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Reducing the expression of *Lap* or *Zydeco* in *Drosophila* causes phenotypes similar to Alzheimer's disease

By:

Thomas Parsons

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Physiology and Pharmacology (MSc by Research) in the Faculty of Life Sciences.

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// Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder, affecting approximately 50 million people worldwide, resulting in the progressive loss of cognition. Although there has been some progress in determining the underlying causes of the pathology, most cases are sporadic, meaning there is an urgent requirement to identify and characterise novel genes implicit in its pathogenesis. One way in which this is achieved is through Genome-Wide and Epigenome-Wide Association Studies. These studies can identity gene loci potentially linked to the disease. However, these loci tend to lie between genes or in non-coding regions of the genome and hence the findings often result in false positives. Therefore, the use of *Drosophila* is integral in these studies; by using *Drosophila,* experiments can be performed in a cost and time efficient way to screen these. Using the GAL4/UAS system, a genetic manipulation tool in *Drosophila,* I expressed *RNAi* against *Drosophila* orthologs of human genes highlighted from Genome-Wide and Epigenome-Wide Association Studies, reducing their expression. I then screened through these mutants using assays set up to identify AD-like behaviour in *Drosophila*. *RNAi* expression in the eye gave a reduction in the surface area, a phenotype associated with the degeneration of photoreceptors. *Zyd,* encoding a potassium dependent sodium/calcium exchanger, and *Lap,* encoding a protein involved in vesicle formation, showed promising results in this assay, and so were chosen to be further investigated. Decreasing expression of *Zyd* or *Lap* in all neurons caused a shortened lifespan and a reduction in the climbing ability of the *Drosophila*. Other AD-like behaviours were observed with these flies; reduced sleep and an impairment in memory. These results are consistent with the effect displayed by human AD causal genes, Tau and Aβ42 overexpressed in *Drosophila*. These results demonstrate that the reduction in both *Lap* and *Zyd* has a potential link to AD pathology.

Lay Abstract

Alzheimer's disease (AD) is a disorder that causes destruction of neurons in key regions in the brain important for cognition. This is why the behaviours observed with human AD patients arise; the breakdown of these neurons results in the improper function of these brain regions. This disease affects approximately 50 million people worldwide and has a massive strain on the lives of sufferers and their families. However currently, there are no treatments for the root cause; only medication to help manage the symptoms. In order to develop effective drugs, a better understanding of the root causes of AD is required. Currently, there are two main proteins involved in the pathology of AD, Tau and Amyloid-β, found by analysing the brains of AD patients post-mortem. These proteins accumulate in the brain and cause the destruction of neurons. However, the lack of new targets for drug treatment means that research is needed into potential new proteins that may be affecting the brain throughout this disease. Recent studies looking at the human genome of AD patients have identified new genes that may be implicit in the disease. In the present study, I used a type of fruit fly, called *Drosophila*, and mis-expressed *Drosophila* genes similar to these potential disease causing genes in order to study their effects. As *Drosophila* only live for a relatively short period of time, the disease progression can be tracked quickly. Tau and Amyloid-β cause degeneration of the neurons in the brain and, when these proteins are expressed in flies, they cause similar AD-like behaviours (eg, impairment in memory and disruption of sleep). When reducing the expression of my two candidate genes, *Lap* and *Zyd* in *Drosophila*, I found these similar behavioural deficits.

Covid-19 statement

Because of the COVID-19 pandemic in early 2020, some of my work in the lab was hindered. In March 2020, during the middle of my allocated time in the lab to collect data for my thesis, I was not allowed into the lab for approximately one month. During this time, I was in the middle of ongoing experiments that were due to last for at least a few more weeks; these experiments were voided and restarted when permission was granted into the building again. The experiments included a climbing assay, in which the flies negative geotaxic response is tested every week for four weeks, and a longevity assay, where the effect of the misexpression of the gene on the fly's lifespan is determined. As I was looking at age dependent effects in these experiments, the flies had to be thrown away and the data were voided. After this period, I was given special access to the lab but this was only to maintain the Drosophila stocks in the lab, and it was specified that no experiments were allowed to be undertaken.

I did not have enough time to carry out qRT PCR experiments on each of my RNAi lines, which would have given me useful information on how much of the gene was knocked down and therefore would have allowed me to better explain the data in my discussion.

Although I was given more time in the lab once we were allowed back in, my progress was hindered. Because of the nature of working with flies, experiments cannot be immediately set up—for example, the flies need to breed. Breeding can take up to three weeks because the correct male and female genotypes need to cross, the females need to lay eggs and then the larvae need to hatch.

Finally, there was the issue of funding, I had originally budgeted to stay in Bristol doing my master's degree until July, when my tenancy at my flat was due to end. However, because of the delays, I had to stay until December to finish my experiments, incurring more living and rental costs. This was an added stress in an already very stressful period.

Acknowledgements

Firstly, I would like to thank to my wonderful supervisor, Dr James Hodge, for all his hard work in allowing this project to succeed (particularly throughout a pandemic). Dr Hodge taught me the basics of working with *Drosophila*, helped with the experimental methods used in this study and gave useful advice throughout the project. His continued passion for the subject is inspiring. I would also like to thank Dr Edgar Buhl for his expertise in teaching me the experimental techniques required for many of the assays used throughout my time in the lab, and for his perseverance when answering the many questions I had for him, especially with regards to statistical analysis. I would also like to thank Drs Sergio Hidalgo and Kiah Tasman for all their support and great advice. Finally, a big thanks to Dr Bangfu Zu for his help in collecting data for figure 13 with me.

Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is my own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: DATE:

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Abbreviations

AD – Alzheimer's Disease

AICD - Amyloid precursor protein intracellular domain

ANOVA - One-way analysis of variance

APOE - apolipoprotein E

APP - Amyloid precursor protein

APP_{SW} – Swedish familial AD mutation

β-CTF – Beta C terminal fragment

CHO - Chinese hamster ovary cells

CNS – Central Nervous System

CS^{W-} - Canton S white -

DAG – Diacylglycerol

DAM – *Drosophila* activity monitor

DIOPT – *Drosophila RNAi* Screening Center Integrative Ortholog Prediction Tool

DNA – Deoxyribonucleic acid

DRSC - *Drosophila RNAi* Screening Center

Elav - embryonic lethal abnormal visual system

EOAD – Early onset Alzheimer's Disease

ER - Endoplasmic reticulum

EWAS - Epigenome-wide association study

FAD - Familial Alzheimer's Disease

GFP – Green Fluorescent Protein

GMR - Glass multiple reporter

GOF – Gain of function

GWAS – Genome-wide association study

HEK – Human embryonic kidney cells

LOAD – Late onset Alzheimer's Disease

LOF – Loss of function

MAPT - Microtubule-associated protein Tau

MB – Mushroom body

MCH – 4-methylcyclohexanol

NCOR2 - Nuclear Receptor Co-Repressor 2

OCT – Octanol

PI – Performance Index

PICALM - Phosphatidylinositol Binding Clathrin Assembly Protein

PLCG – Phospholipase C gamma

qRT PCR – Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

RNA - Ribonucleic acid

RNAi **- Ribonucleic acid interference**

SAD – Sporadic Alzheimer's Disease

SCAMP – Sleep and Circadian Analysis MATLAB Program

SEM – Standard error of the mean

SL – Small wing

SNP – Single nucleotide polymorphism

ST6GAL-1 – Alpha2,6-sialyltransferase

TET1 - Ten-Eleven Translocation 1

Tim – TIMLESS

UAS – Upstream activation sequence

Zyd – Zydeco

Introduction

The pathology Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder that results in the progressive loss of cognition. It is the most prevalent form of dementia, contributing 60–70% of the World Health Organization's estimate of 50 million people affected by dementia worldwide. It is named after Dr Alois Alzheimer who first characterised the disease after examining the brain of a women who suffered from severe memory loss. He noted the presence of large aggregates and tangles in her brain. These are now known to be amyloid-beta (β) plaques and neurofibrillary tangles, respectively (4).

Most AD cases (approximately 95%) are sporadic (SAD). These have no familial genetic link and are caused by a combination of environmental factors and spontaneous gene mutations. The greatest risk of developing SAD is aging, as almost all cases are late onset (>65 years old) AD (LOAD) (5). However, there are six environmental factors: air quality, diet, exercise, gender, smoking and educational attainment, that are also associated with an increased risk of SAD (6). There is no single gene mutation that causes SAD, but one polymorphism in apolipoprotein E (*APOE*) has been found that is associated with an increased risk of developing SAD (7).

Early onset AD (EOAD) (<60 years old) accounts for approximately 5% of AD cases. Some of these cases are characterised as familial AD (FAD). FAD is the form of AD that is heritable, with every case being the result of a mutation passed down from one or both parents (7). Mutations in the amyloid-β pathway is the major cause of FAD, with mutations in amyloid precursor protein (*APP*), and presenilin 1 and presenilin 2 being the most prevalent within these cases (8). Misprocessing of *APP*, as a result of one of these mutations, causes the formation of one of the hallmarks of the disease, Aβ42 (Figure 1). *APP* is cleaved by a series of secretases that determine how it is processed, but the product of this pathway differs depending on what secretases cleave the *APP*. In the non-amyloidogenic (non-harmful) pathway, *APP* is cleaved by α-secretase, followed by γ-secretase (presenilin 1 and presenilin 2 form part of this enzyme) to form the neuroprotective p3 peptide. Harmful Aβ peptides are formed when the first cleavage is by β-secretase, before cleavage by γ-secretase (9). Aβ peptides can range in length but the larger peptides (Aβ42 primarily) correlate with more neurodegeneration as they are more hydrophobic and can therefore more easily form oligomeric complexes with other Aβ monomers (10).

There are two major pathways thought to be central to the pathogenesis of AD. These two pathways result in the deposition of insoluble protein that causes the atrophy associated with AD and forms in the entorhinal cortex, hippocampus and amygdala regions of the brain (11). These three regions (all of which are located in the temporal lobe) are areas associated with forming and processing of memory in humans (12) and hence the loss of neurons here can result in an impairment in memory. The first, amyloid-β, is described above. The other protein now widely associated with AD is encoded by the microtubule associated protein Tau (MAPT) gene. Tau becomes toxic to neurons when it is hyperphosphorylated and forms large insoluble complexes called neurofibrillary tangles (NFTs). Both Aβ peptides and NFTs are hallmarks of AD and are thought to combine to cause the neurodegeneration and synaptic dysfunction in AD. Histological analysis of post-mortem AD brains shows the prevalence of these proteins (11).

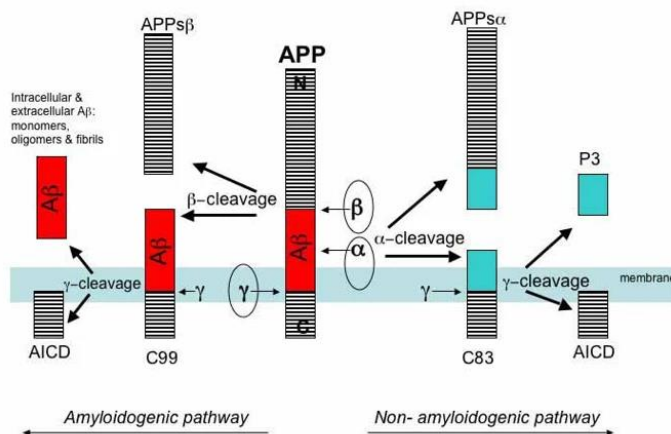


Figure 1. Differential processing of amyloid precursor protein (*APP*). When *APP* is first cleaved by α-secretase, followed by γ-secretase, this is known as the non-amyloidogenic pathway. C83 and soluble *APP* α are formed from the first cut α-secretase makes, the second results in the formation of the protective P3 peptide and *APP* intracellular domain (AICD). The amyloidogenic pathway results in the formation of toxic Aβ isoforms due to *APP* first being cleaved by β-secretase. This leaves the membrane bound fragment, C99, and soluble *APP* β, and C99 is subsequently cleaved by γ-secretase forming amyloid-β and AICD (10).

There is also evidence that links the immune system to AD. The immune system aids in the fight against the build-up of toxic oligomeric Aβ in neurons through the work of microglia. These are the main line of immune defence in the central nervous system. Through phagocytosis, microglia help in the clearance of toxic Aβ42 proteins. It thought that as the body ages, the immune system weakens, and this process is subsequently dampened (13). This clearance of Aβ, however, can bring other problems as it can cause inflammatory responses by the cells that then secrete cytokines which can worsen the damage caused by Aβ (13).

The use of *Drosophila melanogaster* in AD research

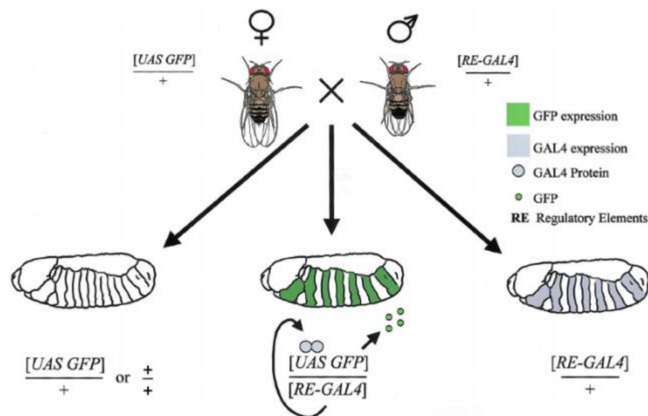


Figure 2. The GAL4/UAS system in *Drosophila.* The UAS line from the female fly, in this example, is crossed with the GAL4 line from the male (this also works the other way around). In the heterozygote offspring, expressing both transgenes, the GAL4 protein is expressed in the specific region depending on the promoter. GAL4 can bind the UAS which initiates transcription of the gene upstream (green fluorescent protein (GFP) here) hence producing green stripes in the fly embryo shown here (3).

The fruit fly, *Drosophila melanogaster,* has been extensively used to study AD (14) (15), as it is a cost effective animal with many genes conserved with humans, including orthologs of 75% of human disease causing genes (16). This makes *Drosophila* a convenient species to study AD risk genes. Their genome is also very easy to manipulate. One way in which this is achieved is through the GAL4/UAS system first described by Brand and Perrimon (17). Briefly, a yeast transcription factor, Gal4, is randomly inserted into the *Drosophila* genome; this is the GAL4 line. The Gal4 will sit downstream of promoter elements that determine where and when the Gal4 protein is produced in the fly. The other line, known as the reporter line, contains the GAL4 binding site and is termed the upstream activation sequence (UAS); this sits upstream of the transgene of interest. When the two lines are crossed, the Gal4 protein can bind UAS, activate it and initiate transcription of the gene of interest (Figure 2), Gal4 or UAS insertions alone should be wild type as there are no such genes endogenous to the fly genome.

To determine where Gal4 was inserted into the genome, the Gal4 line can be first crossed with UAS-green fluorescent protein (GFP); this allows the expression pattern of Gal4 to be visualised. Libraires of Gal4 tissue specific promoters have been characterised and collected in publicly funded stock centres (searchable via flybase.org). Therefore, flies can be selected, depending on where in *Drosophila* you want your gene to be expressed. This system permits specific targeted gene expression in *Drosophila*. GAL4 is used as a driver line for expression in a particular area of interest and the UAS allows misexpression of the gene of interest. This system can be used to mis-express an endogenous gene but can also be used to mis-express exogenous genes of interest (e.g., human MAPT (Tau) or human *APP* (Aβ42)). Using RNA interference (*RNAi*), knockdown of the expression of a gene of interest can be achieved. With *RNAi*, the gene downstream of UAS is an inverted repeat of the gene of interest.

Consequently, when UAS is activated, it causes the expression of double stranded RNA hairpins of this gene, resulting in the knockdown of RNA in the cell (18). This works well when studying gene defects, as 100% knockout (a null) will often result in extreme phenotypes that are potentially lethal when expressed. Another benefit of this system is that it can be used for specific tissue targeted gene expression as well as specific developmental stage targeted expression, both of which are useful in avoiding the confounding adverse effects seen in other models for instance knock outs or constitutively expressed mutations.

Using *Drosophila* is also much less time consuming, cheaper, and easier than creating a loss-of-function mutant in mice. Another benefit of using *Drosophila* is the ability to perform many experiments quickly because of the high fertility of the female (up to 100 eggs per female a day) and the short generation time. Generation time will vary with temperature, but at 25°C, it takes approximately 10 days. *Drosophila* only live for a relatively short time (≈ 60-70 days depending on the strain and sex (19)). This is advantageous when studying neurodegenerative disorders because disease progression is much shorter and therefore the long-term effects of these diseases can be determined more quickly.

Genome wide (GWAS) and epigenome wide (EWAS) association studies

Many GWAS for AD have been carried out to identify potential risk loci linked to the disease (20-27). These studies detect common single nucleotide polymorphisms (SNPs) in gene loci that are shared between a group of individuals with AD and not common in the group without AD. The SNP may be in a gene exon or intron or lie between two genes, making it difficult to determine which gene is associated with the disease; hence further genetic studies are required to determine which genes are linked to the disease and how. Model organism research is useful for this process. Epigenetic changes in AD have also been studied (28, 29). These are non-heritable changes in DNA sequences involving chemical changes to the DNA, including methylation or histone modification. These changes in histones can arise from addition or removal of a methyl, acetyl or phosphoryl group, and may result in changes in DNA expression through activation and inactivation of transcription, DNA damage and repair or changes in chromosome packaging (30). Epigenetic changes can arise as a result of environmental factors, such as age or diet (31). These studies are particularly important when studying AD because more than 90% of AD cases are sporadic, meaning there is no exact gene polymorphism

that is passed through generations. Instead, it is a combination of many gene defects and environmental factors over a life course that play an important role in these cases of AD (7). When analysing these gene loci found to determine potential AD risk genes there are lots of caveats to consider, for example, the SNP may sit between a number of genes or a long way away from the affected gene and hence it is not obvious which is being affected. Also, if the SNP falls in an intron compared to the exon, then this can make it harder to determine which gene is being affected. The type of mutation also needs to be considered, whether the SNP results in a LOF or GOF gene expression.

GWAS highlights genes linked to multiple pathways of AD pathology

The first four GWAS studies for AD were performed between 2009 and 2011 and identified nine different risk loci near 12 different genes; *CLU* and *CR1* (22), *PICALM* (21), *BIN1*, *EXOC3L2*, *BLOC1S3*, *MARK4* (23), *MS4A4*, *MS4A6E*, *CD2AP*, *CD33* and *EPHA1* (24). Lambert *et al* subsequently identified 11 new risk loci in their meta-analysis in 2013 (20), and three new GWAS studies were conducted between 2018 and 2019 (25-27). The GWAS association values (Table 1) from these studies indicate how likely, if the null hypothesis is accepted, the results are found by chance (32). This means that a lower value correlates to a stronger association to an AD related SNP.

By finding *Drosophila* orthologs of these genes identified by GWAS and EWAS (Table 1), their role in *Drosophila* through their misexpression can subsequently be investigated. This lays the foundation for finding their role in human AD pathogenesis as follow up studies in animals of greater brain complexity can then be performed on promising candidates. This is possible because many disease related genes are conserved between *Drosophila* and humans. Potential *Drosophila* orthologs of these genes can be determined through the *Drosophila RNAi* Screening Center Integrative Ortholog Prediction Tool (DIOPT) on FlyBase. This gives a score out of 15, depending on how similar the *Drosophila* gene is to the human gene of interest, based on an algorithm that accounts for sequence homology, phylogenetic trees and functional similarity. Genes that were picked for these studies were those that were consistently highlighted in GWAS, showed strong association values and had a strong *Drosophila* ortholog.

Human gene	Human protein	Fly gene	DIOPT score	GWAS association (P value)	Human expression	*Drosophila* expression	Amino Acid Similarity (%, from FlyBase)
PICALM	Phosphatidylinositol binding clathrin assembly protein	*Lap*	14	$9.3*10^{-26}$	Brain; endothelial cells	Highest expression in brain	51
$PLC_{\gamma 2}$	Phospholipase C gamma 2	*SL*	10	$5.38*10^{-10}$	Ubiquitous	Mostly throughout development, embryo	57
SLC24A4	NCKX4	*Zydeco*	7	$5.5*10^{-9}$	All regions of the brain, aorta, lungs, and thymus	Highest expression in the CNS	56
EphA1	Eph receptor tyrosine kinase	*Eph*	6	$1.1*10^{-13}$	High expression in endocrine tissues	Highest expression in the CNS	51
CR1	Complement receptor 1	*Hasp*	4	$5.7*10^{-24}$	Erythrocytes, T cells, B cells, monocytes, and granulocytes	Brain	34

Table 1. Summary of AD risk genes studied in these experiments, identified from GWAS, and the proteins they encode. Also shown is the most similar *Drosophila* ortholog to these AD risk genes and the similarity between the two genes (DIOPT score). The GWAS association value is also shown (from (20)), as well as tissue expression of the human and *Drosophila* genes, and the amino acid similarity of both genes.

PICALM is linked to synaptic dysfunction and the Aβ pathway.

A promising AD gene found in the GWAS was *PICALM* (21) (Table 1). This gene had the second strongest GWAS association for AD ($9.3*10^{-26}$) when Lambert *et al* performed their meta-analysis of GWAS hits in 2013, and has consistently been identified in GWAS studies for AD.

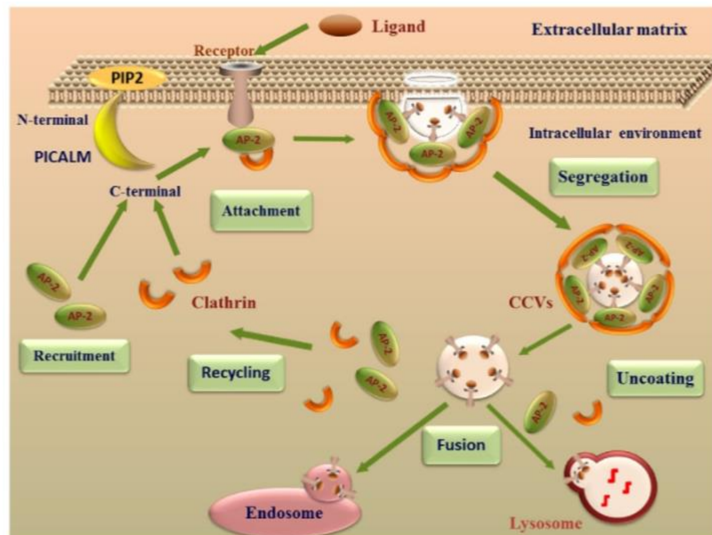


Figure 3. The role of PICALM in clathrin mediated endocytosis. The C-terminal of PICALM can bind clathrin triskelions and adaptor proteins, such as AP2 adaptor complex, in the cytoplasm whereas the N-terminal in the plasma membrane binds to phosphatidylinositol 4,5-bisphosphate (PIP2). This then allows the formation of a clathrin coating and eventually endocytosis (1).

In humans, PICALM encodes phosphatidylinositol binding clathrin assembly protein and is involved in clathrin mediated endocytosis (Figure 3) (1). The closest *Drosophila* ortholog to *PICALM*, is like-AP180 (*Lap*), with 51% amino acid similarity. Similar to the human gene, the *Drosophila Lap* encodes a protein involved in clathrin mediated endocytosis (33). It has a particular role at synapses, where it is expressed presynaptically and mediates the size of synaptic vesicles released, again through a clathrin dependent mechanism (34). In terms of a link to AD, there is conflicting evidence. *PICALM* is thought to mainly affect the Aβ pathway through clearance of Aβ. Aβ is cleared from the brain through the endothelium, where *PICALM* has its highest expression. The endothelium is a layer of cells that lines blood vessels in humans, these provide a barrier between the walls of the blood vessels and the lumen. *PICALM* knockdown studies show the opposite results. Mouse models demonstrated a reduction in Aβ in *PICALM* knockdown by reducing *APP* internalisation (35), while in human cultured cells it worsened the effect of Aβ by depleting Aβ clearance (36). Studies have also been done in glial derived cells and showed that a reduction in *PICALM* expression caused a significant reduction in the harmful Aβ42 without affecting expression of the less toxic form of Aβ, Aβ40 (Figure 3) (37). It has been shown that decreasing the Aβ42/Aβ40 ratio lowers the Aβ congregation and deposition (38). Fly studies have also been performed with *Lap,* the *Drosophila* ortholog of *PICALM*. Here they showed rescue of the neurotoxicity caused by Aβ42 using *Lap* overexpression (39). In human AD patients, *PICALM* mRNA

expression is elevated in the frontal cortex but not in the temporal cortex or thalamus (40). However, other studies have found an increase in *PICALM* expression in the microvasculature associated with an AD protective allele (41). *PICALM* has also been postulated to have a role in tauopathy and synaptic dysfunction in AD (42). This evidence indicates an inconsistent role for *PICALM* in AD with both a reduction and increase in *PICALM* expression correlating with AD pathology in different models. This potentially points towards a region-specific role for this gene in AD.

$PLC\gamma_2$ and *CR1* link immune pathways to AD

Another risk locus identified through the GWAS for AD was near the gene $PLC\gamma_2$, encoding phospholipase C gamma 2. This has a range of functions as it is responsible for the hydrolysation of phosphatidylinositol 4,5-bisphosphate (PIP2) into second messenger molecules inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (43). These molecules cause an increase in intracellular calcium. IP3 is released into the cytosol where it binds to its receptor (inositol trisphosphate receptor) on the endoplasmic reticulum (ER). This opens IP3-gated ion channels, allowing calcium efflux from the ER. The other second messenger, DAG, can activate protein kinase C, causing further downstream effects (44). $PLC\gamma_2$ is ubiquitously expressed. In the brain, however, it is predominantly restricted to microglial cells, specifically in the cortex and hippocampus (43). The closest *Drosophila* ortholog of this gene is called small wing (*SL*), sharing a 57% amino acid similarity with $PLC\gamma_2$ (Table 1). In *Drosophila*, *SL* is involved in the negative regulation of insulin signalling and is implicated in cell growth (45). $PLC\gamma_2$ dysfunction is thought to add to AD risk through the immune pathway linked to AD but the exact mechanism is not clear. It has also been shown that $PLC\gamma$ co-immunoprecipitated with Tau and that this complex was enzymatically active (46). The hypermorph of this gene is associated with a decreased risk of late onset AD (LOAD) (43). It was shown in human embryonic kidney 293 (HEK) cells that the P522R mutation (located in the regulatory region of $PLC\gamma_2$) is protective against AD as it slightly increases enzymatic function (47) but the exact mechanism is yet to be determined.

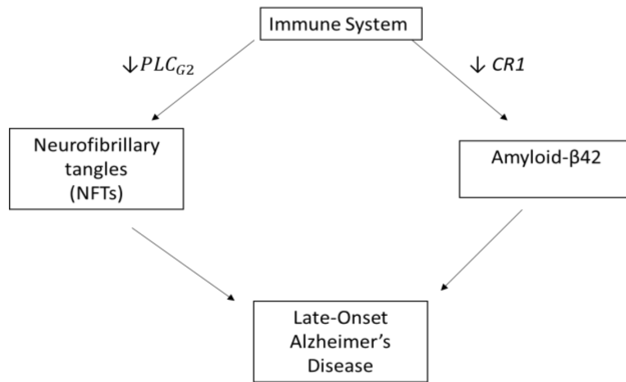


Figure 4. The link between *PLCγ₂*, *CR1* and AD. *CR1* and *PLCγ₂* are thought to be implicated in AD through the immune pathway. *CR1* is thought to be protective against AD as its role in the complement system is thought to help in toxic Aβ42 clearance. A reduction in expression of *CR1* is therefore thought to decrease Aβ42 clearance and contribute to AD pathology. *PLCγ₂* is thought to be linked to AD through its interaction with Tau and NFTs that this protein forms when it is hyperphosphorylated. Increased *PLCγ₂* expression is thought to protective against AD.

CR1 encoding complement receptor 1 was another gene identified in a GWAS for AD and has some sequence homology with the *Drosophila* gene *Hasp* (Hig-anchoring scaffold protein). *CR1* has a DIOPT score of 3 with *Hasp*, which is relatively low but is still the closest *Drosophila* ortholog of this gene. Complement receptor 1 is part of the complement system in humans. The complement system is an innate immune response from the body to fight pathogens. Complement factors bind complement receptors in the plasma in specific regions when pathogens enter the body in an enzymatic cascade to fight infection (48). In *Drosophila*, the hasp protein is localised to cholinergic synapses in the brain. Here it is required to recruit nicotinic acetylcholine receptor subunits (49). *CR1* and the complement pathway are thought to be protective against AD as they aid with the clearance of Aβ plaques, and a reduction in *CR1* expression as a result of the SNP found in GWAS studies (22) reduces the clearance of Aβ plaques (50). This is because CR1 can bind the C3b/C4b complex which recruits and activates microglial cells to the site of the Aβ plaques (51). However, other studies have found that higher expression of CR1 correlated with more cognitive decline in brain tissue from the parietal lobe of AD patients (52). This could be a result of an upregulation in response to increased Aβ plaque concentration.

Other hits from GWAS of AD are less studied and possible mechanisms for their role in AD are yet to be investigated. The *SLC24A* gene, for example, encodes a potassium dependent sodium/calcium exchanger known as NCKX. These transporters work bidirectionally with a stoichiometry of $4Na^+$, $1Ca^{2+}$, K^+ (53). NCKX genes are expressed throughout the body but NCKX4 is the member of the NCKX family that has been associated with AD and has rich expression in the brain (54). There is a *Drosophila* ortholog of these genes, *Zydeco* (*Zyd*) (Table 1). The protein product is a potassium dependent sodium/calcium exchanger, like the human gene. *Zyd*, however, is almost exclusively expressed in glial cells, especially cortex glia, and is thought to control the movement of calcium in membrane

microdomains here (55). So far, the only link to AD with *SLC24A* is the significant association with cognitive decline in older adults (56).

EphA1 belongs to the largest group of tyrosine kinase receptors. These receptors contribute to cell-cell contact and signalling through their membrane bound ligands, known as Ephrins. As their ligands are tethered to the membrane, the two cells containing the ligand and receptor must come together for an interaction to take place (57). This signalling has been shown to be bidirectional, meaning that on binding and activation, both the receptor and ligand on the adjacent cell can stimulate a signalling cascade in their respective cell either forwardly or backwardly (58). Eph receptors are largely found in the central nervous system and endocrine tissues in epithelial cells but are also found in endothelial cells. The SNP near this gene found in GWAS studies is associated with a reduced risk of LOAD (59) but the mechanism by which it does so has not been determined.

Mechanisms of AD pathology for novel genes highlighted by EWAS

TET1, NCOR1, NCOR2 and *ST6GAL-1* are four genes all identified to increase AD risk by EWAS (Table 2). *TET1* encodes ten eleven translocation 1, an enzyme found mostly in neurons, is involved in the demethylation of DNA and therefore is a potential epigenetic marker. TET1 levels have been found to be decreased in the hippocampus (memory centre) in aged (18 months) mice compared with young (3 months) mice. Interestingly, in the same study, reduced expression of this protein was reversed in mice that undertook voluntary exercise (60). Exercise is thought to be one of the best lifestyle choices to reduce the risk of developing AD (61). Conversely, *TET1* levels have also been shown to be significantly upregulated in the nuclear DNA of the hippocampus and parahippocampal gyrus of both preclinical AD and LOAD patients compared with controls (28).

α-2,6-sialyltransferase is encoded by ST6GAL-1. ST6GAL-1 catalyses α2,6-sialylation of Galβ1,4-GlcNAc residues on N-glycans (62). It is expressed in most tissues but its highest expression is in the liver (63). Its link to AD is through the Aβ pathway as it has been shown to be cleaved by β-secretase, the enzyme that is responsible for forming Aβ. When they are co-expressed with the Swedish familial AD mutation (APP_{SW}), ST6GAL-1 expression was decreased (64). In a follow-on study, it was shown that β-secretase knockout mice have a severe reduction in plasma ST6GAL-1 (63). Therefore, upregulation of ST6GAL-1 could outcompete *APP* for β-secretase sites and therefore reduce the production of harmful Aβ. However, it was also shown that increasing the production of ST6GAL-1 increased the concentration of *APP* metabolites in wild type CHO cells (62).

NCOR2 gene encodes nuclear receptor co-repressor 2. This protein binds nuclear receptors and localises histone deacetylases to DNA to inhibit transcription and thus downregulate gene expression (65). NCOR receptors have been knocked out in mice; these transgenic mice showed a reduction in memory compared with controls (66).

Human gene	Human protein	Fly gene	DIOPT score	Human expression	*Drosophila* expression	Amino Acid similarity (%, from FlyBase)
TET1	Ten-eleven translocation	*TET*	10	Skeletal muscle, thymus and ovary	Highest expression in CNS	31
NCOR2	Nuclear receptor co-repressor 2	*SMR*	9	Ubiquitous	Highest expression in eye disc	32
ST6GAL-1	α-2,6-sialyltransferase	*SIAT*	9	Ubiquitous, highest expression in liver	Highest expression in antenna, expression in brain and CNS	55

Table 2. Summary of AD risk genes, identified from EWAS, studied in these experiments, and the proteins they encode. Also shown is the most similar *Drosophila* ortholog to these AD risk genes and how similar the two genes are (DIOPT score), the expression of both the human and *Drosophila* genes and the amino acid similarity between the two genes.

mnb overexpression causes AD-phenotypes in *Drosophila*

Dual specificity tyrosine-(Y)-phosphorylation-regulated kinase 1 A (DYRK1a) is a serine-threonine protein kinase located on chromosome 21 (67). As it was found to be located in a region on chromosome 21 that is thought to be critical for the development of Down syndrome, it was identified

as a risk factor for the disease (68). Recently however, it has been shown to phosphorylate Tau (69), Tau becomes hyperphosphorylated in AD, this causes the formation of large insoluble complexes called neurofibrillary tangles, this is what is thought to cause the destruction of neurons. Although DYRK1A was not found to have a significant association in GWAS studies for AD, it was highlighted as a gene of interest because of its function in phosphorylating Tau. It was also found to have a significant risk of AD in the Japanese population (70). Down syndrome is also linked to Aβ, the other major protein in AD, through the amyloid cascade hypothesis. As the APP gene is found on chromosome 21, it is triplicated in Down syndrome and this is thought to increase Aβ deposits in the brain (71).

Drosophila have an ortholog of DYRK1a, called minibrain (*mnb*), which shares a 58% similarity to DYRK1A. Previous work with the *Drosophila* mnb demonstrated the overexpression of this gene caused AD-like phenotypes (72). When expressing this gene in the eye, it was shown to be neurotoxic, causing neurodegeneration in this area. The overexpression of *mnb* in the *Drosophila* also resulted in other phenotypes like a reduced lifespan and motor ability. As this gene has been implicated in the pathogenesis of AD, future studies should focus on whether an inhibition of this overactivity of this gene could be therapeutic in the treatment of AD as has been hypothesised here (73).

Aims

In this study, using the GAL4/UAS system in *Drosophila,* I will express *RNAi* against *Drosophila* orthologs of the AD candidate genes identified through GWAS and EWAS to knockdown their expression. Due to time constraints, in these studies, only LOF or 'knock down' mutants will be tested to determine their effects in the *Drosophila* model. These will be selected based on genes that were consistently highlighted in GWAS, showed strong association values, and genes that have a good *Drosophila* ortholog. Then, using these mutants, I will screen through assays linked to AD-like behaviours, and characterise the resulting phenotypes. The more rapid assays will be used first to screen for any AD-like phenotypes in the mutant flies. These include the eye degeneration assay, which detects any neurodegeneration in the eye, and the climbing assay, which measures the negative geotaxic response of the flies. Mutants showing AD-like phenotypes in these assays will be tested further for any changes in learning and memory, locomotor activity, and sleep and whether this mutant causes an effect on the lifespan of the *Drosophila*. Finally, I will determine whether a selection of DYRK1A inhibitors can rescue the AD phenotypes caused by *mnb* overexpression. I will then collate these data to determine a role for these genes in the pathogenesis of AD.

Methods

Fly stocks, genetics and *husbandry*

Fruit flies (*Drosophila melanogaster*) were kept at 25°C in bottles or vials containing standard *Drosophila* food: 0.7% agar, 1% soya flour, 8% polenta/maize, 1.8% yeast, 8% malt extract, 4% molasses, 0.8% propionic acid (for increasing egg laying) and 2.3% nipagin (antifungal). They are kept on a 12 hour:12 hour light/dark schedule to maintain their normal wake-sleep patterns. To investigate eye degeneration, the *GMR-GAL4* (Bloomington stock centre line number: BL9146) (74) promoter line was used; this drives transgene expression in the developing photoreceptor neurons of the eye. The *Elav-GAL4* (BL8765) line drives the expression in all neurons and was used to investigate effects on climbing ability and lifespan of the *Drosophila*. The *OK107-GAL4* (BL854) line drives expression in the mushroom body (MB) memory centre of *Drosophila* and therefore was used to investigate any effect of the AD associated mutants on learning and memory. To investigate effects on sleep and rhythmic behaviour, the transgenes were expressed in all clock neurons using *Timeless (tim) -GAL4* (Figure 3, sent from Dr. Ralf Stanewsky, University of Münster, Germany). PST1 was first dissolved in Dimethyl sulfoxide (DMSO), all other drugs were dissolved in water, these solutions were then added to the *Drosophila's* warm food to give a final concentration of 100mM. Mating flies were put into bottles containing the drug food and offspring were raised on the drug food. All experimental genotypes used are listed in Table 3.

Eye degeneration assay

Like many insects, *Drosophila* has a compound eye made up of a series of ommatidia. These are bunches of 20 individual cells (Figure 5). Eight photoreceptive neurons transmit light information to the optic centres of the brain. Four cone cells form the lens, and surrounding pigment cells shared between adjacent ommatidia stop light being lost between neighbouring cells and therefore optically insulates the ommatidium (75) (Figure 5). Here I use this structure to determine whether the mutant genotype causes degeneration in the developing *Drosophila* eye (previously done here (76)).

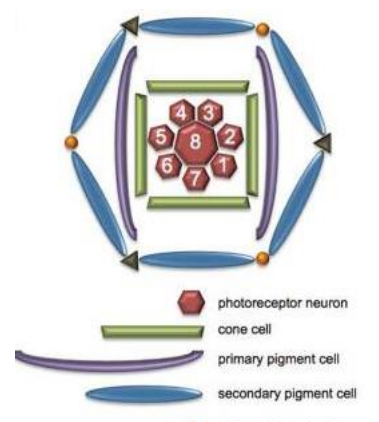


Figure 5. Individual cellular arrangement of the *Drosophila* compound eye, an ommatidium (2).

Human gene	**Drosophila gene**	**DIOPT score**	**AD Link**	**Type of mutant**
PICALM	*Lap RNAi A*	14	GWAS	*RNAi*
PICALM	*Lap RNAi B*	14	GWAS	*RNAi*
$PLC_{\gamma 2}$	*SL RNAi*	10	GWAS	*RNAi*
SLC24A4	*Zyd RNAi A*	8	GWAS	*RNAi*
SLC24A4	*Zyd RNAi B*	8	GWAS	*RNAi*
EphA1	*Eph RNAi A*	6	GWAS	*RNAi*
EphA1	*Eph RNAi B*	6	GWAS	*RNAi*
CR1	*Hasp RNAi*	4	GWAS	*RNAi*
Eph A1	*Human Eph A1*	N/A	GWAS	Human over-expressor
ST6GAL1/2	*SIAT RNAi*	11	EWAS	*RNAi*
NCOR1	*SMR RNAi*	10	EWAS	*RNAi*
Tet1/3	*Tet RNAi*	8	EWAS	*RNAi*
MAPT (0N4R)	*MAPT*	N/A	Tau Pathway	Human over-expressor
APP	*APP*	N/A	Amyloid-β pathway	Human over-expressor
Ephrin	*Ephrin RNAi A*	9	None	*RNAi*
Ephrin	*Ephrin RNAi B*	9	None	*RNAi*
DYRK1A	*mnb*	10	Association in Japanese population	Overexpressor

Table 3. List of all experimental genotypes used in these experiments. The table shows the human gene linked to AD, the closest *Drosophila* ortholog to these genes used in these experiments, the DRSC Integrative Ortholog Prediction Tool (DIOPT) score, which measures homology between the fly gene and human ortholog, where the link to AD arises, and the type of mutant used in the experiments.

Two to five days old male *GMR-GAL4*>UAS mutant and control (GMR/+) flies were collected for each genotype. The GMR line expresses the UAS throughout development exclusively in the eye (Figure 6). They were subsequently anaesthetised using a CO_2 porous pad to avoid movement when analysing. Flies were placed under a microscope and the eyes were imaged and magnified (x8) with a Zeiss Axioplan MRm camera. The surface area of each eye was then calculated using the Zeiss Zen programme. If the mutant genotype caused any degeneration of these photoreceptors in the eye, then the compound eye structure would break down and the surface area would be reduced. A one-way ANOVA was used to test the significance of the results with a Dunnett's multiple comparisons test to compare control with experimental values. All assumptions for the statistical tests were checked and non-parametric tests were used if the assumptions were not met.

GAL4 driver	***Drosophila expression***	**Source**
GMR	Eye (development and adulthood)	BL9146
Elav	Pan-neuronally (development and adulthood)	BL8765
Tim	Clock neurons (development and adulthood)	From Dr. Ralf Stanewsky, University of Münster, Germany
OK107	Mushroom body (development and adulthood)	BL854

Figure 6. Gal4 promoters used in these experiments and their expression patterns in *Drosophila*.

Climbing assay

The climbing assay measures the negative geotaxic response or startle response of *Drosophila*. This is an innate reflex in the fly that causes them to climb upwards, opposing gravity, when they are tapped to the bottom of a vial or similar container. To model this, young, mixed sex *Elav-Gal4* flies were collected and transferred to an empty vial with a line drawn 2 cm from the top. Flies were then left to acclimatise for 15 minutes. The flies were then tapped to the bottom of the vial and the number of flies that crossed the line in 10 seconds was recorded and expressed as a percentage of all flies (77). They were then transferred to a vial of food and tested again every 10 days. For each genotype, 10 vials of flies were collected, giving a total n of 10. Experiments were performed in the afternoon, 12-3

pm, to eliminate any potential bias caused by fluctuations in circadian rhythms. A one-way ANOVA with Dunnett's multiple comparisons was used to calculate differences between the experimental groups and the wild type control.

Longevity assay

To determine whether the mutant genotypes affected lifespan, I collected 2-5 day old, mated female flies. Male flies were not used as they get stuck in the sticky layer on the top of food that forms after 3-4 days when the food gets old and is not turned over by larvae. Ten flies were transferred to a vial with standard food. Every 2-3 days, dead flies were counted as they were transferred to fresh food. Flies were kept at 25°C. Five vials per genotype were used, giving a total of n=5 for each genotype. Mantel-Cox log rank tests comparing median survival between control and tests genotypes were carried out when all flies had died and were plotted onto a Kaplan-Meier survival curve (78).

Sleep and activity experiments

Male 2-5 days old *Tim-GAL4* flies were collected in tubes (65 mm). Food was added to one side of the DAM tube and a bung was placed on top to ensure the food did not dry out. Cotton wool was placed in the opposite end so the flies could breathe but not escape the tube. The tubes were placed in a *Drosophila* Activity Monitor (DAM, TriKinetics Inc), holding 32 flies of the same genotype (Figure 7). They are then placed in an incubator at 25°C attached to a computer. The monitor emits an infrared beam through the middle of the tubes. When flies cross this line, it breaks the beam, and the computer notes this as an activity count with respect to time and light conditions. This

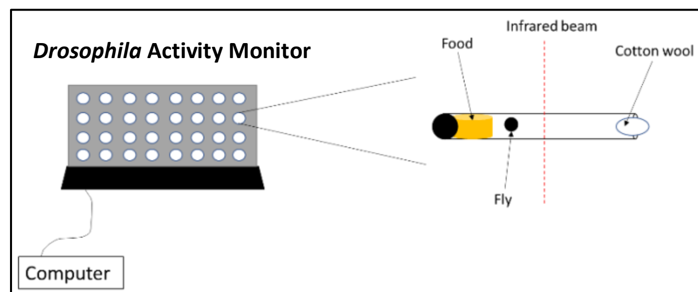


Figure 7. Equipment used for sleep and activity experiments. The *Drosophila* Activity Monitor holds 32 flies and tracks their activity through an infrared beam that is shone through the centre of each individual tube. When a fly crosses this line, the beam is broken, and this is noted on the attached computer. Cotton wool is placed at one end to ensure the flies can breathe but not escape, and a silicon bung is placed over standard food on the other side of the tube to make sure it does not dry out.

provides individual raster plots for each fly in each DAM, which were formatted into 1 minute and 30 minutes bins using DAMFileScan (TriKinetics) and subsequently analysed.

To analyse sleep, flies were kept in a 12 hour:12 hour light/dark cycle (9am-9pm light, 9pm-9am dark) in the incubator. A sleep period is defined as when the fly is inactive for longer than 5 minutes (79). The sleep parameters investigated were: total activity counts throughout the day; total sleep time of the flies; average duration of each sleep episode; and the average number of sleep episodes per day. All parameters were separated into daytime and night-time. All sleep analyses were performed using Matlab and a custom script (Sleep and Circadian Analysis MATLAB Program (SCAMP)). Kruskal-Wallis tests were performed on these data, as the assumptions of the ANOVA test was not met, to determine any differences between the control and experimental groups. The raster plots of flies that died were not recorded for the analysis.

Learning and memory

Intermediate term memory, which is defined as 1 hour memory for *Drosophila*, was tested in the flies using the olfactory shock conditioning assay previously described (80). Briefly, 30-50 mixed sex flies were collected and kept at 25°C and 70% humidity for two days to allow them to acclimatise to the environment. Experiments were performed under red light because the flies cannot see and therefore, they concentrate on the odour cues. It has been shown that *Drosophila* are insensitive to red light (80) but this allows the experimenter to see and conduct the experiment.

Flies were put into a T-maze and were first exposed to one odorant paired with a small shock (1.25 second bursts of 70V and a 3.75 second break) for 1 minute. They were then given a rest for 45 seconds before being given the other odour for 1 minute without shock. After 1 hour, the flies were given a choice between arms of the T-maze containing the separate odours and allowed to distribute in the arms for 2 minutes (see Figure 8 for protocol). Avoiding the shock paired odorant meant the flies had learnt the behaviour. The number of flies on each side were counted by collecting and subsequently anaesthetising the flies on a CO_2 porous pad. The experiment was then repeated with a second set of flies that were shocked with the other odorant to avoid any odour bias and an average of the two memory scores was taken with the memory calculated using the performance index (PI) determined with the following equation:

PI = (No. of correct flies – No. of incorrect flies) / total No. of flies

Therefore, the average performance index was taken between the two pairings to give an n of 1 which consisted of ~100 flies. To ensure the flies had no preference for the odours, odours were chosen that were equally aversive to the flies. Two odorants were chosen that were slightly aversive to flies, they were prepared prior to experiments by mixing mineral oil with 3-octanol (1:1000), Aldrich) or 4-methylcyclohexanol ((MCH 1:500), Aldrich). At these concentrations, the two aversive odours were balanced or equally aversive, so neutral to the fly (i.e., the flies have no odour preference). Sensory controls were performed with octanol and MCH, whereby flies were given a choice between odour and fresh air and showed olfactory behaviour by characteristically avoiding the odour. A separate control was also performed to ensure the flies avoided the shock by presenting the flies with a shock or a fresh air tube. A one-way ANOVA with Dunnett's multiple comparisons was used to determine any significant differences between the control and experimental groups.

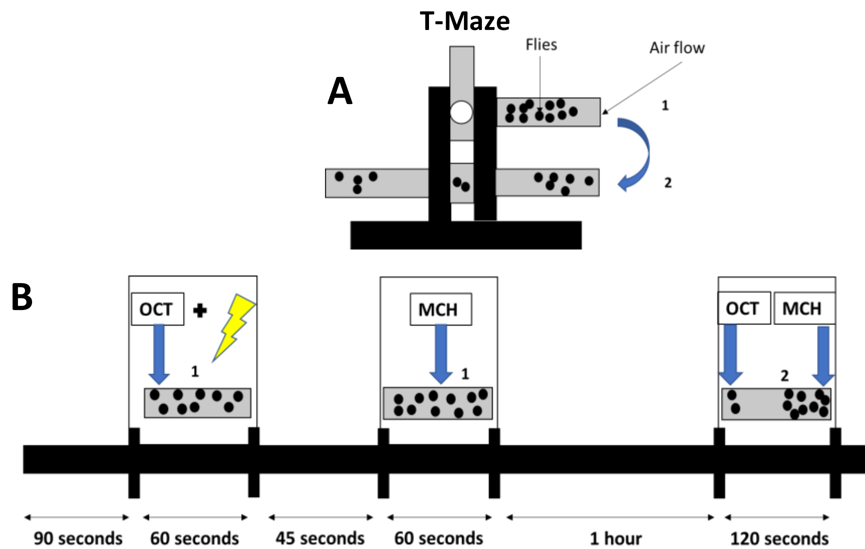


Figure 8. Schematic diagram showing (A) the T-maze apparatus used for the learning and memory experiments and (B) the protocol used for these experiments. Flies are put into the T-maze and allowed to rest for 90 seconds to acclimitise. For the next 60 seconds, they are exposed to odour 1 (octanol) and at the same time given a small shock. After 45 seconds of rest, they are exposed to the second odour (MCH) for a further 60 seconds. The flies are taken out of the T-maze for 1 hour. They are then put into a central chamber and exposed to both odours. They have 120 seconds to decide which side to move towards.

Results

Reducing the expression of *Lap*, *Zyd* or *Siat* in the *Drosophila* eye causes a reduction in eye surface area

The *Drosophila* compound eye consists of approximately 800 ommatidia arranged hexagonally (Figure 5). Each ommatidium is made up of around 20 cells, eight of which are photoreceptive cells that transmit light information to the optic lobes in the *Drosophila* (2). This arrangement is useful for modelling neurodegeneration because the effects that disrupt this alignment like cell death and therefore loss can be easily qualified with a light microscope from what is described as a rough (as in misalignment of regular pattern of the compound) eye phenotype (81, 82). The *GMR* driver line was used to determine the effects of misexpression of fly orthologs of the GWAS and EWAS AD candidate genes on the photoreceptors in the eye. The *GMR-GAL4* line drives expression during development exclusively in the eye and therefore if the protein produced is neurotoxic, this is expected to cause degeneration of the photoreceptor neurons, this can be detected by a reduction in the size of the eye. To quantitatively measure this rough eye phenotype, the surface area of the *Drosophila* eye can be measured. If the protein, or lack of the protein, causes degeneration of developing photoreceptor neurons in the eye, the ommatidia will be disrupted, and the reduction in overall surface area can be quantified.

The two most studied proteins in human AD are Tau and Aβ42. When overexpressed in neurons, these proteins cause AD pathology through a build-up of large insoluble aggregates, causing the cells to die. Tau is hyperphosphorylated in AD leading to the formation of neurofibrillary tangles. Amyloid precursor protein (*APP*) is misprocessed in AD, leading to the formation of harmful Aβ peptides (Aβ42). In both cases, this leads to a build-up of these toxic aggregates in neurons, causing the cells to die. These known neurotoxic and pro-degenerative proteins, Aβ42 and Tau have been shown to cause a similar neurodegeneration in the eye when overexpressed using the *GMR-GAL4* line in *Drosophila* (76, 83). Overexpression of these two genes was used in these studies as a positive control to compare the effect of new putative AD candidate genes. The AD candidate genes that I compared with these AD proteins have been identified through GWAS and EWAS studies for AD but their phenotypes in an AD model are largely unknown and therefore the mechanisms of action have been vastly underexplored. It is therefore useful to use these AD causing genes to compare with my candidate genes to quantify the severity of any AD-like phenotype caused. However, consideration must be

taken when making these comparisons, as with Aβ42 and Tau studies, I am overexpressing human genes in *Drosophila*, whereas the candidate genes used are endogenous *Drosophila* genes that are being mostly knockdown with unverified RNAi transgenes, making my results susceptible to false negative results. I try to mitigate this by measuring the effect of a second RNAi line when available or picking a RNAi line whose effect of expression on the target gene had been verified. In order to mitigate this, in these experiments, to confirm any phenotype that may arise from my gene knockdowns, I used two separate *RNAi* lines or pick a RNAi line whose effect of expression on the target gene had been verified. These should give different results as the level of gene expression in each line will be variable. The *RNAi* technique used also gives a 'knockdown' effect and hardly ever a 'knockout' effect, and hence gene expression will not be completely eradicated. Some *RNAi* lines that I have used in these studies have not yet been verified, therefore, to measure the levels of the gene in the different *Drosophila* lines, quantitative reverse transcription PCR (qRT PCR) should be used, however due to time constraints, I was not able to complete these experiments. This is where the expression levels of a particular target gene mRNA are quantified in reference to a wild type control. However, due to time constraints it was not possible to carry out these studies.

Results from the one-way ANOVA show a significant effect of the genotype on the surface area of the eye (($F_{19,178}$) = 20.37, P<0.001). Consistent with these previous findings, I found a significant reduction in the surface area of the eye of flies overexpressing human *MAPT* (Tau 0N4R, P<0.001) and Aβ42 (P<0.001) (Figure 9). Due to alternate splicing of the RNA of the *MAPT* gene, several isoforms arise, differing in N-terminal inserts (0, 1 or 2N) and microtubule repeats (3 or 4R) (84). These two isoforms exist in the brain in an approximately 1:1 ratio however in human AD patients, this ratio is altered, and the levels of the 4R isoform increase to change the ratio to closer to 2:1 ratio. The extra microtubule repeat in the 4R isoform allows for increased Tau interaction with microtubules, the 4R isoform can also bind stronger to microtubules compared to the 3R isoform (85). These different Tau isoforms relate to different effects in *Drosophila*, with the 3R isoform causing more severe locomotor deficiencies and a reduced lifespan, whereas the 4R isoform has been correlated with worse memory and increased neurodegeneration. *GMR>Tau (0N4R)* caused a 52% reduction in the surface area compared with wild type, whereas *GMR>Aβ42* resulted in a 37% decrease.

RNAi mediated knockdown of the closest *Drosophila* ortholog to the human AD GWAS candidate gene *PICALM* (*GMR* > *Lap RNAi A*, Figure 9A) caused a significant reduction in eye surface area compared with the wild type control (*GMR/+*, P<0.05), although this was not seen in a second separate *RNAi* line

for this gene (*GMR > Lap RNAi B*, P>0.05). Another AD associated GWAS candidate gene *SLC42A*, shares 55% amino acid sequence with the *Drosophila* gene *Zydeco* (*Zyd*). *RNAi* mediated knockdown of *Zyd* resulted in a reduction in the surface area of the eye with one *RNAi* line (*GMR>Zyd RNAi B*) but not with another (*GMR > Zyd RNAi A*) compared with the control (*GMR/+*). Knocking down the expression of *Siat* (*GMR > Siat RNAi*), an AD candidate gene highlighted through EWAS, gave a significantly smaller eye surface area than the control (P<0.05). *Siat* is the closest *Drosophila* ortholog of *ST6GAL-1*, with 55% amino acid homology. *ST6GAL-1* encodes an enzyme which catalyses α2,6-sialylation of Galβ1,4-GlcNAc residues on N-glycans.

Based on the reduced surface area caused by knockdown of *Lap, Zyd* or *Siat* in the eye, these were considered to be candidate genes that displayed some AD relevant phenotype in *Drosophila* which were then investigated further, using different assays that measured behaviours and lifespan reduction associated with AD. These assays are more labour intensive than the quick screen used for the fast test for degeneration of the photoreceptors in the eye. Hence these most promising candidates were chosen to test for any additional AD phenotypes.

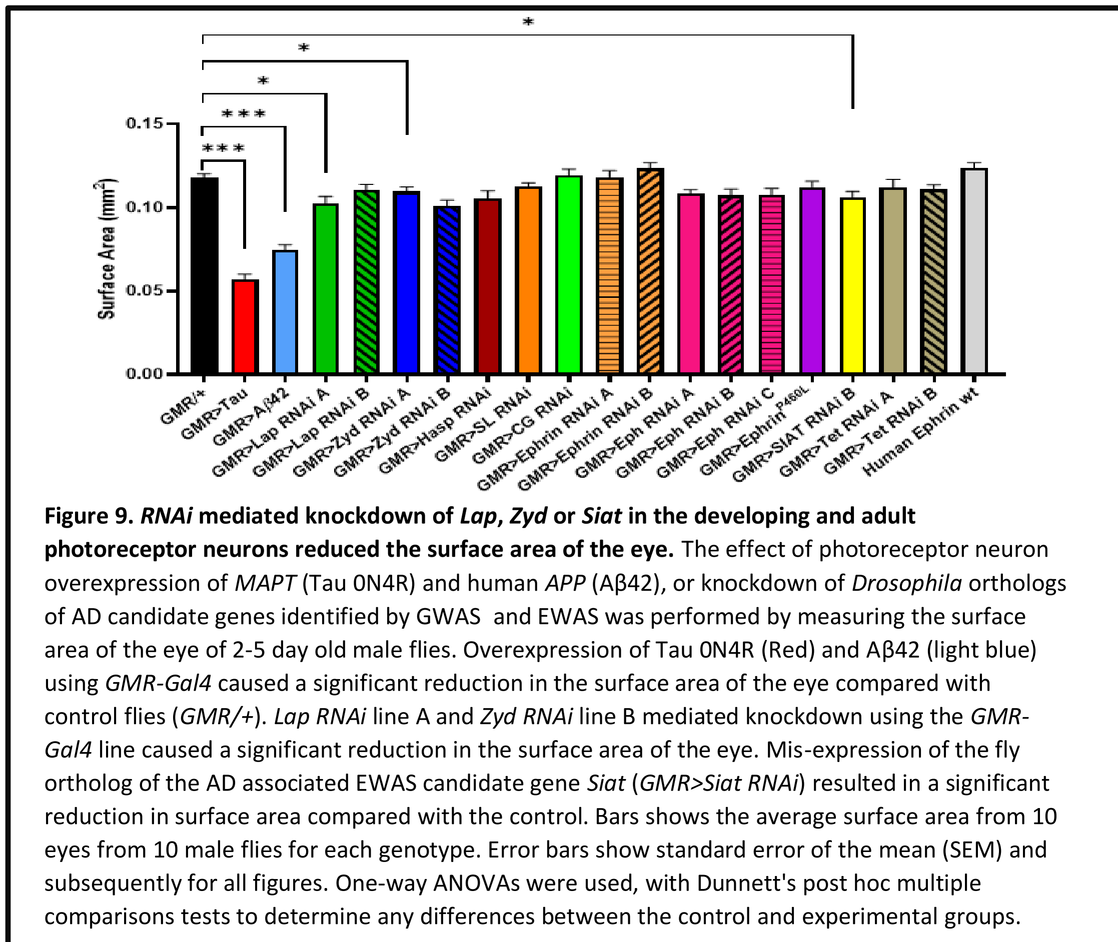


Figure 9. *RNAi* mediated knockdown of *Lap*, *Zyd* or *Siat* in the developing and adult photoreceptor neurons reduced the surface area of the eye. The effect of photoreceptor neuron overexpression of *MAPT* (Tau 0N4R) and human *APP* (Aβ42), or knockdown of *Drosophila* orthologs of AD candidate genes identified by GWAS and EWAS was performed by measuring the surface area of the eye of 2-5 day old male flies. Overexpression of Tau 0N4R (Red) and Aβ42 (light blue) using *GMR-Gal4* caused a significant reduction in the surface area of the eye compared with control flies (*GMR/+*). *Lap RNAi* line A and *Zyd RNAi* line B mediated knockdown using the *GMR-Gal4* line caused a significant reduction in the surface area of the eye. Mis-expression of the fly ortholog of the AD associated EWAS candidate gene *Siat* (*GMR>Siat RNAi*) resulted in a significant reduction in surface area compared with the control. Bars shows the average surface area from 10 eyes from 10 male flies for each genotype. Error bars show standard error of the mean (SEM) and subsequently for all figures. One-way ANOVAs were used, with Dunnett's post hoc multiple comparisons tests to determine any differences between the control and experimental groups.

The negative geotaxic climbing response is reduced in *Drosophila* paneuronally expressing *RNAi* for *Lap* and *Zyd*

Drosophila exhibit an innate negative geotaxis response. This is an escape reflex that occurs when *Drosophila* are knocked down to the bottom of a container, causing them to initiate a reflex response to climb upwards, against gravity. This can used as a measure to test the fly's locomotion, or startle response (86). To do this, flies are collected in a vial and subsequently tapped to the bottom after they have acclimatised. The number of flies that cross a line drawn near the top of the vial in 10 seconds is counted and expressed as a percentage of all flies. In this assay, I used the *Elav-Gal4* promoter. This drives Gal4 expression pan-neuronally throughout development and adulthood and so can be used to determine the effects of gene misexpression on climbing ability. I used the UAS/+ as an extra control. This is important when there are significant differences in an GAL4/UAS line. By showing no effect of the UAS itself, this confirms that it is the GAL4 interacting with the UAS (causing the *RNAi* in this case) that is producing the effect on the genotype, and not the result of a 'leaky' UAS line that expresses the RNAi independent of Gal4 or a insertional effect.

Results from the one-way ANOVA for this assay show a significant effect of the genotype on the performance in the climbing assay ($(F_{12,107}) = 34.03$, $P<0.001$). *Drosophila's* climbing ability declines with age (87, 88), which I confirmed in my tests (Figure 10). Overexpression of human AD causal genes *MAPT* (Tau) and mutant *APP* (Aβ42) (Figure 10A) followed a similar decline in climbing performance, but their climbing performances were significantly smaller ($P<0.001$) across all days compared with controls (Figure 10, also shown in (76, 89)). Therefore, I tested whether the knockdown of my AD candidate genes showed a similar phenotype to the known AD causing genes. Reducing *Lap* expression*,* the *Drosophila* ortholog of the human gene *PICALM*, almost completely eradicated the *Drosophila* negative geotaxic response in one *RNAi* line on the first day tested (*Elav>Lap RNAi A,* Figure 10E). With this line, there was no age dependent decline, as seen with all other genotypes, as the climbing performance of these flies on day 1 was already 10%. The second *RNAi* line (*Elav>Lap RNAi* B), however, had no effect on climbing ability compared with the control (*Elav/+*). Pan-neuronally reducing the expression of two separate *RNAi* lines of the *Drosophila* gene *Zyd* (*Elav>Zyd RNAi A, Elav>Zyd RNAi B,* Figure 10C) demonstrated this age dependent decline in climbing ability. Similar to AD causal genes *MAPT* (Tau) and *APP* (Aβ42), their climbing performance was significantly smaller than the control on all days expect day 10, where *Elav>Zyd RNAi A* showed an increase from day 1 (Figure 10C). *Elav>Siat RNAi* also showed a reduction in the negative geotaxic response compared with

wild type on day 1 (Figure 10D, P<0.05) but this was not significantly smaller than its *UAS/+* control (Figure 10E).

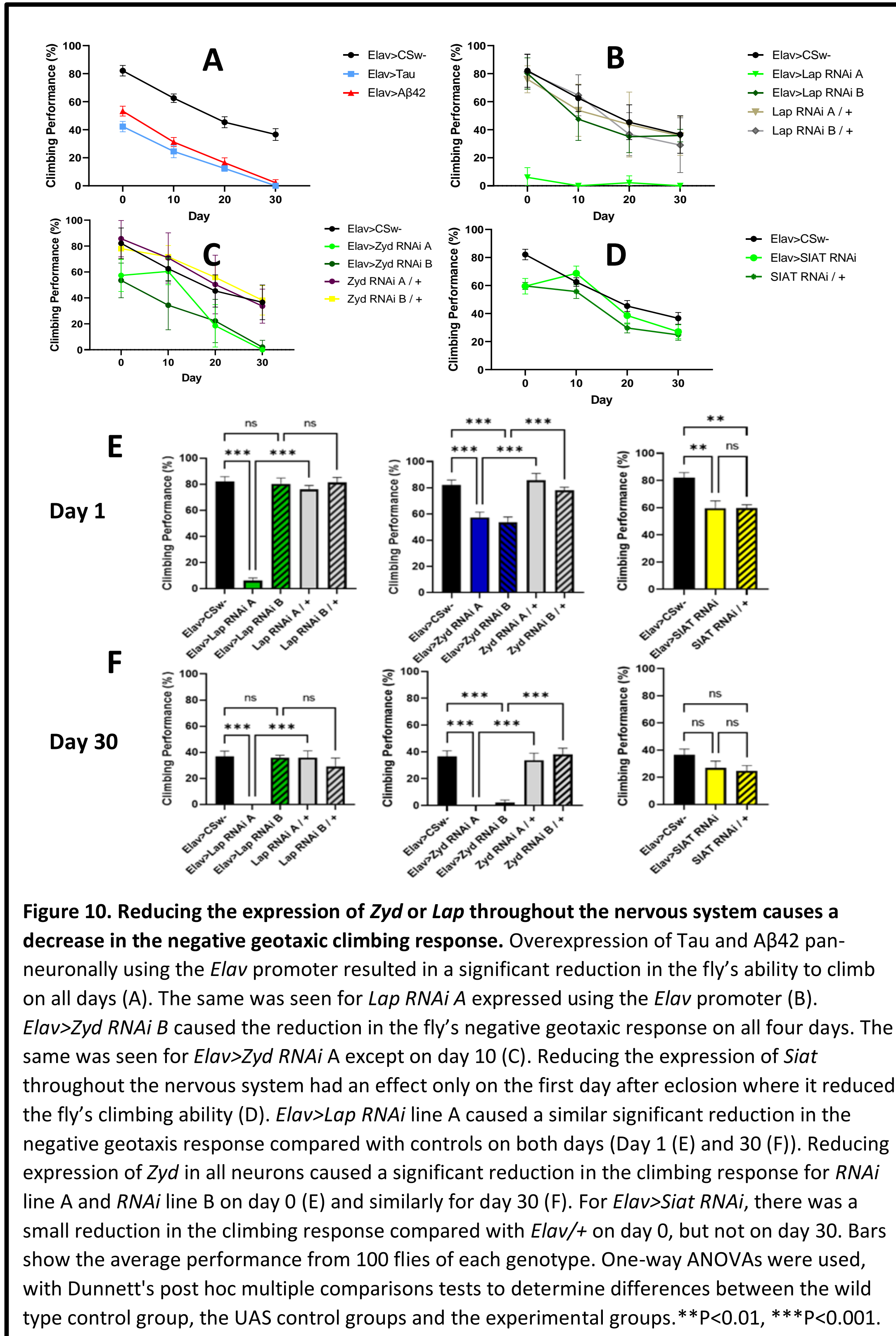


Figure 10. Reducing the expression of *Zyd* or *Lap* throughout the nervous system causes a decrease in the negative geotaxic climbing response. Overexpression of Tau and Aβ42 pan-neuronally using the *Elav* promoter resulted in a significant reduction in the fly's ability to climb on all days (A). The same was seen for *Lap RNAi A* expressed using the *Elav* promoter (B). *Elav>Zyd RNAi B* caused the reduction in the fly's negative geotaxic response on all four days. The same was seen for *Elav>Zyd RNAi* A except on day 10 (C). Reducing the expression of *Siat* throughout the nervous system had an effect only on the first day after eclosion where it reduced the fly's climbing ability (D). *Elav>Lap RNAi* line A caused a similar significant reduction in the negative geotaxis response compared with controls on both days (Day 1 (E) and 30 (F)). Reducing expression of *Zyd* in all neurons caused a significant reduction in the climbing response for *RNAi* line A and *RNAi* line B on day 0 (E) and similarly for day 30 (F). For *Elav>Siat RNAi*, there was a small reduction in the climbing response compared with *Elav/+* on day 0, but not on day 30. Bars show the average performance from 100 flies of each genotype. One-way ANOVAs were used, with Dunnett's post hoc multiple comparisons tests to determine differences between the wild type control group, the UAS control groups and the experimental groups.**P<0.01, ***P<0.001.

Knockdown of *Lap* or *Zyd* in all neurons causes premature death in *Drosophila*

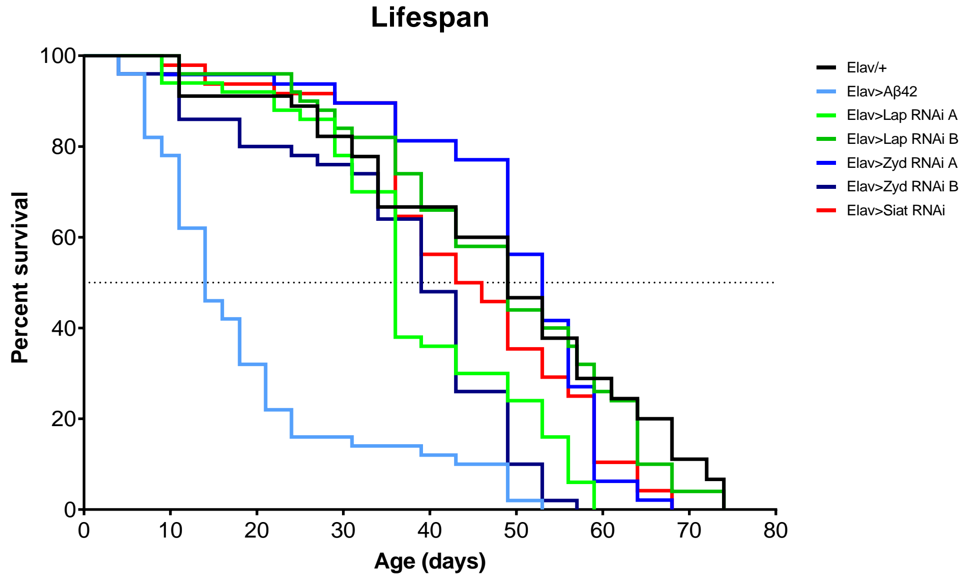
Along with the many challenging symptoms, the neurodegeneration caused in AD ultimately results in premature death in humans. Overexpression of human mutant Tau and Aβ42 are two of the known genes to cause premature death in AD and this has been replicated in a *Drosophila* model (76, 90, 91).

Using this assay, I found the same result when overexpressing Aβ42 with the pan-neuronal promoter (Figure 11). *Elav>Aβ42* caused a significant reduction in the lifespan of the flies compared with the wild type (*Elav/+*), with a 70% reduction in median survival.

I also tested my AD candidate genes to determine whether misexpression caused premature death in *Drosophila*. I used *RNAi* to reduce the expression of these genes across the nervous system of the flies with the *Elav-GAL4* promoter. *Elav>Lap RNAi A* caused a significant reduction in the lifespan of the *Drosophila* compared with controls ($P<0.001$). Median survival was reduced by 27% from 49 days in controls to 36 days. However, this was not replicated in the second *Lap RNAi* line (*Elav>Lap RNAi B*); no difference was seen compared with the control group (*Elav/+,* Figure 11). Reducing the expression of the *Drosophila* gene *Zyd* also caused a shorter lifespan in one *RNAi* line (*Elav>Zyd RNAi B*), reducing median survival from 49 days in control flies to 39 days. I was unable to confirm this effect in the other *RNAi* line tested (*Elav>Zyd RNAi A*).

Clock neuron knockdown of AD candidate genes causes sleep disruption

The link between AD and sleep is bidirectional, with evidence of both sleep disruption giving an increased risk of developing AD and also being a symptom of AD. Although the majority of AD patients report sleep problems, there is conflicting evidence for the link between sleep duration and cognitive impairment in humans. Some studies found lower cognitive performance with less sleep (92) whereas others found no difference in cognitive performance in people who slept less (93). One of the toxic proteins implicit in AD, Aβ42, has been associated with sleep problems in AD. It has been reported that deprivation of sleep increases cerebrospinal fluid levels of Aβ42, and that increased sleep can reduce elevated Aβ42 levels (94). Also, an increase in Aβ42 levels in the brain causes sleep disruption, without any signs of cognitive impairment (94, 95), suggesting that the sleep problems in AD might arise before the onset of cognitive impairment. In *Drosophila* models, it has been shown that circadian rhythms are disrupted when expressing both the two major proteins involved in the pathology of AD, Tau (96) and Aβ42 (97).



B

Genotype	Median survival (days)	Significance level
Elav/+	49	
Elav>Aβ42	14	***
Elav>Lap RNAi A	36	***
Elav>Lap RNAi B	49	ns
Lap RNAi A/+	53	ns
Lap RNAi B/+	49	ns
Elav>Zyd RNAi A	53	ns
Elav>Zyd RNAi B	39	***
Zyd RNAi A/+	56	ns
Zyd RNAi B/+	47.5	ns
Elav>Siat RNAi	46	ns
Siat RNAi/+	39	*

Figure 11. Pan-neuronal misexpression of *Lap* or *Zyd* causes a reduction in the lifespan of *Drosophila*. (A) Kaplan-Meier survival plot showing the effect of overexpression of mutant human *APP* (*Elav>Aβ42*), and knockdown of *Drosophila* genes *Lap* (*Elav>Lap RNAi A* or *Elav>Lap RNAi B)*, *Zyd* (*Elav>Zyd RNAi A* or *Elav>Zyd RNAi B*) and *Siat* (*Elav>Siat RNAi*) on the lifespan of *Drosophila*. (B) Results from Mantel-Cox log rank tests performed to determine significant reductions in the lifespan and median survival (days) as a result of misexpression of these genes. Pan-neuronal overexpression of human Aβ42 using the *Elav-Gal4* promoter caused a significant reduction in lifespan. Reducing the expression of *Zyd RNAi line B* or *Lap RNAi line A* using the pan-neuronal *GAL4* line, *Elav*, caused a significant reduction in the survival of the flies compared with control flies (*Elav/+*). To determine statistically significant differences between the control and experimental groups, Mantel-Cox log rank tests were performed on median survival rates. N=50 for all genotypes. *P<0.01, **P<0.01, ***P<0.001.

Using *Drosophila* activity monitors (DAM), I investigated the effects of my AD candidate genes and human AD causal genes *MAPT* and *APP* on sleep and activity in *Drosophila* and characterised any sleep problems that occurred through the misexpression of these AD candidate genes in *Drosophila*. The clock cell specific promoter sequence, *Tim-Gal4*, was used for these experiments and the flies were loaded into DAMs, as described above (Figure 7). Young flies (2-3 days old) were used in these experiments as it has been shown that *Drosophila* undergo progressive disruption to sleeping rhythms and clock neuron physiology from 29 days old (98).

Results from the Kruskal-Wallis test show a significant effect of the genotype on both sleep (H (7) = 97.73, P<0.001) and activity (H (7) = 107, P<0.001). Overexpression of either mutant *APP* (Aβ42) and *MAPT* (Tau) throughout the clock cells of *Drosophila* caused sleep disruption and increased locomotor activity, with *Tim>Aβ42* showing more severe phenotypes. Activity was higher during the day and night (Figure 12C, P<0.001) for these genotypes compared with the wild type (*Tim/+*). These flies also slept less during the day and night (Figure 12D, P<0.001). Similarly for *Tim>Lap RNAi A*, *Tim>Zyd RNAi A* and *Tim>Zyd RNAi B*, total activity was higher during the day and night (Figure 12C). The decrease in total sleep seen with these genotypes compared with controls (P<0.001, Figure 12B) was due to a decrease in daytime sleep (Figure 12D) as there is no difference in night-time sleep between these and the wild type group.

A problem reported in human AD patients is sleep fragmentation at night (99). I used the mean sleep episode duration (Figure 12E) and the number of sleep episodes (Figure 12F) to determine whether this phenotype was observed in mutant flies. Although there was no difference in total sleep duration at night with *Tim>Lap RNAi A*, these flies, along with *Tim>Aβ42,* displayed sleep fragmentation at night. Both *Tim>Aβ42* and *Tim>Lap RNAi A* showed a reduced in mean sleep episode duration during the night (Figure 12E), implying that the flies had shorter periods of sleep during the night. They also showed an increase in the number of sleep episodes at night compared with the wild type (*Tim/+*). This implies that instead of longer sleep periods at night, their sleep was made up of more frequent, short bursts of sleep, a phenotype associated with human AD.

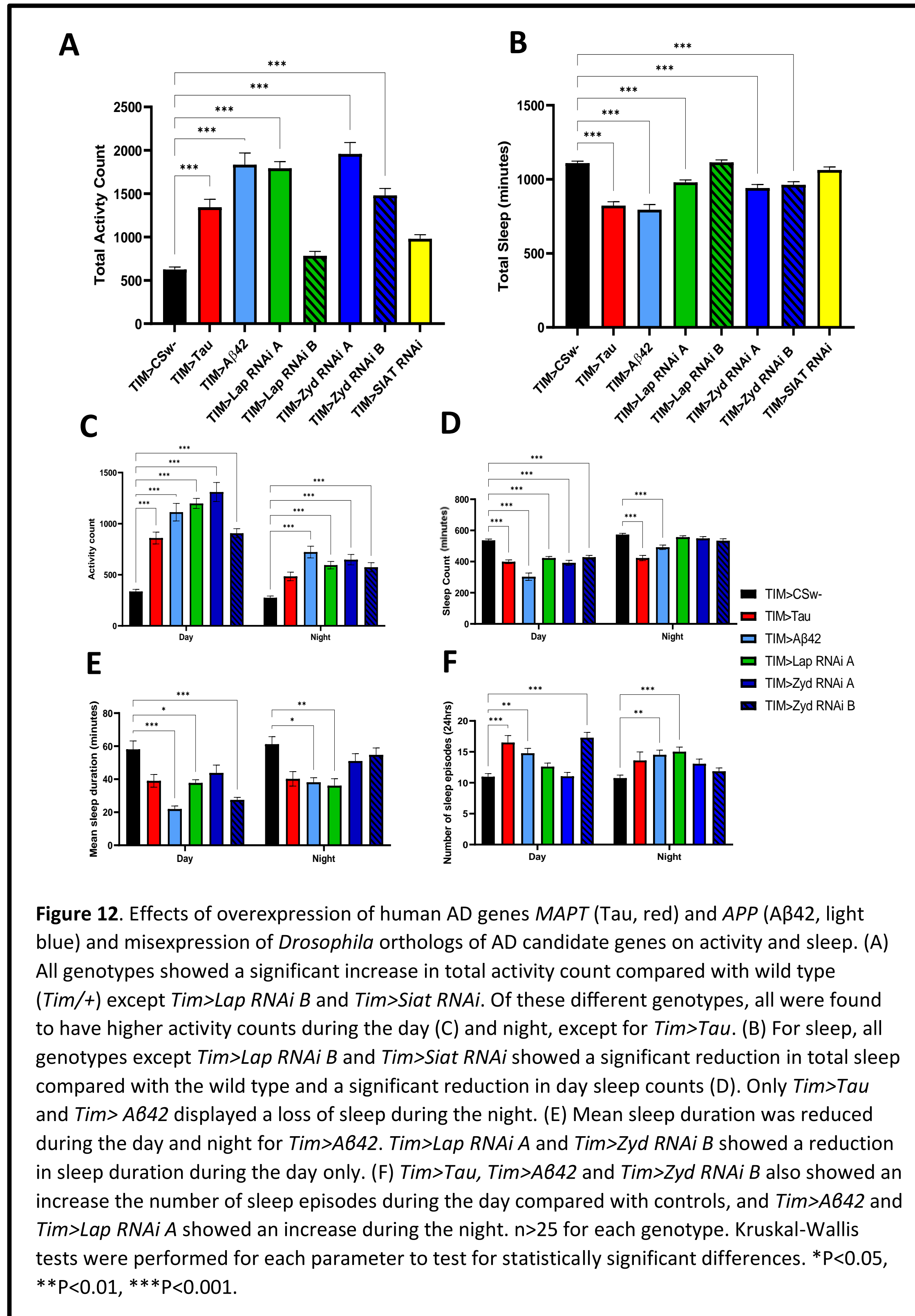


Figure 12. Effects of overexpression of human AD genes *MAPT* (Tau, red) and *APP* (Aβ42, light blue) and misexpression of *Drosophila* orthologs of AD candidate genes on activity and sleep. (A) All genotypes showed a significant increase in total activity count compared with wild type (*Tim/+*) except *Tim>Lap RNAi B* and *Tim>Siat RNAi*. Of these different genotypes, all were found to have higher activity counts during the day (C) and night, except for *Tim>Tau*. (B) For sleep, all genotypes except *Tim>Lap RNAi B* and *Tim>Siat RNAi* showed a significant reduction in total sleep compared with the wild type and a significant reduction in day sleep counts (D). Only *Tim>Tau* and *Tim> Aβ42* displayed a loss of sleep during the night. (E) Mean sleep duration was reduced during the day and night for *Tim>Aβ42*. *Tim>Lap RNAi A* and *Tim>Zyd RNAi B* showed a reduction in sleep duration during the day only. (F) *Tim>Tau, Tim>Aβ42* and *Tim>Zyd RNAi B* also showed an increase the number of sleep episodes during the day compared with controls, and *Tim>Aβ42* and *Tim>Lap RNAi A* showed an increase during the night. n>25 for each genotype. Kruskal-Wallis tests were performed for each parameter to test for statistically significant differences. *P<0.05, **P<0.01, ***P<0.001.

Memory deficits arise from reducing *Lap* or *Zyd* expression

Cognitive decline (including memory loss) is the most severe and prevalent symptom of AD. In humans, the destruction of neurons in the memory centre, the hippocampus, is the hallmark pathology of the disease. To model this symptom of the disease in *Drosophila*, I used the olfactory shock conditioning assay in young flies (2-7 days old) (80). I tested the flies 1 hour after being trained to test the *Drosophila's* intermediate term memory.

This associative odour learning in *Drosophila* arises from a brain region called the mushroom body (MB) (100). The MB consists of groups of cells, Kenyon cells, that form both the calyx and lobes, the two main structures in the MB. The MB receives olfaction input via cholinergic projection neurons into the calyx (101), whereas the lobes form the output region of the MB, synapsing with output neurons and receiving information from input neurons (102-104).

For these learning and memory experiments, I used the *OK107-GAL4* line which drives expression of the UAS transgene throughout the MB of the *Drosophila*. As no more AD-like phenotypes were found from the climbing, longevity and sleep experiments with the *Siat RNAi* line, I decided not to use this line for these labour-intensive experiments. Results from the one-way ANOVA show a significant effect of the genotype on the *Drosophila's* memory (($F_{9,38}$) = 3.837, $P=0.002$). When overexpressing human mutant *APP* (Aβ42) in the MB of the *Drosophila,* I found a significant reduction ($P<0.001$) in the one hour memory of young flies, similar to previous studies with human Aβ42 (105, 106); the latter, however, found this reduction only in 10 day old and not in 5 day old flies. I then determined whether the reduction of these fly orthologs of AD candidate genes showed this characteristic AD phenotype. *OK107>Lap RNAi A* gave a similar reduction ($P<0.01$) in the fly's one hour memory compared with the wild type (*OK107/+*, Figure 13A). There was also a small reduction ($P<0.05$) in the memory of *OK107>Zyd RNAi B* flies (Figure 13A) but this was not significantly different to their UAS controls (Figure 13B). Consistent with the findings from longevity experiments, there was no difference between the one hour memory of *OK107>Lap RNAi B* or *OK107>Zyd RNAi B* and controls.

To ensure these differences were because of a memory deficit, I performed sensory controls on each of the genotypes used in these experiments (Figure 13C). These experiments test whether the flies properly avoid the aversive odours used (octanol (OCT) and methylcyclohexanol (MCH)) and the shock given. All genotypes had an avoidance of >60% for shock, OCT and MCH.

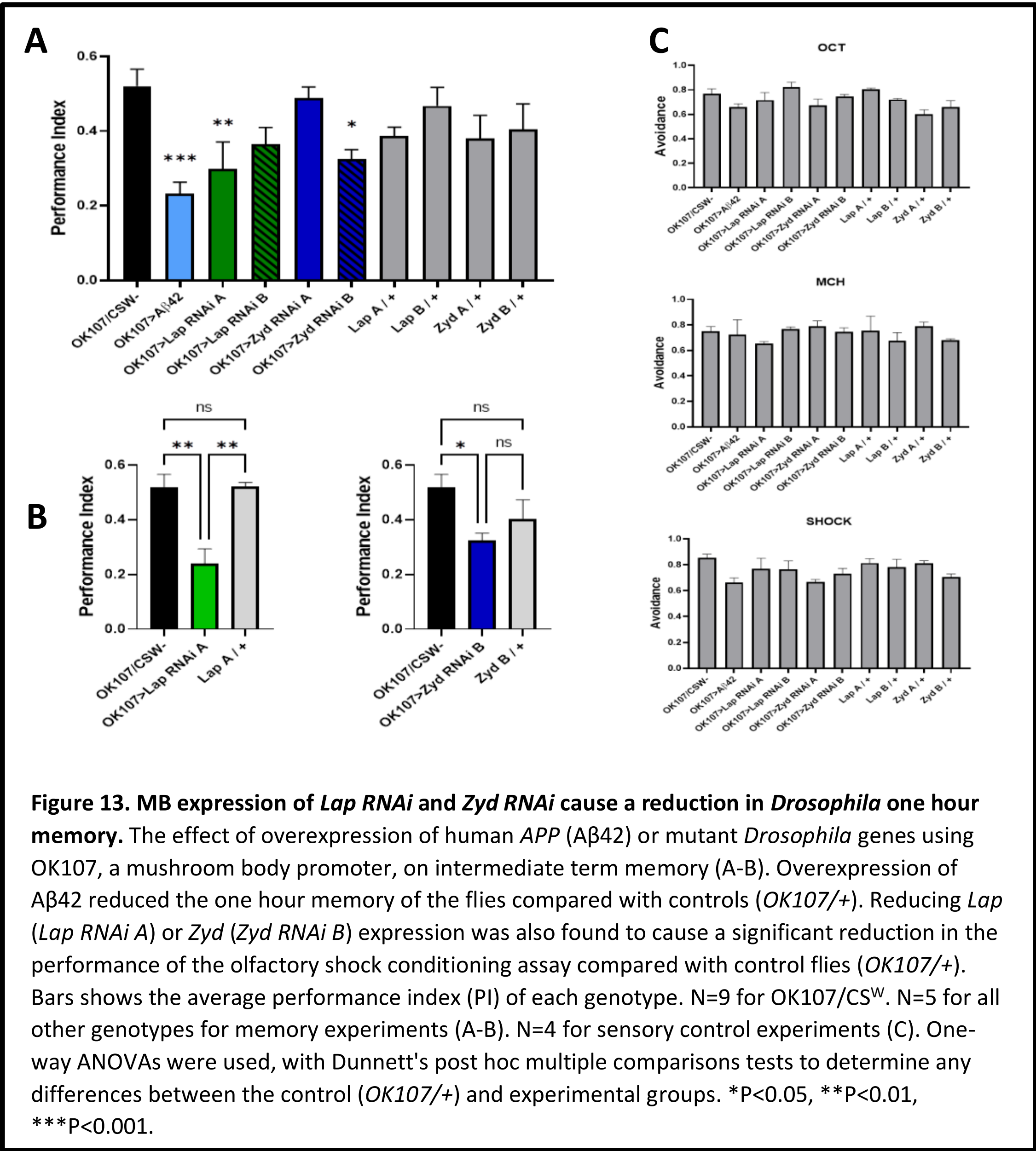


Figure 13. MB expression of *Lap RNAi* and *Zyd RNAi* cause a reduction in *Drosophila* one hour memory. The effect of overexpression of human *APP* (Aβ42) or mutant *Drosophila* genes using OK107, a mushroom body promoter, on intermediate term memory (A-B). Overexpression of Aβ42 reduced the one hour memory of the flies compared with controls (*OK107/+*). Reducing *Lap* (*Lap RNAi A*) or *Zyd* (*Zyd RNAi B*) expression was also found to cause a significant reduction in the performance of the olfactory shock conditioning assay compared with control flies (*OK107/+*). Bars shows the average performance index (PI) of each genotype. N=9 for OK107/CS^{W}. N=5 for all other genotypes for memory experiments (A-B). N=4 for sensory control experiments (C). One-way ANOVAs were used, with Dunnett's post hoc multiple comparisons tests to determine any differences between the control (*OK107/+*) and experimental groups. *P<0.05, **P<0.01, ***P<0.001.

PST1 partially rescues eye degeneration, climbing impairment and sleep disruptions caused by *Drosophila's mnb* overexpression

In order to test the effects of these different DYRK1A inhibitors on the overexpression of *mnb* in *Drosophila*, 100 μl of each drug was dissolved into 10L of fly food (described above) whilst hot and still liquid. Separate flies were then raised on the four different drug food with one set raised on normal food. We then carried out the same assays to see if these drugs had any impact on the *Drosophila's* performance. Results from the two-way ANOVA show a significant interaction between the genotype and the drugs used on the surface area of the eye (($F_{12,120}$) = 9.964, P<0.001). With the eye degeneration assay (Figure 14A), the *mnb* over-expressor flies treated with DYR219, DYR476 and PST1 all showed an increase in surface area of the eye (towards wild type levels) compared to the same flies raised on normal food. Interestingly, using the DYR533 drug on the same *mnb* over-expressor caused a reduction in the surface area. No drug affected lifespan of the *mnb* over-expressor flies (Figure 14B). Results from the two-way ANOVA show a significant interaction between the genotype and the drugs used on the climbing ability of the flies on day 1 (($F_{12,74}$) = 1.961, P=0.04) and on day 21 (($F_{12,73}$) = 3.988, P<0.001). In the climbing assay, when tested on day 1 after eclosion, despite a slight increase in performance, there was no difference between any of the groups. On day 21 however, there was an increase in performance of this assay with flies treated with PST1 (Figure 14D, P<0.01). PST1 also rescued the total sleep back to approximately wild type levels in the *mnb* over-expressor flies (Figure 14E, P<0.001), and also reduced the total activity of these flies back to wild type levels in the DAM experiments (Figure 14F, P<0.001). The two-way ANOVA shows a significant interaction between the genotypes and the drugs used for the *Drosophila's* total sleep (($F_{12,206}$) = 4.254, P<0.001) and total activity (($F_{12,206}$) = 3.187, P<0.001). A similar effect was found using the same *mnb* over-expressor flies treated with DYR219, a reduction in total activity down to wild type levels (Figure 14F, P<0.05) and an increase in total sleep (Figure 14E, P<0.01).

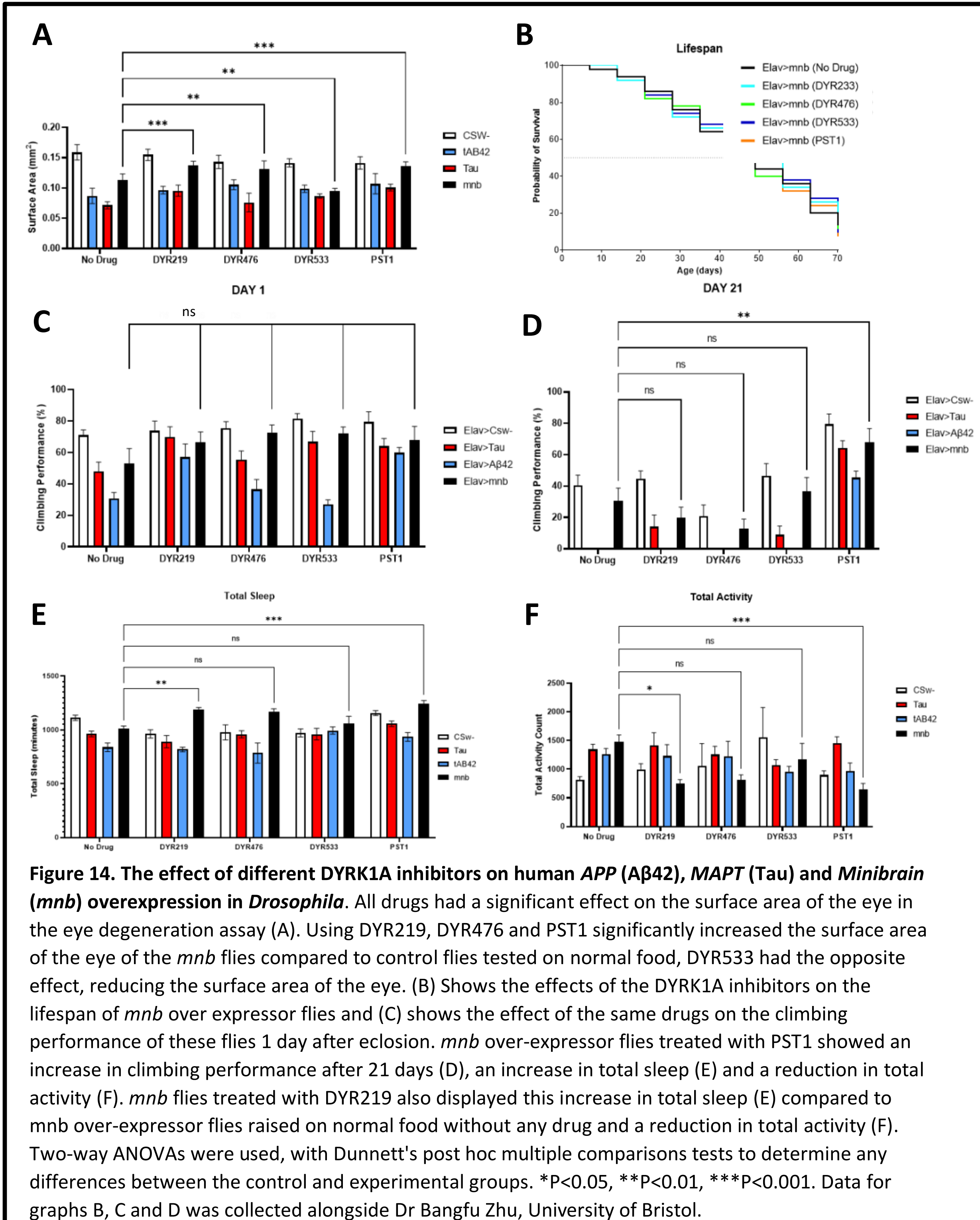


Figure 14. The effect of different DYRK1A inhibitors on human *APP* (Aβ42), *MAPT* (Tau) and *Minibrain* (*mnb*) overexpression in *Drosophila*. All drugs had a significant effect on the surface area of the eye in the eye degeneration assay (A). Using DYR219, DYR476 and PST1 significantly increased the surface area of the eye of the *mnb* flies compared to control flies tested on normal food, DYR533 had the opposite effect, reducing the surface area of the eye. (B) Shows the effects of the DYRK1A inhibitors on the lifespan of *mnb* over expressor flies and (C) shows the effect of the same drugs on the climbing performance of these flies 1 day after eclosion. *mnb* over-expressor flies treated with PST1 showed an increase in climbing performance after 21 days (D), an increase in total sleep (E) and a reduction in total activity (F). *mnb* flies treated with DYR219 also displayed this increase in total sleep (E) compared to mnb over-expressor flies raised on normal food without any drug and a reduction in total activity (F). Two-way ANOVAs were used, with Dunnett's post hoc multiple comparisons tests to determine any differences between the control and experimental groups. *P<0.05, **P<0.01, ***P<0.001. Data for graphs B, C and D was collected alongside Dr Bangfu Zhu, University of Bristol.

Discussion

In this study, my aim was to characterise novel genes associated with AD through GWAS and EWAS using a *Drosophila* model to investigate a role for these genes in the polygenesis and pathogenesis of AD. The cause of AD is still unknown, with 95% of cases being characterised as sporadic, (7) having no familial genetic link. These cases are the result of both environmental factors, such as diet, age and sleep, and sporadic gene mutations (6). This highlights the need to identify and characterise novel genes in relation to SAD to further understand its pathogenesis.

Recently, there has been a rise in the use of *Drosophila* to study AD, and *Drosophila* have become one of the primary organisms to study AD. *Drosophila* only live for a relatively short period of time (≈60-70 days) compared with other model organisms, such as mice or rats. This short lifespan is advantageous when studying AD or any neurodegenerative disorders as the progression of the disease can be studied more quickly. As well as being an inexpensive easy to use organism, *Drosophila* also share 75% of disease causing genes with humans (16), allowing confidence in the translation of the results found with *Drosophila*. With the rise of GWAS and EWAS for AD (29, 107), many novel AD candidate genes are being identified. Through these studies, a gene locus is determined and genes that lie close to these loci are highlighted. However, this does not necessarily implicate that gene because the loci found may lie between numerous genes. Another issue with these studies is that many loci are found near non-coding regions of the genome. This means that experiments need to be performed to easily determine whether there is any link between these genes and AD.

The short generation time (approximately 10 days) and lifespan (approximately 60-70 days depending on strain and sex (108)) of *Drosophila* makes them ideal for experiments like these. *Drosophila* are a powerful tool to screen novel genes quickly in assays set up to identify AD-like behaviours. As well as the wide range of inexpensive transgenes stocks readily available, the *Drosophila* genome (fully sequenced here (109) and very well annotated (flybase.com)) is relatively easy to manipulate in a number of ways. In the present study, I used the GAL4/UAS system (17) (Figure 2), to mis-express an endogenous or exogenous gene (usually attached to the UAS) in a specific targeted tissue or region.

AD-like behaviours arise with *Drosophila* overexpression of human Tau or Aβ42

As previously mentioned, there has been a recent surge in the use of *Drosophila* to model AD. In particular, there have been many advancements in studies with flies expressing the two hallmarks of the disease, Tau and Amyloid-β (reviewed here (110, 111). These models have demonstrated the practicality of this species in studying AD. When overexpressing human Tau or Aβ42 in the eye, they demonstrate the neurodegeneration associated with these proteins in human AD patients (112, 113). Studies also found impaired memory in these mutant flies, a characteristic symptom of the disease. Using the olfactory-shock conditioning assay in *Drosophila*, flies overexpressed mutant Tau or Aβ42 exhibited impaired memory compared with wild-type controls (76). This study also demonstrated a shortened lifespan for these mutants. Because of the AD-like phenotypes demonstrated in previous studies with these genes, in the present study, I used these genotypes as positive controls for each of the assays. This enabled me to make comparisons with my candidate genes to determine the severity of any effect found. Consistent with previous literature, I found a significant reduction in the surface area in the eye of the flies overexpressing human Tau or human Aβ42 (Figure 9). These flies also displayed a ‘rough eye’ phenotype when overexpressing the mutant. This is an effect observed in the *Drosophila* eye where the alignment of the ommatidia is disrupted due to the breakdown of the photoceptor neurons. This effect was only observed with these over-expressor flies and not in any of the *RNAi* lines used. This could be due to the less severe reduction of the eye surface area seen with the other experimental genes.

Expressing these proteins across the whole nervous system, the flies displayed a reduction in their climbing ability of the flies compared with controls with both Tau and Aβ42 (Figure 10A), and overexpression of human *APP* (*Elav>Aβ42*) also caused a shortening of the lifespan of the flies (Figure 11). Expressing these two proteins in the clock neurons throughout development caused sleep problems (Figure 12). *TIM>Aβ42* caused a reduction in total sleep compared with the control; an increase in total activity both in the daytime and night-time. A reduction in the mean sleep episode duration at night was observed along with an increase in the number of sleep episodes at night, meaning the flies sleep for shorter, more frequent bursts during the night-time. This demonstrates a fragmented sleep phenotype in these flies, a behaviour often reported in human AD patients (99). Similarly, *TIM>Tau* flies displayed a reduction in sleep (Figure 12B) and increased total activity (Figure 12C). However, previous studies in these flies have found an increase in the total activity in both the day and night as well as a reduction in total sleep (96). Interestingly, a similar reduction in total sleep

was found in endogenous Tau knock-out *Drosophila* (114). Aβ42 has also been shown to be involved in the memory impairment associated with AD (115). In mice studies Aβ oligomers have been shown to inhibit hippocampal long term potentiation (115) and this is thought to be the basis of the mechanism which drives memory impairment and cognitive decline in human AD. In this study, I showed a reduction in the *Drosophila's* intermediate (1 hour) memory in flies overexpressing Aβ42 in the mushroom body, the memory centre of *Drosophila* (Figure 13). These results are important for forming the benchmark on which I base the performance of my AD candidate gene knock downs, as these were used as my positive controls in these studies. However, caution should be exercised when making comparisons of these results as the positive controls used in the *Drosophila* were overexpressed exogenous genes, conversely the novel genes I was investigating were endogenous genes knocked down in the *Drosophila*, reducing their expression.

***Lap* knock-down in *Drosophila* causes severe phenotypes associated with AD.**

By matching similar phenotypes of the AD causing genes (Tau and Aβ42) in these assays to my novel candidate genes identified from EWAS and GWAS, I could form the basis for characterising these genes in terms of their link to AD. One major consideration and potential limitation of my approach is that the positive controls used in this study were human genes overexpressed in the *Drosophila*, whereas most of the novel genes tested were knock-down of endogenous *Drosophila* genes, chosen based on their similarity to human AD candidate genes. Also, I was not sure if the gene was expressed normally in the Gal4 targeted cells or if RNAi was effective at knocking down the target gene; these are all likely to give false negative results. Despite this, I found many similar phenotypes when reducing expression of *Lap*, the closest *Drosophila* orthologue to human *PICALM*, in *Drosophila* to those found with overexpression of Tau and Aβ42. Like Tau and Aβ42, there was a significant reduction in the surface area of the eye of the fly on reducing *Lap* expression (Figure 9), a phenotype associated with degeneration of neurons in the eye throughout development. It has been reported that 33% of human AD patients have movement difficulties and general locomotion issues (116). Using the climbing assay, I found a similar effect on the locomotor behaviour of flies with reduced *Lap* expression. This was demonstrated by an almost complete elimination of their climbing ability, a measure of their locomotion and startle reflex. This effect was much larger than that seen using Tau or Aβ42 overexpression with the same promoter (Figures 10 and 11). Inevitably, AD in humans results in premature death. This was replicated in my studies with *Drosophila Lap* knock-downs. Like the Aβ42

mutants, when using RNAi mediated knock-down of the *Lap* gene pan-neuronally, the flies had a shorter lifespan than the control (*Elav/+*). The *Lap* knock-down flies, however, had a longer median survival of 36 days, compared with 14 days with the *Elav>Aβ42* line. I also found sleep disruptions similar to those caused by Tau and Aβ42 overexpression; a reduction in overall sleep was found when mis-expressing *Lap* in all clock neurons. Sleep disruption is a major symptom in human AD. In particular, sleep fragmentation is a commonly reported symptom, resulting in an increase in the number and durations of awakenings at night (117). Here I modelled this symptom using two different measures, mean sleep duration and the number of sleep episodes. *TIM>Lap RNAi A* and *TIM>Aβ42* flies showed this sleep fragmentation at night, with a decrease in sleep episode length (mean sleep duration) and an increase in the number of sleep episodes at night compared with my control. This could be the reason for the reduced sleep observed with these flies, as they have shorter, more frequent bursts of sleep.

Interestingly, with these flies, I saw an increase in the activity in experiments using *Drosophila* activity monitors, but there was almost complete abolition of the negative geotaxis response of these flies, a separate measure of locomotion (testing the flies' ability to climb up a vial). These findings suggest that *Lap* could play an integral role in the mechanism of this reflex as their ability to move upwards is hindered but their movement sideways along the DAM tube is not diminished, and in fact this activity is enhanced. However, there is no evidence in the current literature that supports *Lap* involvement in the *Drosophila* negative geotaxis response.

The characteristic phenotype in AD is loss of memory. In the present study, I modelled this symptom using the olfactory-shock conditioning assay (80) and reduced the expression of my candidate genes in the *Drosophila* memory centre, the mushroom body. I used the *OK107-Gal4* promoter line. Reducing the expression of one of my *Lap* RNAi lines (A) caused a significant reduction in the performance in the olfactory-shock conditioning assay compared with control flies. This result translates to impairment of the memory of these flies compared with controls. For most of the genes tested, I used two separate RNAi lines; this is usually done to confirm any phenotype that is found in one. However, all of these positive AD-like phenotypes observed using *Lap* were only found in one RNAi line. *Lap RNAi B* did not give any phenotype in any assay used and consistently gave results not different to the control flies (Gal4/+). One reason for this could be that the RNAi was not working and hence the levels of *Lap* in the second RNAi line were wildtype levels. Due to time constraints, it was not possible to carry out qRT PCR studies to determine the levels of gene expression in each *RNAi* line.

My findings support the theory that it is the reduction in expression of *Lap/PICALM* and therefore a loss of function mutation that is linked with the increased risk of AD. The predominant research implicates *PICALM* to AD through the Aβ pathway. The current literature on this, however, is conflicting (Table 4). Differing evidence emerges with the overexpression or knock-down studies performed with this gene depending on the organism or cell type used. For example, in human cultured cells, there depleted clearance of Aβ following the knock down of *PICALM* (36). In contrast, in mice, there was a reduction in Aβ following the knock-down of *PICALM* as internalisation of APP was reduced (35). There also seems to be differences in race that can affect the predisposition to AD, with different single nucleotide polymorphisms in PICALM (118-120). For example, one study showed only a small proportion of LOAD that PICALM polymorphisms contribute to in the Han Chinese population (making up 92% of the Chinese population) compared with other populations (119). The conflicting evidence with this gene may be attributed to the region of its expression. For example, in human glial derived H4 cells, the reduction in *PICALM* resulted in the reduction of both harmful β-CTF and secreted APPβ, suggesting that a reduced level of *PICALM* in glial cells is protective against AD (121). However, reducing *PICALM* expression in human endothelium cells (where *PICALM* has its highest expression (40)) was found to be detrimental in AD. The study showed the importance of *PICALM* in Aβ clearance through clathrin mediated internalisation by demonstrating that a reduction in *PICALM* consequently reduced Aβ clearance (36). This result was reversed by treatment with *PICALM*.

Another potential cause of the conflicting results with this gene could be that different SNPs found near the *PICALM* gene could result in different effects on its expression. Rs3851179, for example, is an SNP found near the *PICALM* gene and has been shown to be protective against AD. This allele increases *PICALM* expression and enhances Aβ clearance when expressed in human endothelial cells (36).

Using the single cell analysis platform, SCope (122), the expression of *Lap* within *Drosophila* clusters was calculated, these results demonstrate the widespread expression of this protein (Figure 15A), especially in the Kenyon cell clusters. Kenyon cells are situated in the mushroom body, the learning centre of the *Drosophila*. My results suggest that a reduction in Lap/PICALM expression contributes to AD pathology, however, studies using a *Lap* over-expressor would need to be done to confirm this result. Studies should be carried out to see if testing this over-expressor alone in the various assays increases performance compared with wild type (the opposite effect of the knock down mutants). Furthermore, it would be useful to determine if co-expression studies with this and Tau or Aβ42

overexpression showed rescue of the phenotypes caused by these proteins. To identify possible mechanisms of action of *Lap*, further studies could be performed co-expressing the RNAi with Aβ42 or

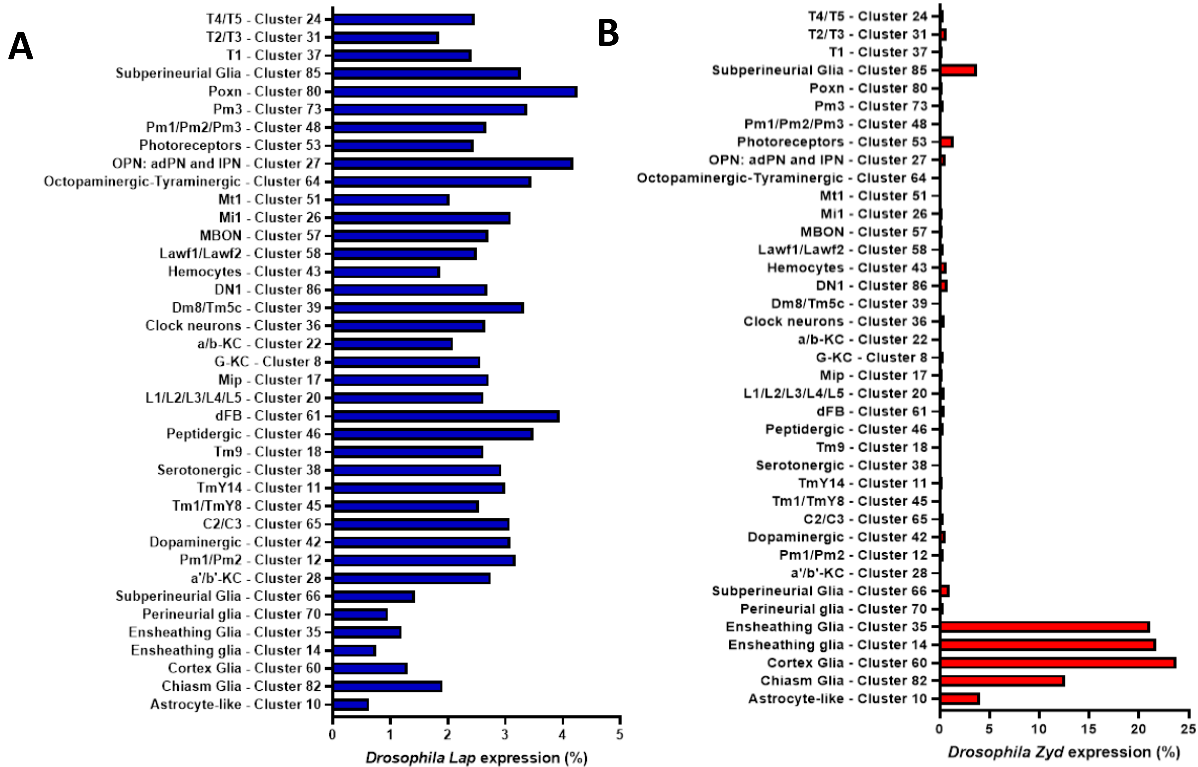


Figure 15. A graph showing the percentage expression of *Lap* (A) and *Zyd* (B) in different areas of the *Drosophila* brain. The clusters are grouped to the cell types based on different markers. The identified marker for each cluster is compared to marker genes previously identified for different cell types (122) .

Tau to see the change in effects compared with expression alone. If the resulting phenotypes are not different, it could indicate a possible overlap of the mechanisms of the genes and may explain their hindered performance in these assays. Also, due to the potential region specific effects of this gene, it would be interesting to determine the effects of knocking down the expression of *Lap* in the glia, similar to work in this study (35).

Over-expression		Reduced Expression		Human observation	
Organism / Cell type	**Effect**	**Organism / Cell type**	**Effect**	**Type of patient**	**Effect**
Fly	Rescued Aβ42-caused neurotoxicity (39)	Mice	↓ Aβ by ↓ APP internalisation (35)	Post-mortem human brains with frontotemporal lobar degeneration	Reduction in PICALM found in these brains (124)
iPSC-derived human astrocytes	↑ PICALM reversed APOE4-induced endocytic defects (125)	Human cultured cells	Depleted Aβ clearance (36)	Human AD patients	↑ PICALM expression in blood of human AD patients (123)
		Glial derived cells	↓ in β-C-terminal fragment and APPβ (121)	Human AD patients	↑ in microvasculature PICALM associated with AD-protective allele (41)
		Mice	Decreasing PICALM expression acceleration Tau pathology (124)	Human AD patients	PICALM ↑ in frontal cortex (40)
				Human Parkinson's patients	↑ PICALM found to give a decreased risk of cognitive impairment (126)

Table 4. List of evidence for the involvement of *PICALM* in AD.

Zydeco knock-down mutants exhibited hyperactivity

The role of Zydeco or its human orthologue, SLC24A, in AD is less studied. Unlike experiments with *Lap*, both RNAi lines used for *Zyd* displayed some AD-like phenotypes. *Drosophila Zyd* mutants caused a significant reduction in the surface area of the eye. This phenotype could be due to the destruction or degeneration of the photoreceptors in the eye as the result of a reduction expression of *Zyd* here. Reducing the expression of *Zyd* resulted in an impairment in the negative geotaxis response of the flies (Figure 10). *Zyd RNAi B* showed a similar age dependent decline in climbing ability similar to the wildtype, however the performance on each day was significantly reduced (Figure 10C). *Elav>Zyd RNAi A*, however, did not show an age dependent decline until day 20, where their climbing ability was reduced to 22%. Although these flies displayed a reduction in climbing ability, they were notably hyperactive. However, this activity was around the bottom on the vial when they were tapped down and so this may have contributed to the poor climbing scores. Locomotor issues is a problem reported in one third of AD patients (116), but this is also a symptom of many other disorders. One line of the *Zyd* mutants (*Zyd RNAi B*) also showed a reduction in the median survival of the *Drosophila* by 10 days compared with control flies (Figure 11), and so reduced the lifespan of these flies.

These flies were hyperactive. Although this is a phenotype previously found with AD causal genes Tau and Aβ42, AD patients mobility often declines quicker than those not suffering with the disease (127). These mutants also displayed sleep problems. Like the phenotype caused by overexpression of AD causal genes, *Zyd* mutants displayed a reduction in total sleep (Figure 12A and 12B, respectively). Consistent with observations in the climbing assay, both of these mutants caused hyperactivity in the sleep experiments. I also found memory impairment when expressing *Zyd RNAi B* with the mushroom body promoter OK107 using the *Drosophila* olfactory-shock conditioning memory assay (Figure 13).

Zydeco encodes a potassium dependent sodium/calcium exchanger. Its highest expression is in the glia of the *Drosophila*. Here it is thought to help manage neuronal excitability, particularly within the cortex glia (128), but the mechanism has yet to be established. Previous work with *Zyd* knock-down flies demonstrated a predisposition to seizure-like activity (55) when exposed to certain external stress, a phenotype I observed when using these flies in the climbing assay. This could explain the poor performance of these flies in this assay; instead of an impaired negative geotaxic response, the banging at the bottom of the vial could have induced seizure activity. The seizure-like activity observed with these mutants is thought to be the result of the dysregulation of cortex glial excitability, through enhanced basal Ca^{2+} levels (128). Epilepsy and seizures are linked to AD. It has been found that AD

patients have a 10 times higher risk of developing epilepsy (129); this is particularly troubling for these people as it can result in worsening of other symptoms of AD, such as cognitive decline (130). Seizures seem to be more likely to occur in the earlier stages of AD and in people with EOAD (131). There is also evidence to suggest that this relationship is bidirectional, with research suggesting that older epilepsy patients are more likely to develop dementia (132). The principle finding in these *Zyd* mutants was the hyperactivity displayed in the DAM activity experiments and climbing assay. This suggests that this gene may not be directly involved in the pathogenesis of AD, but its reduced expression may contribute to some severe symptoms connected to the disease, such as seizures. Although I did not investigate the mechanism of action, previous research with this mutant suggests this could be due to dysregulation of glial excitability, possibly resulting in the destruction of these cells through hyperexcitability.

Drosophila Zyd protein was predominantly expressed in glial clusters, with some expression elsewhere in the photoreceptors and clock neurones (Figure 15B). As this gene seems to be primarily localised in the glial clusters in the *Drosophila*, determining the effects of knocking down its expression should be investigated, to see whether this exacerbates the phenotypes expressed in its neurons or gives different results. I found more severe phenotypes with one of the two RNAi lines used. *Zyd RNAi* B consistently displayed a stronger effect compared with the wild-type. This is probably due to RNAi not working as efficiently in this line but to determine expression levels of both lines, qRT PCR experiments should be carried out.

PST1 partially rescued AD-like phenotypes caused by mnb overexpression

Results from the drug studies carried out with the *mnb* over-expressor indicated some promise of their effectiveness in rescuing disease phenotypes caused by the mutant flies. PST1 demonstrated the most effective recovery of wild-type phenotypes of all the DYRK1A inhibiters tested. Using this drug, the surface area of the *Drosophila* eye was significantly larger than without the drug (Figure 14A), indicating a reduction in the neurodegeneration caused by the mutant. When testing the negative geotaxis response of these flies on the PST1 drug, their performance in the climbing assay was enhanced only on day 21 (Figure 14D). There were also promising results with this drug in the sleep and activity studies. I found that the reversal of the reduced sleep caused by the *mnb* over-expressor exceeded the level of the wild type (Figure 14E), and there was a reduction in the hyperactivity caused

(Figure 14F). Although this was not confirmed by looking at the day and night figures separately, it suggests that the flies treated with PST1 had a reduction in their activity at night and were therefore sleeping better. Two other DYRK1A inhibitors also increased the surface area of the flies overexpressing *mnb*, DYR219 and DYR476. These drugs did not affect the performance in the climbing assay or have any effect on the lifespan of the flies. DYR 219 however did reverse the AD-like behaviours in the DAM experiments, by increasing the amount of sleep, and reducing the activity of these flies towards wild-type levels (Figure 14E). DYR533 did not have any effect on the overexpressed *mnb* fly's behaviour except that it caused a reduction in the surface area of the eye compared with the *mnb* overexpressed flies eating normal food. These results suggest that PST1 would be an ideal candidate to investigate in higher animal models, but there are factors to consider. All of the drugs used were at the same relatively high concentration, to correctly determine the effects on the overexpressed *mnb* flies. A range of concentrations of drugs should retested in the different assays to look at the impact on performance. One issue with these experiments is that although the concentration of drug added to food can be controlled, it is difficult to determine how much food the flies eat, and therefore how much of the drug is in their system. There is also a potential issue if the drug makes the food unpalatable; however, if this was the case a lower median survival would be expected, and this was not seen for any of the drugs used. One further study could be to determine the effect of the drugs on the interaction of *mnb* and Tau co-expression. As DYRK1A phosphorylates Tau, and overexpression of this may lead to hyperphosphorylation, determining the effects of the DYRK1A inhibitors in this system would better reflect *in vivo* conditions.

Despite strong trends in the data, there were no significant changes in the performance of the Tau or Aβ42 over-expressor flies following treatment with any of the DYRK1A inhibitors. Although these drugs don't directly work on either of these proteins, there has been recent evidence to suggest an interaction between them. As stated earlier, it was found that DYRK1A phosphorylates tau. In AD, tau becomes hyperphosphorylated and therefore you would expect an inhibition of this phosphorylation with the drugs used to have some effect on the mutant. This could be explained by the concentrations of the drugs used, more experiments, with differing concentrations of the drugs would have to be carried out in order to determine whether an effect would be seen with the over-expressed tau mutants.

Future directions and limitations

My results demonstrate a success in using *Drosophila* to better understand novel genes that may be implicit in the pathogenesis of AD. Using the assays demonstrated here shows a quick, cheap, and reliable method for identifying potential new disease-causing genes in humans. More work can be done with these mutants, however, to better determine their role in the pathogenesis of AD. For example, except for one, all of the novel mutants that were tested in these studies were knock-down mutants performed using RNAi. This was done because of time constraints, I decided to test the effects of LOF of the candidate genes of interest. However, many SNPs result in a GOF mutation, and this could explain the negative results from some of the genes in the assays. Another possible cause of false negatives results is that the genes may not be expressed in the region where I reduced expression for each of the assays. For example, although there is some expression in neurons, *Zyd* is predominantly expressed in glial cells and therefore using RNAi in this region would probably give a larger effect than using the *Elav-Gal4* promoter, expressing in just neurons. Also, some genes may have region specific effects, and despite being expressed in these regions, reducing their expression may not have an effect in one area but play a more important role in another. Also, the RNAi can have off target effects on other genes, this highlights the requirement to confirm any phenotype found with two different RNAi lines for each genotype.

To further investigate the mechanism by which these genes cause their effects, co-expression studies could be carried out with AD causal genes, such as Tau and Aβ42, and the performance of these mutants could be compared with the single knock-down genes to see if the phenotype is stronger. If there is no change in the effect, this could indicate that the two proteins may work by a similar mechanism to cause the defect. Because of the phenotypes found with *Lap* and *Zyd* knock-down in *Drosophila*, it would then be interesting to use an over-expressor line for my candidate genes in these assays to see if this has an effect on their performance. Further, co-expression experiments with these over-expressors and Tau or Aβ42 would be useful to try to replicate previous studies (39) that rescued the phenotypes caused. Circadian analysis would also give further useful information. After acclimatisation to a light-dark cycle, the flies are kept for a further five days in the incubator in constant darkness. This shows how intact the circadian rhythm of the fly is and how well it can persist without external light cues. These studies were due to be carried out with my mutant flies after the sleep experiments, however the majority of the flies became too sick after the first five days in the incubator and hence the N of these results was too small and variable to analysis.

Before the start of these experiments, qRT PCR experiments should be performed on each of the different experimental lines. This would allow exclusion of the RNAi not working as a potential cause for no effects seen with a genotype. Furthermore, knowing the expression levels in different RNAi lines could also help determine why you may be getting more of an effect with one line. One assumption made in the present study was to further test only the genotypes that gave a phenotype in the eye degeneration assay. This assay is quick and so it is easy to screen different genotypes in this experiment. Although this seems to be a good indicator for genes that give more AD-like phenotypes, confirmation that the gene is not necessary for the development of the eye is needed first; this could lead to false positive results as the gene knock-down effect would not be due to the neurodegenerative effect associated with AD, but rather a developmental defect. Also, the expression of the gene needs to be considered before the start of these studies. If this gene is not expressed in the developing eye, no effect will be observed. Another problem with this assumption is that it can lead to false negative results; because the gene knock-down in the eye does not cause neurodegeneration does not mean it will not cause other AD-like phenotypes in other assays. What also needs to be considered is that the proteins being compared (in *Drosophila* and humans) only have an approximately 50% amino acid similarity to each other. Although these *Drosophila* genes chosen for the study were the most similar orthologs, the genes could have different functions in the two species and so could dampen the translation of the results.

AD is a neurodegenerative disorder; this means that the disease worsens as it progresses. In AD, for the majority of sAD cases, patients are symptomatic after about 60 years of age. In the present study, I expressed my candidate proteins throughout development and for the whole of the fly's lifespan. However, to properly model this disease, these proteins should be mis-expressed in the adult aged brain to reflect when these proteins are causing their effect in human AD patients. Another benefit of conducting the studies in this way is that the possibility that the effect seen is not a result of the experimental gene impairing the development of a particular brain region can be eliminated. As the gene is not mis-expressed throughout development, it allows the proper formation of that region and therefore confirms that the phenotype must be a result of misexpression on the neurons and not an improperly formed structure. However, accumulation of Tau and amyloid-β has been found to manifest 20 years before the onset of symptoms of the disease (133), and so this should also be considered when performing these studies. This method of modelling AD in flies has drawbacks because in many of the assays used in this study, the flies display an age dependent decline in performance (Figure 10A for example). This makes it difficult to distinguish between the effects of age

versus the effect of the genotype. However, looking at differences in age dependent decline could solve this problem; if a certain genotype accelerates this age dependent decline, then this effect could be compared.

Conclusion

These studies show that *Drosophila* can be used successfully to study the pathogenesis of AD. In particular, this study highlights the advantage of using this organism to quickly screen novel genes that may be linked to AD, identified through GWAS and EWAS, for example. There are, however, limitations to using *Drosophila* in studying this disease; for example, the difficulty in modelling more complex symptoms of the disease, such as cognitive impairment. This limitation highlights the need to use animals of greater brain complexity in any studies that follow-up on these results. Another consideration for further experiments is the need to reflect disease progression more accurately. Because AD is a neurodegenerative disorder, usually developing after 60 years of age, a system in which the flies are tested in old age would better represent the disease state in humans. There are difficulties when using this system, however, because in most of the assays used, there tends to be an age dependent decline in performance. Gene expression needs to take account of fluctuations in gene levels throughout adulthood across different regions of the *Drosophila*. These fluctuations could mask the true effect of the gene knock-down.

This study gives promising preliminary evidence for the potential role of both *Zyd* (*SLC24A* in humans) and *Lap* (*PICALM*) in the pathogenesis of AD and highlights the need to further investigate these two genes in relation to AD.

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