that warrants further investigation. We demonstrated, however, that the lifespan extension seen in dGBA1a/H11002/H11002 flies is not associated with any improvement in locomotor ability.

Mitochondrial integrity is necessary for normal autophagy-lysosomal system functioning (Palikaras and Tavernarakis, 2012; Ashrafi and Schwarz, 2013). Indeed, mitochondrial dysfunction occurs in GD mouse models (Osellame et al., 2013; Xu et al., 2014), likely due to defects in the autophagic removal of damaged mitochondria (Osellame et al., 2013). In keeping with this, the deficiency of the Drosophila autophagy gene atg7 is associated with sensitivity to oxidative stressors (Juhász et al., 2007). Accordingly, we demonstrated mitochondrial abnormalities in dGBA1b/−/− flies, with giant mitochondria, reduced ATP and hypersensitivity to oxidative stress. Enlarged mitochondria and decreased ATP occur in neuronopathic GD mice (Xu et al., 2014), and giant mitochondria are directly linked to Drosophila autophagy defects (Bouche et al., 2016). Therefore, the accumulation of autophagic substrates and giant mitochondria in dGBA1b/−/− flies is consistent with autophagy block (Fig. 10C).
As previously demonstrated, rapamycin also extended the lifespan of dGBA-deficient flies (Kinghorn et al., 2016). Furthermore, we demonstrated that the neuropathological features of dGBA-deficient flies (rapamycin was also able to increase survival of control flies in response to subsequent exposure to starvation conditions from 15 d of age. *p = 0.023. The bar chart represents survival after 8 d of exposure to starvation.

Figure 9. The mTOR inhibitor rapamycin partially ameliorated the lifespan, locomotor, oxidative stress, and starvation phenotypes in dGBA-deficient flies. A, Day 7 dGBA1b−−/− fly heads had significantly decreased phosphorylated S6K (p-S6K) levels compared with age-matched control flies. *p = 0.0414. Treatment of control flies with 200 μM rapamycin led to a reduction in phospho-S6K levels. "**p = 0.0009. Although there was a trend for rapamycin to further decrease phospho-S6K levels in dGBA1b−−/− flies, this was not statistically significant (p = 0.327, one-way ANOVA with Bonferroni correction). B, The 200 and 400 μM of rapamycin significantly extended the lifespan of dGBA1b−−/− flies (p = 0.005 and p < 0.05; n = 110–160 flies per genotype and condition). As previously demonstrated, rapamycin also extended the lifespan of w1118 control flies (p = 0.007 and p = 0.02). C, Treatment with 400 μM rapamycin partially rescued the age-related climbing defects in day 35 dGBA1b−−/− flies and dGBA1ab−−/− flies ("**p = 0.0005 and "***p = 0.0006), but did not have a significant effect on the climbing ability of age-matched control flies (p = 0.23; n = 45 flies per genotype and condition, average of 3 repeats). D, Treatment of dGBA1b−−/− flies from 2 to 15 d of age with 200 μM rapamycin partially rescued the survival on subsequent exposure to food containing 5% H2O2 (p = 2.5 × 10−7). This protective effect was specific to dGBA-deficient flies as 200 μM rapamycin was toxic to control flies under similar conditions (p = 0.0007; n = 60 flies per group). E, The 200 μM rapamycin was also able to increase survival of dGBA1b−−/− flies in response to subsequent exposure to starvation conditions from 15 d of age. *p = 0.004. The time point shown is after 3 d of starvation stress (n = 60 flies per condition). Under similar conditions, 200 μM rapamycin also rescued the survival of control w1118 flies (n = 60 flies per group). *p = 0.023. The bar chart represents survival after 8 d of exposure to starvation.

Furthermore, we demonstrated that the neuropathological defects in dGBA1b−−/− flies occur independently of α-synuclein, confirming that DGBA deficiency is sufficient to cause neurodegeneration (Fig. 10C). A recent study using a deletion mutant harboring imprecise removal of the dGBA1b gene, as well as the incomplete removal of the dGBA1a gene and excision of the intergenic CG31413 gene, provides further validation of our model for studying GBA1-associated neurodegeneration. Loss of 60% of GCase activity was also associated with reduced survival and climbing ability in addition to accumulation of autophagy sub-
strates (Davis et al., 2016). GCase deficiency was also shown to enhance H9251-synuclein pathology in the fly (Suzuki et al., 2015; Davis et al., 2016). Our model has the advantage over existing models, as it allows the precise and complete knock-out of each of the dGBA genes, and the dissection of the role of dGBA in different tissues. The ability to knock out only brain-specific dGBA1b is particularly relevant given our finding that loss of dGBA in the gut causes prolongevity effects. Our model also avoids the production of truncated, possibly pathogenic, variants of dGBA.

The nutrient-sensing mTOR is localized to the lysosomal surface (Efeyan et al., 2012; Betz and Hall, 2013), where it modulates autophagy (Yu et al., 2010; Zoncu et al., 2011). It has thus been speculated that abnormal lysosomal function leads to activation of autophagy by mTOR downregulation (Li et al., 2013). Indeed, a recent study reported increased expression of mTOR signaling pathway genes in neuronopathic GD mice (Dasgupta et al., 2015). In view of the autophagy defect in dGBA1b−/− flies, we probed mTOR signaling by analyzing S6K phosphorylation downstream of mTORC1. Interestingly, we detected a decrease in S6K phosphorylation and hence downregulation of mTORC1 compared with controls. Furthermore, although not statistically significant, there was a trend for mTOR to be further downregulated in dGBA1b−/− flies treated with rapamycin, a known mTORC1 inhibitor. Because rapamycin has protective effects in animal

Figure 10. Knock-out of dGBA activity in the fly brain was associated with upregulation of Mitf and its downstream target Atg8a. A, qRT-PCR data analysis revealed that day 15 dGBA1b−/− fly heads had increased expression levels of Mitf compared with controls. ***p = 9.7 × 10−5. In addition, treatment with 200 μM rapamycin from 2 d of age had no significant effect on Mitf gene expression in dGBA1b−/− flies (p = 0.32). However, rapamycin treatment led to a significant increase in the expression levels of Mitf in control flies. (p = 0.016). B, Atg8a gene expression levels were greater in dGBA1b−/− fly heads compared with controls. **p = 2.8 × 10−7. Treatment with 200 μM rapamycin resulted in a decrease in Atg8a gene levels in dGBA1b−/− fly heads (**p = 0.002); and although there was a trend toward increased Atg8a gene expression in control flies with rapamycin treatment, it did not reach statistical significance (p = 0.054). The mRNA expression levels of all genes are shown relative to the expression levels of a control gene, RP49. C, Schematic diagram showing the downstream effects of impaired autophagy flux in dGBA-deficient flies.
models of PD and other neurodegenerative diseases (Malagelada et al., 2010; Cheng et al., 2015), we treated dGBA1b−/− flies with rapamycin and found that, despite the autophagy block, it was able to partially ameliorate many neurotoxic phenotypes. Rapamycin treatment led to an improvement in the lifespan and climbing phenotypes, in addition to the hypersensitivity to oxidative and starvation stressors seen in dGBA1b−/− flies. These results are somewhat surprising given that rapamycin treatment of iPSC-derived neuronal cells from GD patients caused cell death (Awad et al., 2015), demonstrating that drug effects in vitro vary to those at the organismal level. Furthermore, although rapamycin increases the lifespan and protects against starvation conditions in control flies (Bjedov et al., 2010), the rescue of age-related climbing defects and oxidative stress appeared to be specific to dGBA-deficient flies. Indeed, rapamycin had no effect on the climbing ability of control flies, and rapamycin-treated control flies were more sensitive to H2O2 than untreated flies. The reason for the opposing effects of rapamycin on the oxidative stress responses of dGBA1b−/− and control flies is unclear, but may be related to the differential activation of oxidative stress pathways between dGBA1b−/− and healthy controls.

Lastly, we demonstrated that knock-out of dGBA in the brain results in an upregulation of Mitf and its downstream target, Atg8a, suggesting a compensatory response to the autophagy block. This is in contrast to findings in a recent study showing downregulation of TFEB expression in GD iPSC-derived cells (Awad et al., 2015). The reasons for these differing results is not clear, but possible explanations may relate to variations in autophagy block between models, and the fact that neuronal cells are newly differentiated and may reflect early disease compared with adult flies. Mitf gene expression may also vary in the presence of mutant dGBA. Our fly model displays very little dGBA expression compared with iPSC cells, which are derived from mutant carriers, and therefore, despite showing very low levels of GCase activity, express mutant GBA. Mutant GCase is known to exert gain-of-function toxic effects, including in Drosophila, with upregulation of the unfolded protein response (Maor et al., 2013, 2016). Knockdown of Mitf in Drosophila leads to similar phenotypes to those in dGBA1b−/− flies, including autophagy substrate accumulation, upregulation of Atg8-II, giant mitochondria, as well as enlarged lysosomes (Bouché et al., 2016). Thus, the autophagy defects seen in dGBA deficiency appear to phenocopy Mitf downregulation in the fly. This suggests that upregulating Mitf as a potential therapeutic strategy in GD and GBA-linked PD may be limited in its efficacy due to the inability of GBA-deficient neuronal tissue to stimulate autophagy. Further studies are warranted to explore the complex interaction between mTORC1 and TFEB/Mitf in GD.

Rapamycin treatment of dGBA1b−/− flies did not upregulate Mitf gene expression, suggesting that rapamycin does not act through Mitf signaling to rescue the neurotoxic phenotypes of dGBA-deficient flies. We hypothesize, therefore, that mTOR is downregulated in dGBA-deficient flies as a compensatory response to lysosomal-autophagy block. Possibly as a consequence of lysosomal dysfunction in dGBA-deficient flies, the normal interaction between mTORC1 and TFEB/Mitf at the surface of the lysosome is disrupted, leading to changes in TFEB/Mitf gene expression. Therefore, by exerting small additional effects on mTORC1 signaling, rapamycin may mediate its beneficial effects (Fig. 10C). Furthermore, Mitf also plays a critical role in lipid metabolism and recapitulates the function of TFEB in mammals in this regard (Bouché et al., 2016). Mitf expression may therefore contribute to the reduced TAG that we see in dGBA1b−/− flies.

In conclusion, the autophagy defects seen in our GD flies, likely as a result of failure of the fusion of autophagosomes and lysosomes, may also be relevant to PD linked to GBA1 mutations. Our results raise the possibility that therapies aimed at ameliorating not only lysosomal dysfunction, but also at autophagic abnormalities, including lowering mTOR activity, may be effective in treating GBA1-associated disease. They also suggest that rapamycin may offer significant health benefits in neurodegenerative GD and in GBA1-related synucleinopathies. Together, our data demonstrate that these Drosophila models of dGBA-deficiency are a useful platform to further study the downstream effects of lysosomal-autophagic dysfunction and to identify genetic modifiers and new therapeutic targets in neurodegenerative GD and GBA1-associated PD.

References


de la Mata M, Cotán D, Oropesa-Avila M, Garrido-Maraver J, Cordero MD,