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Alzheimer’s disease risk gene *Wwox* controls amyloid pathology through metabolic reprogramming

Short title: *Wwox* links metabolism and amyloid

Hannah J Lucas-Clarke^{1,2,†}, Daniel C Maddison^{1,2,†}, Leonardo Amadio^{1,2,3}, Edgar Buhl⁴, Clement Regnault⁵, Owen Peters^{2,3}, James J L Hodge⁴, Gaynor A Smith^{1,2*}

1. Division of Psychological Medicine and Clinical Neurosciences, School of Medicine, Cardiff University, Cardiff CF24 4HQ, UK
2. UK Dementia Research Institute at Cardiff University, Cardiff CF24 4HQ, UK
3. School of Biosciences, Cardiff University, Cardiff CF10 3AX, UK
4. School of Physiology, Pharmacology and Neuroscience, University of Bristol, Bristol BS8 1QU. UK
5. College of Medical Veterinary Life Sciences Shared Research Facilities University of Glasgow, Glasgow G12 8QQ, UK.

[†] Hannah J Lucas-Clarke and Daniel C Maddison contributed equally to this work.

*Correspondence to: Gaynor Smith

Hadyn Ellis Building
Cardiff University
Maindy Road
Cardiff
Wales, UK, CF24 4HQ
Smithga@cf.ac.uk

Abstract

Genome-wide association studies have identified multiple loci that contribute to late-onset Alzheimer's disease risk, yet the functional mechanisms of many candidate genes remain unclear. WW-domain containing oxidoreductase (*WWOX*) is one such gene associated with disease and downregulated in patient brains, but its role in neurodegeneration is poorly understood.

Here, we use *Drosophila* models to investigate the cell-type-specific and mechanistic roles of *Wwox* in ageing and amyloid- β_{42} ($A\beta_{42}$)-induced pathology. Neuronal and glial knockdown of *Wwox* reduced lifespan and increased sleep drive, indicating a role in brain homeostasis. In an $A\beta_{42}$ transgenic model, neuronal *Wwox* knockdown exacerbated toxicity, shortening lifespan, impairing locomotion, and increasing soluble $A\beta_{42}$ levels.

Transcriptomic and metabolomic analyses revealed that *Wwox* knockdown in an $A\beta_{42}$ transgenic model promotes metabolic reprogramming, characterised by elevated lactate dehydrogenase (*Ldh*) expression and increased lactate levels, driven by activation of the stress-responsive transcription factor Atf4. Genetic reduction of *Ldh* rescued locomotor deficits, identifying dysregulated pyruvate metabolism as a key mediator of *Wwox*-dependent neurotoxicity.

Conversely, overexpression of *Wwox* in neurons markedly reduced amyloid burden, improved survival and locomotor function, and reversed $A\beta_{42}$ -induced transcriptional changes. Both $A\beta_{42}$ and the protective effects of *Wwox* were associated with altered amino acid metabolism, including changes in the cysteine and L-methionine pathway.

Together, these findings identify *WWOX* as a regulator of neuronal metabolic state, linking genetic risk to amyloid pathology via metabolic reprogramming, and highlight *WWOX* activation as a potential therapeutic strategy in Alzheimer's disease.

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Key words: Alzheimer’s disease; WWOX; risk genes; amyloid; *Drosophila*

Introduction

Alzheimer’s disease (AD) is the most common form of dementia, characterised by pronounced neurodegeneration, causing memory impairment, cognitive decline, and sleep disruption. On a cellular and molecular level, these symptoms follow the accumulation of extracellular amyloid β ($A\beta$) plaques, hyperphosphorylated tau pathology and reactivity of astrocytes and microglia. AD manifests in two forms, early- (EOAD) and late-onset (LOAD). Whilst EOAD can be caused by monogenic mutations in amyloid precursor protein (*APP*), presenilin-1 (*PSEN1*) or presenilin-2 (*PSEN2*), LOAD is considered sporadic and a result of the convergence of environmental and genetic risk factors. Single nucleotide polymorphisms (SNPs) at approximately 90 genomic loci have been discovered to contribute to LOAD risk.¹ Determining how genes associated with these SNPs contribute to disease onset and progression is vital to our understanding of LOAD and how it may be prevented or treated.

An intergenic SNP located on chromosome 16 between WW domain-containing oxidoreductase (*WWOX*) and MAF bZIP transcription factor (*MAF*) has been associated with an increased the risk of developing LOAD.² Genome-wide expression quantitative trait locus (eQTL) mapping between LOAD patients and controls identified *WWOX* as a significant eQTL in the brain with a negative β value, indicating a decrease in *WWOX* mRNA in individuals with the associated SNP.³ A genome-wide significant association has also been made between a SNP within a predicted *WWOX* enhancer region and a key disease biomarker, $A\beta_{42}/A\beta_{40}$ ratio and decreased *WWOX* has been observed in post-mortem hippocampal tissue of AD patients compared to controls.^{4,5} Interestingly, *WWOX* has been implicated in a protein aggregation

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3 cascade and may influence the hyperphosphorylation of tau.^{6–8} However, the mechanisms
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5 underlying *WWOX* modulation of AD pathology are not well understood.
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11 *WWOX* is an oxidoreductase and tumour suppressor that plays a significant role in cancer cell
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13 metabolism, influencing glycolysis, mitochondrial integrity, signalling and oxidative stress.^{9–13}
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15 It contains two N-terminal WW domains, characterised by highly conserved adjacent
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17 tryptophan residues, binding to proline-rich regions in protein ligands and is thus responsible
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19 for the mediation of interactions between *WWOX* and multiple protein partners.¹⁴ In
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21 addition, *WWOX* loss of function has been postulated to mediate a Warburg-like effect, a
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23 switch to glycolysis despite aerobic conditions, via modulation of HIF1 α , a transcription factor
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25 with a variety of metabolic targets including glucose transporter 1 (*GLUT1*) and lactate
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27 dehydrogenase (*LDH*).^{9,15,16} A similar metabolic shift has been observed in *Drosophila* and
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29 human stem cell-derived neuronal models of AD,^{17,18} but whether this is influenced by
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31 *WWOX*, in the context of AD, has not yet been explored. Thus far *WWOX* has only been linked
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33 to glycolysis in fibroblasts, cancer cells and peripheral metabolic tissues.^{9–13}
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43 We sought to address this question by altering *Wwox* levels in the *Drosophila melanogaster*
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45 brain, via neuronal or glial-specific RNA interference (RNAi) and CRISPR activation (CRISPRa).
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47 We explored the age associated effects on AD-relevant phenotypes with or without the
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49 overexpression of the human A β ₄₂ peptide.¹⁹ We show that both neuronal and glial
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51 knockdown of *Wwox* shortened lifespan and increased sleep, in the absence of A β ₄₂.
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53 However, in the presence of A β ₄₂, *Wwox* knockdown exacerbates lifespan and locomotor
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55 deficiencies in a neuron-specific manner, whilst also increasing amyloid load. Transcriptomic
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57 and metabolomic analyses identified that elevated *Ldh*, as a consequence of A β ₄₂
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3 overexpression, was exacerbated by *Wwox* knockdown and was accompanied by increased
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5 levels of lactate and the integrated stress response (ISR)/unfolded protein response (UPR)
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7 regulator Atf4. Strikingly, upregulation of *Wwox* in neurons significantly reduced amyloid
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9 load, rescuing lifespan and locomotor defects but did not alter lactate levels, instead altering
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11 L-methionine and reversing the transcriptional dysregulation seen in the A β ₄₂ model. Taken
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13 together, these findings provide insight into the mechanisms underlying LOAD risk conferred
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15 by genetic variation at the *WWOX* locus and identify *WWOX* activation as a strategy for
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17 reducing A β ₄₂ pathology.
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26 **Materials and Methods**

27 **Fly husbandry**

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29 *Drosophila* stocks (Supplementary Table 1A) were housed at room temperature and
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31 transferred to fresh standard ‘Cornmeal-Molasses-Yeast’ media, set with agar, once every 3
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33 weeks. Crosses and experimental lines (reported in Supplementary Table 1B for each
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35 experiment) were housed at 25 °C on a 12:12 hour light-dark schedule.
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44 **Evaluation of *Wwox* expression in a single cell atlas of the ageing *Drosophila* brain**

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46 *Wwox* expression was assessed using a single cell transcriptomic dataset of the ageing
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48 *Drosophila* brain available on SCOPE.²⁰ We calculated the percentage of cells within
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50 annotated clusters that expressed detectable levels of *Wwox*.
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Lifespan

Newly eclosed females (Supplementary Table 1B) were allowed to mate for < 48 hours, then were separated into vials of $n < 11$ and passed to fresh food two to three times a week, whilst scoring deaths. Lifespan and other phenotypic analyses were not directly compared to males because of the hyper X activation effects.²¹ Alive flies that did not successfully transfer into a new vial were included as censored flies in the analysis. Kaplan-Meier plots were generated using GraphPad Prism v.10.

Rapid Iterative Negative Geotaxis (RING)

Newly eclosed females (Supplementary Table 1B) were allowed to mate for < 48 hours and then divided into vials of $n < 11$. Progeny were flipped onto fresh food two to three times a week and assayed at 7-, 14-, 21- and 28-days post eclosion (± 1 day). After 5 minutes of acclimatisation, the apparatus was raised to a fixed height (8 cm) and dropped, images were captured once a second for 10 seconds. After one minute recovery, this procedure was repeated for a total of five drops. Using FIJI, the mean distance climbed by each fly at the 4 second time point was measured.

Assessment of sleep and circadian rhythms

Males (0-6 days, Supplementary Table 1B) were individually housed within *Drosophila* activity monitors (DAM, Trikinetics Inc) and subjected to a 12-hour light:dark (LD) cycle at 25 °C for 5 days, followed by 5 days of 24-hour darkness (DD). For each run (> two runs), $N = 31-80$ individual males were assayed per genotype. Activity (beam crosses) was calculated in 1- and 30-minute bins using DAMFileScan v1.11 and an in-house script (https://github.com/Fly-Cardiff/sleep_analysis) and subsequently analysed using "Sleep and Circadian Analysis

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3 MATLAB Program - SCAMP" (MATLAB, vR2020b).^{22,23} A bout of sleep was defined, as per
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5 convention, as more than five minutes without activity.^{24,25} Data from the LD phase was
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7 derived after exclusion of day one, to allow for entrainment, and after removal of any deaths.
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13 Seventeen metrics of sleep and wakefulness were exported from SCAMP for subsequent
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15 analyses: total wake episodes, mean wake episode duration, total activity counts, total time
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17 awake, latency to sleep, mean wake duration minus latency, mean activity a minute whilst
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19 moving, activity normalised to time awake, the probability of transitioning to an inactive state
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21 or active state, the number of sleep episodes, the total, max, mean and median sleep episode
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23 duration and sleep stability. All metrics were assessed for inter-correlations (Pearson) and
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25 strongly correlated ($r > |0.8|$) metrics were excluded from further analysis. Of the remaining
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27 metrics, normality of residuals was assessed with Kolmogorov-Smirnov tests and quantile-
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29 quantile plots and consequent Box-Cox transformations were performed where data was
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31 deemed sufficiently non-gaussian (RStudio v2022.12, GraphPad Prism v.10).
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40 Assessment of circadian strength was performed for the DD regime, including flies that
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42 survive to day ten. Rhythmicity statistic (RS) and period length were derived from SCAMP
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44 autocorrelograms. The RS describes the ability of a fly to maintain a cycle, with an RS > 1.5
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46 being rhythmic, RS = 1-1.5 being weakly rhythmic and flies with an RS < 1 being arrhythmic.
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48 Periodicity was calculated for rhythmic and weakly rhythmic flies only. If a fly had a RS < 0, it
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50 was set to zero for analysis of rhythmicity.
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Immunohistochemistry

Day 14 *Drosophila* heads were dissected and fixed in 4 % PFA in PBS with 0.1 % Triton X-100 (PTX) for 10 minutes (Supplementary Table 1B). Brains were then dissected from the head capsule and fixed for a further 10 minutes. Brains were blocked in 10 % normal goat serum (in PTX) for 1 hour at room temperature, then incubated with primary antibodies – anti-A β ₄₂ (1-16, 6E10, mouse, 1:400, BioLegend #803001), anti-Atf4 (rat, 1:200, gifted by Bateman lab)²⁶ – for 60 and 16 hours respectively at 4 °C in 10 % normal goat serum (in PTX). Brains were washed five times in PTX, then incubated with fluorophore-conjugated secondary antibodies AlexaFluor-647 (anti-mouse, 1:500, Invitrogen #A-21235), AlexaFluor-594 (anti-rat, 1:400) for 1-2 hours at room temperature in PTX. Brains were then washed five times in PTX, then mounted in VECTASHEILD Antifade (Vector Laboratories, H-1000-10) before imaging on a Cell Observer Spinning Disk Confocal Microscope (ZEISS) with Plan Apo 20X/0.8 dry objective and Axiocam 503 monochromatic camera, using Zen Blue software (ZEISS). Images were processed in FIJI - A β aggregates were detected by the *Analyze Particles* function and Atf4 fluorescence intensity (mean gray value) was averaged across four equal sized region of interests (ROIs, 227.5 μm^2) per brain. Data was analyzed with Mann-Whitney tests or t-tests dependent on normality (GraphPad Prism v.10).

Imaging of sima:GFP fluorescent reporter

Brain sima levels were measured using the genetically encoded reporter line *UAS-sima:GFP* in day 14 flies (Supplementary Table 1B). Unfixed brains were dissected and imaged on a Zeiss Cell Observer spinning disk confocal using 488 nm excitation, Plan Apo 20X/0.8 dry objective and Axiocam 503 camera. Fluorescence intensity (mean gray value) was averaged across four equal sized (415.1 μm^2) ROIs (FIJI).

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Electrochemiluminescence quantification of Aβ₄₂

Day 14 flies were snap-frozen in liquid nitrogen and heads (n = 25 per replicate) separated by vortexing (Supplementary Table 1B). Heads were then homogenized for 3 minutes in 150 µl of soluble extraction buffer (50 mM HEPES pH 7.3, 5 µM EDTA and 1X protease inhibitor) on ice, vortexed, then incubated for 10 minutes at room temperature. Homogenates were sonicated at 4 °C for 4 minutes (30 seconds on 30 seconds off), then centrifuged at 21,000 x g for 5 minutes at 4 °C. The supernatant was separated and used as the soluble fraction. 55 µl of insoluble extraction buffer (soluble extraction buffer with 5 mM Guanidinium HCl) was added to the remaining pellet. The pellet was then homogenized for 2 minutes, vortexed, and incubated for 10 minutes at room temperature. Homogenates were sonicated in a 4 °C water bath for 4 minutes (30 seconds on 30 seconds off), then centrifuged at 21,000 x g for 5 minutes at 4 °C. The supernatant was separated and used as the insoluble fraction.

The Meso Scale Discovery (MSD) V-plex assay was performed according to manufacturer’s instructions. Briefly, the plate was blocked with diluent 35 for 1 hour at room temperature. Standard calibrators were serial diluted in Diluent 35. After blocking, the plate was washed three times with 150 µl/well of 0.05 % Tween-PBS. Next, 25 µl of detection antibody (anti-Aβ 17-24, 4G8, mouse, MSD) was added to each well, followed by 25 µl of standard or 0.5 µg/µl brain homogenate from soluble or insoluble fractions. The plate was sealed and incubated whilst shaking for 2 hours. The plate was subsequently washed six times with 150 µl/well of 0.05 % Tween-PBS. The final wash was discarded and 150 µl of 2X read buffer was added to each well, before immediately reading the plate on an MSD instrument.

Bulk transcriptomics

Heads of 14-day old, mated females (n = 10, Supplementary Table 1B) were removed and pooled into N = 4 replicates per genotype. RNA extracted using the RNAqueous-Micro kit (AM1931) according to the manufacturer's instructions and sent to the Cardiff University School of Biosciences Genomics Research Hub for 6.5 million reads paired-end mRNA sequencing on an Illumina NextSeq500 with a fragment length of 75 base-pairs. Quality was screened using FastQC (0.11.8); Poly-G tails, Truseq3 adapters and low-quality bases/regions (phred < 3 or sliding window average of < 15) were trimmed using Illumina clip (Trimmomatic).²⁷ Any remaining reads < 20 bases in length were also removed. Reads were mapped to the *Drosophila melanogaster* genome (BDGP6.32) using STAR and reads counted using featureCounts.^{28,29} Non-coding RNAs, transposons and unassigned features were removed using SARTools.³⁰ Differential gene expression analysis was then performed using DESeq2 with log₂ fold-change shrinkage using the ashR algorithm.^{31,32} Dimensionality of variance stabilised data was assessed via principal component analyses (PCA, prcomp). Volcano plots were generated plotting -log₁₀ adjusted p-value against log₂ fold-change (threshold = ± 0.58) (ggplot2, RStudio v.4.4.1).³³ Overrepresentation analysis was performed between differentially expressed genes and gene ontology molecular function (GO:MF), gene ontology biological process (GO:BP), KEGG, Wiki Pathways and TRANSFAC databases, via the functional enrichment tool g:Profiler (version e112_eg59_p19_25aa4782).^{34 35}

Metabolomics

Heads of 14-day old, mated females were removed and separated into N = 5 replicates of n = 10 (Supplementary Table 1B). Metabolites were extracted by homogenising in 1:3:1 chloroform (SLS, C2432), methanol (Fisher, 10284580) and water, followed by an hour

agitation at 4 °C. Hydrophilic Interaction Liquid Chromatography (HILC) based metabolomics was performed (UltiMate 3000 RSLC, Thermo Fisher Scientific) using a hydrophilic ZIC-pHILIC column (Merck) on samples and standards (Metabolomics Standards Initiative). Samples, were ionised using a Thermo Orbitrap QExactive (Thermo Fisher Scientific) and mass spectrometry performed in both ionisation modes, detecting metabolites in range of 70-1050 m/z. Data processing, feature annotation (based on exact mass match to compounds in databases) and identification (based on exact mass and retention time match to an authentic standard) was performed using IDEOM, mzMatchISO, PiMP and Metaboanalyst 5.0.³⁶⁻³⁸ Fragmentation analysis of the pooled samples was performed using in-house software FrAnK.

Statistical Analysis

Normality was assessed using Kolmogorov-Smirnov tests and quantile-quantile plots. Dependent on normality and the number of experimental groups, different tests were performed with an $\alpha = 0.05$. For data deemed gaussian with just two groups, unpaired two-tailed t-tests were performed. If data was matched and two experimental variables were being assessed, we performed mixed effect models. If two experimental variables were being assessed, but unmatched, we performed two-way ANOVAs. If just one experimental variable was being assessed, with more than two groups, we performed one-way ANOVAs. Significant effects, where appropriate, were explored further using Šidák, Tukey's, Dunnett's or Bonferroni multiple comparisons, dependent on the effect being explored. If multiple tests were performed, p values were further corrected with 5 % false discovery rate (FDR). If data was deemed non-gaussian we performed non-parametric Mann-Whitney tests. Lifespan was analysed using Mantel-Cox and Cox proportional hazard-model tests (GraphPad Prism v.10).

RNA sequencing was analysed with DESeq2 with Fisher Exact Tests to assess similarities between gene sets using the GeneOverlap package.^{32,35} Pearson correlations were used to assess the relationship between log₂ fold-changes. Log-transformed relative metabolite intensities were analysed via two-way ANOVAs on the top 200 features (based on variance) followed by multiple testing correction with 10 % FDR using Metaboanalyst 5.0.³⁷

Results

***Wwox* knockdown in neurons or glia shortens lifespan**

WWOX is highly conserved between species, with *Drosophila Wwox* sharing 65 % sequence similarity with human WWOX, and a score of 16/16 using the DRC Integrative Ortholog Prediction Tool (DIOPT).³⁹ Equivalent to its mammalian orthologue,⁴⁰ *Drosophila Wwox* is expressed throughout the brain. Using a single cell transcriptomic dataset from the ageing *Drosophila* brain,²⁰ *Wwox* expression was detectable in both neuronal and glial cell-types (Fig.1A).

To explore the cell-type specific roles of *Wwox* and mimic the subtle expression changes observed in AD, we expressed a *Wwox* targeted RNAi construct under control of *elav-GAL4* (pan-neuronal) or *repo-GAL4* (pan-glial) drivers. *Wwox* knockdown in neurons (Supplementary Figure 1),^{10,11,41} significantly reduced lifespan in comparison to control (median lifespans = 57 and 42 days respectively, N = 91-105, $\chi^2 = 30.76$ p < 0.0001 Fig.1B). Startle-induced locomotor function was significantly decreased with age (F = 6.106, p = 0.0026) but unaffected by neuronal knockdown of *Wwox* (F = 0.018, p = 0.8942) or the interaction between the two (F = 2.316, p = 0.0982, Fig.1C, N = 3-6). Glial knockdown of *Wwox*

caused a reduction in lifespan in comparison to control (median lifespan = 70 vs. 74 days, N = 98-101, $\chi^2 = 13.07$ p = 0.0003, Fig.1D), albeit milder than that of neuronal knockdown. Like neuronal knockdown, age significantly worsened startle-induced locomotor activity (F = 7.454, p = 0.0033), however glial knockdown of *Wwox* had no effect (F = 3.830, p = 0.0820), nor was there an interaction (F = 0.7327, p = 0.5142, Fig.1E, N = 4-6). These results suggest that both neuronal and glial *Wwox* play a role in longevity but are dispensable for startle-induced locomotor function up to 28 days old.

***Wwox* knockdown in neurons or glia decreases wakefulness and increases sleep**

A common symptom of AD is disruption to circadian rhythms and sleep homeostasis,⁴⁴⁻⁴⁸ leading us to next to assess the contribution of neuronal or glial *Wwox* expression to sleep and circadian activity regulation. Sleep metrics were derived from activity of individually housed flies using the MATLAB toolbox SCAMP.^{22,23} The effect of *Wwox* manipulation was investigated on: total activity counts, activity counts normalised to time awake, max wake episode duration, mean wake episode duration, sleep latency, probability to transition from an active to inactive state (sleep pressure) and number of sleep episodes. Other strongly correlated sleep metrics ($r > |0.8|$) are shown in Supplementary Table 2.

Neuronal knockdown of *Wwox* decreased activity normalised to time awake in the light phase (t = 4.969, $p_{adj} < 0.0001$, Fig.2A) and increased sleep pressure (t = 7.146, $p_{adj} < 0.0001$, Fig.2B), whilst increasing mean sleep episode duration, both in the light and dark phase (t = 8.978 and t = 6.066 respectively, both $p_{adj} < 0.0001$, Fig.2C, N = 31-80 over > two runs). Similarly, pan-glial *Wwox* knockdown reduced activity normalised to time awake (F = 29.81, $p_{adj} < 0.0001$, Fig.2D), increased sleep pressure in the light and dark phases (t = 7.268 $p_{adj} < 0.0001$, t = 2.870

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$p_{\text{adj}} = 0.0129$, respectively, Fig.2E) and increased mean sleep episode duration ($F = 5.768$, $p_{\text{adj}} = 0.0258$, Fig.2F, $N = 53$ -56 over > two runs). Additional sleep metrics are summarised in Supplementary Figure 2A-J. To understand whether these changes in sleep were due to underlying circadian differences, we evaluated the periodicity of flies in constant darkness without light entrainment cues. After neuronal knockdown of *Wwox*, all flies remained strongly rhythmic ($t = 0.1766$, $p = 0.8606$, Fig.2G, $N = 17$ -34) with circadian periods comparable to control ($U = 178$, $p = 0.8329$, Fig.2H-I, $N = 12$ -31). Glial knockdown of *Wwox* had no effect on rhythmicity ($t = 1.058$, $p = 0.2955$, Fig.2J, $N = 6$ -42) or period length ($U = 50$, $p = 0.3052$, Fig.2K-L, $N = 4$ -37). *Wwox* knockdown driven by the clock specific driver *timeless-GAL4*, resulted in a similar decrease in wakefulness (Supplementary Figure 2K-R) to pan-neuronal and pan-glial knockdown, with flies remaining strongly rhythmic (Supplementary Figure 2S) with a slightly increased circadian period (Supplementary Figure 2T). These results suggest that *Wwox* knockdown, irrespective of cell type, increases sleep, without disrupting circadian rhythmicity.

As *Wwox* is an oxidoreductase and has been linked to alterations in reactive oxygen species (ROS) formation,^{10,11} which in turn can alter sleep homeostasis,^{42,43} we explored whether the sleep alterations witnessed in our model were due to increases in ROS. Utilising the roGFP2-Orp1 reporter for mitochondrial hydrogen peroxide, we witnessed an increase in ROS after neuronal *Wwox* knockdown ($t = 3.221$, $p = 0.0047$, Supplementary Figure 2U, $N = 9$ -11) – suggesting that *Wwox* knockdown may increase sleep, at least in part, through an increase in ROS.

***Wwox* knockdown modifies A β ₄₂-induced phenotypes and increases A β ₄₂ load**

Next, we explored the impact of *Wwox* knockdown on amyloid pathology, a hallmark feature of AD. We employed a model of amyloid toxicity whereby neuronal overexpression of aggregate-prone human A β ₄₂ harboring the Arctic mutation (E693G) and secretion signal-peptide from the *argos* gene, drives secretion of the peptide to the extracellular milieu. This causes accumulation of amyloid deposits, leading to brain vacuolation, locomotor deficits and reduction in lifespan in the fly.¹⁹ Neuronal knockdown of *Wwox* combined with overexpression of human A β ₄₂ did not affect expression of A β ₄₂ mRNA (Supplementary Figure 3A), but caused a further reduction in lifespan compared to A β ₄₂ expression alone (median lifespan = 41 vs. 26 days, $\chi^2 = 103.7$, $p_{\text{adj}} < 0.0001$, Fig.3A, N = 90-99). Cox-proportional hazard modelling of the effects revealed a significant interaction between A β ₄₂ overexpression and *Wwox* knockdown ($Z = 4.815$, $p < 0.0001$), indicating that the decrease in lifespan is not simply the additive effect of the two variables but synergistic and that *Wwox* knockdown is modulating amyloid toxicity directly. As expected, startle-induced locomotor ability was significantly reduced with age ($F = 11.77$, $p < 0.0001$, Fig.3B), from ~3.5 cm climbed at day 7 compared to only ~1.0 cm climbed at day 28 in RNAi Control A β ₄₂ flies. *Wwox* knockdown led to a significant reduction in locomotion ($F = 12.51$, $p = 0.0019$, Fig.3B), climbing just ~0.5 cm by day 21 (representative images available in Supplementary Figure 3B). We found no significant interaction between age and genotype ($F = 0.3165$, $p = 0.8133$, Fig.3B, N = 3-5 vials), suggesting that *Wwox* knockdown in the presence of amyloid significantly worsened locomotor ability at all four time points assessed. Utilising a dual-binary transgene expression system, we restricted *Wwox* knockdown to glial cells (*repo-GAL4*), whilst simultaneously restricting A β ₄₂ expression to neurons (*nSyb-QF2*). *Wwox* knockdown mildly, albeit

significantly, decreased lifespan (median = 22 vs. 24 days, $\chi^2 = 6.720$ $p = 0.0095$, Supplementary Figure 3C, $N = 100-115$) but had no effect on startle-induced locomotion ($t = 0.9876$, $p = 0.3491$, Supplementary Figure 3D, $N = 5-6$ vials). These results suggest that neuronal *Wwox* may be more important than glial *Wwox* for modulation of $A\beta_{42}$ toxicity.

To understand if neuronal *Wwox* knockdown directly impacts endogenous amyloid accumulation, we probed intact brains from day 14 flies using the anti- $A\beta$ [6E10] antibody, choosing this time-point due to clear locomotor defects but before a substantial proportion of flies were dead. Specificity of the immunostaining was confirmed by lack of visible deposits in LacZ-expressing control brains at the same timepoint (Supplementary Figure 3E). Visible $A\beta_{42}$ deposits were quantified in $A\beta_{42}$ -expressing flies, however no significant changes in aggregate number ($U = 9$, $p = 0.2020$, Fig.3C-D) or median size ($t = 0.2313$, $p = 0.8218$, Fig.3C & E, $N = 5-7$) were observed upon *Wwox* knockdown. To quantify changes in $A\beta_{42}$ deposition with increased sensitivity, we assessed soluble and insoluble fractioned extracts of 14-day old fly heads by an electrochemiluminescence immunoassay (MSD) using the anti- $A\beta$ [4G8] antibody (Fig.3F). Total $A\beta_{42}$ was significantly increased upon neuronal *Wwox* knockdown ($q = 3.997$, $p_{adj} = 0.0127$ Fig.3G). Interestingly, this effect was led by an increase in soluble $A\beta_{42}$ ($q = 4.126$, $p_{adj} = 0.011$ Fig.3H), whereas insoluble fractions were non-significantly changed ($q = 1.666$, $p_{adj} = 0.2412$, Fig.3I). However, the ratio between soluble/insoluble $A\beta_{42}$ was not significantly changed ($q = 2.131$, $p_{adj} = 0.1307$, Fig.3J, $N = 3$). Taken together, these results indicate an increase in $A\beta_{42}$ load upon neuronal *Wwox* knockdown, without an associated increase in detectable aggregate size or number.

Neuronal *Wwox* knockdown in the presence of Aβ₄₂ alters pyruvate metabolism

To investigate the mechanisms underlying the effect of neuronal *Wwox* knockdown on Aβ₄₂ associated phenotypes, we performed bulk transcriptomics from flies overexpressing Aβ₄₂ or a LacZ control construct, with or without neuronal *Wwox* knockdown (N = 4 replicates of n = 10 pooled heads). Transcriptomics were performed at day 14, an age at which the flies showed a clear motor phenotype but before significant mortality. The effects of *Wwox* knockdown and Aβ₄₂ expression could be clearly separated along PC1 and PC2, explaining 23 % and 15 % of variance respectively (Supplementary Figure 4). Knockdown of *Wwox* in a control background significantly downregulated 54 genes and upregulated 44 (Fig.4A, Supplementary Table 3). Overrepresentation analysis determined these genes were enriched for oxidoreductase activity and metabolism of ascorbate, aldarate, and porphyrins (Fig.4B, Supplementary Table 3). Overexpressing human Aβ₄₂ in neurons downregulated 23 genes and upregulated 51 (Fig.4C, Supplementary Table 3); overrepresentation analysis revealed a significant association of these genes with fatty acid and pyruvate metabolism (Fig.4D, Supplementary Table 3), suggesting amyloid expression induces metabolic changes. Knockdown of *Wwox* in neurons also overexpressing Aβ₄₂, significantly downregulated 58 genes and upregulated 54 genes (Fig.4E, Supplementary Table 3). These genes were enriched within pyruvate metabolism, lipid metabolism and pentose and glucuronate interconversion pathways (Fig.4F, Supplementary Table 3), suggesting that knockdown of *Wwox* in the presence of Aβ₄₂ may alter the metabolome further via transcriptional regulation.

Wwox* knockdown in the presence of $A\beta_{42}$ increases lactate concentrations via *Atf4

Since various metabolic pathways were identified by our transcriptomic analysis, we performed Hydrophilic Interaction Liquid Chromatography (HILC)-based metabolomics to assess the effect of overexpression of $A\beta_{42}$ and or *Wwox* knockdown on metabolic profiles of the *Drosophila* head (N = 5 replicates of n = 10 pooled heads). Of 2,355 LC-MS peaks, 85 compounds mapped to known standards by m/z and retention time. Neuronal knockdown of *Wwox* differentially expressed ($P_{adj} < 0.1$) eight standard-mapped metabolites, namely, reduction of L-lysine (two peaks, $F = 50.176$ and $F = 63.950$ both $p_{adj} < 0.0001$), orotate ($F = 18.036$, $p_{adj} = 0.0046$) and 4-trimethylammoniobutanoate ($F = 10.659$, $p_{adj} = 0.0263$) and upregulation of D-glucuronate ($F = 15.385$, $p_{adj} = 0.0069$), 2-hydroxyglutarate ($F = 10.672$, $p_{adj} = 0.0263$), L-lactate ($F = 7.524$, $p_{adj} = 0.0636$) and itaconate ($F = 6.310$, $p_{adj} = 0.0885$, Fig.5A, T Supplementary Table 4). $A\beta_{42}$ expression upregulated two standard-matched metabolites: 2-hydroxyglutarate ($F = 49.551$, $p_{adj} = 0.0006$) and L-lactate ($F = 27.846$, $p_{adj} = 0.0050$, Fig.5B, Supplementary Table 4). Both lactate and 2-hydroxyglutarate are generated from pyruvate and α -ketoglutarate, respectively, by *Ldh*.^{26,44} This is consistent with our transcriptomics analysis, which revealed a 1.19 log₂ fold-change upregulation of *Ldh* after overexpression of $A\beta_{42}$ (Fig.4C, Supplementary Table 3). Crucially, the interaction between neuronal *Wwox* knockdown and $A\beta_{42}$ overexpression differentially expressed just one standard-mapped metabolite, L-lactate ($F = 23.606$, $p_{adj} = 0.0174$, 1.30 log₂ fold-change Fig.5C-D, Supplementary Figure 5A, Supplementary Table 4). L-Lactate increased in the response to *Wwox* knockdown, only when $A\beta_{42}$ was also present (Fig.5C & S5A), consistent with the 1.29 log₂ fold-change increase in *Ldh* transcripts compared to $A\beta_{42}$ overexpression alone in our transcriptomics experiment (Fig.4E, Supplementary Table 3). In addition to this increase in *Ldh* and lactate, we observed corresponding transcriptional decreases in the pyruvate metabolism pathway -

Pepck2, *CG11052* and *Adh* - further supporting the role of pyruvate metabolism in the phenotypes observed (Fig.5D). An increase in *Ldh* and lactate suggests a Warburg effect-like shift towards glycolytic energy production. Interestingly however, we observed no differences in ATP levels in the brain as a consequence of *Wwox* knockdown ($F = 0.3376$, $p = 0.5720$), $A\beta_{42}$ overexpression ($F = 0.3811$, $p = 0.5486$) or their interaction ($F = 2.206$, $p = 0.1633$, Supplementary Figure 5B, $N = 4$), suggesting that the observed shift towards glycolytic metabolism is sufficient to maintain energy supply and may be a protective response.⁴⁵

Given the consistent changes in lactate/*Ldh* across our omic analyses, previous literature implicating *Ldh* in amyloid toxicity,⁴⁵ and our results suggesting that *Wwox* modulation may be an upstream regulator of this toxicity, we next investigated the physiological consequences of elevated *Ldh* and lactate seen in $A\beta_{42}$ -transgenic flies with reduced *Wwox* expression. We utilized RNAi to mediate combined downregulation of both *Ldh* and *Wwox* in neurons expressing $A\beta_{42}$ (Supplementary Figure 5C) and assessed startle-induced locomotion. Multiple comparisons testing revealed that *Ldh* knockdown rescued climbing deficits seen in $A\beta_{42}$ -transgenic flies with downregulated *Wwox* (at 14-, 21- and 28-days $t = 3.182$ $p_{adj} = 0.0081$, $t = 6.832$ $p_{adj} < 0.0001$ and $t = 5.198$ $p_{adj} < 0.0001$, respectively, Fig.5E, $N = 4-6$ vials). These results suggest that *Wwox* knockdown in the presence of $A\beta_{42}$ elevates lactate levels via neuronal *Ldh*, which is sufficient to exacerbate locomotor defects.

Both HIF1 α and Atf4 are transcription factors known to promote *Ldh* expression. We investigated the levels of these proteins upon *Wwox* knockdown to further understand *Ldh* regulation in our model. No changes in endogenously tagged *sima:GFP* (HIF1 α homolog) levels were observed in the adult (day 14) fly brains after neuronal *Wwox* knockdown in $A\beta_{42}$

overexpressing flies ($t = 0.6623$, $p = 0.5185$, Fig.5F-G, $N = 7-9$). However, immunocytochemistry revealed a significant upregulation of Atf4 levels in $A\beta_{42}$ flies upon *Wwox* knockdown ($U = 0$ $p = 0.0022$, Fig.5H-I, $N = 6$), which were not observed in LacZ-expressing *Wwox* knockdown flies (Supplementary Figure 5D-E). Consistent with the notion that Atf4 is minimally expressed at rest in the adult brain, but upregulated upon stress.²⁶ Since Atf4 activation occurs downstream of protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) through the ISR and UPR,⁴⁶ we assessed if the Ire1-Xbp1 branch of the UPR was affected, by measuring Ire1-mediated *Xbp1* splicing with a GFP-based reporter expressed in neurons.⁴⁷ However, we observed no differences in neuronal spliced *Xbp1* because of *Wwox* knockdown in $A\beta_{42}$ flies ($t = 1.365$, $p = 0.1954$, Supplementary Figure 5F-G, $N = 6-9$). These data suggest that neuronal *Wwox* knockdown specifically upregulates the PERK-Atf4 branch of the ISR/UPR in $A\beta_{42}$ flies and this could be the mechanism via which *Ldh* and lactate are upregulated, leading to exacerbated $A\beta_{42}$ -induced phenotypes (Fig.5J).

Upregulation of neuronal *Wwox* rescues $A\beta_{42}$ related pathology

Having described the detrimental effect of *Wwox* knockdown on $A\beta_{42}$ -induced phenotypes, we wanted to explore whether *Wwox* upregulation could also affect $A\beta_{42}$ toxicity. We employed CRISPRa upregulation of *Wwox* by expressing *UAS-dCas9-VPR* and tandem gRNAs targeting the *Wwox* transcriptional start site (*Wwox*^{gRNA}) or non-targeting (control^{gRNA}), under pan-neuronal *elav-GAL4* control. This was sufficient to achieve two-fold increase in *Wwox* mRNA levels in the fly head (Supplementary Figure 1), whilst not affecting the mRNA levels of $A\beta_{42}$ when it was overexpressed (Supplementary Figure 3A). In the absence of $A\beta_{42}$, *Wwox* upregulation had no significant effect on survival (median = 60 vs. 58 days, $\chi^2 = 2.156$, $p = 0.1420$, Supplementary Figure 6A, $N = 76-86$) or startle-induced locomotion ($F = 0.02434$, $p =$

0.8770, Supplementary Figure 6B, N = 3-6 vials). However, in the presence of neuronal A β_{42} , upregulation of *Wwox* significantly improved lifespan (median = 47.5 vs. 29 days, $\chi^2 = 104.5$, $p < 0.0001$, Fig.6A, N = 78-91) and startle-induced locomotion ($F = 42.33$, $p < 0.0001$) at days 14 and 21 ($t = 5.669$ and $t = 6.169$ both $p_{\text{adj}} < 0.0001$, Fig.6B, N = 3-7 vials). We observed a similar rescue of lifespan (median = 41 vs. 51 days, $\chi^2 = 9.493$ $p = 0.0021$, Supplementary Figure 6C, N = 85-106) when overexpressing a *UAS*-cDNA construct of human *WWOX* (*hWWOX*) in neurons. Since different posttranslational modifications to *WWOX* have been previously implicated in Tau pathology,^{48,49} we generated phosphomimic and phosphomutant cDNA variants of *hWWOX* at residues Ser14 and Tyr33. These variants performed similarly to wildtype *hWWOX* in their rescue of A β_{42} induced locomotor function (Supplementary Figure 6D). Notably, *dWwox* cDNA overexpression provided a better rescue of locomotor ability than *hWWOX* and *hWWOX* Ser14 phosphomutant (Supplementary Figure 6D).

Overexpression of *Drosophila* or *hWWOX* cDNA in glial cells whilst simultaneously overexpressing A β_{42} in neurons had no effect on survival ($\chi^2 = 0.0644$ $p_{\text{adj}} = 0.7997$ and $\chi^2 = 3.768$ $p_{\text{adj}} = 0.1045$ respectively, Supplementary Figure 6E, N = 84-154) or locomotion ($F = 0.4914$, $p = 0.6654$, Supplementary Figure 6F, N = 4-7 vials) in comparison to controls, further indicating the importance of neuronal, compared to glial *Wwox*, in the modulation of neuron-derived A β_{42} toxicity. In the brains of flies with *Wwox* upregulation in neurons, we found a dramatic reduction in the median size ($t = 5.485$, $p = 0.0003$, Fig.6C-D) and number ($t = 3.374$, $p = 0.0071$, Fig.6C & E, N = 5-7) of A β_{42} deposits with confocal microscopy. Similarly, by MSD assay, we found reduced total A β_{42} ($t = 3.252$, $p = 0.0313$, Supplementary Figure 6G) in both soluble ($t = 3.114$, $p = 0.0357$, Fig.6F) and insoluble ($t = 2.975$, $p = 0.0409$, Fig.6G, N = 3) head lysate fractions of day 14 flies, without altering the ratio of insoluble/soluble A β_{42} ($t = 1.334$,

p = 0.2530, Supplementary Figure 6H, N = 3). This suggests that increasing neuronal *Wwox* can protect against A β ₄₂ associated pathology by lowering total amyloid load.

Neuronal upregulation of *Wwox* reverses the transcriptional profile induced by A β ₄₂

To understand how neuronal upregulation of *Wwox* may provide protection against A β ₄₂ and since *Wwox* knockdown substantially altered the transcriptomic profile of A β ₄₂ flies, we performed bulk transcriptomics on the heads of flies with *Wwox* upregulation in neurons (N = 3-4 replicates of n = 10 pooled heads). PCA only partially distinguished the effects of *Wwox* overexpression and A β ₄₂ with PC1 explaining 29 % and PC2 16 % of variance (Supplementary Figure 7A). This lack of separation between control and overexpression in the LacZ background was reflected by no upregulated genes and downregulation of only six genes (Fig.7A, Supplementary Table 5) – these downregulated genes were enriched for fatty acid metabolism terms (Supplementary Figure 7B, Supplementary Table 5). Overexpression of A β ₄₂ significantly downregulated 51 genes and upregulated 28 (Fig.7B, Supplementary Table 5), of which *Ldh* was the second most significantly upregulated gene with a 2.85 log₂ fold-change compared to control. Overrepresentation analysis revealed dysregulation of translation repression, membrane transport and oxidative phosphorylation (Supplementary Figure 7C, Supplementary Table 5). There was a significant overlap between the differentially expressed genes identified upon A β ₄₂ expression in the knockdown and overexpression experiments, including *Ldh*, *Hsc70-3*, *CG11842*, *GNBP-like3*, *BomBc3* and *Oat*, thus implicating ER stress response, Toll receptor activation and energy metabolism as reproducible changes on the transcriptional level in this amyloid model (Supplementary Figure 7D, OR = 28.52, p < 0.0001, Fisher Exact Test)

Strikingly, upregulation of neuronal *Wwox* in the presence of $A\beta_{42}$, downregulated 305 genes and upregulated 59 (Fig.7C, Supplementary Table 5). Overrepresentation analysis revealed metabolic and immune enrichments, including a dysregulation of cysteine and methionine metabolism lead largely by these downregulated genes (Supplementary Figure 7E, Supplementary Table 5). Markedly, the expression of the 404 unique genes differentially expressed appear to shift in directional change, implying *Wwox* overexpression reverses amyloid related transcriptional signatures (Fig.7D). Specifically, 40 of these genes were common between the analyses of $A\beta_{42}$ vs. LacZ and *Wwox*^{gRNA} $A\beta_{42}$ vs. control^{gRNA} $A\beta_{42}$ (Supplementary Table 6), the log₂ fold-changes of these genes were significantly negatively correlated ($r = -0.8341$, $p < 0.0001$, Fig.7E). Notably, every gene differentially expressed in $A\beta_{42}$ vs. LacZ comparison was differentially expressed in the opposing direction by the *Wwox*^{gRNA} $A\beta_{42}$ vs. control^{gRNA} $A\beta_{42}$ comparison. Overrepresentation analysis revealed that those genes upregulated by $A\beta_{42}$ and downregulated by *Wwox* overexpression were enriched within immune response terms, whereas genes with the opposing change were enriched for synaptic terms (Fig.7E). Taken together, these results suggest that upregulation of *Wwox* reverses the transcriptional reprogramming of metabolic, immune and synaptic pathways caused by $A\beta_{42}$ upregulation.

Neuronal upregulation of *Wwox* reverses transcriptional pathways dysregulated by $A\beta_{42}$.

To understand how this transcriptional reprogramming may impact neuronal metabolism of these flies, we performed unbiased metabolomic profiling on flies expressing $A\beta_{42}$, overexpressing *Wwox*, or overexpressing *Wwox* in the presence of $A\beta_{42}$ (N = 5 replicates of n = 10 heads). Of 2414 peaks, 86 compounds were exact matches to known standards by m/z

and retention time. Expression of $A\beta_{42}$ differentially altered four standard-matched metabolites ($p_{adj} < 0.1$), including a reduction in sn-glycero-3-phosphocholine ($F = 35.42$, $p_{adj} = 0.0006$) and an increase in betaine ($F = 8.164$, $p_{adj} = 0.0667$), 2-hydroxyglutarate ($F = 47.94$, $p_{adj} = 0.0001$) and citrate ($F = 8.197$, $p_{adj} = 0.0667$, Fig.8A, Supplementary Table 7). This increase in 2-hydroxyglutarate is consistent with our previous finding of a 2.85 $\log_2$ fold-change increase in *Ldh* transcripts in $A\beta_{42}$ expressing flies compared to control – the second most significant differentially expressed gene in this comparison (Supplementary Table 5). Neuronal *Wwox* overexpression downregulated five standard-matched metabolites: L-leucine ($F = 9.949$, $p_{adj} = 0.0545$), sn-glycero-3-phosphocholine ($F = 9.311$, $p_{adj} = 0.0667$), L-methionine ($F = 22.15$, $p_{adj} = 0.0047$ and $F = 17.38$, $p_{adj} = 0.0091$) and a suspected isomer of methyl-L-histidine (N-pi) ($F = 9.93$, $p_{adj} = 0.0545$, Fig.8B, Supplementary Table 7). However, the interaction between *Wwox* overexpression and $A\beta_{42}$ revealed just the L-methionine metabolite ($F = 19.79$, $p_{adj} = 0.0073$ and $F = 14.92$, $p_{adj} = 0.0133$, Fig.8C, Supplementary Table 7). L-methionine levels were decreased after *Wwox* overexpression in the control background but were unaltered by overexpression of *Wwox* in the presence of $A\beta_{42}$ (Fig.8C, Supplementary Figure 8A-B). Surprisingly, the reduction of amyloid load and improvement in lifespan and locomotion of these flies therefore occurred without restoring *Ldh* transcript or 2-hydroxyglutarate levels but instead caused a reduction in transcripts encoding numerous enzymes within the cysteine and methionine metabolism pathway (Fig.8D). Reduced L-methionine levels may represent a protective response to $A\beta_{42}$ -induced stress, while *Wwox* overexpression appears to regulate the same pathway transcriptionally to maintain these low L-methionine levels.

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Discussion

In this study we investigated the multifaceted, cell-type specific effects of *Wwox* manipulation in the *Drosophila* brain, in combination with the effects of ageing and human Aβ₄₂ overexpression. Like mammals,⁴⁰ *Drosophila Wwox* is expressed throughout the brain in both neuronal and glial cell types. Using RNAi, we reduced *Wwox* levels in these cell types to recapitulate the subtle dysregulation of expression observed in LOAD.^{3,5} Whilst startle-induced locomotion was unaffected by *Wwox* RNAi in neurons or glia, knockdown in either cell type reduced lifespan, suggesting its important role in both neuronal and glial health. However, it is important to note that startle-induced locomotor phenotypes may emerge later in the flies' lifespan as reduction in *Wwox* caused more subtle changes in general activity captured at baseline in the longitudinal DAM experiment. Additionally, *Wwox* knockdown in either cell type affected sleep homeostasis, decreasing activity and increasing sleep, without disrupting circadian rhythmicity. These results suggest an involvement of *Wwox* in both age-related sleep decline and abnormalities seen in early-stage LOAD.^{50,51} Supporting our findings, a recent study also reported sleep defects in *Wwox* knockout *Drosophila* whilst also demonstrating a genome wide association between SNPs at the *Wwox* locus and self-aware sleep duration.⁴² Furthermore, a genome wide analysis for gene-sleep length interactions in the context of hypertension identified a SNP mapping to *Wwox* associated with short sleep (rs542745170).⁵² Taken together these results suggests a role of *Wwox* modulating sleep duration across diseases.

Wwox knockdown in the Aβ₄₂ model of AD exacerbated neurotoxicity, leading to increased Aβ₄₂ load, impaired locomotor function and reduced lifespan. However, *Wwox* knockdown in glia had no effect on the toxicity of neuron-derived Aβ₄₂, in the context of startle-induced

locomotor function, suggesting that neuronal *Wwox* plays a cell-autonomous protective role against $A\beta_{42}$ -mediated toxicity. Metabolic exploration of $A\beta_{42}$ expression revealed an increase of 2-hydroxyglutarate and L-lactate – the products of *Ldh* conversion of α -ketoglutarate and pyruvate, respectively.^{26,44} Consistently we found *Ldh* transcripts significantly increased with $A\beta_{42}$ expression. This enzymatic upregulation could explain previous reports of increased lactate levels in $A\beta_{42}$ -expressing *Drosophila* and AD patients.^{53,54} Keeping *Ldh* levels controlled in the brain could be key to maintaining normal metabolic homeostasis and *Ldh* overexpression has been found to shorten lifespan, disrupt circadian rhythms and induce neurodegeneration.^{55,56} Interestingly, *Ldh* knockdown has also been shown to cause detrimental effects in fly $A\beta_{42}$ models, suggesting that *Ldh* expression and lactate levels need to be tightly controlled.^{45,57} Here we identified that *Wwox* knockdown combined with $A\beta_{42}$ expression further elevated L-lactate levels, likely mediated by the further increase in *Ldh* expression observed in our transcriptomics data. Taken together, these data provide evidence that some genetic risk loci, like *WWOX*, may be acting upstream of transcriptional regulation of the metabolome to control pathology, thereby influencing disease progression.

The transcription factors Atf4 and HIF1 α are both known to increase transcription of *Ldh*.^{15,45} While *WWOX* has been shown to inhibit HIF1 α ,⁹ our data suggest that Atf4, rather than the HIF1 α orthologue *sima*, is primarily responsible for the observed *Ldh* upregulation in this fly model. Indeed, Atf4-dependent, *sima*-independent upregulation of *Ldh* has been previously reported in $A\beta_{42}$ -expressing *Drosophila*,⁴⁵ further supporting these results. Atf4 is upregulated by stimulation of the PERK arm of the ISR/UPR pathway via phosphorylation of eif2 α which suppresses global translation but specifically promotes translation of specific stress response factors, including Atf4. Whilst we did not determine whether *WWOX* is

activating this pathway directly in this study, previous research has shown that WWOX can interact in proximity with PERK and could be directly affecting its activation.^{58atf} Furthermore, WWOX has also been shown to directly bind GCN1,⁵⁶ an activator of Eukaryotic Translation Initiation Factor 2 Alpha Kinase 4 (GCN2) – a kinase known to phosphorylate eif2 α .⁵⁹ GCN2 depletion has previously been shown to be detrimental in the 5xFAD mouse model of A β ₄₂ toxicity, via enhanced PERK activation and its elevation.⁶⁰ WWOX regulation of GCN2 could thus also explain the elevated Atf4 and aggravation of A β ₄₂ toxicity in our *Wwox* knockdown model. Interestingly, this pathway is not linear – previous research has shown that hippocampal neurons exposed to A β ₄₂ oligomers can increase transport of *Atf4* mRNA in axons, where it is locally translated before retrograde transport to the soma, leading to prolonged CHOP expression and CHOP-dependant cell-death.⁶¹ Whilst no *Drosophila* CHOP orthologue has been identified, prolonged PERK/Atf4 signalling has been shown to cause cell death in *Drosophila* overexpressing Presenilin, which encodes the catalytic subunit of A β ₄₂ liberating γ -secretase.⁶² Our findings suggest that the combined effect of *Wwox* knockdown and A β ₄₂ leads to chronic ISR/UPR activation, characterized by elevated Atf4, *Ldh* and lactate, above that of A β ₄₂ overexpression alone. Previous work has demonstrated that A β expression induces *Xbp1* splicing and activation of the ER stress response, and that the active isoform Xbp1 exerts a neuroprotective effect against A β toxicity.⁶³ However, we do not find that this pathway is linked to *Wwox* regulation of the amyloid phenotypes.

Notably, we found that reducing *Ldh* levels in neurons rescued *Wwox* knockdown-induced locomotor deficits in the presence of A β ₄₂, suggesting that elevated neuron-derived lactate levels, downstream of Atf4 signalling, could be driving the detrimental effects of *Wwox* knockdown in A β ₄₂ overexpressing flies. Lactate has been shown to be detrimental in amyloid

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models previously, for instance, lactic acid accumulation may increase APP-Grp78 binding, subsequent amyloidogenic cleavage and UPR disinhibition.⁶⁴ Additionally, aerobic glycolysis has been suggested to directly modulate A β ₄₂ accumulation,^{65,66} implying a direct link between lactate and amyloid through *Ldh*. Accordingly, *Ldh* knockdown provided a rescue in our model. Taken together these results place *Wwox* upstream of Atf4, *Ldh* and Lactate, and suggest it is this increase in lactate that may increase amyloid toxicity. However, the rescue we witnessed was partial, suggesting that other alternative pathways may also be contributing to the phenotypes. The change observed in 2-hydroxyglutarate levels is of particular interest and is only partially controlled by *Ldh*. 2-hydroxyglutarate has been extensively explored in the context of cancer and enantiomers can be generated via action of numerous enzymes including isocitrate dehydrogenase, malate dehydrogenase and phosphoglycerate dehydrogenase.⁶⁷ Therefore, further research is needed to understand the contribution of several converging metabolic pathways in the context of amyloid pathology.

In addition to identifying deleterious effects of *Wwox* knockdown, recapitulating human risk, we found that upregulation of *Wwox* remarkably reduced amyloid load, rescued lifespan and locomotion of A β ₄₂ overexpressing flies. Surprisingly however, omics analyses show this rescue occurs without a reduction in *Ldh* or 2-hydroxyglutarate, suggesting a different mechanism of protection. We observed a dramatic reversal of transcriptional changes after *Wwox* overexpression in the presence of amyloid, identifying a downregulation of metabolic and inflammatory related genes. The reduction in L-methionine observed in the A β ₄₂ model brain may reflect a protective adaptation to A β ₄₂-induced stress, whereas *Wwox* overexpression appears to transcriptionally regulate this pathway to sustain lower L-methionine levels. This suggests that *Wwox* may play a critical role in maintaining metabolic

brain homeostasis and regulating amino acid pathways under normal physiological conditions. L-methionine is an essential amino acid; however, it has been suggested that diets high in methionine can result in worsened memory,^{68,69} neurodegeneration and increased A β ₄₂ deposition in mouse models.⁶⁹ Interestingly chronic L-methionine administration has also been shown to increase inflammatory cytokine release from microglial cells.⁷⁰ Therefore, there is a potential that overexpressing neuronal *Wwox*, and thus altering neuronal metabolism, is conferring protection through glia – an area for future research. In this study we suggest differing mechanisms conferring vulnerability and protection in the context of amyloid pathology via modulation of *Wwox*. Our findings support the growing literature implicating WWOX in numerous metabolic pathways,⁷¹ many of which lead to the generation of acetyl-CoA from degradation of proteins, lipids or carbohydrates.⁵⁶ *Wwox* can be seen as involved in broad metabolic reprogramming and fuel selection in neurons, with metabolomic analysis revealing alterations in glucose (lactate, 2-hydroxyglutarate), amino acid (L-lysine, 4-Trimethylammoniumbutanoate, D-Glucuronate, L-methionine, methyl-L-histidine (N-pi), and L-leucine), lipid (sn-glycero-3-phosphocholine), and nucleotide metabolism (D-Glucuronate, orotate).

In conclusion, our study provides new insights into the multifaceted relationship between A β ₄₂ expression and *Wwox* levels in neurons. We demonstrate that *Wwox* plays a critical role in neuronal health and the modulation of A β ₄₂ toxicity. Additionally, we identified metabolic alterations associated with A β ₄₂ expression and *Wwox* knockdown. Excitingly, increasing *Wwox* expression in neurons caused a dramatic rescue of amyloid load. Future studies should explore the potential therapeutic implications of targeting these pathways for treatment of AD.

Data availability

Data analysis code is available at <https://github.com/Fly-Cardiff>. Raw reads and feature counts for the transcriptomics experiment can be accessed on Array Express, for both the knockdown (E-MTAB-14948) and overexpression (E-MTAB-14949) experiments. The metabolomics data have been deposited to MetaboLights repository with the study identifier MTBLS12344.

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Competing interests

The authors report no competing interests.

Supplemental Material

Supplementary material is available at *Brain Communications* online.

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Figure Legends

Figure 1. Knockdown of neuronal and glial *Wwox* reduces longevity. **A)** Analysis of single cell RNAseq data acquired through SCOPE,²⁰ demonstrates that *Wwox* is expressed in both glia and neurons in the *Drosophila* brain. Data is presented as percentage of cells from each cluster expressing detectable levels of *Wwox* mRNA. Total number of cells in the whole dataset was 57,000 from N = 40 adult brains. **B)** Lifespan is reduced upon pan-neuronal *Wwox* knockdown (N = 91-105 per genotype). **C)** Distance climbed after 4 seconds (s) in a startle induced locomotion assay (7-, 14-, 21-and 28-day old flies) is unaffected by neuronal *Wwox* knockdown at (N = 3-6, n~10 flies per N). **D)** Glial knockdown of *Wwox* reduces median lifespan (N = 98-101). **E)** Startle induced locomotion (7-, 14-, 21-and 28-day old flies) is unaffected by glial knockdown of *Wwox* (N = 4-6, n~10 flies per N). Statistics = Lifespan was assessed via Mantel-Cox tests. Locomotion was assessed via mixed effect models (matching by vial, panel E) or two-way ANOVA with factors of age and genotype, followed by Šidák multiple comparisons (panel C). Data is represented as mean ± SD. For lifespan (panels B and D) N represents biological replicates of individual flies. For locomotion (panels C and E) N represents independent vials (data points plotted) of n individual flies (females). *p < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001, ns = non-significant. Abbreviations (panel A): DN1 = Dorsal clock neurons 1, OPN = olfactory projection neurons, adPN = antero-dorsal olfactory projection neurons, IPN = lateral olfactory projection neurons, Pm1/Pm2/Pm3 = proximal medulla neurons 1/2/3, Dm8 = Distal medulla neurons 8, Tm5c = transmedullary 5c neurons, a/b-KC = α/β lobe kenyon cells, a'/b'-KC = α'/β' lobe kenyon cells, G-KC = γ lobe kenyon cells, dFB = dorsal fan-shaped body neurons, T1 = T1 neurons, Lawf1/2 = lamina wide-field neurons 1/2, MBON = mushroom body output neurons, Mip = Myoinhibitory peptide neurons, TmY14 = transmedullary Y 14 neuron, Tm1 = transmedullary 1 neuron, TmY8 = transmedullary Y 8 neuron, Tm9 = transmedullary 9 neuron, T4/T5 = T4/T5 neurons, C2/C3 = centrifugal interneurons 2/3, Mt1 = medulla tangential 1, Mi1 = Medulla intrinsic neuron, L1/L2/L3/L4/L5 = lamina monopolar neurons 1/2/3/4/5.

Alt text: Graphs summarising statistical analyses, labelled A to E. A is a bar graph of *Wwox* expression across cell types, revealing high chiasm glia expression. B and D are Kaplan-Meier survival plots (age versus percentage survival). C and E are line graphs (age versus distance climbed after 4 seconds in centimetres) depicting a decrease in climbing with age but not *Wwox* knockdown.

Figure 2. Knockdown of *Wwox* decreases wakefulness and increases sleep. Pan-neuronal *Wwox* knockdown decreases **A)** locomotor activity, **B)** increases sleep pressure and **C)** increases mean sleep episode duration in minutes (mins). **D)** Pan-glial *Wwox* knockdown decreases locomotor activity, **E)** increases sleep pressure and **F)** increases mean sleep episode duration. Sleep metrics were collected over n > two independent runs, N = 31-80 individual flies per genotype. **G)** Rhythmicity statistic (RS) and **H)** period length of circadian behaviour was unaffected upon pan-neuronal *Wwox* knockdown in dark conditions. **I)** Representative double plotted actograms over 24 hours (hrs) for pan-neuronal *Wwox* knockdown. **J)** RS and **K)** period length were unaffected upon pan-glial *Wwox* knockdown. **L)** Representative actograms for pan-glial *Wwox* knockdown. Dotted lines at RS = 1 (weakly rhythmic threshold), RS = 1.5 (strongly rhythmic threshold) and period length = 24 hrs. Circadian rhythmicity was analysed from N = 17-34 (panel G) and N = 6-42 (panel J) individual flies per genotype, due to

removal of deaths. Whereas circadian period was assessed from N = 12-31 (panel H) and N = 4-37 (panel K), due to the further removal of arrhythmic flies. Statistics = Sleep metrics were analysed via two-way ANOVA followed by Bonferroni multiple comparisons, further corrected for multiple comparisons across all sleep metrics by 5 % FDR. $p_{adj} * < 0.05$, **** < 0.0001 . Circadian data was analysed via two-tailed unpaired t-test or non-parametric Mann-Whitney tests (non-significant). Data points represent individual flies (0-6 day old males), mean $\pm$ SD.

Alt text: Graphs summarising statistical analyses of sleep and circadian metrics, labelled A to L. A to C and D to F are bar graphs depicting the effect of *Wwox* knockdown and light phase on sleep. G, H, J and K are bar graphs of circadian rhythmicity and period. I and L are four representative double plotted actograms from control or knockdown in the neuronal or glial experiments (time in hours on the x axis and days on y axis).

Figure 3. *Wwox* knockdown exacerbates $A\beta_{42}$ related phenotypes. **A)** Lifespan is reduced upon pan-neuronal *Wwox* knockdown in the presence of $A\beta_{42}$ overexpression (N = 45-68 individual flies). **B)** Distance climbed after 4 seconds (s) in a startle induced locomotion assay (7-, 14-, 21- and 28-day old flies) is reduced upon pan-neuronal *Wwox* knockdown in the presence of $A\beta_{42}$ overexpression (N = 3-5 vials of n~10 flies). **C)** $A\beta_{42}$ immunocytochemistry (cyan) from day 14 *Drosophila* brains upon *Wwox* knockdown in neurons. **D)** Quantification of $A\beta_{42}$ staining revealed no changes in number (N = 5-7 brains) or **E)** plaque size (N = 5-7 brains) after neuronal *Wwox* knockdown. **F)** Schematic of $A\beta_{42}$ quantification in head soluble and insoluble fractions by MSD assay (day 14). Created in BioRender. Maddison, D. (2026) <https://BioRender.com/d4bip72>. **G)** Total and **H)** soluble $A\beta_{42}$ levels quantified by MSD assay increase with *Wwox* knockdown in neurons (N = 3 pooled replicates of n = 25 heads each). **I)** But identified no changes in insoluble or **J)** the insoluble/soluble ratio (N = 3 pooled replicates of n = 25 heads each). Statistics = Lifespan was analysed by Mantel-Cox and Cox proportional hazard tests. Startle-induced locomotion was analysed by two-way ANOVA with factors of age and genotype (Šídák's multiple comparisons correction). Plaque number was assessed by Mann-Whitney tests and size by unpaired t-test. MSD assay was analysed by one-way ANOVA followed by Dunnett's multiple comparisons tests $p/p_{adj} * < 0.05$, ** < 0.01 , *** < 0.001 , **** < 0.0001 . Mean $\pm$ SD. Scale bar = 100 μ m. All experiments were performed on females. Datapoints represent N: independent vials (panel B), independent brains (panels C-E), independent pools of brains (panels G-J).

Alt text: Graphs summarising statistical analyses of reducing *Wwox* in the presence of amyloid, labelled A-J. A is a Kaplan-Meier plot (age in days versus percentage survival). B is a line graph (age in days versus distance climbed after four seconds in centimetres). C are two representative images of brains stained for amyloid beta 42. D and E are bar charts. F is a schematic describing preparation of soluble and insoluble fractions. G to J are bar plots summarising MSD results for each genotype with LacZ control at 0 for each metric.

Figure 4. Neuronal *Wwox* knockdown in the presence of $A\beta_{42}$ vastly alters the metabolic transcriptome. **A)** Knockdown of *Wwox* in a control background differentially expressed ($p_{adj} < 0.05$, $\log_2$ fold-change threshold of ± 0.58) 98 genes, 54 down (red) and 44 up (blue). **B)**

Pathway analysis revealed these were enriched in within metabolic pathways. **C)** Volcano plot summarising the significant up (51) and down regulated (23) genes after $A\beta_{42}$ expression. **D)** These were enriched within pyruvate and fatty-acid metabolism pathways. **E)** Knockdown of *Wwox* in the presence of $A\beta_{42}$ differentially expressed 112 genes (58 down, 54 up), **F)** enriched within lipid and pyruvate metabolism terms. Dotted lines on volcano plots (panels A, C and E) represent $\log_2$ fold-change threshold of ± 0.58 . Overrepresentation analysis (panels B, D and F) was performed using g:Profiler, size of datapoints represents the number of genes identified in within the term. Abbreviations represent unique databases which were used for overrepresentation analysis GO:BP Gene ontology biological processes, GO:MF Gene ontology molecular functions, KEGG Kyoto Encyclopedia of Genes and Genomes, TF TRANSFAC, WP Wiki pathways. Transcriptomics was performed on N = 4 replicates of n = 10 female heads per genotype. Differential expression was performed using DEseq2, overrepresentation pathway analysis was performed using g:Profiler. Non-sig = non-significant.

Alt text: Volcano plots and overrepresentation analyses for transcriptomics experiment investigating the effect of *Wwox* knockdown. A, C and E are volcano plots (adjusted p value versus $\log_2$ fold-change) for each contrast. B, D and F are pathway enrichment dot plots for each respective contrast, split by database, summarising enriched metabolic pathways.

Figure 5. Neuronal *Wwox* knockdown in the presence of $A\beta_{42}$ overexpression increases L-lactate levels via increased lactate dehydrogenase expression. **A)** Volcano plots summarising the significant ($p_{adj} < 0.1$) up- and down-regulated metabolites for the main effect of *Wwox* knockdown or **B)** the main effect of $A\beta_{42}$ (day 14). **C)** Metabolite counts for L-lactate in each condition reveals a large increase, after knockdown of *Wwox* in the presence of $A\beta_{42}$ (N = 5 replicates of n = 10 pooled heads, mean $\pm$ SEM). **D)** Schematic of changes in pyruvate metabolism revealed by the statistical interaction between *Wwox* knockdown and $A\beta_{42}$ overexpression from omic analyses. Differentially expressed transcripts and metabolites are depicted with arrows showing their direction of change, transcripts are represented in boxes. Created in BioRender. Lucas-Clarke, H. (2026) <https://BioRender.com/fwthumj>. **E)** Knockdown of lactate dehydrogenase (*Ldh*) rescued startled induced locomotor deficits in *Wwox* knockdown (+ $A\beta_{42}$) flies at 14-, 21- and 28 days (N = 4-6 of n = 5-10 flies). **F)** Representative images of brains expressing the endogenously tagged Sima:GFP reporter in day 14 flies. **G)** Quantification revealed no differences in sima levels (N = 7-9 brains). **H)** Atf4 immunocytochemistry in day 14 fly brains, **I)** demonstrated an upregulation of Atf4 in the brain after *Wwox* knockdown in the presence of $A\beta_{42}$ (N = 6 brains). **J)** Schematic representation of the effect of *Wwox* knockdown on pyruvate metabolism and $A\beta_{42}$ aggregation via Atf4. Created in BioRender. Lucas-Clarke, H. (2026) <https://BioRender.com/4ljuoko>. Statistics = Two-way ANOVA followed by multiple testing correction with 10 % FDR (panels A-C), two-way ANOVA mixed effect model (Šídák's multiple comparisons correction) (panel E), two-tailed unpaired t-test (panel G) and Mann-Whitney Test (panel I). $p/p_{adj}^* < 0.05$, $** < 0.01$, $*** < 0.001$, $**** < 0.0001$. Mean $\pm$ SD, unless otherwise stated. Scale bars = 100 μ m. Dotted line in panels A-C represents $p_{adj} = 0.1$. All experiments were performed on females. Datapoints represent N: independent replicates of pooled heads (panel C), independent vials (panel E) and independent brains (panels G, J).

Alt text: Graphs summarising statistical analyses and schematics related to the effect of *Wwox* knockdown on the metabolome, labelled A to J. A and B are volcano plots for in each contrast of interest ($\log_2$ fold-change versus adjusted p value). C is a bar plot of lactate levels in each group LacZ, Amyloid, RNAi control and *Wwox* knockdown. D is a pathway schematic. E is a line graph (age versus distance climbed after four seconds in centimetres). F and H are representative images of brains. G and I are bar plots quantifying fluorescence. J is a schematic summarising the proposed pathway.

Figure 6. Neuronal upregulation of *Wwox* improves longevity, behaviour, and reduces amyloid load. **A)** Upregulation of *Wwox* in neurons expressing $A\beta_{42}$ significantly improved lifespan (N = 78-91 individual flies), **B)** and distance climbed after 4 seconds (s) in a startle induced locomotion assay locomotion (N = 3-7 vials of n = 5-10 flies) at days 14 and 21. **C)** Representative images of $A\beta_{42}$ staining (cyan) in *Drosophila* brains (day 14). **D)** Quantification revealed a significant decrease in median plaque size and **E)** number (N = 5-7 brains). Using the MSD assay for amyloid we revealed decreased **F)** soluble and **G)** insoluble $A\beta_{42}$ (N = 3 replicates of n = 25 heads). $p/p_{adj}^* < 0.05$, $** < 0.01$, $*** < 0.001$, $**** < 0.0001$. Mean $\pm$ SD. Scale bar = 100 μ m. Statistics = Mantel-Cox tests (panel A), two-way ANOVA mixed effect model (Šídák's multiple comparisons correction) (panel B) and two-tailed unpaired t-tests (panels D-G). All experiments were performed on females. Datapoints represent N: independent vials (panel B), independent brains (panels D-E) and independent replicates of pooled heads (panel F-G).

Alt text: Graphs and representative images exploring the effect of *Wwox* overexpression in the presence of amyloid, labelled A to G. A is a Kaplan-Meier plot (age versus percentage survival). B is a line graph (age versus distance climbed after four seconds in centimetres). In C are two representative images of brains stained for amyloid. D to G are bar graphs revealing decreased amyloid burden with *Wwox* overexpression via two methods.

Figure 7. Neuronal overexpression of *Wwox* reverses the metabolic, immune and synaptic transcriptomic reprogramming caused by $A\beta_{42}$ expression. **A)** Volcano plot for the effect of *Wwox* overexpression in a control background reveals dysregulation of six genes (down), **B)** overexpression of $A\beta_{42}$ downregulated 51 genes and upregulated 28, and **C)** overexpression of *Wwox* in the presence of $A\beta_{42}$ differentially expressed 364 genes (305 down, 59 up). **D)** Heatmap of z-scores of all the uniquely differentially expressed genes and their expression in each condition reveals a clear reversal of the amyloid signature with *Wwox* overexpression. **E)** 40 genes were commonly dysregulated between $A\beta_{42}$ expression alone and $A\beta_{42}$ expression with *Wwox* overexpression. The $\log_2$ fold-change of each of these genes were significantly negatively correlated suggesting a reversal of the transcriptional profile. Those genes that were upregulated by $A\beta_{42}$ and subsequently downregulated with *Wwox* overexpression were enriched within immune pathways (left) whereas genes that change in the opposing direction were enriched with synaptic transmission. Datapoints in panel E represent each of these individual 40 genes. Dotted lines on volcano plots (panels A-C) represent $\log_2$ fold-change threshold of ± 0.58 and p_{adj} threshold of 0.05. N = 4 replicates of n = 10 pooled heads. Statistics

= Differential expression was performed using DEseq2 (panels A-C), pathway analysis was performed using g:Profiler (panel E), z-scores (panel D), Pearson correlation coefficient (panel E). All experiments were performed on day 14 females. Abbreviations: LFC = log₂ fold-change, Non-sig = non-significant.

Alt text: Graphs summarising transcriptional alterations after *Wwox* overexpression in the presence of amyloid, labelled A to E. A to C are volcano plots for each contrast (log₂ fold-change versus adjusted P). D is a heatmap (blue to red) of gene z-scores across the genotypes. E is a scatter plot of log-fold change *Wwox* overexpression in amyloid background versus log₂ fold-change amyloid main effect. To the left and right of this scatter plot are overrepresentation analyses bar plots for pathway enrichment (colour = p value, x = number of genes, y = pathway).

Figure 8. Neuronal overexpression of *Wwox* alters L-methionine levels. Volcano plots **A)** illustrating the differentially expressed metabolites due to the main effects of A β ₄₂ expression or **B)** *Wwox* overexpression. Labelled metabolites significantly altered after FDR correction ($p_{adj} < 0.1$) and standard matched. **C)** Log₂ fold-changes were plotted for the effect of *Wwox* overexpression in a LacZ background or A β ₄₂ background, revealing differing effects on L-methionine levels. **D)** Schematic of enzymes encoded by downregulated transcripts (pink boxes) within the cysteine and methionine metabolism KEGG pathway revealed in our analysis of *Wwox* overexpression in the presence of A β ₄₂. L-methionine metabolites were reduced in the A β ₄₂ model (pink arrow) and remain low when *Wwox* is overexpressed in the presence of A β ₄₂ (interaction effect). Created in BioRender. Lucas-Clarke, H. (2026) <https://BioRender.com/zz1zyt8>. N = 5 replicates of n = 10 pooled day 14 female heads. Statistics = two-way ANOVA followed by multiple testing correction with 10 % FDR.

Alt text: Graphs and schematics outlining metabolic changes, labelled A to D. A and B are volcano plots (log₂ fold-change against adjusted p value). C is a scatter plot of log₂ fold-changes of genes in the control background versus amyloid background. D is a schematic revealing downregulation of transcripts and metabolites within the methionine pathway.

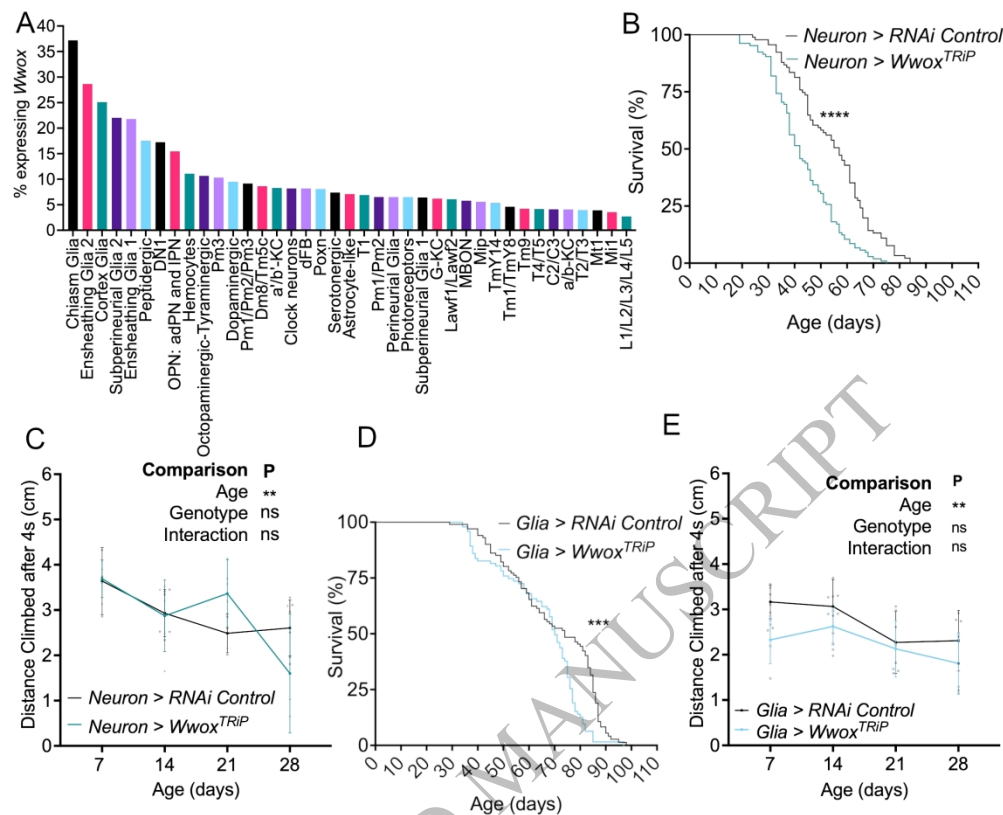


Figure 1

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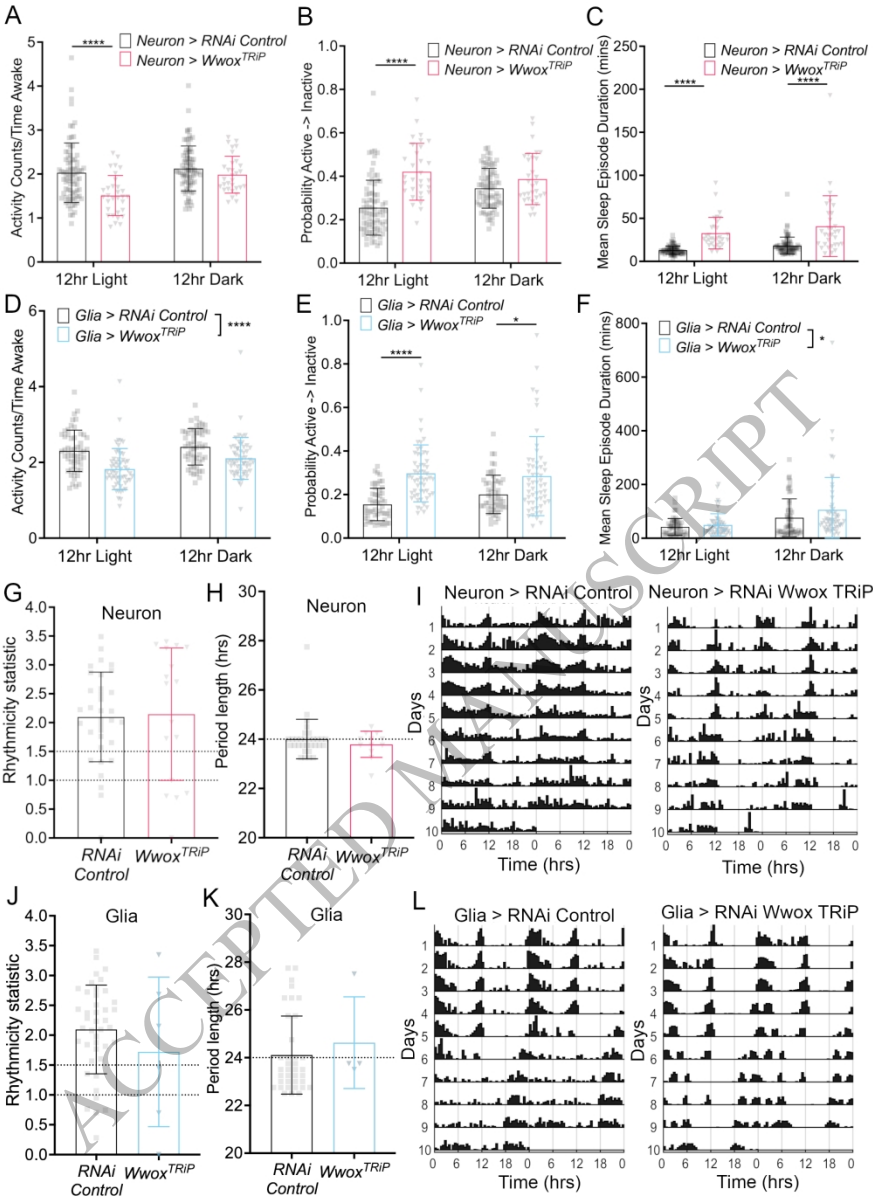


Figure 2

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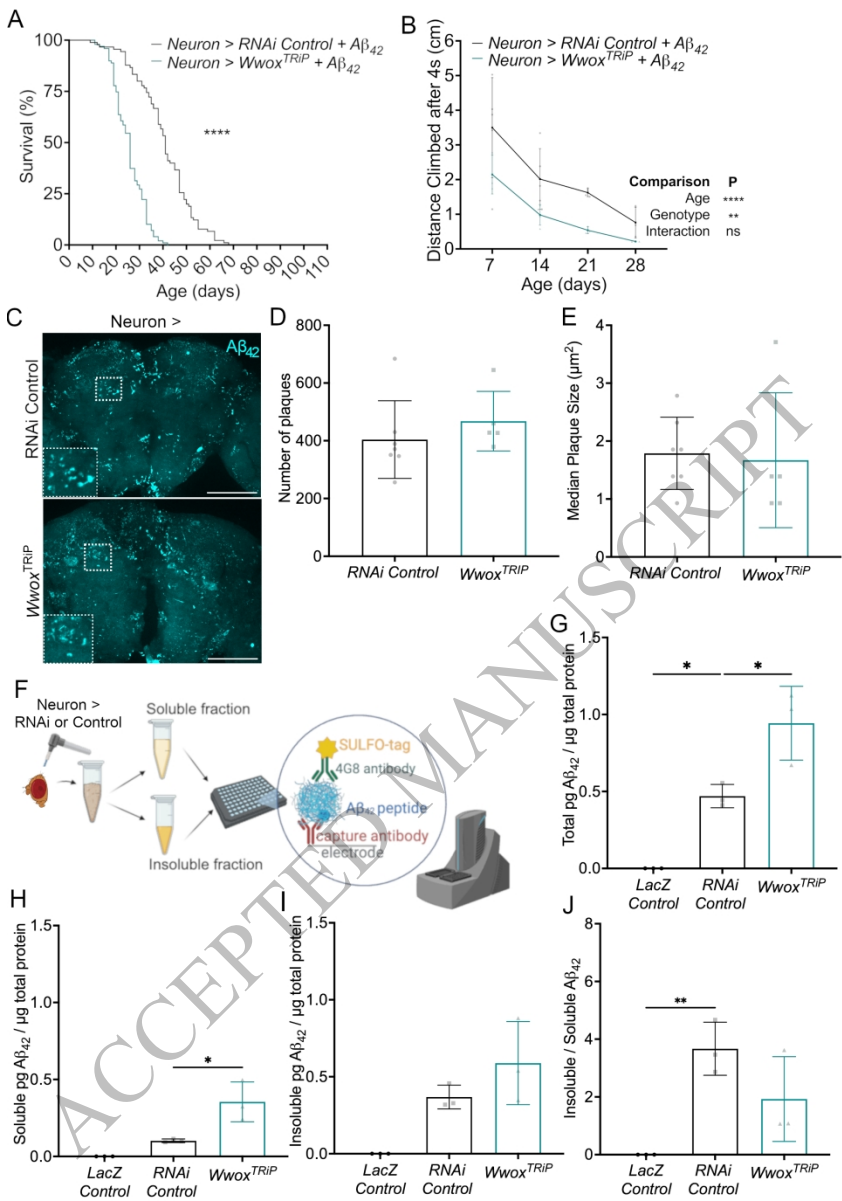


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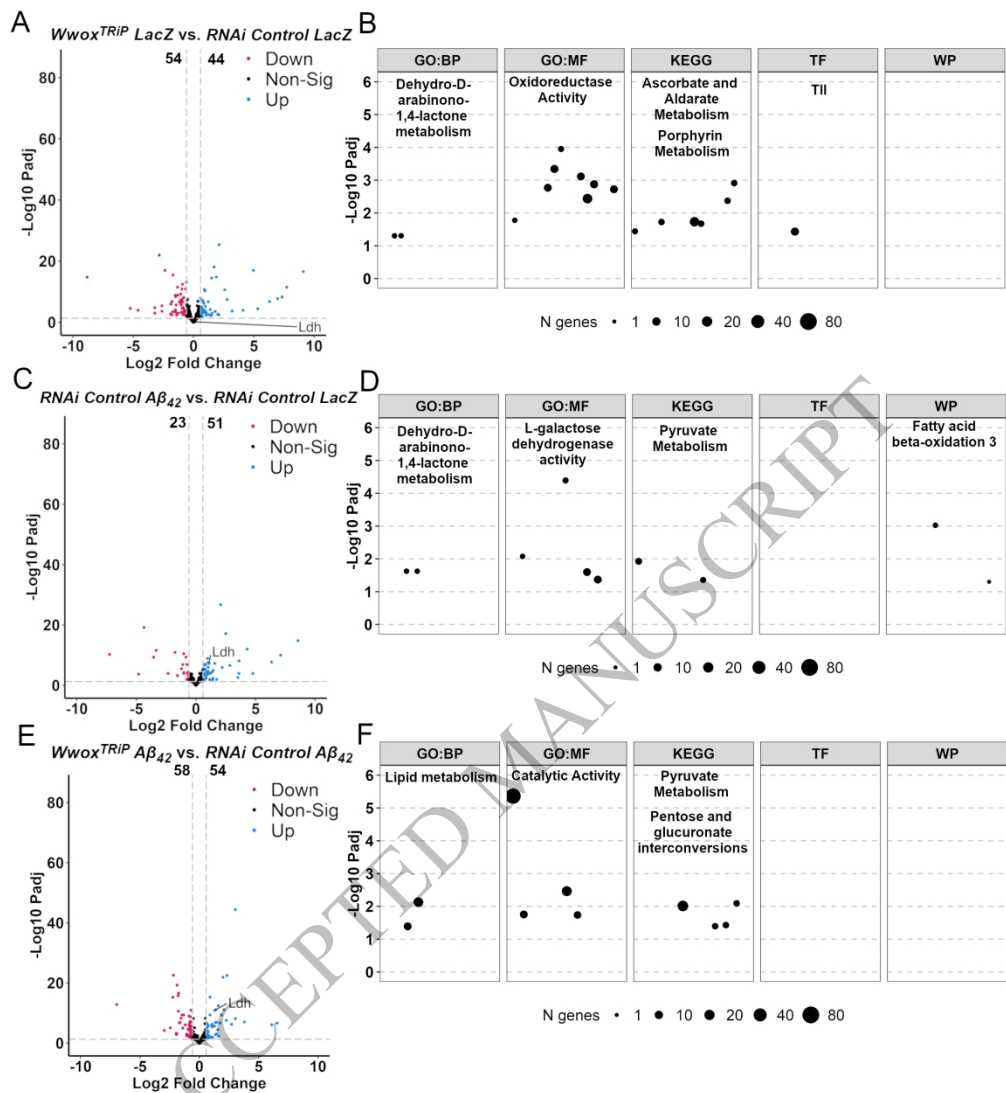


Figure 4

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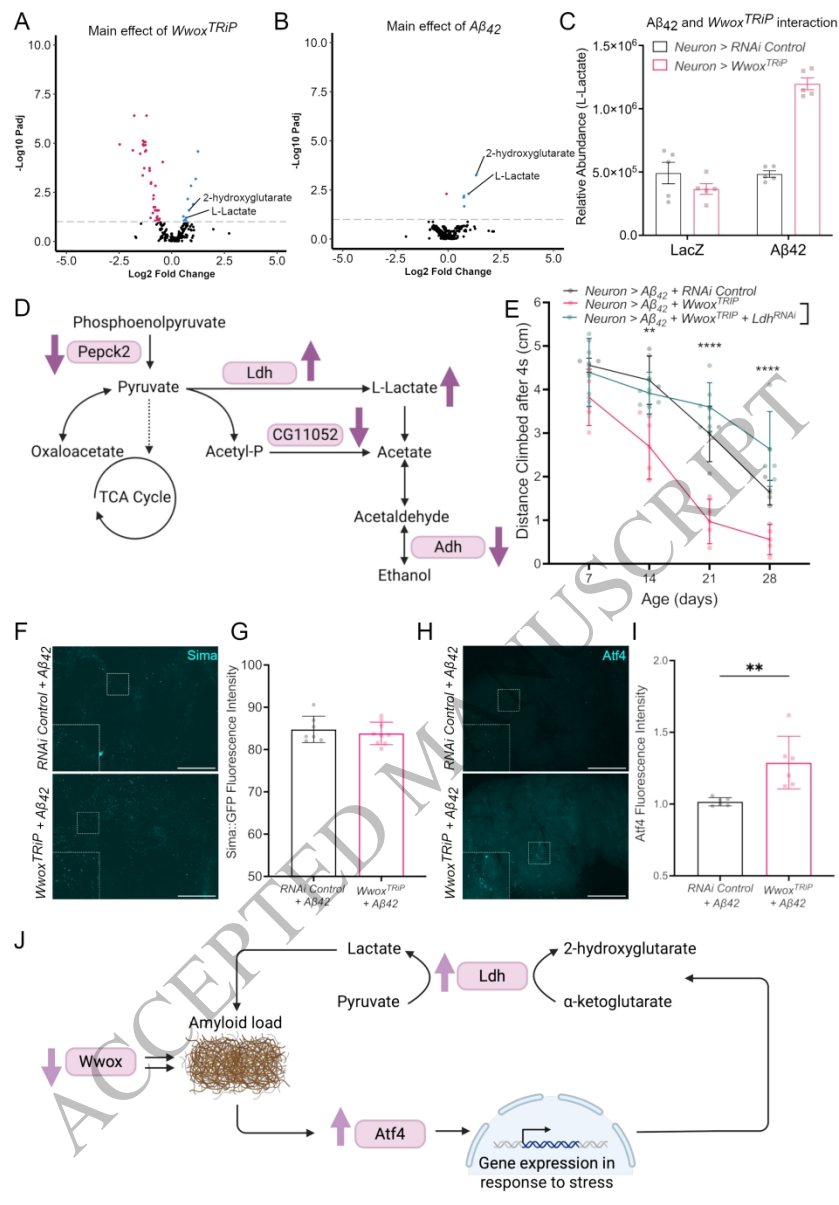


Figure 5

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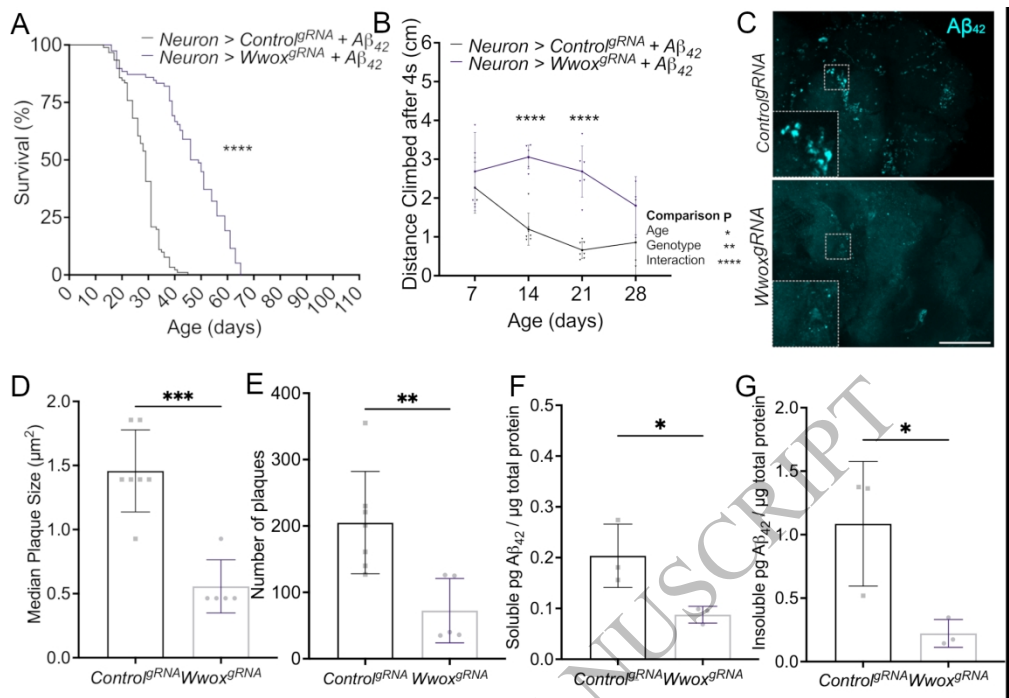


Figure 6

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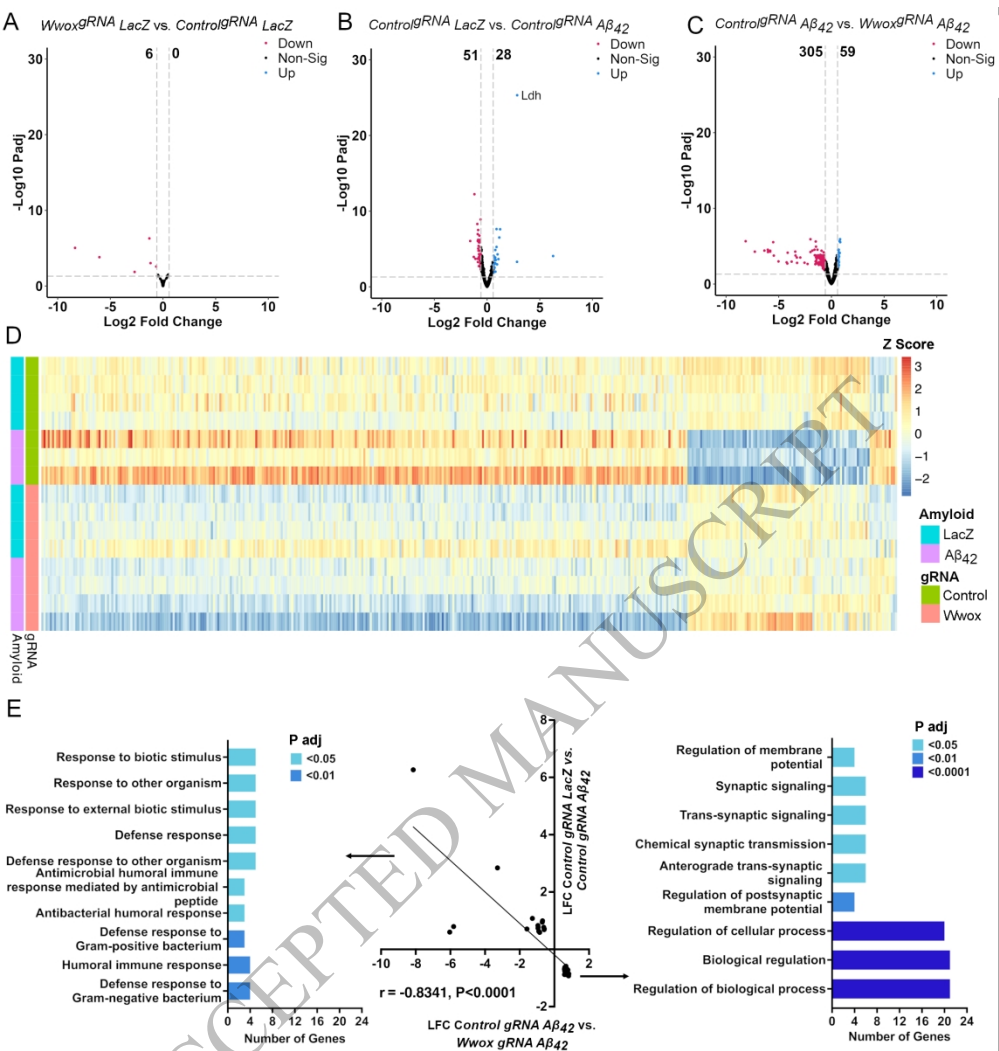


Figure 7

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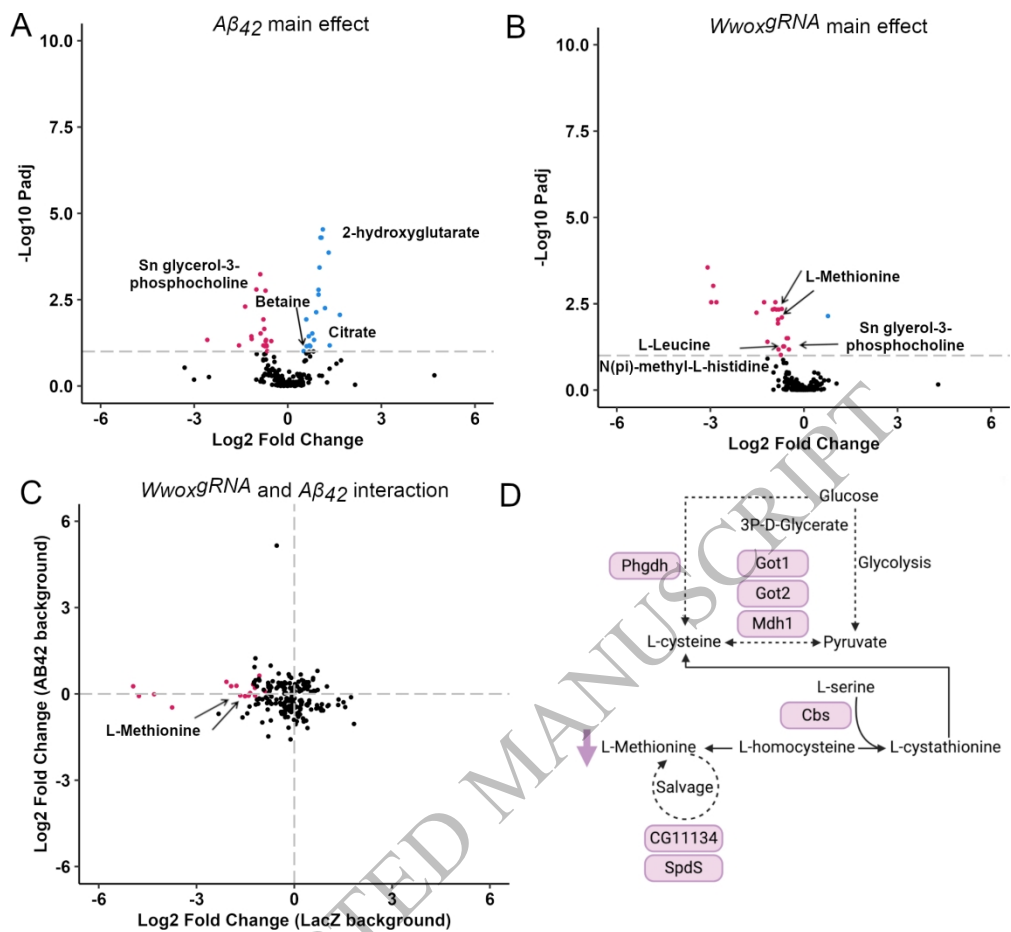
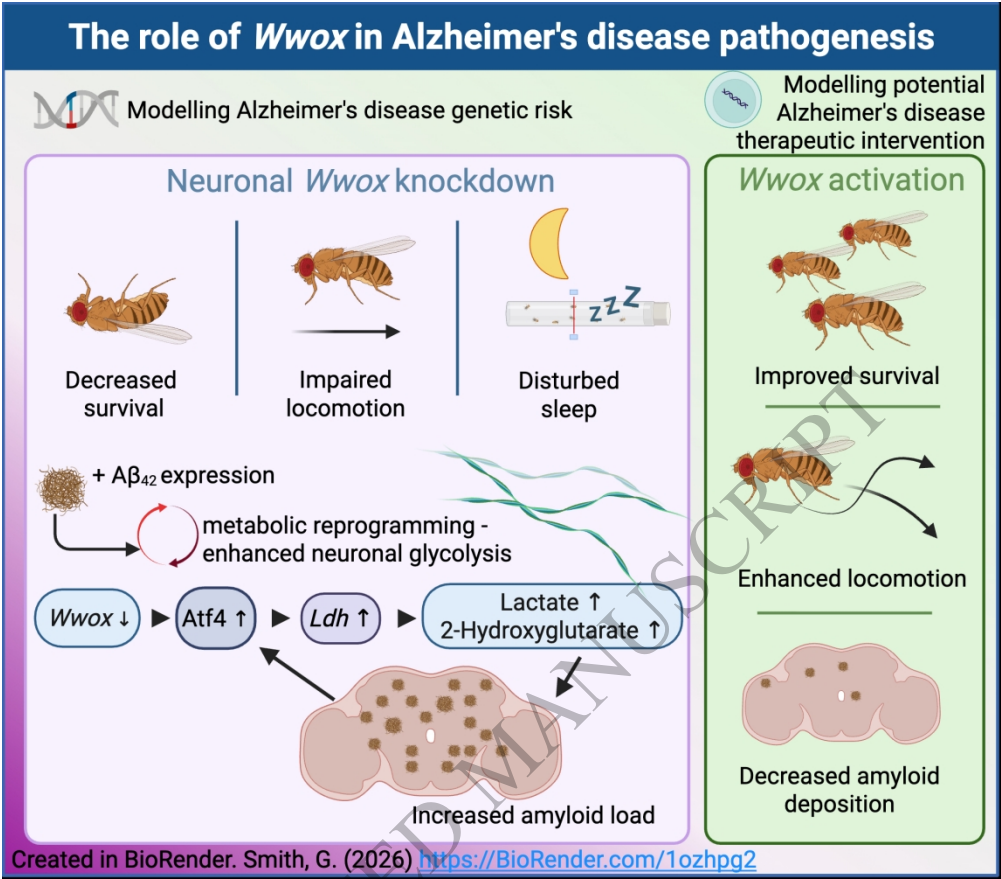


Figure 8

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Graphical abstract

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