Investigating the role of *herzog* in the *Drosophila melanogaster* brain

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Abstract

Memory and cognitive disorders, such as Alzheimer's disease and age-associated memory loss, are common, but currently there is no cure or effective treatment. Better understanding of the pathways involved in memory and how disruptions within these occur may assist in the development of new treatments. To further dissect normal memory and how disruptions in these pathways affect long-term memory, it is vital to first understand which proteins are involved and their function within memory. Histone deacetylase 4 (*HDAC4*) plays an important role in memory and brain development in *Drosophila melanogaster*. Mutations in *HDAC4* result in intellectual disability in humans and overexpression causes memory deficit in *Drosophila*. The phosphatase *herzog* was identified as a potential gene target of *HDAC4* via a previous RNA-seq experiment. We found *herzog* to be highly expressed in the brain, especially in the mushroom body. No nuclear localisation of *herzog* was seen which contrasts to the localisation of human CTDSP1 suggesting they have diverged in regard to their functions and phosphatase targets. *Drosophila* eye development requires *herzog*, however mushroom body development, courtship learning and short-term memory does not require *herzog*.

Acknowledgements

The year 2020 was quite an eventful and carrying out research within a global pandemic was quite an experience but the year has flown by and here we are.

I would first like to thank my supervisor Helen Fitzsimons for all her support, kindness, and expert guidance throughout the project. Her advice and instruction during the experimental and writing process have been invaluable. So again, thank you.

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Another huge thank you to my family, especially my parents who have always encouraged me and without whom I would never have been brave enough to commence this journey. To all my friends who have been so consistent in their support and kindness, thank you. Lastly I'd like to thank Albert for all his love, encouragement, and patience throughout the process.

SARS-CoV-2 Impact Statement

My master's thesis research commenced at the beginning of 2020 with a thesis due date of 28th February 2021. My research outline was planned with these dates in mind aiming to be completed my lab work by December 2020. Due to the global pandemic and the New Zealand wide lockdowns the University completely shut down on the 23rd of March and a full lock down was implemented. During this time only staff who were necessary were allowed on campus, so no lab work was able to be done during this time. Also due to the nature of working with *Drosophila*, crosses and lines cannot be halted in their development and be preserved via freezing. Due to this all samples experiments in progress had to be discarded.

Shortly after New Zealand entering Level 2, on the 13th May we were permitted to enter campus again and were able to continue with lab work. The lock down and level 3 resulted in just under 8 weeks of lost lab time. After getting back into the lab we were able to resume relative normalcy while maintaining precautions. To get lines ready to work with fly stocks were expanded, which requires approximately 3 weeks, from these crosses could be set or further expansion carried out which took another 3 weeks. Prior to lockdown I had various crosses coming out ready for various assays. To get back to where I was before lockdown, 6 weeks were required after returning to the lab.

Overall given the lockdowns and loss of perishable work it was approximately13 weeks from the beginning of the lockdown to when I was able to resume my work I had lost. This impacted the following objectives:

- Objective 1: PCR analysis to confirm *herzog::GFP* line was unable to be carried out and optimized.
- Objective 3: western blotting to confirm RNAi knockdowns of herzog would have ideally been repeated to determine whether RNAi 4 knocked down herzog to a statistically significant level.
- Objective 4: Long-term memory assays were unable to be carried out due to time constraints.
- I would have also liked to investigate Herzog aggregation in the brain using Thioflavin T staining.

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Abbreviations

°C	Degree Celsius
ANOVA	Analysis of Variance
ATF4	Activating Transcription Factor 4
BCA	Bicinchoninic Acid
BDMR	Brachydactyly Mental Retardation
BDNF	Brain Derived Neurotrophic Factor
BDSC	Bloomington *Drosophila* Stock Centre
CAMK	Calcium/Calmodulin Dependent Kinase
cAMP	Cyclic Adenosine Mono Phosphate
CI	Courtship Index
CNS	Central Nervous System
CTD	Carboxy Terminal Domain
CRE	cAMP Response Element
CREB	cAMP Response Element Binding protein
CS	Canton Special
Cy	Curly
DAPI	4’,6-diamidino-2-phenylidole
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose Nucleic Acid
DSHB	Developmental Studies Hybridoma Bank
ECL	Enhance Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
Elav	Embryonic Lethal Abnormal visual system
FasII	Fasciclin II
GFP	Green Fluorescent Protein
GMR	Glass Multiple Reporter
HAT	Histone Acetyl Transferase
HCL	Hydrochloric Acid
HDAC4	Histone Deacetylase 4
HSD	Honestly Significance Difference

HRP	Horseradish Peroxidase
Hzg	Herzog
kDa	Kilodalton
L	Litre
LI	Learning Index
LTM	Long-term Memory
M	Molar
MAPK	Mitogen Activated Protein Kinase
MEF2	Myocyte Enhancer Factor 2
mg	Milligram
MI	Memory Index
ml	Millilitre
mm	Millimetre
mM	Millimolar
MPI	Ministry of Primary Industries
mRNA	Messenger RNA
ng	Nanogram
OE	Overexpression
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PKA	Protein Kinase A
RE1	Repressive Element 1
REST	Repressive Element Silencing Transcription Factor
RIPA	Radio-Immunoprecipitation Assay Buffer
RNA	Ribonucleic Acid
RNAi	RNA Interference
RNAseq	RNA Sequencing
RPM	Revolutions Per Minute
Sb	Stubble
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
STM	Short-Term Memory

TARGET	Temporal and Regional Gene Expression Targeting
TE	Tris EDTA
Ts	Temperature Sensitive
UAS	Upstream Activating Sequence
V	Volt
VDRC	Vienna *Drosophila* Resource Centre
WT	Wild Type
µg	Microgram
µl	Microlitre
µm	Micrometre
YFP	Yellow Fluorescent Protein

1 Introduction

1.1 Memory disorders – a growing problem

The population of the world is becoming increasingly older, with the proportion of the population aged 60 and over expected to increase from 11% to 22% by 2050 (Newgard & Sharpless, 2013). An ageing population increases the number of people suffering from dementia, as after the age of 65 the chance of developing dementia doubles every five years. Currently an estimated 35.6 million people suffer from dementia globally, with this set to increase to 115 million by 2050 (Jindal et al., 2014; Zeisel et al., 2020). Alzheimer's disease is the most common form of age-related dementia (Mayeux et al., 2011; Ott et al., 1998) and currently no effective treatment, to slow down the progression or cure exists (Chiang & Koo, 2014; Thies & Bleiler, 2012). The primary characteristics of Alzheimer's are the loss of memory and cognitive function (Grober et al., 1988; Mayeux & Sano, 1999; Shankar et al., 2008). Ageing can also cause memory loss without dementia, this age-associated memory loss is present in 18.5% of people between the ages of 50-95 (Barker et al., 1995). How memory is disrupted in such disorders is still unknown and there are many gaps within our collective knowledge of memory. A better understanding of the molecular pathways involved in memory will allow further understanding research into how they are disrupted in memory disorders. Understanding what leads to memory disorders, will aid in development of new strategies for treatment.

1.2 Molecular basis of memory

1.2.1 Short-term memory

Memory consists of both long-term and short-term phases, the formation of which requires distinct molecular pathways within neurons. Short-term memory lasts minutes to hours and is due to covalent modifications of pre-existing proteins (Hawkins et al., 2006). Much of the current knowledge of the molecular basis of memory stems from research in the marine snail *Aplysia californica* by Eric Kandel and colleagues during the 1970s, in which they studied the gill withdrawal reflex. In this model, if the snail's tail is shocked at the same time as the siphon of the gill is touched, the gill will withdraw. When the gill is subsequently touched it will withdraw despite this being an innocuous stimulus, therefore the snail has been sensitised. The sensitisation lasts for a short time, i.e. is transient in nature and results in short-term memory. With training by repetition of tail shocks, long-term memory can be formed in which the snail is sensitised for days to weeks. Thus, *Aplysia* is an ideal model to investigate the molecular processes required

for short-term and long-term memory. Kandel and colleagues found that the molecular basis for short-term memory in *Aplysia* is that a stimulus (tail shock) induces a release of serotonin, a neurotransmitter, from the facilitating neuron that forms a synapse with a sensory neuron (Castellucci et al., 1970; Glanzman et al., 1989; Marinesco & Carew, 2002) (Figure 1.1). Activation of the serotonin receptors results in an increase in cyclic adenosine monophosphate (cAMP) (Brunelli et al., 1976), which in turn activates Protein kinase A (PKA), allowing it to phosphorylate various targets, including potassium (K+) channels (Kandel, 2012). The phosphorylation of the potassium channels causes them to remain closed, generating an action potential, which opens voltage-dependent calcium channels, and allows an influx of Ca^{2+} ions into the neuron (Benfenati, 2007). This increased Ca^{2+} induces a transient increased release of neurotransmitter by the sensory neuron at the synapse with the motoneuron (Castellucci & Kandel, 1976) (Figure 1.1). This transient increase in release of neurotransmitter results in a short-term increase in synaptic strength and therefore, increases post-synaptic response of the motor neuron to the same stimulus. Since this mechanism was discovered, subsequent research in other model systems including *Drosophila* and higher vertebrates have found that the genes and molecular pathways involved in memory formation are conserved (Kandel, 2001, 2012).

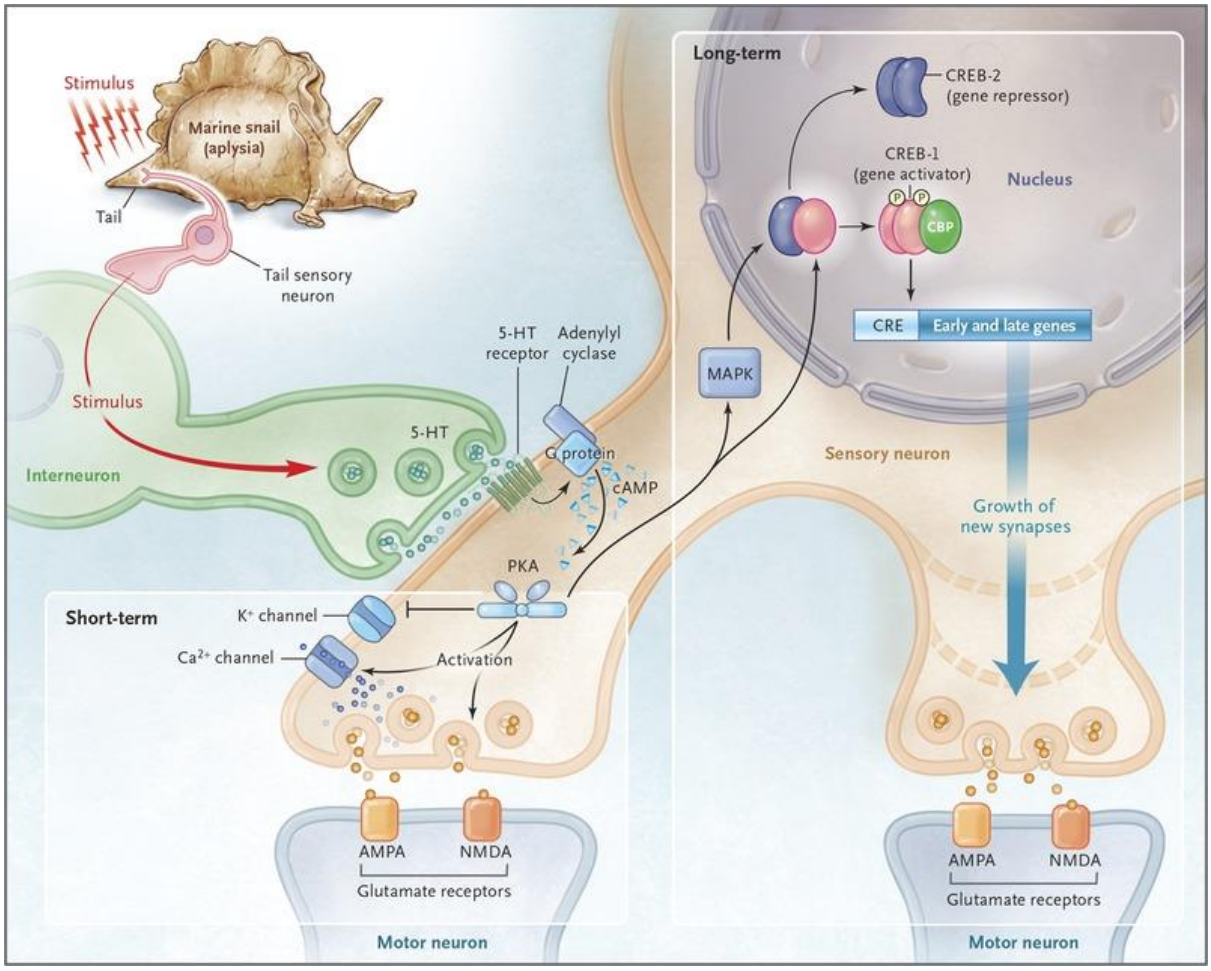

Figure 1.1 Molecular pathways of short-term and long-term memory

A stimulus results in increased serotonin release (5-HT), inducing increased cAMP levels. Short-term memory results in transient activation of PKA which phosphorylates ion channels, leading to a transient increase in neurotransmitter release. Long-term memory requires repetition of a stimulus resulting in persistent activation of PKA, which translocate to the nucleus with MAPK, where it activates the expression of genes required for synaptic plasticity. Reproduced with permission from (Kandel & Kandel, 2014), Copyright Massachusetts Medical Society.

1.2.2 Long-term memory

Long-term memory can be divided into three stages: acquisition of information; consolidation to long-term memory, via repetition or training; and retrieval, where the learning is recalled after a time period. Long-term memory is formed after repeat exposure to a stimulus or training (Frost et al., 1985; Kandel et al., 1986). Formation of long-term memory requires new synapses therefore changes in gene expression and protein synthesis are essential (Bailey et al., 1996). Repeat activation by a stimulus produces a continuous increase in cAMP and PKA. Consistent high levels of PKA results in the formation of complex with mitogen activated protein kinase 42 (MAPK42) and

translocate to the nucleus (Bacskai et al., 1993; Martin et al., 1997). In the nucleus PKA phosphorylates transcription factors, including cAMP Response Element-Binding protein (CREB). Activated CREB is able to bind cAMP response elements (CRE) in promoters to induce transcription of the downstream genes that are necessary for synaptic plasticity (early and late genes) and long-term memory formation (Yin et al., 1995; Yin et al., 1994) (Figure 1.1). Along with differential expression and epigenetic changes to chromatin, modification of neuron shape, morphology and growth are vital for long-term memory formation and storage. During formation of new long-term memories, neurons undergo growth of new synaptic connections, which is facilitated by a range of proteins. One such protein is Moesin, a cytoskeletal regulator which is critical for this process. Both increased or decreased levels of Moesin cause defects in long-term memory in *Drosophila* due to axon growth deformities (Freymuth & Fitzsimons, 2017). There are many other proteins which have been identified to be essential for memory formation, storage, and retrieval. However, more research is required to determine the specific roles of these genes and how they are regulated, small model organisms, such as *Drosophila,* are ideal for this type of research.

1.3 *Drosophila* as a neurogenetic model

Drosophila is an ideal model for neurogenetic analysis due to the ease of genetic manipulation (Venken & Bellen, 2007; Venken et al., 2011) and short reproduction cycles of approximately 21 days, which allows the ability to generate thousands of offspring within a few weeks. Importantly for the study of neurodegenerative or neurodevelopmental disorders, they can learn and form memories which can be assessed by quantitative, reproducible assays (Ejima et al., 2005; Koemans et al., 2017; Mehren et al., 2004). The *Drosophila* genome is also conserved, sharing of 87% genes that have been implicated in human neurological disorders (Inlow & Restifo, 2003). Although the *Drosophila* brain is simpler than a human brain, it is a bilateral structure that contains approximately 100,000 neurons, with hundreds of different types of neurons organised into specific centres. The similarities and shared evolutionary origin of human and *Drosophila* central nervous systems, makes *Drosophila* a good model for human neurological function (Aso, Hattori, et al., 2014; Hirth & Reichert, 1999; McGurk et al., 2015). The mushroom body has emerged as a structure within the *Drosophila* brain that is necessary for learning and memory, as chemical ablation of the mushroom body causes impaired courtship learning and memory (McBride et al., 1999). Chemical ablation of the

mushroom body has also been shown to cause loss of olfactory learning (de Belle & Heisenberg, 1994). This shows both olfactory and courtship memory require the mushroom body and there is evidence that different forms of memory require different neuronal circuitry within the mushroom body, for example gamma lobe output neurons are specifically required for courtship memory (Redt-Clouet et al., 2012; Zhao et al., 2018), which is the assay that is used throughout the project to assess memory. Memory of a females courtship behaviour is important for male *Drosophila* as virgin females are usually willing to mate whereas mated females are usually not, so a male learns and remembers rejection behaviour from mated females and rather focuses courting virgin females which he will have more chance of successfully mating. Recognition of a mated female verses a virgin female is due to the male pheromones left behind by the male with which the female mated (Keleman et al., 2012).

1.3.1 The mushroom body

The intrinsic neurons of the mushroom body are the approximately 2200 Kenyon cells, of which there are three subtypes; the α/β, α'/β' and γ neurons (Aso, Sitaraman, et al., 2014). The cell bodies of Kenyon cells are clustered at the posterior of the brain and project dendrites into the calyx, which surrounds the cell bodies. The axons of all the Kenyon cells are bundled into a fibre termed the peduncle which projects towards the anterior of the brain where the axons split into 3 lobes. The γ neurons form the γ lobe which projects laterally, the α/β and α'/β' axons bifurcate vertically to form the α and α' lobes and horizontally to form the and β and β' lobes, respectively. A visual representation of the mushroom body is shown in Figure 1.2.

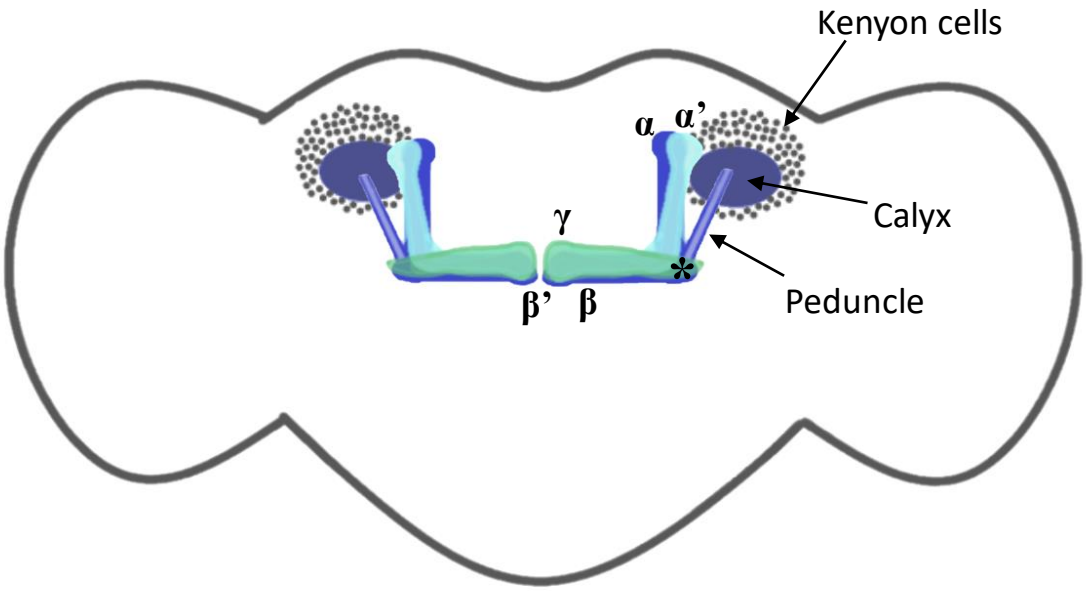

Figure 1.2 Schematic model of a *Drosophila* mushroom body.

Calyx, Peduncle and Kenyon cells are labelled by name and the lobes are labelled and organised by colour. Dark blue, α/β; light blue, α'/β' and green, γ. * indicates where the α and β lobes bifurcate.

The lobes are both structurally and functionally distinct and investigation into which lobes of the mushroom body are required for olfactory memory showed that a mutant causing the mushroom body to develop lacking the medial (γ and β/β') lobe did not affect long-term memory, however a mutant lacking vertical lobes (α/α') had impaired long-term memory. The loss of both medial and vertical lobes had no effect on short-term memory (Pascual & Preat, 2001). There are various studies into the specific role of the mushroom body during different phases of memory; it has been demonstrated that retrieval of memory requires synaptic signalling in the mushroom body, but formation and consolidation of memory does not (McGuire et al., 2001). Similar results were seen in another study where neurotransmission within the mushroom body was required for retrieval of memories but not the acquisition or storage (Dubnau et al., 2001). Gene expression and protein synthesis changes were shown to occur within mushroom body neurons during long-term memory (Wu et al., 2017). Halting of ribosomes and therefore gene expression did not affect formation of long-term memory, however, it did effect retrieval of the memory (Chen et al., 2012). Together these data show that the mushroom

body is a vital centre for memory in the *Drosophila* brain, however its exact role is not yet fully understood.

1.4 Tools available to study *Drosophila* neurogenetics

A vast number of tools have been developed over the last two decades to genetically manipulate *Drosophila*. One example is the well-established and relatively simple process of generating transgenic fly lines via the PhiC31 system (Groth et al., 2004; Venken et al., 2006). Another widely used system which allows tissue specific control is the UAS/GAL4 system, which represented a huge advance in the field as it provided the ability to express any construct, such as a transgene for overexpression or misexpression, RNAi knockdown or expression of a reporter or effector, in a tissue specific manner.

1.4.1 UAS/GAL4 system

This system is based on the yeast GAL4 transcriptional activator, which binds to the upstream activating sequence (UAS) to activate expression of a downstream transgene. Neither the UAS promoter or GAL4 protein are present endogenously in *Drosophila* so are able to act without GAL4 targeting endogenous *Drosophila* genes (Brand & Dormand, 1995; Brand & Perrimon, 1993). GAL4 expression can be driven in a tissue specific manner by inserting a tissue specific promoter upstream of the GAL4 gene. The utility of the UAS/GAL4 system is that it is bipartite, with one fly line carrying the UAS-transgene construct and the other carrying the promoter-GAL4 construct. When the lines are crossed, the F1 progeny carry one copy of each transgene, resulting in tissue specific expression (Figure 1.3). Libraries of driver-GAL4 lines are available from commercial stock centres that drive expression in the majority of *Drosophila* tissues. This allows a researcher to drive expression in tissues or cell-types of interest by crossing the selected driver with their UAS-transgene line without requiring the cloning of a promoter-transgene construct. The *Elav-GAL4* driver is particularly useful for neurogenetic research as it drives expression in all neurons. It makes use of the *Elav* promoter, which endogenously drives expression of the *Elav* gene in all neurons throughout development and in the adult brain (Robinow & White, 1988, 1991). The tissue-specific expression avoids potential whole organism-wide effects which may be lethal or may impair physiological functions.

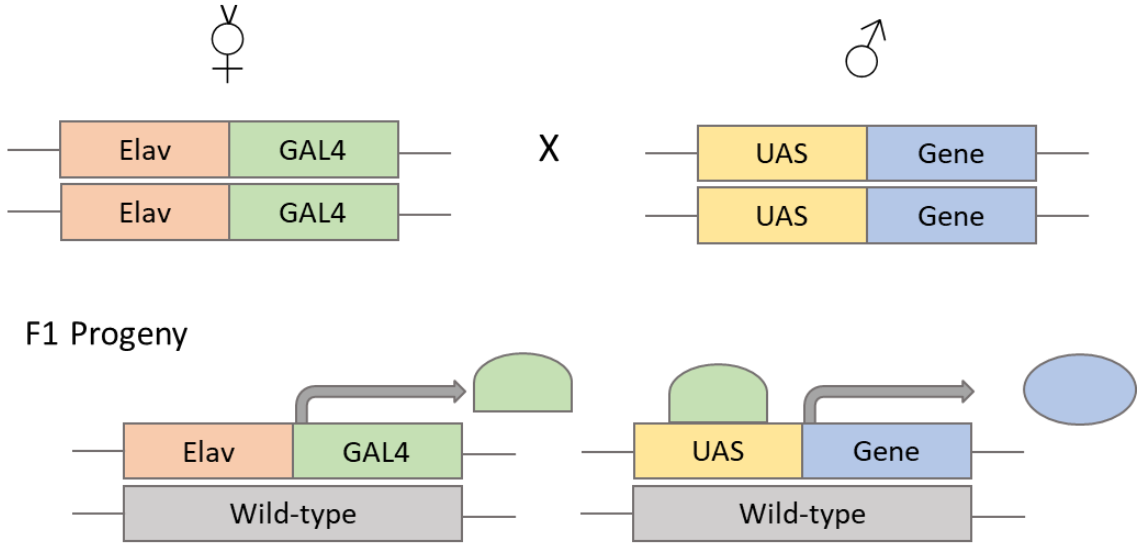

Figure 1.3 Schematic of the UAS GAL4 system.

A line containing the *elav-GAL4* construct is crossed with a line carrying the *UAS-Gene* construct. Progeny of the cross contain a single copy of each construct, in which GAL4 activates transcription at the UAS site and the target gene is expressed in tissue specific manner, in this case all neurons.

To investigate whether a specific protein is essential for a specific physiological process or behaviour, a common strategy is to reduce expression of the gene and examine the effect on the phenotype of interest. In *Drosophila*, this can be carried out via RNA interference (RNAi), in which a small inverted repeat of the mRNA corresponding to the target gene is expressed. The inverted repeat binds to the mRNA of target gene which results in degradation of the mRNA (Kennerdell & Carthew, 2000; Lam & Thummel, 2000; Martinek & Young, 2000). This method of RNA knockdown results in depletion of the mRNA and therefore reduced protein levels, however, the level of knockdown can be variable between RNAi constructs and the majority do not result in a complete knockdown of mRNA (Dietzl et al., 2007).

1.4.2 DeGradFP system for knockdown

Another method of reducing protein levels in *Drosophila* is the deGradFP system (Figure 1.4) which takes advantage of the vast library of GFP-trapped proteins (see section 1.4.3). The NSlmb-vhhGFP4 protein is a fusion between the vhh-GFP4 nano-body (vhh-GFP4) and the F-box protein NSlmb. Vhh-GFP4 recognises and binds GFP and NSlmb recruits the poly-ubiquitination machinery, thus NSlmb-vhhGFP4 binds GFP-tagged proteins and tags them for degradation by the proteasome (Caussinus & Affolter, 2016). The deGradFP construct expression is driven by a UAS promoter region so it able to be tissue specifically controlled.

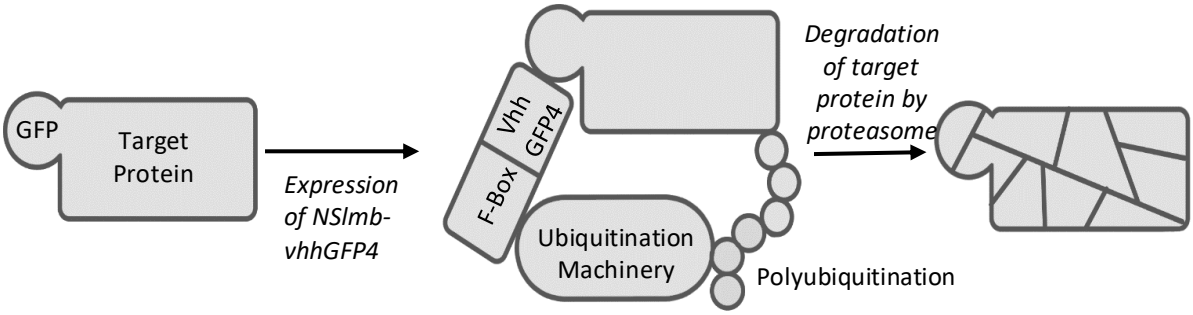

Figure 1.4 Schematic of the deGradFP system.

The *Nslmb-vhhGFP4* construct expresses the fusion protein VhhGFP4::F-Box (a nanobody for GFP::Ubiquitin machinery recruiting protein). Vhh-GFP4 binds GFP and F-Box recruits the Ubiquitination machinery then the target protein is polyubiquitinated and degraded by the proteasome.

1.4.3 Protein trapping

Libraries of such protein-trapped lines have been developed in order to examine expression and function of proteins for which there is no antibody available, as well as for the many proteins that are expressed in the brain but their expression is as yet uncharacterised. One such library was developed by a laboratory at the University of Cambridge, to identify genes that were expressed in the *Drosophila* brain. A transposon containing GFP flanked by splice sites was randomly inserted in frame into the introns of endogenous genes. This allows the gene to be expressed endogenously and during mRNA splicing GFP will remain and be incorporated into the protein when translated, resulting in the GFP-tagging of the endogenous protein (Figure 1.5). They then screened to identify which lines resulted in expression of GFP in the brain and the GFP insertion loci of these genes were identified. The BrainTrap database carries confocal images of the *Drosophila*

brain for each GFP-tagged protein to provide preliminary neuronal expression data (Knowles-Barley et al., 2010). Another library is the Minos Mediated Integration Cassette collection, which contains in-frame GFP trapped insertions into 400 genes (Nagarkar-Jaiswal, DeLuca, et al., 2015; Nagarkar-Jaiswal, Lee, et al., 2015).

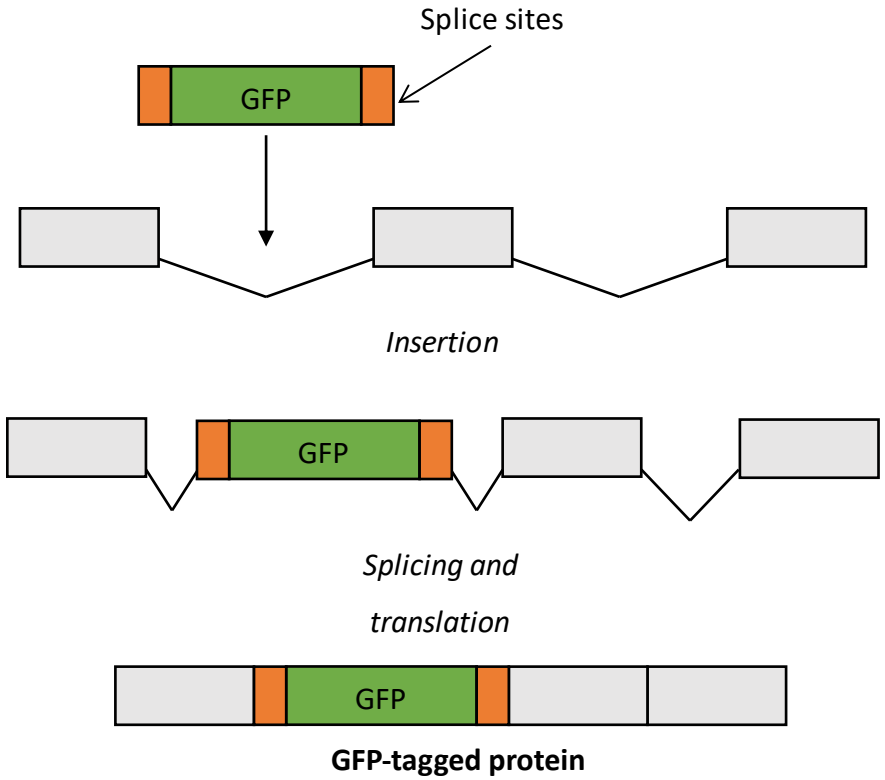

Figure 1.5 Protein trapping of a *Drosophila* gene.

The GFP gene, flanked by splice sites and tags is inserted into the intron of the endogenous *Drosophila* gene. Post-transcription splicing and translation results in expression of GFP-tagged endogenous protein. Grey boxes are representative of the endogenous gene exons and \/s are endogenous gene introns. Green is the GFP gene, orange are the splice sites.

1.4.4 Quantitative analysis of memory

There are several quantitative assays that can be used to test memory and learning in *Drosophila*. The two most common training paradigms used are olfactory training, in which an odour is trained to be associated with a shock (Tully & Quinn, 1985), and the other is the courtship training paradigm and courtship suppression assay, which were employed in this project. The courtship suppression assay measures a male fly's ability to remember that he was previously rejected by a mated female. Male *Drosophila* carry out specific behaviours, such as licking, tapping, chasing, and orientating, to initiate

copulation with a desired mate (Figure 1.6). These behaviours can be recorded to analyse normal courtship, learning, short-term and long-term memory (Ejima et al., 2005; Koemans et al., 2017; McBride et al., 1999; Mehren et al., 2004).

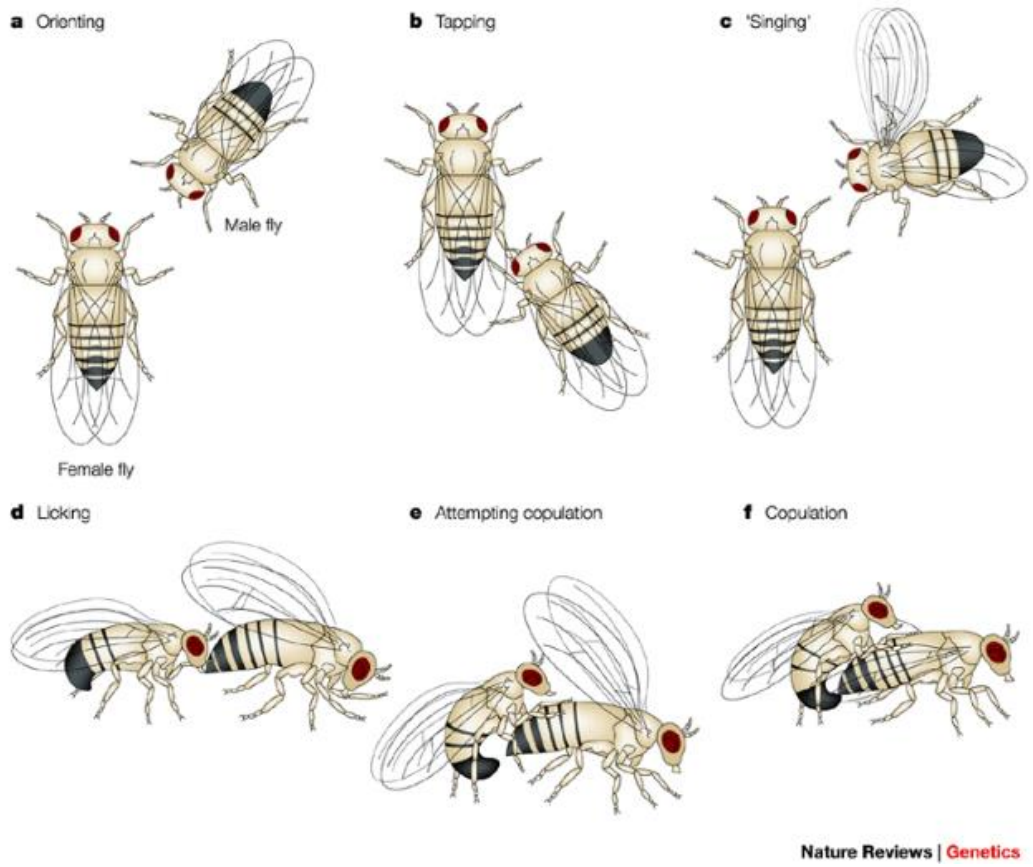

Figure 1.6 Courtship behaviours displayed by the male when attempting to initiate copulation.

a. the male orientates his body in front of the female where he is visible to her. b. the male taps the female's abdomen attempting to get her attention and initiate copulation. c. singing is the act of the male vibrating one of his wings to gain the attention of the female and show his intentions. d. male licks the female's lower abdomen in attempt to prepare for copulation. e. attempted copulation where the male attempts to initiate copulation however is unable due to the female being unreceptive. f. if the female is receptive to the male's courting then copulation will occur. Reprinted with permission from Marla B. Sokolowski: Springer, Nature Reviews Genetics, *Drosophila*: Genetics meets behaviour, Sokolowski, 2001 (Sokolowski, 2001).

Females who are newly mated will reject males exhibiting these behaviours and males with intact memory will remember the rejection and reduce their courtship behaviours towards subsequent females. Procedurally, a male is placed with a mated female for a

defined length of time. To assess learning, the percentage of time spent displaying courtship behaviour in the first ten minutes and the last ten minutes over an hour is assessed. A male with normal learning ability will diminish his attempts at courting over the hour. To assess memory, after the training period the female is removed. The male fly's memory can be tested immediately or after a specific length of time such as an hour for short-term memory or 24 hours for long-term memory. The testing protocol involves placing the male with a different mated female and scoring the amount of time he spends performing courtship behaviours over ten minutes. A memory index can then be calculated by comparing courtship activity of trained and naïve males for each genotype (i.e. a trained male with intact memory will display reduced courtship compared to a naive untrained males), and the memory indices of control vs test groups can be quantitatively compared to determine whether manipulation of a gene of interest has an impact on learning or memory. (Fitzsimons et al., 2013; Freymuth & Fitzsimons, 2017; Mehren et al., 2004; Schwartz et al., 2016).

1.4.5 The *Drosophila* eye as a model for neurogenetic research

The *Drosophila* compound eye is made up of 800 individual ommatidia, each of which contain eight photoreceptor neurons, four cone cells and two primary pigment cells (Freeman, 1996; Freeman, 1997). Due to the precision required for development of the highly organised ommatidial arrays, the eye is particularly vulnerable to perturbation – many mutations or alterations in gene expression that affects the development or survival of photoreceptors or pigment cells can be easily visualised as disorganisation ommatidial arrays, malformation of interommatidial bristles, or loss of pigmentation or necrosis (Iyer et al., 2016). Moreover, the eye is much more accessible than the brain and detailed images are relatively easy to obtain through light microscopy and scanning electron microscopy. Due to the detailed images that can be acquired, scoring systems can be detailed and thorough, allowing small differences between abnormal eye phenotypes to be observed and analysed. In addition, as photoreceptors employ many of the same molecular pathways as CNS neurons, they are an ideal model for the study of neuronal development, survival and patterning (Cutler et al., 2015; Ghosh & Feany, 2004). The *Drosophila* eye is often used as a model to test for genetic interactions, e.g. whether two genes interact in the same molecular pathway, by determining whether the phenotype of both genes is worse than the additive effect of their individual phenotypes (Schwartz et al., 2016). The *Glass multimer reporter (GMR)-GAL4* driver is used to drive expression

of any UAS construct in the eye (Brand & Dormand, 1995; Brand & Perrimon, 1993). *GMR* gene expression is activated at the pupal stage in the cells posterior to the morphogenetic furrow in the eye disc. Low levels of expression also occur in other tissues including the brain, trachea and leg disks (Li et al., 2012).

1.5 HDAC4 and its role in neurological disorders

1.5.1 Histone acetylation and memory

Histone deacetylases (HDACs) remove the acetyl groups from histones (Inoue & Fujimoto, 1970; Taunton et al., 1996), and work in opposition to histone acetyl-transferases (HATs) which add acetyl groups to histones (Kleff et al., 1995; Seto & Yoshida, 2014). The removal of acetyl groups is usually associated with a reduction in transcription (Braunstein et al., 1993). More highly acetylated sections of chromatin tend to be more actively transcribed (Hebbes et al., 1988), as the acetyl groups maintain a less compact chromatin structure, allowing access of transcription factors. Therefore, the removal of acetyl groups from chromatin results in more compact packing of those sections of chromatin, causing decreased transcription of the region (Wolffe, 1996). It has been established that long-term memory is associated with epigenetic changes and one such change is an increase in histone acetylation during memory formation (Levenson et al., 2004). This difference in histone acetylation indicates that HATs and HDACs have a role within memory (Levenson & Sweatt, 2006; Peixoto & Abel, 2013), and investigation of the roles of HDACs in regulating memory is a key area of research into the molecular pathways that underlie normal and disrupted memory.

1.5.2 Domain structure of histone deacetylase 4

Histone Deacetylase 4 (HDAC4) is a class IIa histone deacetylase that has been implicated in several memory and neurodegenerative disorders (Wu et al., 2016), including dementia (Whitehouse et al., 2015) and Alzheimer's disease (Anderson et al., 2015). *HDAC4* is conserved between species, sharing an overall 59% sequence similarity between Human and *Drosophila*, with 84% similarity across the deacetylase domain (Fitzsimons et al., 2013). The deacetylase domain of HDAC4 is well conserved across species but vertebrate HDAC4 has a tyrosine to histidine mutation in the active site (Bottomley et al., 2008) resulting in it being catalytically inactive (Lahm et al., 2007). It can however interact with SMRT/N-Co3-HDAC3 to confer enzymatic activity through the catalytic activity of HDAC3 (Fischle et al., 2002). HDAC4 also contains a conserved

nuclear-localisation sequence (NLS), which allows HDAC4 to be imported into the nucleus (Wang & Yang, 2001). A conserved MEF2 binding domain is also present in HDAC4, allowing it to bind the transcription factor and inhibit its function by blocking its DNA binding (Miska et al., 1999). HDAC4 also contains three conserved lysine residues which conserve the ability to bind the chaperone protein, 14-3-3ζ(Wang et al., 2000). These domains are conserved in HDAC4 throughout different organisms allowing the study of HDAC4 in *Drosophila* to correspond to its action in Humans.

1.5.3 Expression of HDAC4

Expression of HDAC4 in humans is high throughout the body, especially within the brain and skeletal muscle, the exception is no expression is found in the liver and kidneys (Wang et al., 1999). In mammals HDAC4 is expressed throughout the brain, with high expression in the hippocampus (Darcy et al., 2010), which is an important region for memory formation in mammals (Milner et al., 1998; Scoville & Milner, 1957). At a subcellular level HDAC4 is present primarily in the cytoplasm, however is present within the nucleus in subsets of some types of neurons (Darcy et al., 2010). The subcellular distribution of HDAC4 can differ from neuron to neuron as it is able to be shuttled between the nucleus and cytoplasm. This shuttling is regulated by calcium/calmodulin dependent kinase II (CAMKII) phosphorylation of three serine residues in HDAC4. CaMK is activated by Ca^{2+}/calmodulin (Swulius & Waxham, 2008), which is increased in response to serotonin and synaptic activity (Benfenati, 2007). When the serine residues are phosphorylated, they are able to bind 14-3-3ζ, which chaperones HDAC4 out of the nucleus and retains it in the cytoplasm (Bolger & Yao, 2005; Grozinger & Schreiber, 2000; Wang et al., 2000). In *Drosophila* HDAC4 is expressed in the mushroom body of the brain, and similarly to mammals, is primary localised to the cytoplasm with only a subset of Kenyon cells having nuclear localisation of HDAC4 (Fitzsimons et al., 2013).

1.5.4 HDAC4 and memory disorders

HDAC4 has been implicated as the underlying cause of 2q37 deletion syndrome, previously known as Brachydactyly Mental Retardation syndrome (BDMR) (Le et al., 2019; Williams et al., 2010). The symptoms of 2q37 deletion syndrome are varied and include autism, intellectual disability, developmental defects as well as various physical disabilities. One individual with the disorder was found not to have a complete deletion of HDAC4, instead a frame shift mutation caused a truncated version of the protein which lacked a nuclear export signal. The result was that truncated HDAC4 accumulated in the

nucleus and still resulted in the typical features of 2q37 deletion syndrome, this suggesting that loss of the cytosolic protein or the higher nuclear abundance may be causing the phenotype (Williams et al., 2010).

Long-term memory in mice has also been shown to be reliant on the presence of HDAC4, when transgenic mice were generated with a brain-specific knockout of HDAC4, long-term memory was impaired as assessed by the fear conditioning and Morris water maze (Kim et al., 2012). Similarly in *Drosophila*, RNAi knockdown of HDAC4 in the mushroom body impaired formation of long-term courtship memory (Fitzsimons et al., 2013). Overexpression of HDAC4 also has been shown to cause detrimental effects; in *Drosophila* it impairs both axon morphogenesis in the mushroom body and long-term memory formation (Fitzsimons et al., 2013; Schwartz, 2016; Schwartz et al., 2016). A mutant version of HDAC4 lacking deacetylase activity showed the same phenotypes suggesting that the effects of HDAC4 overexpression on memory is independent of its enzyme activity.

1.5.5 Mechanism of action of HDAC4

The mechanism(s) by which HDAC4 affects memory and brain development remains unknown and research continues to find new insights into possible modes of action. In wildtype *Drosophila* brains, the transcription factor protein MEF2 is usually is evenly distributed throughout Kenyon cell nuclei. However, when HDAC4 is overexpressed in these cells, MEF2 localisation is disrupted and co-localises with HDAC4 in puncta within the nucleus. The other contents of these puncta is still unknown but previously a physical interaction between HDAC4 and MEF2 has been identified in which binding of HDAC4 impairs MEF2-dependent transcriptional activity (Miska et al., 1999; Wang et al., 1999). The repression of MEF2 gene targets is a possible mechanism through which HDAC4 impairs long-term memory, which is dependent on changes in gene expression. Another possible mechanism of HDAC4 is via an interaction with Ubc9, a SUMO conjugating protein. This interaction was identified through a genetic screen which shows a genetic interaction between HDAC4, Ubc9 and two other proteins. Further research into Ubc9 as a possible pathway of effect showed reduced Ubc9 levels caused impaired long-term memory (Schwartz et al., 2016). There is evidence that changes to normal sumoylation has effects on memory (Yang et al., 2012) and normal protein function within neurons (Wilkinson & Henley, 2010). A subsequent study was carried out in this laboratory to identify possible transcriptional targets of HDAC4. RNA-sequencing was carried out on

heads of flies in which human HDAC4 mutants were overexpressed (Main, 2019; Main et al., 2021). The mutants included a cytoplasmic-localised and a nuclear-localised mutant to shed light on the relative roles of nuclear vs cytoplasmic pools of HDAC4. The RNA-sequencing revealed only a small number of transcriptional changes resulting from expression of nuclear localised HDAC4, however interestingly, several hundred were differentially regulated in the presence of increased cytoplasmic HDAC4.

The mechanism by which cytoplasmic HDAC4 affects gene expression is unknown, however, physical interaction between HDAC4 and various transcription factors have been found, such as the interaction with MEF2 (Miska et al., 1999; Wang et al., 1999). There is evidence that HDAC4 interacts with transcription factors and can alter the subcellular localisation of such proteins. One such protein is ATF4, which HDAC4 has been shown to directly interact with and has an effect on the subcellular location of AFT4 by sequestering it in the cytoplasm (Zhang et al., 2014). It is possible that by sequestering transcription factors in the cytoplasm, such as ATF4, they are unable to act in the nucleus resulting in changes to gene expression. Whether this is the mechanism by which cytoplasmic HDAC4 causes changes to gene expression still requires further investigation. One gene found to be differentially expressed at a significant level in brains expressing the cytoplasmic-localised human *HDAC4* mutant was *herzog*, which showed a 2.5-fold increase in transcription (Main, 2019). In summary HDAC4 has been shown to be essential for memory and knockdown of HDAC4 has caused impaired memory in *Drosophila* (Fitzsimons et al., 2013). The loss of cytoplasmic HDAC4 may be the cause of the impaired memory therefore the potential targets of HDAC4 are of interest to identify how HDAC4 functions to regulate memory.

1.6 *herzog*

One gene found to be differentially expressed in the previously described RNA-seq experiment was *herzog* (*Drosophila* gene annotation *CG5830*), which is an uncharacterised gene which shares 54% sequence identity and 64% sequence similarity with human Carboxy-terminal domain small phosphatase 1 (CTDSP1), (FlyBase, 2008; Weiss et al., 2012), seen in Figure 1.7. From the Herzog protein sequence, two known domains were identified including the FCP1 domain, a RNA polymerase II CTD phosphatase domain, first identified in fungi (Ghosh et al., 2008). Herzog protein sequence was found to have 73% identity with FCP1. This domain is also found within

the Human CTDSP1 protein. Human CTDSP1 is expressed throughout all tissues in the body, however it is minimally expressed in neurons (Yeo et al., 2005). Its role in human tissue is to assist in the repression of neuronal genes in non-neuronal tissues via the dephosphorylation of RNA polymerase II at repressive element 1 (RE1) (Yeo et al., 2005). This is intriguing as RE1 and its repressor REST (RE1 silencing transcription factor) have been implicated in Huntington's disease. REST has been shown to interact with the wildtype huntingtin protein and is sequestered in the cytoplasm, the mutated version of huntingtin protein does not interact with REST resulting in its accumulation in the nucleus in Huntington's disease (Zuccato et al., 2003). This increased nuclear REST results in increased repression of genes containing RE1, such as brain derived neurotrophic factor (BDNF), which has decreased expression in Huntington's disease (Rigamonti et al., 2009; Zuccato et al., 2007).

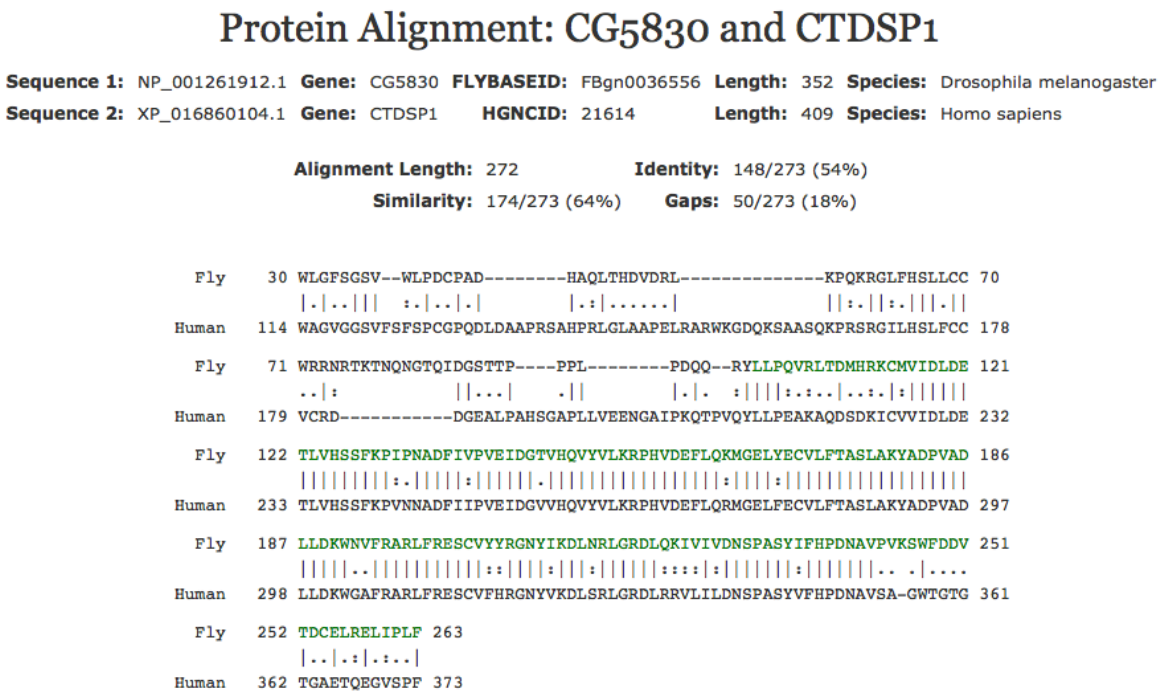

Figure 1.7 Protein alignment of *Drosophila* Herzog (CG5830) and Human CTDSP1.
Alignment was carried out using the *Drosophila* RNAi Screening Centre (DRSC) integrative ortholog prediction tool (Hu et al., 2011). https://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl

No Herzog antibody was available to examine the expression pattern of Herzog in the brain, however a *herzog::GFP* protein trap line is commercially available, and low resolution images of *Drosophila* brains for each of the protein trapped lines is available to the public (http://braintrap.inf.ed.ac.uk/braintrap/). Interestingly, unlike the human

non-neuronally expressed *CTDSP1*, *Herzog* is expressed in the brain and a preliminary analysis from these images indicates that it is highly expressed in the lobes of the mushroom body (circled in Figure 1.8) and synaptic neuropil (Figure 1.8). Its expression in the lobes indicates that Herzog is localises to axons, however detailed subcellular localisation (such as whether it is also present in the nucleus like CTDSP1) cannot be seen in these images, due to the low resolution. Another collection of YFP protein trapped lines is also available and shows YFP-trapped Herzog is localised to the nervous system, however there are no images available to public (Lye et al., 2014).

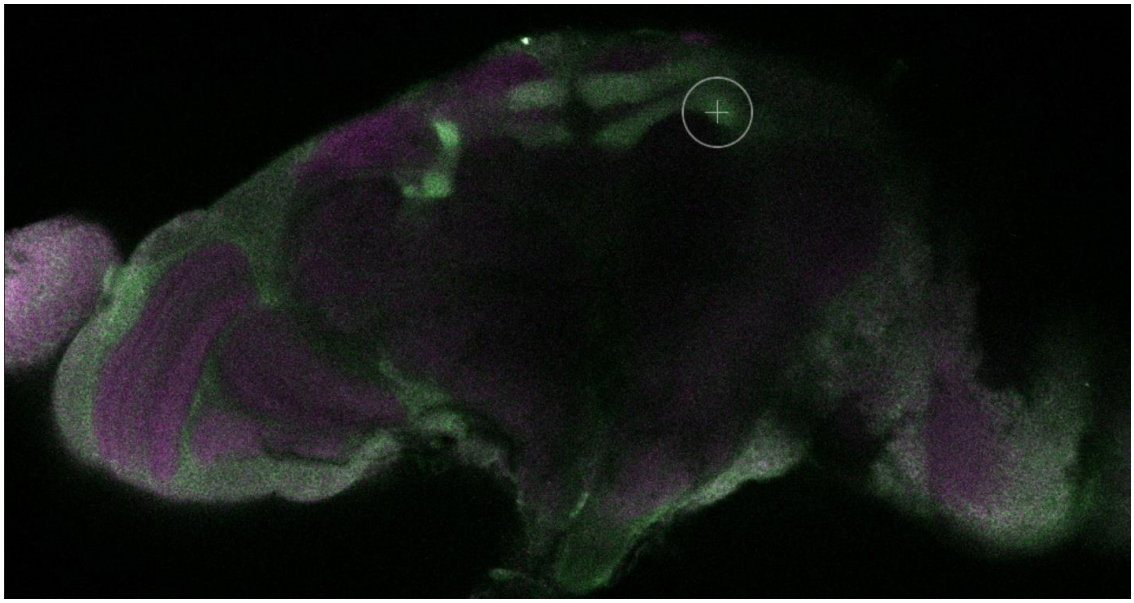

Figure 1.8 Preliminary imaging of *herzog* expression in the brain.
Images from Brain trap showing preliminary images of *herzog::GFP* expression in the brain. Immunohistochemistry has been used to stain for GFP, shown in green, and bruchpilot as a background stain, shown magenta. The cross within the circle indicates the medial lobes of the mushroom body. No scale bar is included, image shows a whole *Drosophila* brain. Image from http://braintrap.inf.ed.ac.uk/braintrap. (Knowles-Barley et al., 2010)

The opposing subcellular distribution patterns suggests Herzog's role differs from that of CTDSP1 and warrants further investigation. In addition, the high level of expression in the mushroom body suggests it may play a role in memory formation and is a potential target of HDAC4. At the outset of this project, very little information was available about the role of *herzog* in neuronal function. It was identified in a screen for genes that which are differentially expressed after memory formation; an RNA sequencing experiment carried out to identify transcriptional changes in the mushroom body after memory formation showed a 4-fold increase in *herzog* expression after one hour of courtship

training and learning, which also suggests a possible role within memory formation (Jones et al., 2018). In another study, trained algorithms were used to predict the role of genes based on their sequence and Herzog was predicted to play a role in synaptic formation and assembly (Pazos Obregon et al., 2015). Whether Herzog does have a role in synaptic reconstruction is yet to be confirmed.

Another screen investigating modifiers of Tau, a microtubule stabilising protein, showed Herzog as a possible enhancer of Tau-induced neurodegeneration in the *Drosophila* eye (Blard et al., 2007). This is intriguing as Tau hyperphosphorylation has been implicated in the pathogenesis of Alzheimer's disease (Roberson et al., 2007) and frontotemporal dementia (Baker et al., 2006; Garcia & Cleveland, 2001; Lee et al., 2001). Tau accumulation in the *Drosophila* mushroom body has been shown to severely impair olfactory learning and memory (Mershin et al., 2004). Further indications that Herzog plays an important role in neuronal function arise from a screen for proteins which modulate the aggregation of huntingtin, a protein which contains a polyglutamine repeat that, when expanded forms toxic aggregates leading to neurodegeneration. Herzog appears to be a repressor of huntingtin aggregation in this case, suggesting a possible protective role (Weiss et al., 2012).

Taken together, the high expression of Herzog within the mushroom body, its upregulation after learning and memory formation and transcriptional regulation by HDAC4 suggest it may play a role in learning and memory. In addition, Herzog has been implicated in various neurodegenerative disorders which is of further interest, however its function and localisation within neurons is yet to be characterised.

Aims and Objectives

1. **Characterise the expression pattern of *herzog* in the brain and determine the subcellular and neuronal-cell type distribution of Herzog.**
   The expression and subcellular distribution of *herzog* within the brain will be determined using immunohistochemistry and confocal microscopy. Various counterstains, such as DAPI (nuclear marker), FasII (axonal marker), and nc82 (synaptic neuropil), will be used to characterise the cellular and subcellular localisation of *herzog* in the brain. As no Herzog antibody is available, a *herzog*::*GFP* protein trap line which carries an internal fusion of GFP within the endogenous *herzog* gene will be used.
2. **Determine via western blot whether overexpression of HDAC4 alters *herzog* expression.**
   To determine the normal level of *herzog* expression, western blots will first be carried out on *herzog::GFP* lines using an anti-GFP antibody to optimise the detection protocol. HDAC4 will be overexpressed in a *herzog::GFP* background to confirm RNAseq data that HDAC4 regulates *herzog* expression.
3. **Investigate the impact of reduced *herzog* on mushroom body development.**
   Knockdown of *herzog* will be achieved via RNAi or the deGradFP system. The impact of the knock-down will be analysed using immunohistochemical staining of the mushroom body and confocal microscopy and assessed for defects in axon elongation, termination and guidance using a semi-quantitative scoring system.
4. **Determine whether a reduction of Herzog protein in the brain impairs courtship behaviour, learning and short-term memory.**
   herzog will be knocked down in the *Drosophila* brain, either by RNAi or deGradFP, then courtship activity, learning and short-term memory will be quantitatively assessed using the courtship suppression assay.

2 Material and Methods

2.1 *Drosophila melanogaster* strains

All *Drosophila* lines were either made within the lab or purchased from stock centres and imported under New Zealand MPI (Ministry of Primary Industries) and HSNO (Hazardous Substances and New Zealand Organisms) regulations and approvals (GMO10/MU002 and GMC00017s167a). All lines used within the project are listed in Table 1.

Table 1 *Drosophila melanogaster* stock lines

Integrated DNA	Chromosomal Linkage	Genotype	Source
GAL4-Tubulin	3	*y1w*; P{w+mC=tubP-GAL4} fis LL7/TM3, Sb1 r*	Bloomington *Drosophila* Stock Centre (BDSC) 5138
elav^{C155}-GAL4	1	*w[CS10];P{w[+mW.hs]=GawB}elav[C155]*	BDSC 458
UAS-hHDAC4	3	*y[1] w[67c23]; P{y[+t7.7]=CaryP}attP2, w[m+C]=UAS-hHDAC4*	Genetivision, USA
UAS-hHDAC4 3SA	3	*y[1] w[67c23]; P{y[+t7.7]=CaryP}attP2, w[m+C]=UAS-hHDAC4 3SA*	Genetivision, USA
UAS-hHDAC4 L175A	3	*y[1] w[67c23]; P{y[+t7.7]=CaryP}attP2, w[m+C]=UAS-hHDAC4 L175A*	Genetivision, USA
UAS-DmHDAC(WT)-myc	3	*y[1] w[67c23]; P{y[+t7.7]=CaryP}attP2, UAS-DmHDAC4(WT)-myc*. Insert into P2:(3L) 68A4	Genetivision, USA
UAS-DmHDAC4-3A-myc	3	*y[1] w[67c23]; P{y[+t7.7]=CaryP}attP2, UAS-DmHDAC4-3A-myc*. Insert into P2:(3L) 68A4	Genetivision, USA
UASDmHDAC4-dNLS-myc	3	*y[1] w[67c23]; P{y[+t7.7]=CaryP}attP2, UAS-DmHDAC4-dNLS-myc*. Insert into P2:(3L) 68A4	Genetivision, USA

UAS-CG5830 RNAi	3	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01077}attP2*	BDSC 34079
CG5830::GFP	3	*y[1] w[*]; Mi{PT-GFSTF.1}CG5830[MI02112-GFSTF.1]*	BDSC 63163
UAS-CG5830 RNAi #2	3	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC04526}attP2*	BDSC 64474
UAS-Nslmb-vhhGFP4	3	*w[*]; P{w[+mC]=UAS-Nslmb-vhhGFP4}3*	BDSC 38421
UAS-Nslmb-vhhGFP4	2	*y[1] w[*]; P{w[+mC]=UAS-Nslmb-vhhGFP4}2*	BDSC 38422
UAS-CG5830 RNAi #3	3	*w[1118]; P{GD11782}v40611 CG5830*	VDRC 40611
UAS-CG5830 RNAi #4	2	*w[1118]; P{attP,y[+],w[3`v101539] CG5830*	VDRC 101539

2.2 Maintenance of *Drosophila* stocks

2.2.1 Stock maintenance

Drosophila were raised at 20ºC in 15 ml vials on standard food made of 10 g agar, 40 g yeast, 110 g cornmeal, 20 mL molasses, 130 g sugar, and 3.3 g methyl 4-hydroxybenzoate dissolved in 37 mL ethanol per 1 L of water. *Drosophila* stocks were turned onto new food every three weeks. The adults were transferred onto fresh food and left to lay eggs for four days. The adults were removed, and the progeny were left for 16-18 days to pupate and eclose at which point they were turned again. At a given time three vials of each stock are maintained, including the newly turned vial and the two prior generations.

2.2.2 Collection of virgins

Virgin females for crosses were collected from vials or bottles of the required strain. Female *Drosophila* do not reach sexual maturity until 8-10 hours post eclosion. Bottles were checked for virgins in the morning (8-9am), which were identified by the presence of a meconium along with other signs including: swollen and pale abdomen; folded wings; and a swollen head. Virgin females were collected into a fresh vial and mature adults were discarded. All females that eclosed within the next eight hours were considered virgins and were collected.

2.2.3 *Drosophila* genetic crosses

Each genetic cross was set in a 100 mL bottle, with approximately 40 mL of food. 10-15 virgin females and 10-15 males of each strain were added to bottle and were housed at 25ºC. Adults were then removed after 3-4 days and the next generation emerged approximately 10 days later. These heterozygous F1 progenies were then either further crossed/backcrossed or used for assays.

2.3 Immunohistochemistry

2.3.1 Dissection of *Drosophila* brains

Whole flies were first pre-fixed in PFAT-DMSO (4% paraformaldehyde in 1X PBS +0.1% Triton X-100 + 5% DMSO) prior to dissection for 1-2 hours. Brains were dissected in 1X PBST (1X PBS + 0.5% Triton X-100) then fixed for 20 minutes in PFAT-DMSO. They were then transferred to 100% methanol and stored at -20°C. The brains were rehydrated for 5 minutes in 1:1 methanol and 1X PBST then washed for 3 X 5 minutes in PBST in preparation for immunohistochemical staining.

2.3.2 Immunohistochemical staining

Brains were blocked for two hours in 5% normal goat serum in 1XPBST (1XPBS + 0.1% Triton X-100), then incubated overnight in primary antibody diluted in 5% normal goat serum in 1XPBST. Following 3 X 5-minute washes in PBST, brains were incubated overnight at 4°C with the appropriate secondary antibody in 5% normal goat serum in 1XPBST. If DAPI stain was being used, brains were washed for 3 X 5 minutes in PBST after secondary antibody and incubated with DAPI (1:20,000 dilution in PBST) for 10 minutes before washing for 20 minutes, three times in PBST. The brains were mounted on a slide in Anti-fade (1XPBS, 9 ml Glycerol, 20% n-propyl gallate) and kept at 4°C in the dark until they were ready to be imaged.

Table 2 Primary antibodies and respective dilutions used for immunohistochemistry

Protein	Cat no.	Host	Source	Dilution
Bruchpilot	nc82	Mouse	Developmental Studies Hybridoma Bank (DSHB)	1:100
Fasciclin II	1D4anti-Fas II	Mouse	DSHB	1:20
GFP	ab290	Rabbit	Abcam	1:20,000
MYC	9E10	Mouse	DSHB	1:50

Table 3 Secondary fluorescent antibodies used for immunohistochemistry and respective dilutions

Product Name	Fluorophore	Cat No.	Source	Dilution Used
Goat anti-rabbit IgG	AlexaFluor® 555	A21428	Sigma-Aldrich	1:100
Goat anti-rabbit IgG	AlexaFluor® 647	A21244	Sigma-Aldrich	1:100
Goat anti-mouse IgG	AlexaFluor® 488	A11001	Sigma-Aldrich	1:100
Goat anti-mouse IgG	AlexaFluor® 555	A21422	Sigma-Aldrich	1:100
Goat anti-mouse IgG	AlexaFluor® 647	A21236	Sigma-Aldrich	1:100

2.3.3 Confocal microscopy imaging

Brains were imaged using a Leica TCS SP5 Confocal Laser Scanning Microscope (objectives 10x, 20x and 40x oil) in the Manawatu Microscopy and Imaging Centre (MMIC). Optical sections (1 μm for whole brains and 0.5 μm through the mushroom body) were taken and z-stack images were collected through the appropriate brain regions. Each image was them processed using the ImageJ application, to artificially change the colours and generate maximal intensity projections.

2.3.4 Thioflavin T staining

Thioflavin T staining was used in conjunction with immunohistochemistry to stain for β-amyloid. Immunohistochemistry was carried out as per section 2.3.2, with an additional step after the final wash before mounting, in which brains were transferred to 0.25% Thioflavin T in 50% ethanol and were incubated overnight at room temperature. Thioflavin T was then removed, and brains were de-stained in 50% ethanol for ten minutes. Following de-staining brains were washed three times for five minutes each in PBT, and then mounted as described above in Section 2.6.2. Imaging was carried out in the same manner as in Section 2.5.3 with thioflavin T emission spectrum peak detection at 482nm and peak excitation at 450nm. Protocol was adapted from: (Chiang et al., 2010; Iijima et al., 2004; LeVine, 1999; Nil et al., 2019; Saeed & Fine, 1967) .

2.4 Western blotting

2.4.1 Isolation of *Drosophila* heads

Drosophila were snap frozen, either using a -80°C freezer or a dry ice and ethanol bath. The heads were removed by vortexing then separated from the bodies with a paintbrush on a piece of transparency over dry ice. The heads were then collected for use in a western blot.

2.4.2 Western blotting

To generate a whole cell lysate, heads were homogenized with a disposable mortar and pestle in RIPA buffer (150 mM sodium chloride, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0), then centrifuged for two minutes at 13,000g at 4ºC and the supernatant was collected. The protein concentration of lysates was then quantified using the Pierce BCA Assay Kit (Thermo Fisher Scientific), as per manufacturer's instructions. 2.4 µl of Laemmli buffer (2% sodium dodecyl sulphate, 5% 2-mecaptoethanol, 10% glycerol, 0.01% bromophenol blue, 60 mM Tris HCl pH 6.8) was added to each of the lysates (30 µg of protein). Samples were boiled for five minutes at 95ºC, then 10 µl of sample was loaded onto a 4-20% SDS-PAGE gel and resolved at 180V for 40 minutes in 1X Running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS). The gel was then transferred to nitrocellulose membrane for 1 hour at 100V in chilled 1X Transfer buffer (25 mM Tris, 190 mM glycine, 0.1% SDS, 20% methanol). To confirm transfer the membrane was stained in Ponceau (0.1% Ponceau Stain-S in 5% acetic acid) for five minutes and then distained using milli Q water. The membrane was blocked for 3 hours at room temperature in blocking buffer (5% skim milk powder in TBST (1X TBS, 0.1% Tween-20)) then washed three times for five minutes each with TBST. The membrane was then incubated in primary antibody (Table 4), diluted in 1% skim milk powder in TBST, overnight at 4°C. The membrane was washed three times, for five minutes each, with 1X TBST and then incubated for one hour at room temperature with secondary horseradish-peroxidase conjugated antibody (Table 5), diluted in 1% skim milk powder in TBST. The membrane was washed three times for five minutes each using TBST. Detection was then performed using the ECL Prime (GE) reagent (1 ml Reagent A, 1 ml Reagent B, per membrane) which is pipetted onto the membrane and incubated for a minute. Excess liquid is removed, and the membrane was imaged using the Azure

c600 imaging system. The membrane was then washed then re-blocked and re-probed for α-Tubulin as a loading control, using the same protocol from the blocking step.

Table 4 Primary and secondary antibodies and respective dilutions used for western blotting

Protein	Cat. no	Host	Brand	Dilution
GFP	Ab290	Rabbit	Abcam	1:4000
α-Tubulin	12G10	Mouse	Abcam	1:500

Table 5 Secondary horseradish peroxidase conjugated antibodies used for western blotting

Type	Cat. no	Dilution
Anti-Mouse ECL	NA931VS	1:20000
Anti-Rabbit ECL	NA934VS	1:20000

2.5 Evaluation of eye phenotypes

2.5.1 Light microscopy

Light imaging of the eyes was carried out using a stereomicroscope (Olympus SZX12, DP controller imaging software). Flies were immobilised using FlyNap and then positioned using card and blue tac. Each fly was positioned so one eye was clearly visible and the microscope was then focused, and image was captured at a magnification of 108x.

2.5.2 Preparation of samples for scanning electron microscopy

Flies of the desired genotype were collected and 20 were placed in a vial containing a disk of paper soaked with water. They were housed as such overnight allowing them to clean their eyes of any food residue. Flies were then fixed in a modified Karnovsky's fixative (3% glutaraldehyde 2% formaldehyde in 0.1 M phosphate buffer, pH 7.2, with Triton X-100) for minimum of 8 hours. Samples were then washed and dehydrated in graded ethanol before being dried using liquid CO_2 in Polaron E3000 series II critical point drying apparatus.

After fixation and drying, the heads of each fly were removed and collected. The heads were mounted on aluminium stubs with one eye facing outwards. Each stub was then coated in gold using the Baltec SCD 050 sputter coater for 200 seconds. Once the stubs

were coated they were then stored in a moisture regulated sample safe until imaging was carried out. During imaging if there was excess flaring the stub was coated for a further 100 seconds. Fixation and drying were carried out by technician staff at MMIC.

2.5.3 SEM imaging

Imaging was carried out using the FEI Quanta 200 Environmental Scanning Electron Microscope at an accelerating voltage of 20kV. Stubs were inserted into the platform and the stage was rotated to 61º, so the edges of the stub were visible for imaging. The brightness, contrast and focus were then adjusted to give the best image for each eye while avoiding any flaring which may occur. The eye was then imaged at a uniform magnification of 200x.

2.6 Behaviour assays

2.6.1 Preparation for behaviour assays

To generate freshly mated females for the courtship behaviour assays, the wildtype *Canton S* (*CS*) strain was expanded, from which virgin males and females were collected. *CS* virgin males and females were housed in separate vials. Two days prior to carrying out the behaviour assay, 25 virgin females and 30 virgin males were put together and mated for two nights. The morning of the behaviour testing, the females and males were separated via brief CO_2 anaesthesia. Males were discarded and females were placed into a new vial and given a least an hour to recover from anaesthesia before behaviour assaying commenced. The crosses of the test genotypes were set in parallel timing to the expansion of the *CS* flies, such that males of the test genotypes were ready for testing at the same time as the freshly mated CS females were generated. Virgin males of the test genotypes were collected, and each was housed individually so that their first exposure to a mature fly was the freshly mated female. Male virgins were allowed to mature in their individual vials, and the behaviour assays were carried out on males aged between 4 and 8 days old.

2.6.2 Courtship activity

Courtship activity of the lines was recorded by placing a freshly mated female and a virgin male of chosen genotype into a testing chamber. These were then placed immediately under the GoPro Hero 6 camera and were recorded for 10 minutes. Each chamber was then scored to find the time spent by the male courting the female. Scoring was carried out over 10 minutes from GoPro6 recordings by a blinded individual. Courtship

behaviours recorded were orientating, 'singing', tapping, licking, attempted copulation and chasing (Figure 1.6). If the pair mated during training or recall then they were excluded from the trial. The timing of the beginning and end of each of these behaviours was recorded and the proportion of time spent courting was calculated. The score was then converted to a courtship index using the proportion of the ten minutes the male spent courting.

$$Courtship\ Index = \frac{Time(courting)}{10min}$$

2.6.3 Learning assay

To determine the learning index for each line, virgin males of each genotype were placed into a testing chamber with a freshly mated female. They remained in these chambers for an hour, with the first and last 10 minutes of the hour recorded. For each of the two recordings, the proportion of time the male spent courting over the 10 minutes was recorded. The courtship activity of the ten minutes immediately after being introduced to the female and after 50 minutes of rejection are then compared to determine whether the fly learned the rejection behaviours or not. A learning index was calculated using the courtship indices of the first and last 10 minutes. The learning index gives a number between zero and one. A learning index of zero suggests no learning occurred, with higher numbers indicating increased learning.

$$Learning\ Index = 1 - \frac{CI\ (last10)}{CI\ (first10)}$$

2.6.4 Short-term memory assay

Immediate memory was assayed using a one-hour training session in training chambers which contain food. Males of each genotype were placed in the training chambers with a freshly mated female and were left for an hour during which time the male attempted to court the unreceptive female. An equivalent number of sham males were placed in the training chambers and remained alone until testing.

Testing was carried out in testing chambers. To test each trained or sham male, a freshly mated female was placed into a testing chamber. The male was then removed from the training chamber and placed into the test chamber with the new female. The flies were then recorded for 10 minutes using a GoPro Hero6 camera. The behaviour was then

scored, and the courtship index of the test vs sham males was compared and used to calculate a memory index. The memory index gives a score between zero and one, with zero indicating no memory and higher numbers indicating increased memory.

$$Memory\ Index = 1 - \frac{CI(trained)}{CI(naïve)}$$

2.7 Statistical analysis

To determine whether differences between results of multiple groups were statistically significant or not a one-way ANOVA was used, followed by a post-hoc analysis with a Tukey's HSD test. A post-hoc Tukey's HSD test was only carried if ANOVA provided a K value of more than 2 and analysis of variance gave a significant F-ratio. Statistical significance of difference between groups was classed as a p value of less than 0.05. Statistical analysis was carried out using Vassar stats (http://vassarstats.net/), however exact P values are not given only a range. If p values were expected to be close to the cut off for significance then a R script was used to find exact p-values.

3 Results

3.1 Characterising the expression pattern of *herzog::GFP*

The first step in investigating Herzog's role in the brain was to characterise its expression pattern in the brain. There is currently no antibody available to detect Herzog, so in order to detect the protein allowing analysis of expression and subcellular localisation, a *Drosophila* line was purchased. This line contained an in-frame insertion of GFP (*herzog::GFP*) into the endogenous *herzog* gene (Knowles-Barley et al., 2010). This was done via the process of protein trapping that is described in Section 1.4.3.

3.1.1 Expression of *herzog* in the *Drosophila* brain

A preliminary analysis of *herzog::GFP* expression via immunohistochemistry on whole brains and imaging using confocal microscopy, showed moderate expression in the brain, particularly within the mushroom body (Knowles-Barley et al., 2010). These images were only to provide initial confirmation of expression and overview of the expression pattern as part of a library of protein-trapped genes for subsequent analysis Therefore, the images are of low quality and detail such as subcellular localisation cannot be observed. To that end, immunohistochemistry was carried out on *Drosophila* brains to further characterise expression of Herzog. The key features of the *Drosophila* brain from both anterior and posterior perspective are shown in Figure 3.1 A & B and Figure 3.2 A. The nc82 antibody binds the bruchpilot protein, which localises to the synaptic neuropil, which are present throughout the brain, therefore this antibody allows visualisation of the whole brain as a background. Herzog expression as visualised with a GFP antibody and appeared at moderate levels throughout the brain with a high level of Herzog concentration in all lobes of the mushroom body in the anterior brain (Figure 3.1 C-E) and in the Kenyon cells and calyx in the posterior of the brain (Figure 3.1 J-L). To further characterise the expression pattern in the mushroom body, immunohistochemistry using a Fasciclin II (FasII) antibody as a counterstain was carried out. FasII is highly expressed in the α/β and γ lobes of the mushroom body. The α-GFP staining co-localises with the α-FasII in the mushroom body (Figure 3.1 F-I), showing high localisation of Herzog to all the lobes of the mushroom body. The GFP staining also appears to be present in the α' lobe, as indicated in Figure 3.1 I. Brains were also counterstained with DAPI to investigate the subcellular distribution of Herzog. DAPI is a common stain used to visualise cell nuclei as it binds chromatin in the nucleus of cells. DAPI stained nuclei were visible in the Kenyon cells of the mushroom body (Figure 3.2 F-I) as well as other neuronal nuclei throughout the brain (Figure 3.1 M-O). However, there was no GFP co-localisation with

DAPI in any of the nuclei (Figure 3.2 B-E) and cytoplasmic halos were observed around nuclei (Figure 3.2 B-I). To confirm this lack of co-localisation, DAPI and GFP localisation was analysed via a cross section over a Kenyon cell taken from Figure 3.2 I. The fluorescence of DAPI and GFP were plotted (Figure 3.2 J), which clearly shows GFP staining does not co-localise with DAPI but instead localises either side of the DAPI fluorescence spike. This confirms that Herzog is not present in the nucleus and only within the cytoplasm. Taken together these data indicate that Herzog is expressed throughout the brain, with high expression in the mushroom body, and localises to the cell bodies of axons of Kenyon cells but is absent from the nuclei. High expression is also able to be seen in the calyx where the axons of the Kenyon cells bundle to project anteriorly into the peduncle.

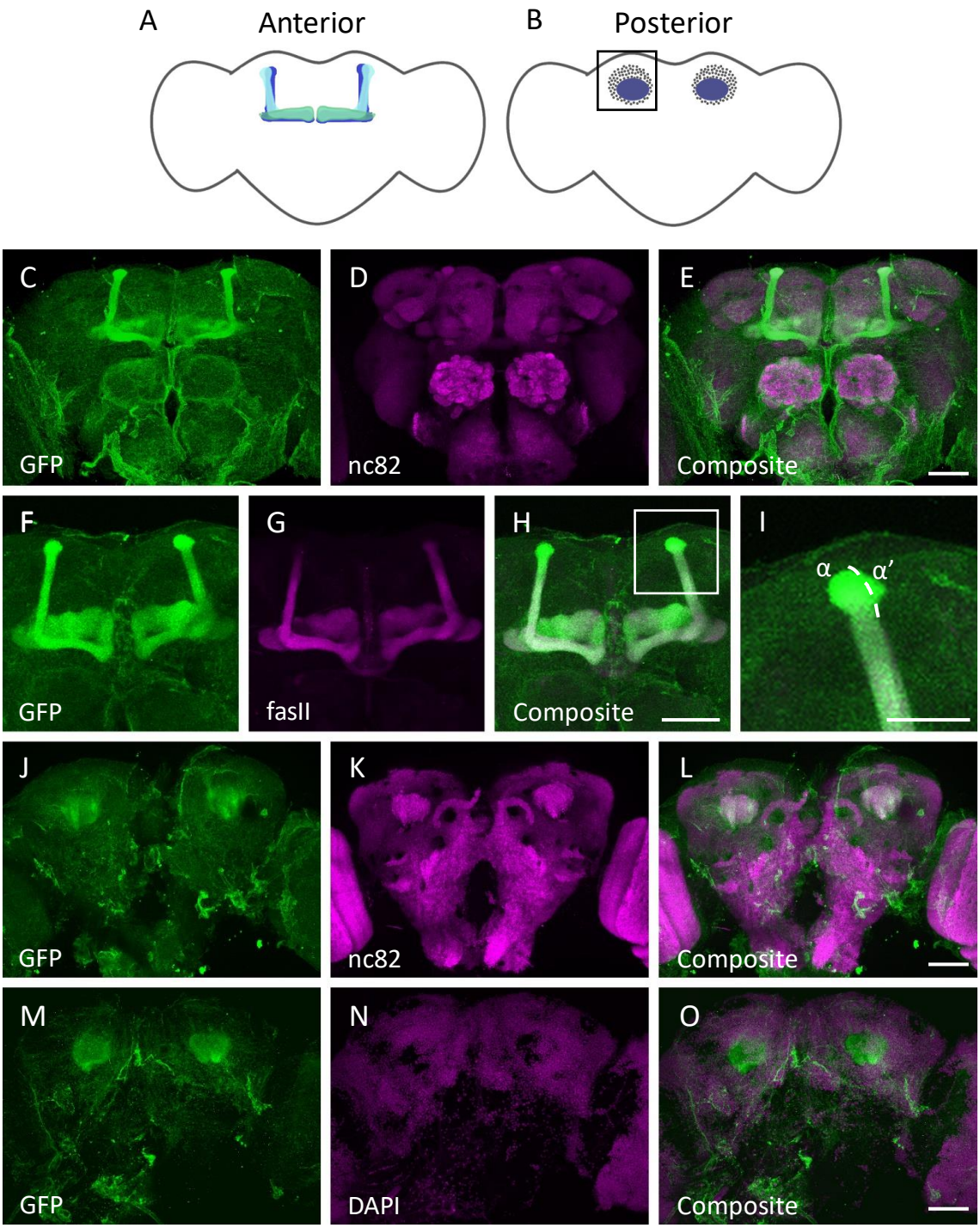

Figure 3.1. Endogenous expression of Herzog in the *Drosophila* brain.

A. Schematic of the anterior *Drosophila* brain, showing the mushroom body, dark blue represents α/β lobes, light blue represents α'/β' lobes, and green represents γ lobes. B. Schematic showing the anterior of the *Drosophila* brain showing the calyx, in dark blue and Kenyon cells, black. C-O. Immunohistochemistry using anti-GFP (green) and either anti-Bruchpilot (nc82), anti-Fasciclin II (FasII) or DAPI stain (magenta). C-E. Anterior images showing the endogenous expression of *herzog*, with background nc82 staining, showing high expression in throughout the brain, scale bar = 50μm. F-H. Anterior images showing expression of *herzog* localising with FasII

staining in the mushroom body, scale bar = 50µm. I. Magnification of boxed area shown in H α and α' lobes are indicated by labels, scale bar = 25 µm. J-L. Posterior images of Herzog expression in relation to nc82 background, scale bar = 50 µm. M-O. Posterior images showing Herzog expression and DAPI showing neuronal nuclei, scale bar = 50 µm.

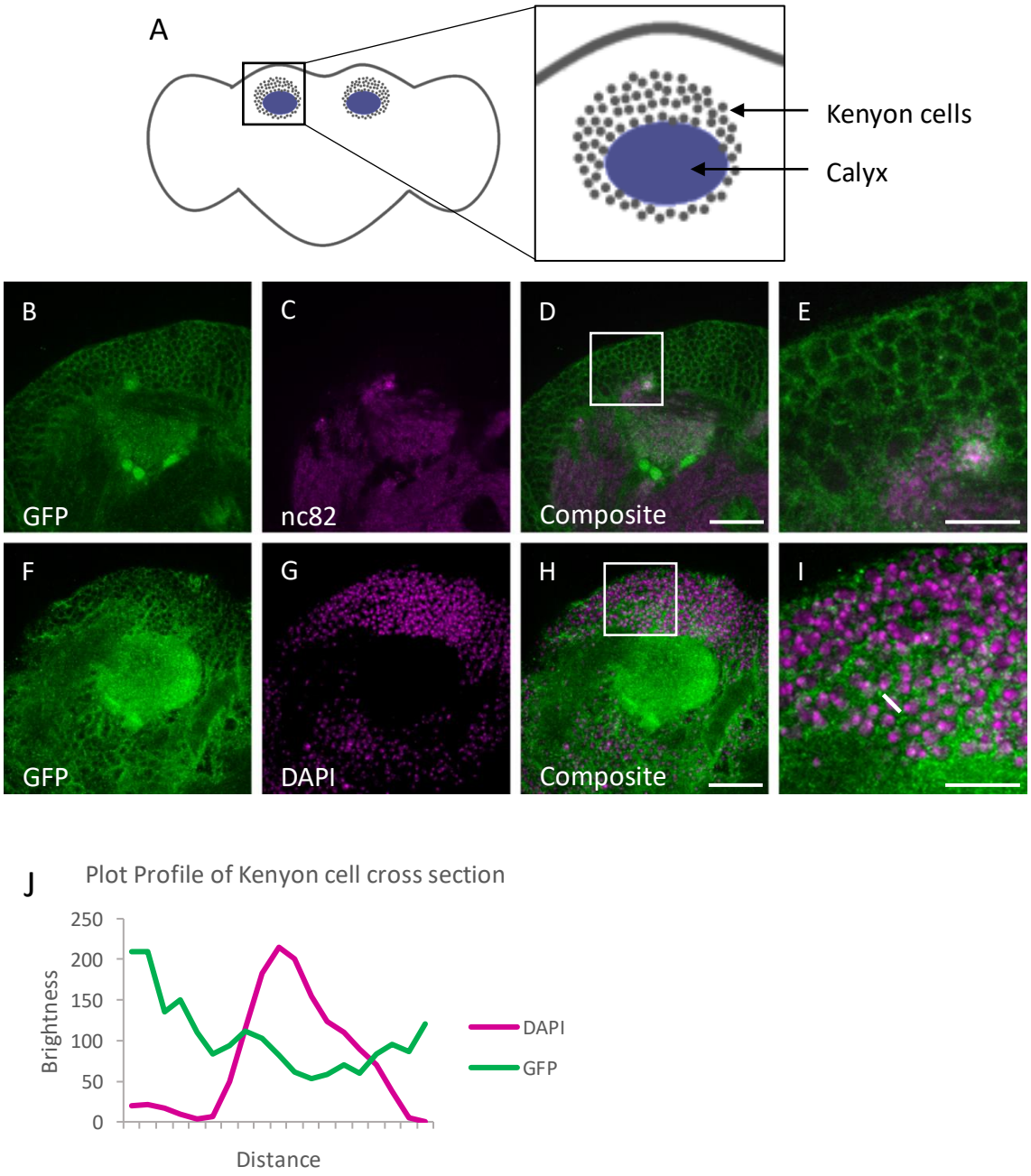

Figure 3.2 Endogenous expression of Herzog in the Kenyon cells of the mushroom body.
A. Schematic of posterior *Drosophila* brain, boxed area indicates Kenyon cell area analysed by immunohistochemistry and confocal imaging. Calyx and Kenyon cells are labelled. B-I.

Immunohistochemistry carried out on whole mount brains; optical slices through the mushroom body at the level of the calyx indicated by magnified area in A. B-E. GFP and nc82 co-staining shows Herzog expression in calyx and Kenyon cells. D. Brightly GFP stained spots in calyx are the origins of bundled axons which project into the peduncle, scale bar = 25 μm. E. Magnified image of boxed area indicated in D, cytoplasmic halos can be seen, scale bar = 10 μm. F-H. GFP and DAPI co-staining, sale bar = 25 μm. I. Shows magnification of area indicated in H. Cytoplasmic halos of GFP staining are observed and no colocalization of GFP and DAPI staining seen. Line indicates where plot profile cross section, J, was taken. Scale bar = 10 μm. J. Fluorescence plot profile from Kenyon cell cross section in I. Brightness indicates the intensity of fluorescence, Green = GFP and Magenta = DAPI. Distance is the distance from the origin of the cross section line.

3.2 Investigating the regulation of *herzog* by HDAC4

3.2.1 Generation of a new line homozygous for *elav-GAL4* and *herzog::GFP*

Previous RNAseq data suggested that human cytoplasmic restricted HDAC4 resulted in a 2.5-fold increase in *herzog* mRNA expression (Main, 2019). Therefore it was imperative to confirm that overexpression of *HDAC4* alters Herzog protein expression levels. In order to express *HDAC4* in a *herzog::GFP* background a genetic crossing scheme had to be devised to generate flies carrying genomic copies of both the *elav-GAL4* driver and *herzog::GFP*. This will allow *elav-GAL4* mediated expression of *UAS-HDAC4* transgene in the presence of *herzog::GFP*. This line was made using the crossing scheme (Figure 3.3), utilizing the *Tm3, Sb* (stubble) balancer chromosome, which contains a large inversion to prevent recombination and loss of the desired construct, and the stubble marker, which allows tracking of the balancer, therefore the construct. The new line contains the *herzog::GFP* construct and an *elav-GAL4* construct, which when crossed with another line allows co-expression of *UAS*-transgenes in a *herzog::GFP* background in a tissue specific manner, as described in Section 1.4.1. Two *elav-GAL4 herzog::GFP* lines were generated by the crossing scheme as described in Figure 3.3.

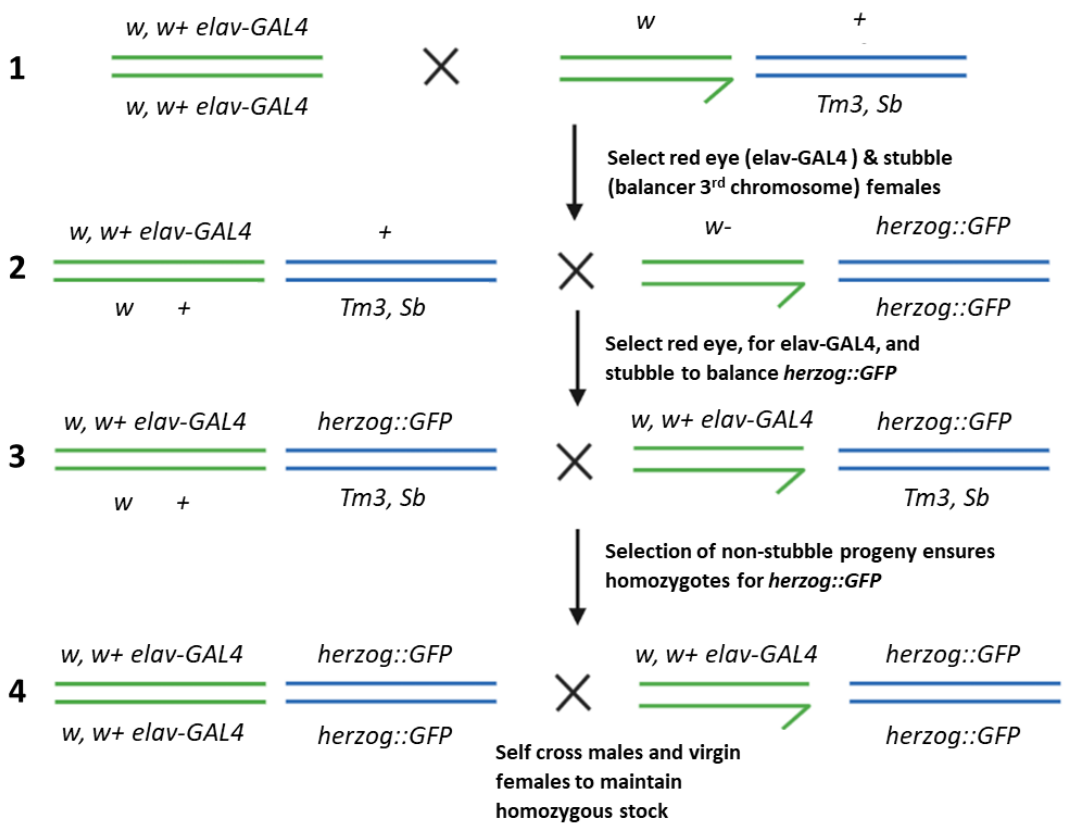

Figure 3.3 Crossing scheme to create *elav-GAL4;Herzog::GFP* line.

All flies are in a w^- genetic background, which is phenotypically observed as white eyes. The *elav-GAL4* construct also carries a copy of the w^+ gene, the presence of which restores the wildtype red eye colour; thus, their presence can be tracked in subsequent generations by the presence of red eyes. Firstly, flies homozygous for *elav-GAL4* (an X-linked insertion) are crossed to *w-* males heterozygous for the *Tm3* balancer chromosome (cross 1). A balancer chromosome carries inversions that prevents recombination, as well as a dominant marker that can be visualized (in this case the *Sb* mutation that causes stubble bristles) in order the monitor the presence of the balancer. The progeny of this cross carry a balanced third chromosome, such that when crossed to *herzog::GFP* homozygotes (cross 2) , the red eyed, stubble bristled progeny carry one copy of *elav-GAL4*, and one copy of *herzog::GFP* over the balancer. Males and females are self-crossed (cross 3), and progeny homozygous for *herzog::GFP* can be identified as having red eyes and wildtype bristles. These can then be crossed to themselves (cross 4) to maintain the homozygous fly stock. Note that following cross 3, females homozygous for *elav-GAL4* can be identified by their darker red eyes, as a result of carrying two copies of the w^+ gene.

To confirm the homozygous presence of *herzog::GFP* western blotting was performed on whole cell lysates of heads of the two new lines alongside *herzog::GFP* (*without elav-GAL4*) and a control (*Canton S*) lines (Figure 3.4). The size of the Herzog::GFP fusion protein was estimated to be 73-75kDa given that the size of GFP and epitope tags is ~35kDa and the two Herzog isoforms are 38.7 and 40.3 kDa (UniProt-Consortium, 2018). The resulting band, seen in lane 2, ran at 75-80 kDa, relatively close to our estimates. The band was seen clearly after a one second exposure and five seconds was found to be optimal to see a clear band with no over exposure (Figure 3.4). The detection at short exposures suggests a high level of endogenous expression of Herzog within the heads and brains of *Drosophila*. Two independently generated *elav-GAL4;herzog::GFP* lines (A and B) in lanes 3 and 4 show a band the same size and brightness as the homozygous *herzog::GFP* sample seen in lane two. This confirms that the *herzog::GFP* construct is homozygous in both the lines that have been made and either would allow for neuron specific expression of *UAS-HDAC4* in the presence of *herzog::GFP*.

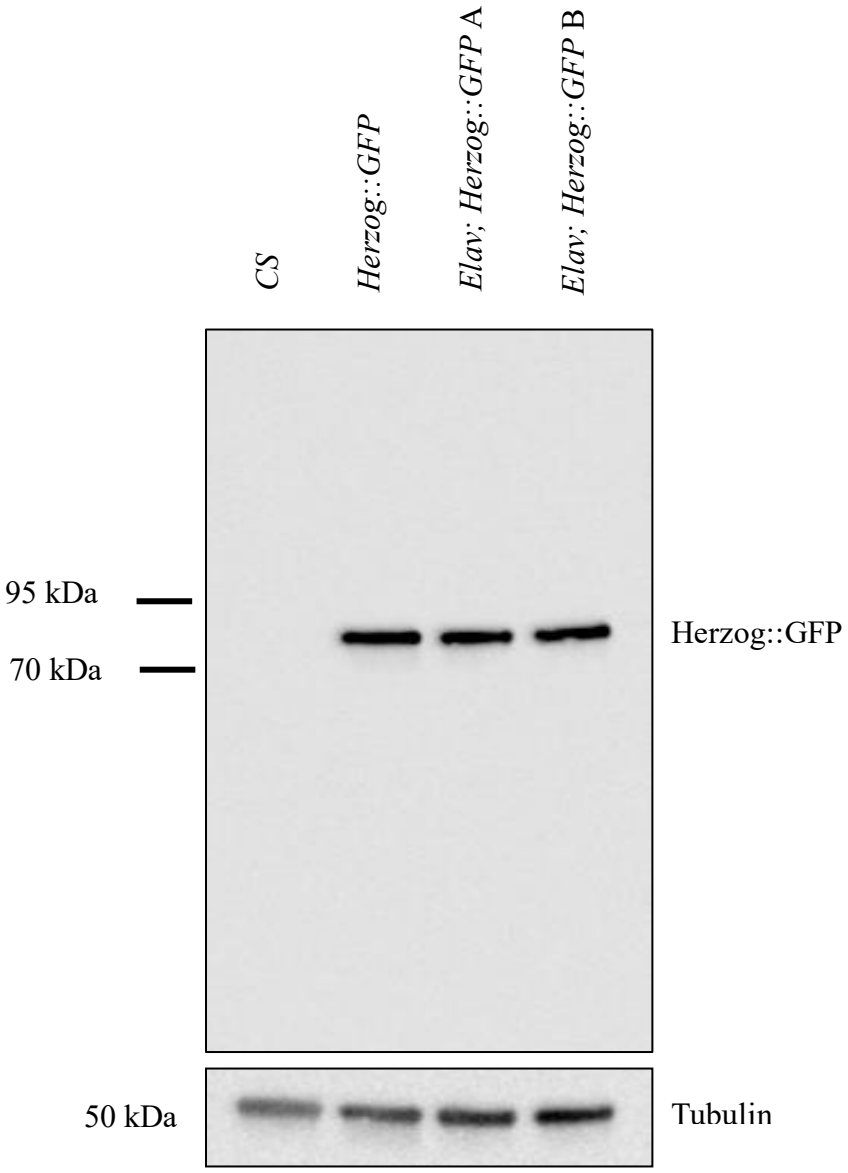

Figure 3.4. Confirmation of *herzog::GFP* homozygotes.

Western blot of whole cell lysates from *Drosophila* heads. Lane one. control line. Lane 2. Homozygous GFP-trapped Herzog line. Lane 3 and 4. New lines containing both *GFP-trapped herzog* and *elav-GAL4*. Tubulin was used as a loading control.

3.2.2 Human and *Drsosophila HDAC4* mutants do not effect Herzog protein expression

It was previously shown that expression of a cytoplasmically-restricted mutant of HDAC4 upregulated *herzog* expression via RNA-seq (Main et al., 2021). To confirm the effect of *HDAC4* on *herzog* expression and to determine whether *Drosophila HDAC4* also regulates *herzog* expression; various lines containing *UAS* driven *HDAC4* constructs were crossed to the newly made *elav-GAL4;herzog::GFP* line. The lines selected for this

experiment were; *CS* (Control); H WT (Human wildtype HDAC4), H 3SA (Human 3SA nuclear restricted HDAC4); H L175A (Human L175A cytoplasmic restricted HDAC4); DM WT (*Drosophila* wildtype HDAC4); DM 3A (*Drosophila* 3A nuclear restricted HDAC4); DM dNLS (*Drosophila* dNLS cytoplasmic HDAC4).

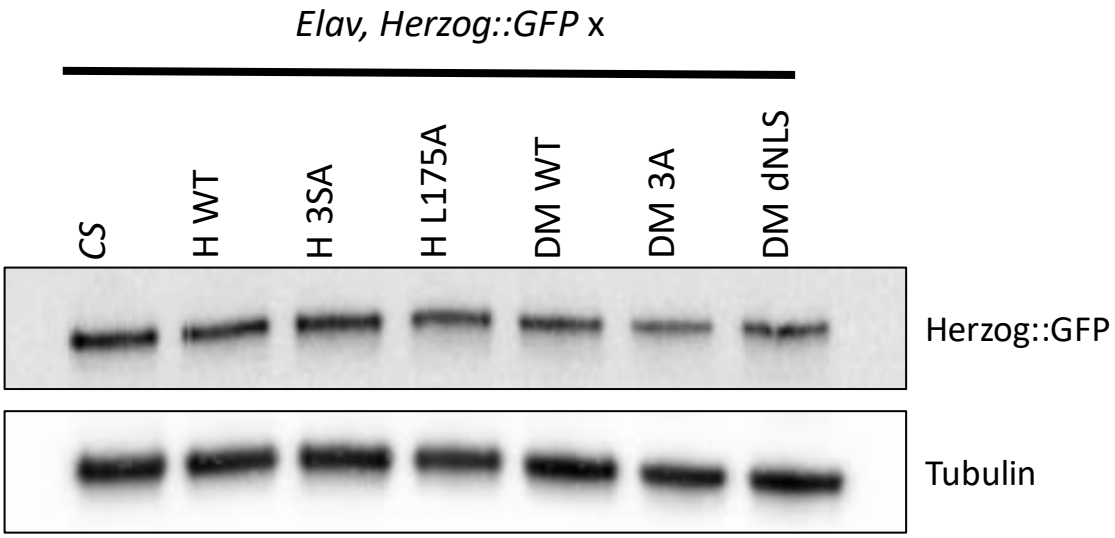

Figure 3.5.HDAC4 mutants do not affect Herzog protein levels.
Western blot using whole cell lysates from *Drosophila* heads to determine *HDAC4* mutant effects on Herzog protein expression. Tubulin was used as a loading control.

Whole cell lysates from the heads of progeny were subjected to western blotting to determine the levels of Herzog protein (Figure 3.5). The crosses, protein extraction and blots were repeated three times and each of the bands were quantified using ImageJ then normalised against the loading control (tubulin). The resulting quantification was graphed and showed no significant difference in the amount of Herzog protein between any of the seven lines, seen in Figure 3.7. These data indicate that neither human nor *Drosophila* wildtype *HDAC4*, nor nuclear or cytoplasmic mutants of each, alter the level of Herzog protein in the brain.

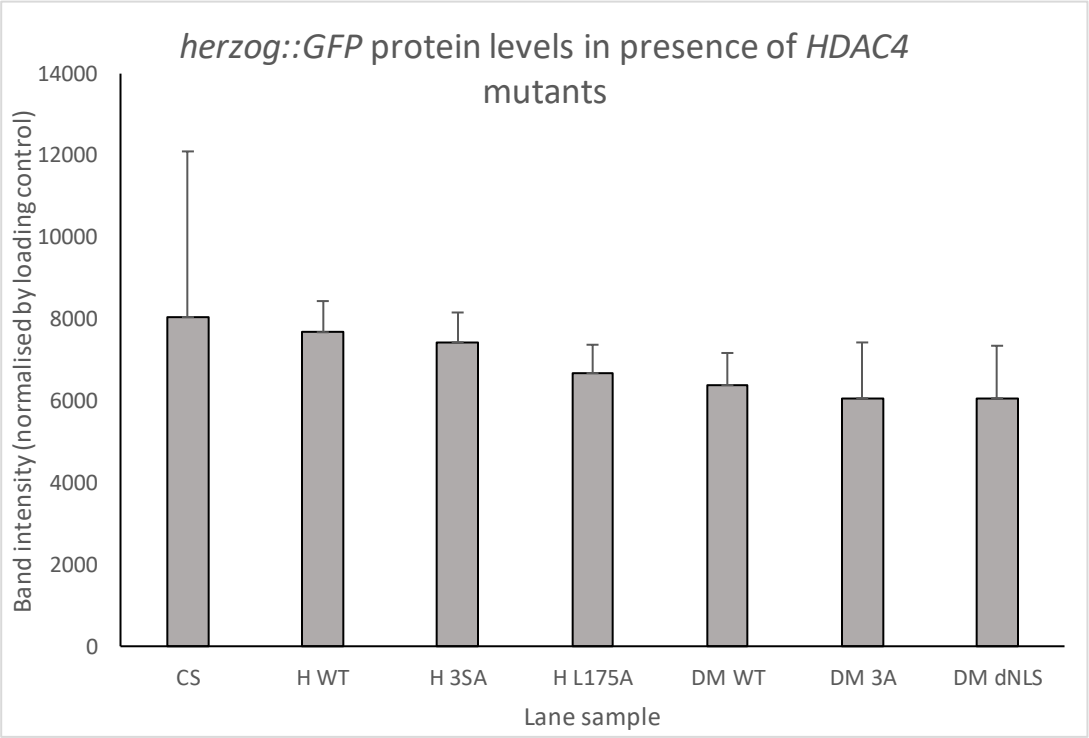

Figure 3.6. Herzog protein levels were unchanged by human and *Drosophila* HDAC4 overexpression.

Quantification of Herzog protein levels from three western blots (such as seen in Figure 3.7), normalised to loading control. No difference in Herzog protein levels between the seven lines. (ANOVA, P = 0.181). Error bars = standard error.

3.3 Assessment of the efficiency of reducing *herzog* expression via RNAi and deGradFP

To investigate the role that *herzog* may play in neuronal function in *Drosophila,* knocking down of *herzog* was attempted using two different approaches. Two *herzog UAS-RNAi* lines (*herzog* RNAi 1 and *herzog* RNAi 2) and a *UAS-DeGradFP* line were initially assessed for their ability to reduce Herzog protein. Both RNAi lines contain small inverted repeats targeted to different sequences within the *herzog* transcript, that bind and target *herzog* mRNA for degradation. The deGradFP construct contains a fusion gene of a GFP nano-body and F-box. The resulting protein is able to bind GFP-tagged proteins and then recruit the poly-ubiquitination machinery, causing the GFP-tagged protein to be targeted for degradation (Section 1.4.2). These lines were crossed to the *elav-GAL4;herzog::GFP* line, resulting in offspring which were heterozygous for both

constructs and therefore expression of the constructs was driven by UAS specifically in neurons.

Using protein lysates of the heads of the offspring of these crosses, a western blot showed that there was little to no difference in expression of the Herzog protein following knockdown with either of the RNAi lines, indicating that they were not effective (Figure 3.7). There was also only modest reduction of the Herzog protein by deGradFP. The homozygous *herzog::GFP* lysates displayed 2-fold higher Herzog protein than the heterozygotes (*elav;herzog::GFP* crossed to *CS*) indicating that a 2-fold change in expression could be detected by this method (Figure 3.7).

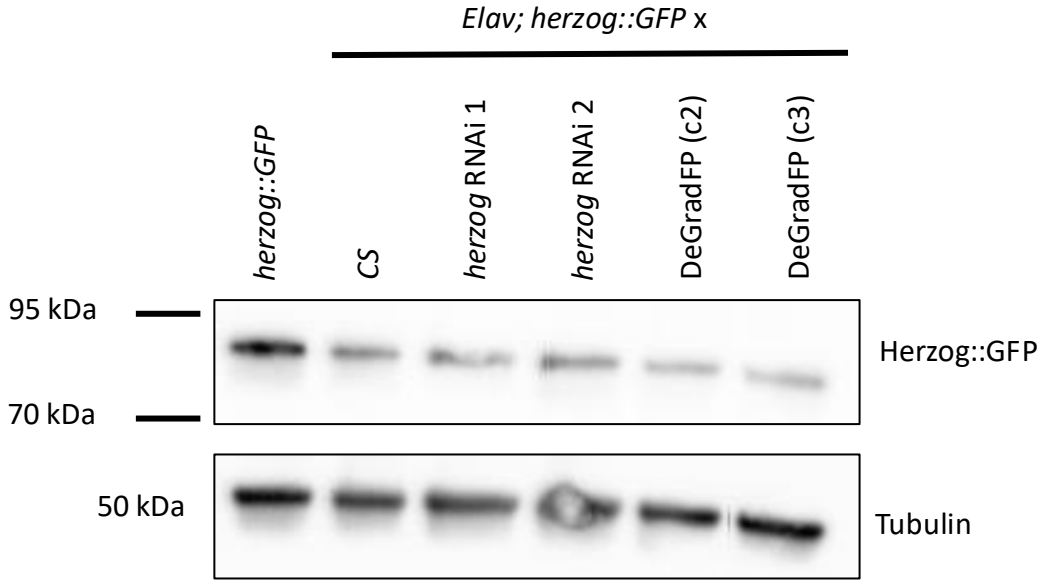

Figure 3.7. Western blot of knockdown attempt using first two RNAi lines and DeGradFP. The membrane was probed with anti-GFP for Herzog detection and anti-tubulin for Tubulin detection, as a loading control. Lane 1. Homozygous *herzog::GFP*, shows endogenous protein expressed from two copies of *herzog.* Lane 2. *Elav;herzog::GFP* crossed to *CS*, endogenous protein levels with a single copy of *herzog*, approximately 50% of lane 1, this is the control for the comparison of RNAi and deGradFP crosses. Lane 3 and 4. *Elav;herzog::GFP* crossed to *herzog* RNAi lines 1 and 2. Lane 5 and 6. *Elav;herzog::GFP* crossed to DeGradFP lines, minimal reduction of Herzog protein. c2/c3 = which chromosome construct is present on.

A further two *herzog* RNAi lines (RNAi 3 and RNAi 4) were purchased from the Vienna *Drosophila* Resource Centre (VDRC). Similarly, these contain inverted repeats of the *herzog* gene which binds the *herzog* transcript and targets it for degradation. These lines were tested for their efficiency along with the DeGradFP lines and the initial two *herzog* RNAi lines. All lines were crossed to *elav-GAL4;herzog::GFP* then the offspring were

collected and heads were processed to generate whole cell protein lysates. Both RNAi 3 and 4 knocked down the Herzog protein considerably more efficiently that the others tested (Figure 3.8).

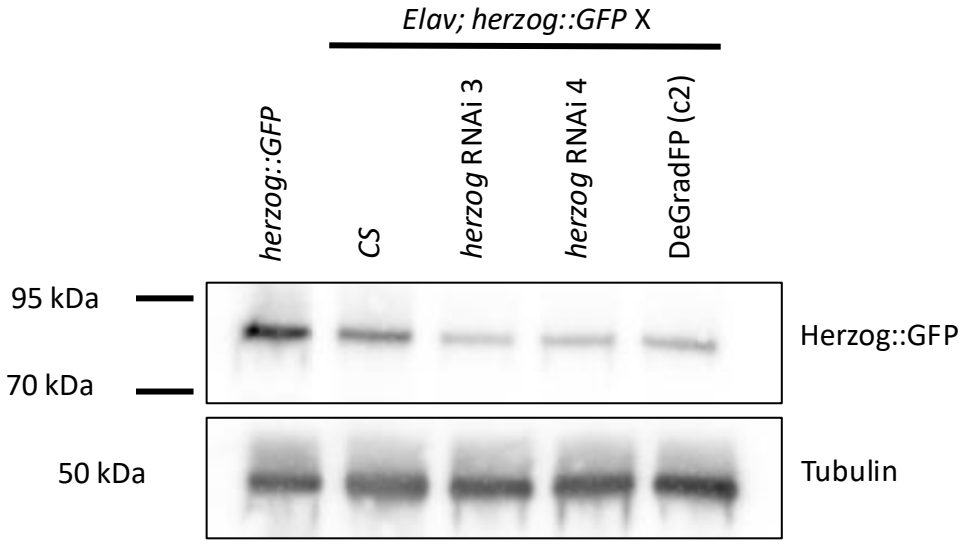

Figure 3.8 Successful knockdown of *herzog* in *Drosophila.*

Western blot of knockdown of Herzog with RNAi 3 & 4 lines and DeGradFP. The blot was probed with anti-GFP for Herzog detection and anti-tubulin for Tubulin detection, as a loading control. Lane 1. Homozygous *herzog::GFP*, shows endogenous protein expression from 2 copies of herzog. Lane 2. *Elav;herzog::GFP* crossed to CS, endogenous protein levels with a single copy of *herzog*, approximately 50% of lane 1. Lane 3 and 4. *Elav;herzog::GFP* crossed to herzog RNAi lines 3 and 4, both show reduction of Herzog protein, compared with lane 2. Lane 5. *Elav;herzog::GFP* crossed to DeGradFP (ch2), minimal reduction of Herzog protein.

DeGradFP, RNAi 3 and 4 crosses, protein extractions and western blots were repeated a further two times and all bands were quantified and normalised against loading control (Figure 3.9). There was a significant reduction in Herzog when comparing the control cross (*Elav;herzog::GFP* x *CS*) and RNAi 3 cross ($p < 0.05$). Expression appeared lower in RNAi 4, however, this was not quite sigificant ($p = 0.114$). From here on it was decided that *herzog* RNAi 3 and *herzog* RNAi 4 would be selected for further study due to higher knockdown efficiency. While RNAi 4 was not significant it was included as the error bar was large and it was possible it still efficiently knocked down Herzog. SARS-CoV-2-related delays to research prevented further repetitions of this blot to reduce the variability in this sample.

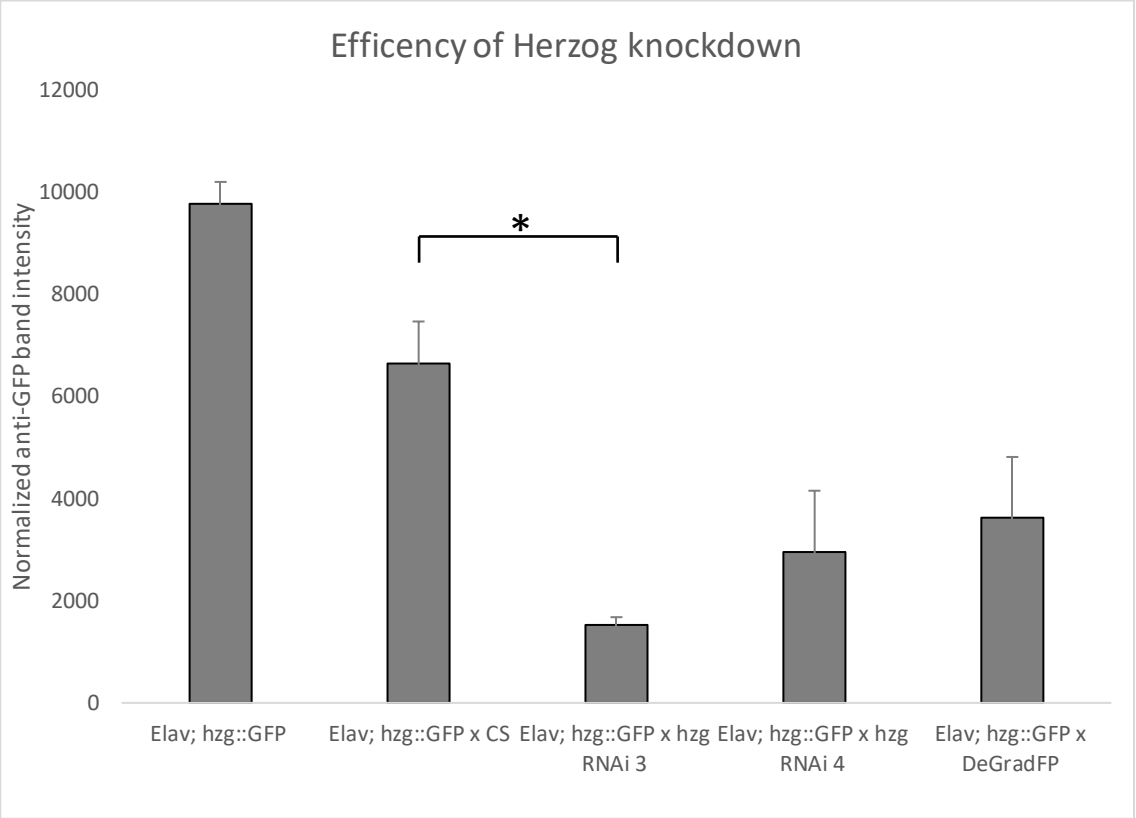

Figure 3.9. Herzog protein levels were successfully reduced by RNAi 3.
Quantification of Herzog protein from three western blots, normalised to loading control. ANOVA, Post Hoc Tukey's HSD, * = $p<0.05$, ** = $p<0.01$. Error bars = standard error.

3.4 Investigating the role of *herzog* in mushroom body development

Normal memory formation requires an intact mushroom body in *Drosophila*, therefore it was investigated whether *herzog* is required for normal mushroom body development. Developmental defects in the mushroom body can be easily detected as the axons are bundled into fibres (lobes) that can be easily visualised by confocal microscopy. The nature of the defects observed can inform not just that a specific gene is required for normal development but also gives clues as to the processes it is in involved in, such as axon elongation, termination and guidance. *Elav-GAL4;herzog::GFP* was crossed to *CS* (control), RNAi 3 and RNAi 4 flies and brains of progeny were dissected. Mushroom

body development was analysed using immunohistochemistry and confocal imaging, as described in Section 2.5.

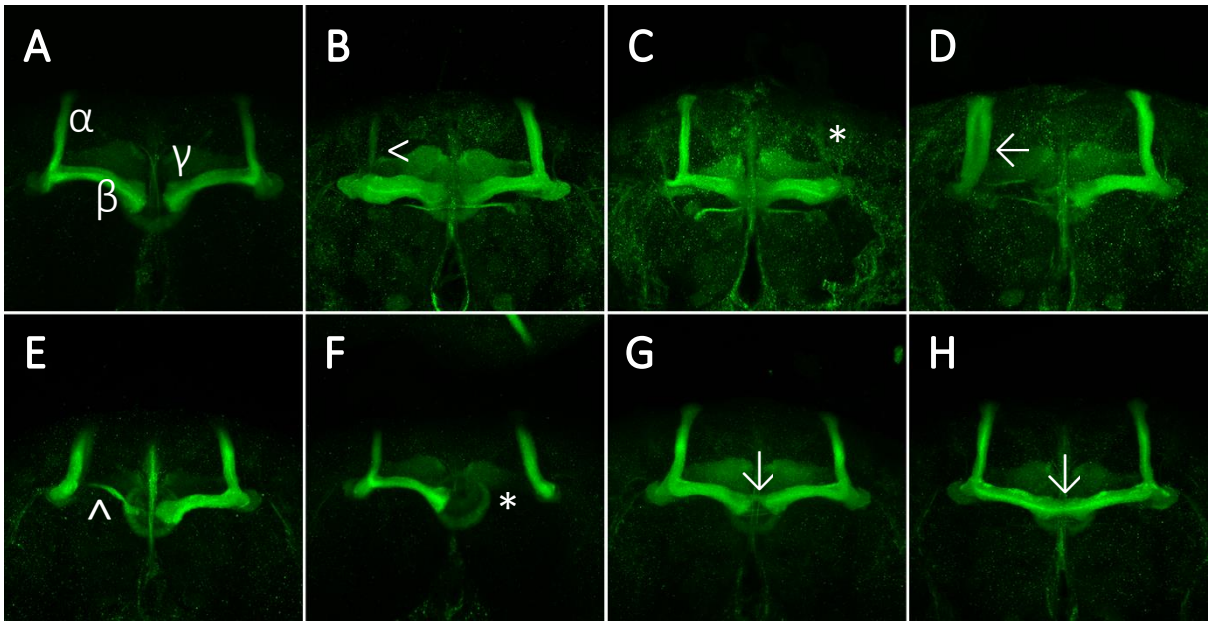

Figure 3.10. Mushroom body phenotypes commonly seen in knockdown or overexpression of neuronal genes.

A. A wildtype mushroom body, alpha (α), beta (β) and gamma (γ) lobes are labelled. B. Mushroom body with thin α-lobe indicated by >. C. Missing α-lobe as indicated by *. D. Guidance defect causing β-lobe to project vertically, indicated by →. E. Thinned β-lobe indicated by >. F. Missing β lobe, normal location indicated by *. G. Minor β lobe fusion indicated by ↓. H. Major β-lobe fusion indicated by ↓. All images provided by Hannah Hawley. Brains are either wildtype (A) or overexpression of *Drosophila HDAC4.*

A scoring system was used to semi-quantitatively assess developmental defects in the mushroom body. The scoring system considers the different types of developmental defect that can occur in the mushroom body and categorises them into groups based on their severity. Commonly observed phenotypes resulting from impaired axon morphogenesis are shown in Figure 3.10. A mushroom body that appeared normal resulted in a score of zero. A thinned lobe indicates fewer individual axons projected medially or horizontally to form a complete lobe and was scored as 0.25. Fusion of the β-lobes is a result of lack of axon termination and was scored as 0.25. The presence of both lobe thinning and β-lobe fusion is scored 0.5, as a sum of the two separate phenotypes. Complete absence of a lobe (i.e. all axons terminated prematurely) or mis-pathing (defects in axon guidance) were scored as 0.75. If two or more lobes are mis-pathed or absent then this was scored as 1. Immunohistochemistry was carried out on whole brains of the

heterozygous flies and then imaged using confocal microscopy. A representitive image of each of the 3 crosses was selected (Figure 3.11). A minimum of 20 brains of each the RNAi and control lines were imaged and scored (Table 6 & Figure 3.12). The average scores were graphed and no significance difference was seen between the control cross and the two RNAi line crosses (Figure 3.12), indicating that Herzog is not required for mushroom body devlopement.

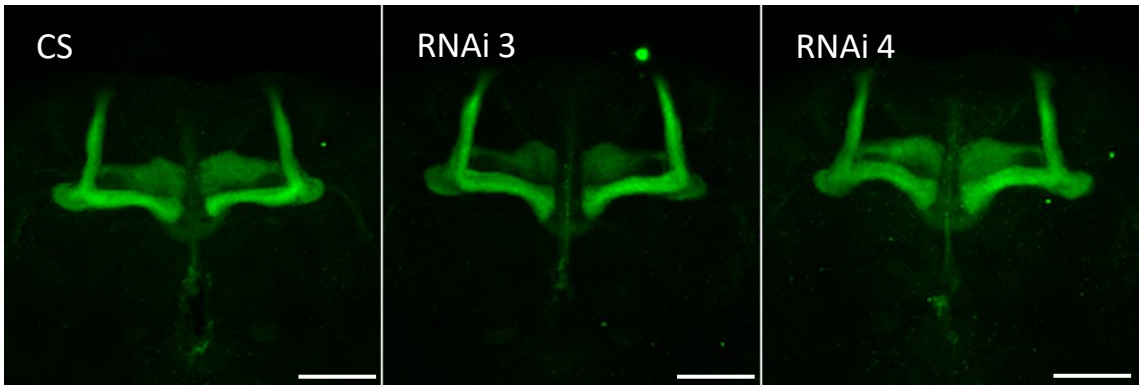

Figure 3.11. Knockdown of *herzog* did not result in mushroom body defects.
Representative images of mushroom bodies from knockdown and control. Stained with anti-FasII to visualise the α/β and γ lobes of the mushroom body. Scale = 50 μm

Table 6 Prevalence of mushroom body phenotypes in *herzog* knockdown

Mushroom body phenotype	***CS***	***herzog* RNAi 3**	***herzog* RNAi 4**
No defect	95%	92%	92%
Lobe thinning	0%	0%	0%
β-lobe fusion	5%	8%	8%
Lobe missing	0%	0%	0%
Lobe mis-pathed	0%	0%	0%

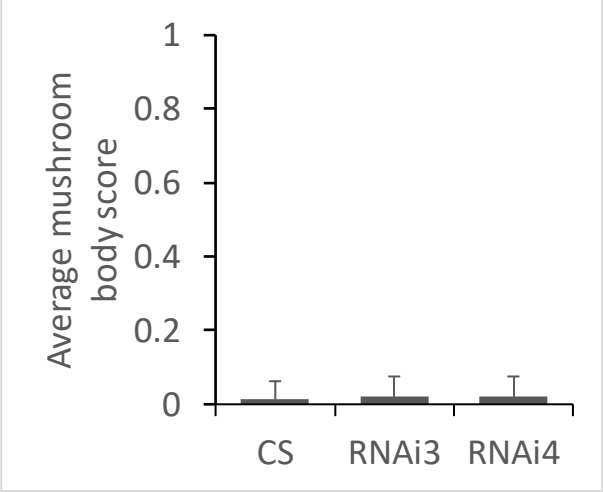

Figure 3.12. Knockdown of Herzog did not disrupt mushroom body development
CS, RNAi 3 and RNAi 4 were all crossed to *elav-GAL4;herzog::GFP*, resulting in progeny which had reduced Herzog protein in neurons. Imaging and scoring of 20 to 25 brains from each genotype were carried out and the scores for each group averaged and graphed. No difference was seen between the three genotypes. (ANOVA, P = 0.914). Error bars = standard error.

3.5 Investigating the role of *herzog* in eye development

It was next investigated whether *herzog* is required for eye development, with the rationale that *HDAC4* overexpression impairs eye development in *Drosophila* (Schwartz et al., 2016), and is also required for development of the retina in mice (Chen & Cepko, 2009). Thus, if HDAC4 regulates *herzog*, then *herzog* may also play a role in eye development. In addition, the eye is an excellent model system for examining neuronal development (see Section 1.5 of Introduction). To knockdown *herzog* in the eye, the UAS-RNAi 3 and UAS-RNAi 4 lines as well as the *CS* control line were crossed to the *GMR-GAL4* driver line. The *GMR-GAL4* driver promotes expression in all post-mitotic cells posterior to the morphogenetic furrow in the eye (Freeman, 1996), therefore if *herzog* is required for morphogenesis of photoreceptors or accessory cells, this will be visualised as defects in the adult eyes. The eyes of the F1 progeny of these crosses were examined via light and scanning electron microscopy (SEM). The light microscopy allowed visualisation of any discolouration or gross disorganisation of the eyes. SEM allowed for closer analysis of the nature of any defects in ommatidial patterning. For both SEM and light microscopy, 20 or more individual eyes were imaged for each genotype. A semi-quantitative scoring system was developed to provide an overall indication of the degree of phenotypic severity. Light microscopy images were scored based on three

criteria: colour, ommatidia organisation and ommatidia fusion. The presence of each phenotype was given a score of one and the overall score was assigned as the sum of the scores, with each eye scoring between zero and three. A score of zero indicates that the eye appeared wildtype, whereas a score of three indicates that the eye has discolouration, ommatidia disorganisation and fusion. Scores for each eye were then averaged to provide a mean score for each genotype (Figure 3.14).

For the SEM images of the eyes, a more detailed scoring system was used, which allows for further characterisation of less severe phenotypes. The scoring system was as follows: each eye image was scored from zero to four, with zero indicating an eye that was wildtype in appearance and four indicating the most severe phenotype. Scores were assigned depending on the severity and number of phenotypic defects seen which were classified from minor to severe (Table 7). The presence of a minor defect of extra bristles, in wildtype eyes each ommatidia is surrounded by three mechanosensory bristles, or two or less instances of fused ommatidia resulted in a score of one. A moderate defect or two minor defects results in a score of two. A score of three was recorded when a major or multiple moderate defects were detected. Finally, a score of 4 was given if all major defects were observed along with a severe phenotype, such as the presence of necrosis or a collapsed ommatidial array (observed as a "caved in" phenotype.)

Table 7 Defects observed in SEM images of *Drosophila* eyes

Minor defect	**Moderate defect**	**Major defect**	**Severe defect**
- Missing or extra bristle - 2 ≥ instances fused ommatidia	- Disorganisation of ommatidia - 3 ≤ fused ommatidia	- > 3 fused ommatidia - Major disorganisation of ommatidia	- > 3 fused ommatidia - Necrosis - Collapse of ommatidial array

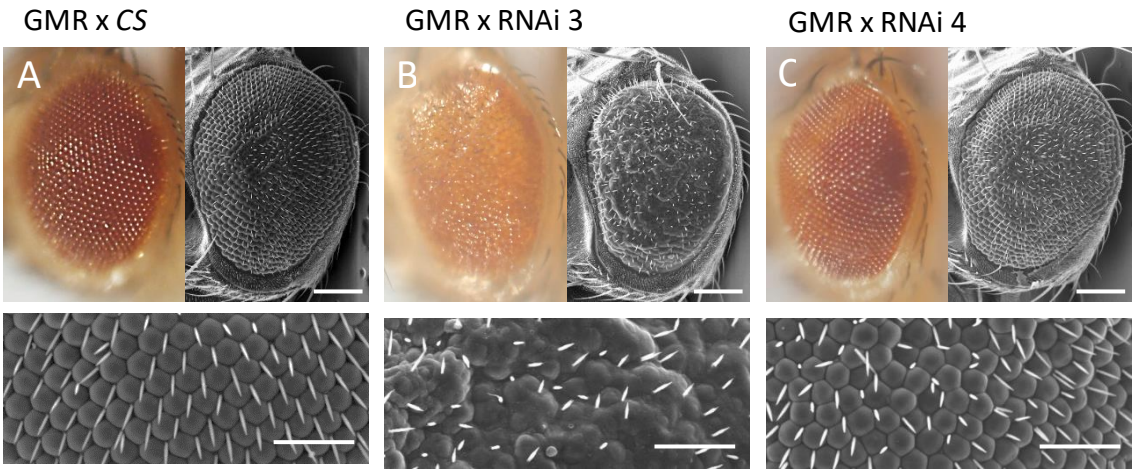

Figure 3.13 Light and scanning electron microscopy images of *Drosophila* eyes

GMR-GAL4 was crossed to: *CS*, *herzog* RNAi 3 and *herzog* RNAi 4, the F1 progeny were assessed. Top left panel, light microscopy image, top right panel SEM image, scale bar = 150 µm. Bottom panel, magnified image of ommatidia, scale bar = 50 µm. A. *GMR-GAL4* x *CS* normal eye colour and normal ommatidial array observed in light microscopy and SEM, some minor defects are observed. B. *GMR-GAL4* x *herzog* RNAi 3, discolouration and defects observed in light and SEM images. C. *GMR-GAL4* x *herzog* RNAi 4, normal colour seen in light microscopy image but irregular ommatidial array.

Progeny of *GMR-GAL4* crossed to the wildtype *CS* line displayed occasional minor defects of fusion of two ommatidia or a missing or extra bristle. The colour of the eye as seen in the light microscopy image (Figure 3.13 A), was dark red. Knockdown of *herzog* with RNAi 3 displayed the most severe phenotype (Figure 3.13 B). The light microscopy images show the reduced pigmentation of the whole eye, appearing yellow in colour. It also has a glossy appearance which is an indication of severe ommatidial fusion. This was confirmed in the SEM images in which there are no clearly defined ridges between the ommatidia resulting in fusion across the whole eye surface. The bristles are also in disarray with areas of the eye where there are no bristles present and others where there is an excess of bristles. In addition, all eyes expressing RNAi 3 showed buckling of the fused surface of the eye resulting in ridges and dips. There was a statistically significant difference between the CS cross and the RNAi 3 cross, when scored with both SEM and light microscopy (Figure 3.14). RNAi 4 eyes showed a less severe phenotype and there tended to be more variation in the phenotype as compared to RNAi 3 (Figure 3.13), however there was still a statistically significant perturbation of the normal eye development (Figure 3.14). The eyes did not show any loss of pigmentation in the light microscopy, however some disorganisation of the ommatidia was observed. More

detailed phenotypic analysis of the SEM images revealed that most eyes had disorganisation of the ommatidia, and majority also contained several points of ommatidia fusion as well as missing or extra bristles. The RNAi line also showed a significant difference than the control cross, when scored with both light and SEM microscopy. A difference was also observed between the two RNAi lines (Figure 3.14), suggesting a dose dependant effect in the eye phenotype, given the variation in knockdown efficiency. Depletion of the Herzog protein results in a severe rough eye phenotype suggesting it plays a role within *Drosophila* eye development.

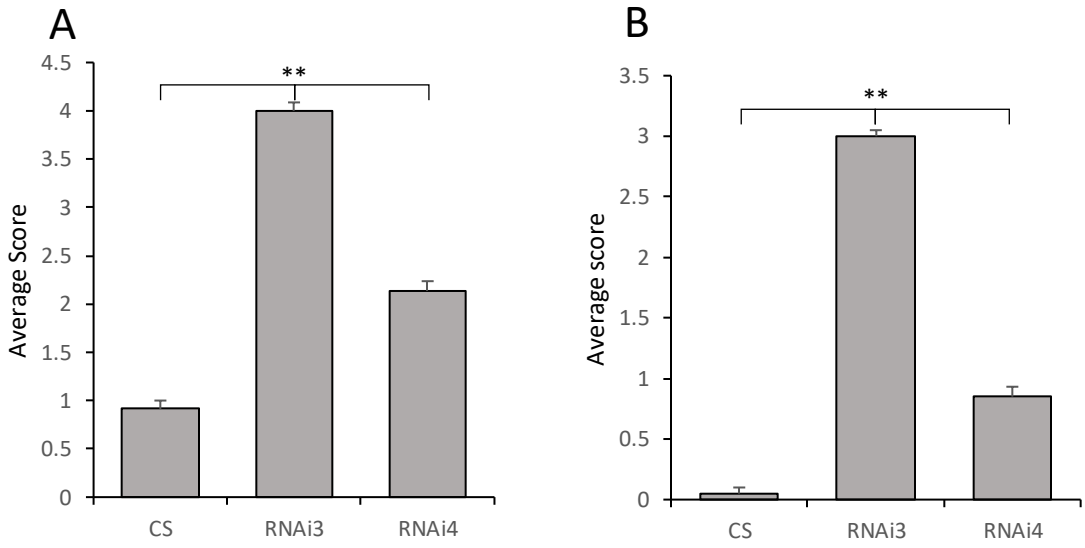

Figure 3.14. Phenotype scores resulting from knockdown of *herzog* in the eye.
GMR-GAL4 was crossed to *CS*, UAS-*herzog* RNAi3, and UAS-*herzog* RNAi4, and the eyes of F1 progeny were assessed A. Average scores for SEM images of eyes, between 22-27 eyes of each genotype were scored. B. Average scores for light microscopy images of eyes, 20 eyes of each genotype were scored. One-way ANOVA was used with a post-hoc Tukey-HSD test. Statistical significance: * = $p<0.05$, ** = $p<0.01$. Error bars = standard error.

3.5.1 Investigating the role of *herzog* in learning and memory

The data indicates that wildtype levels of *herzog* are not required for mushroom body development, however the fact that it is robustly expressed in the adult brain suggests it may play a post-developmental role. Since it is highly expressed in the mushroom body, it was investigated as to whether *herzog* is required for learning or memory. *herzog* was initially knocked down in all neurons with the *elav-GAL4* driver, with the rationale that

if any deficits were observed, the expression could then be restricted to different regions of the brain with tissue-specific GAL4 drivers. *elav-GAL4* females were crossed to *herzog* RNAi 3 males (*elav/Hzg3*) and to *CS* males as a control for the presence of *elav-GAL4 (elav-GAL4/+)*. In addition, *herzog* RNAi 3 females were crossed to *CS* males (*HzgRNAi3/+)* as a control for the presence of the (unexpressed) *herzog* RNAi3 construct. Only the RNAi 3 line was used due to its superior efficiency of knockdown.

3.5.2 Evaluation of courtship activity and learning

As the courtship suppression assay relies on the flies displaying normal courtship behaviours, courtship activity of all the genotypes was measured. For each assay, a freshly mated *CS* female was placed with a male, from one of the test genotypes, into a testing chamber where they would remain for an hour. The first ten minutes of the hour was recorded and scored for the proportion of time the male spent courting, to provide a courtship index (pre-training/first10) for each male. It is expected that a male with a normal capacity for learning will experience the females' rejection behaviours and learn that his courtship attempts are fruitless. After forty minutes, the last ten minutes of the hour was also scored, which allowed the calculation of a learning index (proportion of time spent in courtship in first ten minutes [pre-trained CI]/ proportion of time spent in courtship in last ten minutes[post-trained CI]).

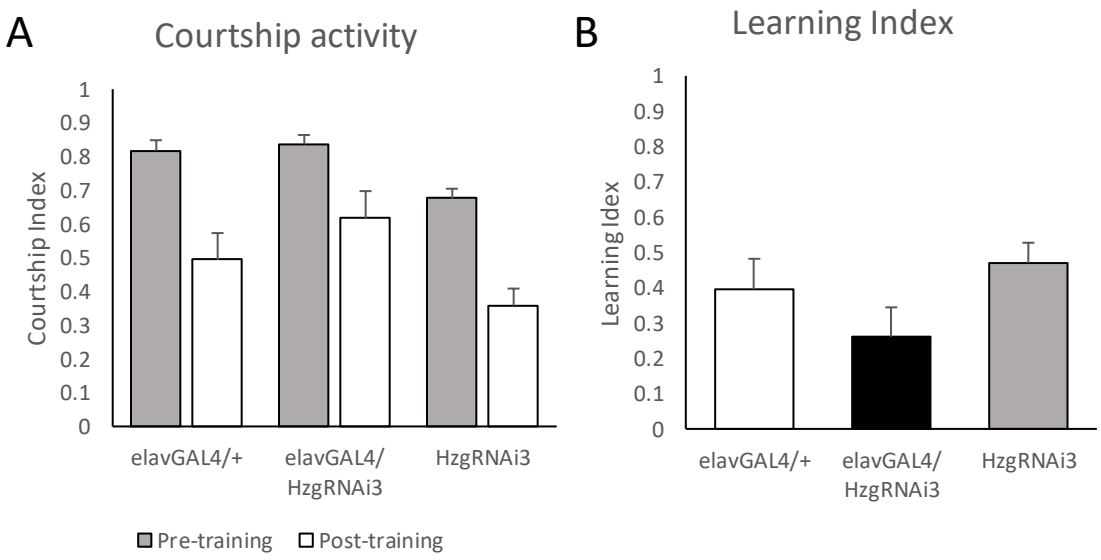

Figure 3.15 Knockdown of Herzog does not affect courtship or learning.

A. Average courtship index of the first ten minutes. Grey bars show virgin male's courtship index at first exposure, prior to training. White bars show male's courtship index after undergoing

training. For each genotype 16 to 20 male flies were scored. B. Average learning index. No difference was seen in learning between the genotypes (ANOVA, p = 0.425). For each genotype 16 to 20 male flies were scored. Error bars = standard error.

The courtship index of the pre-trained group of *elav-GAL4/+* was 0.818, meaning on average the males of that line spent 8.18 minutes out of ten courting the female. The *elav-GAL4/Hzg RNAi3* line had an average courtship index of 0.836 and the *Hzg RNAi3/+* an index of 0.76. Although the *HzgRNAi3/+* had a courtship lower than the others it was not of statistical significance. These data show that expression of *herzog* is not required for normal courtship behaviour (comparison of the grey bars in Figure 3.15 A). The absence of an effect on courtship means that the courtship suppression assay can be used to assess learning and memory without a potentially confusing effect on courtship itself. The learning index is the ratio of change of the post-training courtship index over the courtship index of pre-training. The learning index falls between zero and one with learning index of zero indicating that no learning has taken place, as the courtship index post-training is the same as before training. The closer to one the learning index is the better the learning in the post-training group. The *elav-GAL4/+* line had a learning index of 0.395, *elav-GAL4/HzgRNAi3* a learning index of 0.261, and *HzgRNAi3/+* an index of 0.469 (Figure 3.15 B). There is no significant difference between the three groups' learning index suggesting that *herzog* is not required for courtship learning in *Drosophila*.

3.6 Evaluation of short-term courtship memory

Short-term memory is measured by training a fly with a freshly mated female for an hour and then testing the memory immediately post training (as described in Section 1.4.4). The male flies are subjected to an hour in the training chambers either with a freshly mated female or alone. Those housed alone are referred to as the "sham" males and those with a female are the "trained" males. Both males after an hour are placed in testing chambers with a freshly mated female and their behaviour is recorded for ten minutes and courtship scored. Male flies that have intact memory will remember the rejection behaviours, associate them with the new freshly mated female and will reduce the amount of time he spends courting her. Flies of the same genotypes tested in the learning assay were evaluated for short-term memory. The courtship indices of the sham males for each group were not statistically significant (Figure 3.16 A), indicating again that *herzog* is not required for courtship behaviour.

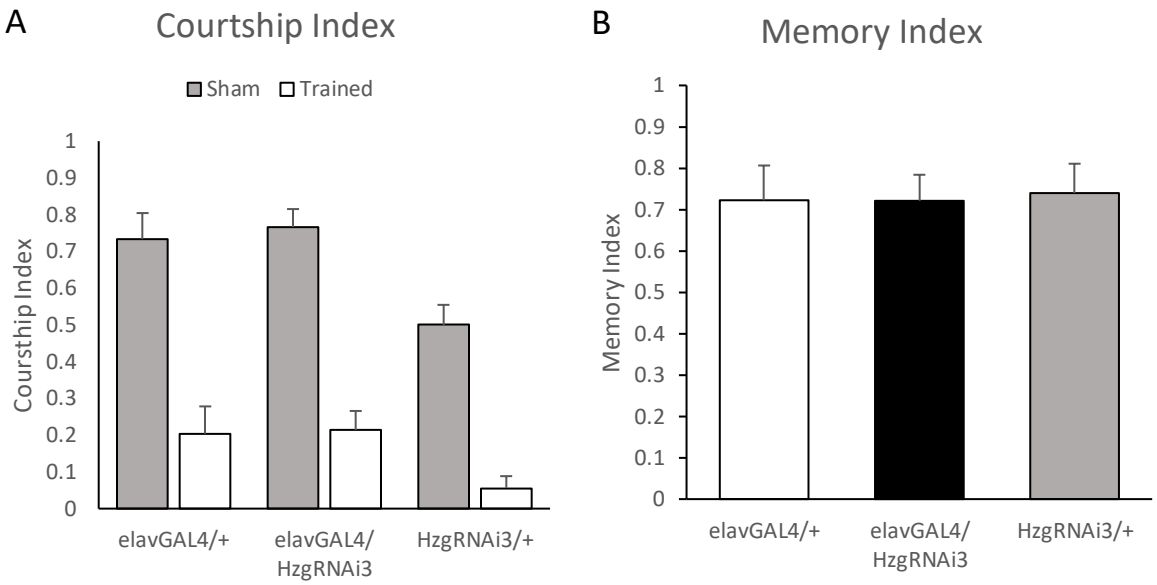

Figure 3.16. Knockdown of Herzog has no effect on short-term memory.

A. Courtship index is proportion of time spent courting over the ten-minute scoring time. Average courtship indices of sham (grey bars) and trained (white bars) male for each genotype. A significant difference between the trained and sham males of the same genotype was recorded as $p<0.01$ for each genotype. For each genotype 8 to 12 male flies were scored. B. Short-term memory appears un-effected by the knockdown of Herzog in neurons. No difference occurs between memory indices of the three genotypes (ANOVA P = 1). For each genotype 8 to 12 male flies were scored. Error bars = standard error.

All three genotypes, however showed a significant reduction of courtship by the trained males, indicating that all the males can form short-term memory. A memory index can be calculated by dividing the CI of the trained flies over the CI of the sham males to provide a quantitative comparison of memory between the three groups. The memory index gives a number between zero and one, and like the learning index the closer to one the better the memory. A memory index of zero indicates short-term memory is no different to untrained sham controls, i.e. no formation of short-term memory. The memory indices of the three lines were very similar: *elav-GAL4/+* was 0.723, *elav-GAL4/HzgRNAi3* a MI of 0.721 and *HzgRNAi3/+* was 0.74 (Figure 3.16 B). The was no significant difference between any of the three lines memory indices therefore *herzog* is not required for short-term memory formation.

4 Discussion and Future Directions

4.1 Summary of findings

The research presented in this thesis has increased understanding about the role of Herzog in the brain of *Drosophila*. Characterisation of *herzog* expression showed that it is expressed highly in the *Drosophila* brain, specifically in the mushroom body, where it is localised to the axons but is absent from the nuclei of the Kenyon cells. *herzog* expression does not appear to be regulated by HDAC4 therefore, it is unlikely to act in the HDAC4 memory pathway. *herzog* knockdown also had no effect on the development of the mushroom body or on learning and short-term memory. However, *herzog* knockdown does have a severe effect on the development of the *Drosophila* eye. More research is required to fully elucidate *herzog's* role within the *Drosophila* brain.

4.2 The expression pattern and subcellular distribution of Herzog indicate that it is not a functional orthologue of CTDSP1

A previous preliminary analysis of *herzog* expression as part of a protein-trap screen suggested that *herzog* was expressed in the brain, with high expression within the mushroom body (Knowles-Barley et al., 2010; Lye et al., 2014). In this research presented, a more detailed analysis confirmed the expression pattern by both western blot and immunohistochemistry. This expression pattern indicates that the human gene with the highest sequence similarity, CTDSP1, is not a functional orthologue. Unlike Herzog, CTDSP1 is excluded from neurons, (Yeo et al., 2005), thus this opposite pattern of expression indicates different functions of the two proteins despite the sequence similarities. CTDSP1, localises to the nucleus where it represses the expression of neuronal genes in non-neuronal tissues via de-phosphorylation of RNA polymerase II. Despite the difference in tissue specificity it was still considered that Herzog may function in a similar way to CTDSP1 by transcriptionally regulating genes via RNA polymerase II. The subcellular localisation of Herzog was also investigated by immunohistochemistry which revealed that there was no co-localisation of Herzog and DAPI in Kenyon cells. This confirmed that Herzog is not present in the nucleus and instead is largely localised to the lobes (bundled axons) of the mushroom body, showing it is present in the cytoplasm.

The lack of nuclear localisation further distances the possibility of Herzog and CTDSP1 being functional homologs (Yeo et al., 2005). The protein sequence similarity between Herzog and CTDSP1 was 65% and during the course of this project, a study was

published that characterised the role of Herzog in the developing embryo and confirmed that Herzog has a functional phosphatase domain. Similarly to its subcellular distribution in the brain, Herzog was also not localised to the nucleus, rather, in embryos Herzog specifically localises to the cell membrane (Nil, 2019). Together this data suggests that Herzog and CTDSP1 have evolved different roles with respect to their phosphatase targets.

4.3 Overexpression of HDAC4 does not alter Herzog protein levels

This study into the role of Herzog in the brain was initiated following the identification of *herzog* as a potential gene target of HDAC4 following a RNA sequencing experiment which showed *herzog* mRNA to be up-regulated by 2.5-fold upon expression of a cytoplasmically restricted mutant of *HDAC4* (Main, 2019). This suggested *herzog* as a potential target of *HDAC4*, which are of interest due to the requirement of *HDAC4* for memory, but the pathway in which HDAC4 acts is unknown. Investigating potential targets of HDAC4 will allow better understanding of how it functions in normal and altered memory.

To confirm the increased expression of *herzog* with cytoplasmic human HDAC4 and to investigate whether *Drosophila* HDAC4 also regulates *herzog*, western blotting was carried out on heads of flies in which various human and *Drosophila HDAC4* mutants were expressed. Each mutant was expressed in the presence of *herzog*::*GFP* which allowed quantification of Herzog protein levels with an antibody for GFP. There appeared to be no change in the protein levels of Herzog in any of the *HDAC4* mutant lines including in the presence of cytoplasmically-restricted human HDAC4, which had increased *herzog* mRNA levels in the previous RNA-seq experiment. In this experiment the 2.5-fold increase in expression of *herzog* was one of the most significant changes, with an adjusted p-value of $9.81E^{-69}$. A subsequent RNA-seq experiment has also shown that overexpression of *Drosophila HDAC4* in the brain results in increased transcription of *herzog* by 1.86-fold (padj $=7.22x10^{-21}$) (Wei Jun Tan and Helen Fitzsimons, unpublished data). These low p-values and the reproducibility of the increase in *herzog* mRNA with both human and *Drosophila* HDAC4, suggest that the increase in *herzog* mRNA levels when *HDAC4* is overexpressed is real. However, RT-qPCR should be carried out on these samples to confirm the RNA-seq data.

The fly crosses and western blots performed here were carried out multiple times and the results remained consistent and were statistically significant, therefore this does appear to be representative of the true Herzog protein levels. It may be that HDAC4 increases the transcription of *herzog* but there is post-transcriptional regulation controlling the transcription of RNA to protein. HDAC4 has been shown to affect the levels of *herzog* mRNA however, it does not affect Herzog protein levels. Due to this it is unconfirmed whether or not HDAC4 regulates transcription of *herzog*, either directly or indirectly. However, it does not affect the amount of Herzog protein, therefore regulation of *herzog* is unlikely to be a mechanism through which HDAC4 regulates memory.

It is still of interest to determine the role of *herzog* in the brain due to its high expression in the mushroom body, which is vital for memory formation. A knockdown of *herzog* also resulted in a severe rough eye phenotype suggesting that *herzog* has a role within neurological function. There have also been implications for *herzog* in multiple neurological disorders including Huntington's disease (Weiss et al., 2012) and Alzheimer's disease (Blard et al., 2007).

4.4 Herzog expression can be efficiently reduced by RNAi and deGradFP

To investigate the role of *herzog* in neuronal development, learning and memory, tissue specific drivers were used to deplete expression in the brain and eye. Two methods were compared for their efficacy of knockdown. The first was the use of RNAi, for which four different RNAi lines were tested (Section 1.4.1). The other method was using DeGradFP, which is a nanobody for GFP fused to an F-box, which recruits the poly-ubiquitination machinery, therefore causing GFP-tagged proteins to be poly-ubiquitinated and sent to the proteasome for degradation (Section 1.4.2). The *elav-GAL4* driver was selected for expression of the RNAi and deGradFP constructs to knockdown *herzog* in neurons. The efficacy of knockdown was assessed via western blot to quantify the level of Herzog protein. The first two RNAi lines were ineffective, however the second pair reduced Herzog expression. This variability in efficacy of RNAi lines from the VDRC has been previously documented (Dietzl et al., 2007).

The deGradFP system was also successful in reducing Herzog protein, although not quite to the level of the most efficient RNAi line. The deGradFP system targets GFP-tagged proteins and the progeny of these initial crosses carried a single copy of the GFP-trapped

herzog gene. However, the second *herzog* allele was un-trapped, therefore its expression was unaffected by deGradFP (Figure 4.1 A). For the deGradFP to target both *herzog* alleles, a line would need to be generated in which both alleles are GFP-trapped. This would require a new line to be generated, which is homozygous for both *herzog::GFP* and *UAS-deGradFP* constructs. This could then be crossed to the *elav-GAL4;herzog::GFP* line and resulting progeny would contain one copy of the *elav-GAL4* and *deGradFP* constructs and two copies of the *herzog::GFP* construct, therefore allowing deGradFP to act upon both copies of *herzog* (Figure 4.3 B).

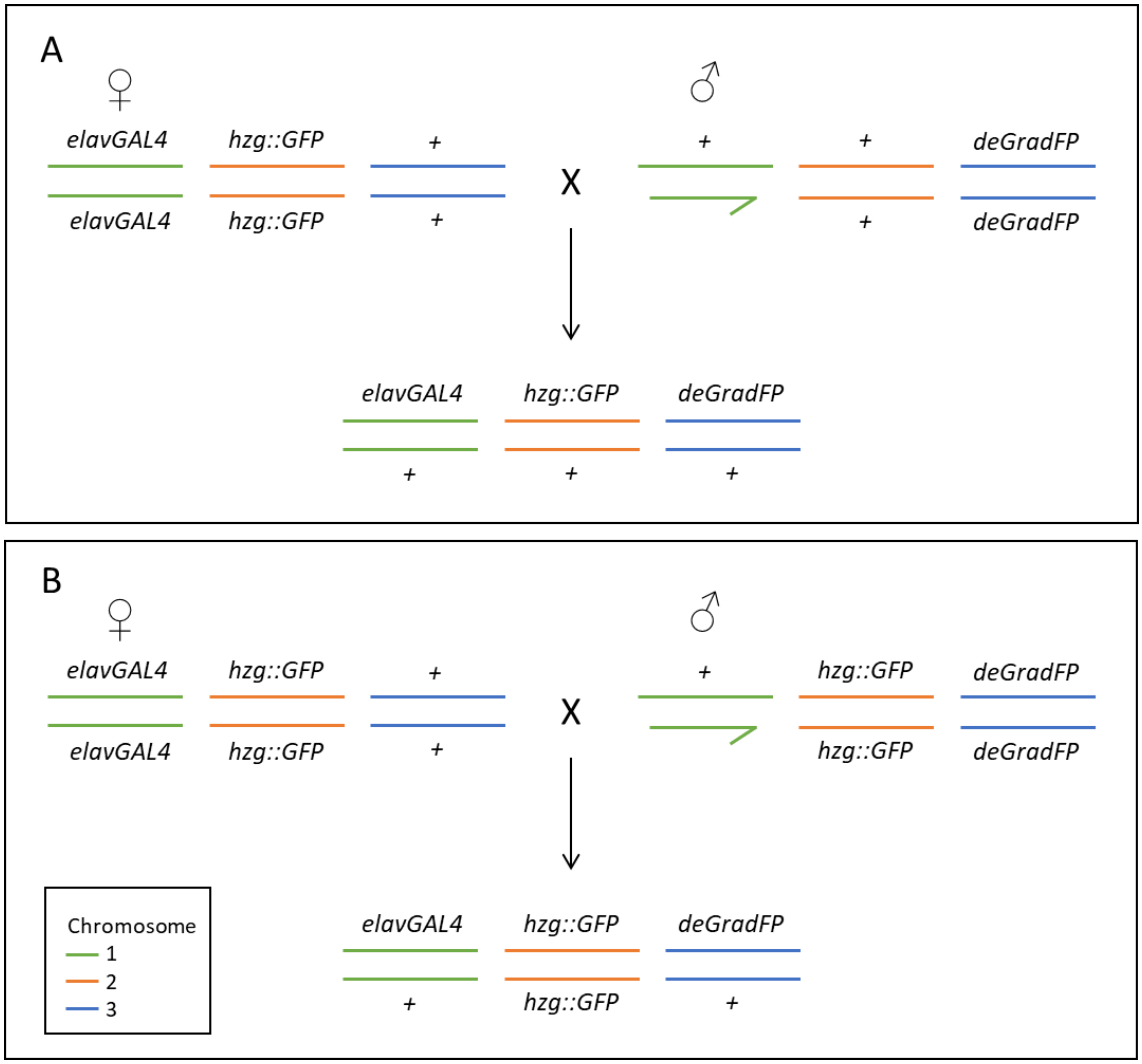

Figure 4.1 Current deGradFP cross and proposed method of crossing.

A. Current cross that was carried out to deplete Herzog protein. Female *elav-GAL4;herzog::GFP* flies were crossed to deGradFP males, resulting in progeny that are heterozygous for both deGradFP and *herzog::GFP*. The deGradFP protein is only able to deplete GFP-tagged protein so the wildtype *herzog* (on the + chromosome) expression is unaffected. B. *elav-GAL4;herzog::GFP* females are crossed to males from a proposed new line which is homozygous

for both *herzog::GFP* and deGradFP. The progeny then contains a copy of deGradFP and two copies of *herzog::GFP*, allowing all Herzog protein to be depleted via deGradFP.

This system works at the protein level causing degradation of the Herzog protein and is reliant on the extra step of DeGradFP protein being generated. There was the possibility that in later experiments it may be desirable to use an inducible system (such as GAL80ts which represses GAL4 in a temperature specific manner (McGuire et al., 2004)) to induce knockdown of *herzog* in the adult brain. In this case the extra step to knockdown *herzog* was considered to be a hindrance as Herzog protein would be expressed before then being degraded, resulting in a delayed knockdown time from activation. Given that the RNAi lines were efficient, it was decided to continue with this approach for knockdown of *herzog*. The RNAi lines used (3 and 4), appeared to knockdown *herzog* however RNAi 3 was more consistently reproducible with a smaller variation between experiments. No significant difference was found between the efficiency of knocking between the two lines, however further repetitions of this experiment may elucidate the difference more clearly. The phenotypes seen in the eyes showed a significant difference between the two RNAi lines. Therefore, further experiments to investigate the difference in knockdown efficiency between the two lines could be valuable for future experiments investigating the effects of reduced *herzog* and determining whether the dosage of *herzog* changes the severity of the phenotype. Another alternative approach to determining *herzog's* function in the brain would be to overexpress *herzog* in various tissues to examine its effect.

4.5 Herzog is not essential for mushroom body morphogenesis

The high expression of *herzog* within the brain, and in particular the expression in the mushroom body, suggests that *herzog* plays an important role in neuronal function. It was therefore investigated as to whether expression of *herzog* was required for mushroom body development. Knockdown of *herzog* with the pan-neuronal driver *elav-GAL4* did not cause any obvious alterations in overall behaviour, with locomotor activity and ability to fly appearing normal when observed in their vials. Analysis of brain development revealed that the knockdown of *herzog* did not affect the gross development or structure of the mushroom body lobes, which appeared no different to controls. This lack of any discernible affect does not rule out *herzog* involvement in neuronal development as there may be more subtle abnormalities in neuronal structure that are not observable with FasII staining. To further investigate this, future experiments could include examination of

synaptic structure and synaptic transmission. This could be done by using double labelling for synaptic markers or via electrophysiology (Atchison, 1988; Kandel & Spencer, 1961; Rohrbough & Broadie, 2002). It is also possible that Herzog was not reduced sufficiently to cause a phenotype and that the remaining protein is sufficient for its function. Further reduction or complete knock-out of the *herzog* gene may resolve whether it plays a role in the development of the mushroom body structure.

4.6 Herzog is required for normal eye development

Next it was investigated whether Herzog was required for eye development. The eye is often used as a model to investigate neuronal development and function for a variety of reasons: The *Drosophila* eye is made of types of neurons called photoreceptor neurons which form structures called the ommatidia (Freeman, 1996; Freeman, 1997). The array of ommatidia is highly organised and requires precise development which makes the eye vulnerable to disruption. Compared with the brain, the eye is accessible and easy to image, while it still a neuronal structure. These traits allow it to be an ideal model for the study of neuronal development (Cutler et al., 2015; Ghosh & Feany, 2004). In the eye, knockdown of *herzog* resulted in a severe rough eye phenotype with RNAi 3 and a less severe phenotype with RNAi 4, however quite severe developmental abnormalities including fused ommatidia and abnormal bristle orientation were still evident. If the knockdown with RNAi 4 is less efficient than RNAi 3 (which requires further analysis as described above), then it goes to reason that the remaining Herzog protein may be sufficient to carry out some of the regular function and that a full knock out of *herzog* may cause an even more severe phenotype.

The phenotype resulting from knockdown of *herzog* in the eye contrasted with that of the mushroom body. The phenotype variation between the different tissues suggests that *herzog* may have different roles in different tissues. As described above it is still possible that while *herzog* isn't required for axon elongation or termination it could still play a developmental role in neurons. It would be of interest to further examine the defects in the eye, such as the arrangement of the photoreceptors and accessory cells, or whether the axons of the photoreceptors have developed correctly (Oliva & Sierralta, 2010). Another aspect to investigate would be whether there was increased apoptosis in the eye (Miller & Cagan, 1998; Sawamoto et al., 1998) which could cause irregular patterning of the ommatidia due to excess cells (Carthew, 2007).

Due to the strong phenotype seen in the eye further investigation should be carried out determine to how *herzog* is functioning within the eye. A rescue experiment could be carried out with a knockdown of *herzog* and reintroduction of wildtype *herzog* as well as mutants, such as a mutant with an inactive phosphatase domain. This would allow elucidation of whether the reintroduction of wildtype *herzog* is sufficient to rescue the knockdown and whether the phosphatase domain is required for eye development. Another experiment to further investigate the role of *herzog* would be to express a tagged wildtype *herzog* and carry out co-immunoprecipitation followed by mass spectrometry to determine the binding partners of *herzog*. The identification of binding partners may provide clues and new avenues of investigation into the function of *herzog* in neurons.

4.7 Herzog is not required for learning or short-term immediate memory

To test whether Herzog has a role in the formation of learning or memory, the courtship suppression assay was carried out. It was imperative to first determine that knockdown of *herzog* did not alter courtship activity itself, otherwise this assay would not have been informative. In this case, the olfactory memory paradigm would have been another way to test memory, by training them to associate an olfactory stimulus with a shock and determine whether they remember the association (Tully & Quinn, 1985). Flies in which *herzog* was knocked down displayed a very similar courtship activity to the first control. However, the *herzogRNAi3/+* line (the control for the presence of the *herzog* transgene, which was not expressed) showed a lower level of courtship than the knockdown and the second *elav/+* control. This lower courtship activity is very likely due to the reduced eye pigmentation of these flies, which was the result of the direction the genetic cross was performed in (Figure 4.2 A).

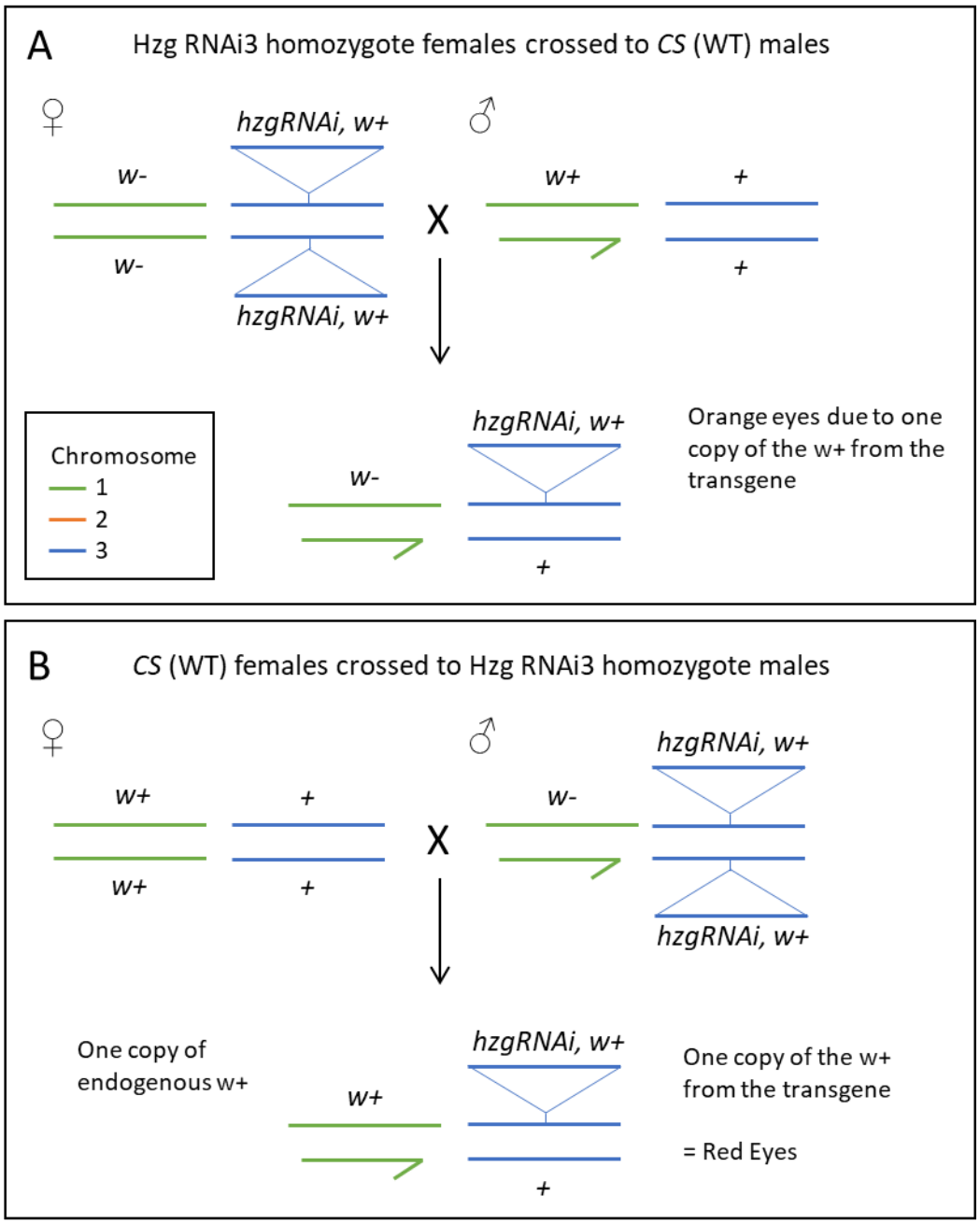

Figure 4.2 Current compared to proposed cross to knockdown *herzog* for behaviour analysis.

Transgenic flies are generated by micro injection of a plasmid containing the mini-white gene into *w-* (*white*) embryos. Transgenics are selected by the expression of white which can range from yellow to WT red, depending on position of insertion. The eye colour is dependent on the level of expression of white in a dose dependent manner. A single copy of *w+*, results in yellow to orange eyes and two copies restores the wildtype red colour. A. female RNAi line crossed to the wildtype male results in orange eyed progeny. B. Alternative approach where wildtype females are crossed to RNAi males, resulting in progeny with an endogenous copy of *w+* and a copy of *w+* from the RNAi transgene, therefore progeny will have red eyes.

The cross between *herzogRNAi3* females and *CS* males resulted in male offspring containing only one copy of the *white* gene, due to not inheriting an X chromosome (with wildtype *w+* gene) from their *CS* father (Figure 4.2 A). These progeny had orange eyes, rather than the wildtype brick red, and flies with reduced pigmentation have reduced vision, resulting in lower courtship vigour (Krstic et al., 2013). For normal courtship, male flies integrate visual as well as mechanosensory and olfactory information. Thus, the control *herzogRNAi3/+* group likely had reduced courtship activity due to reduced vision, however they still displayed normal learning and memory as described in the following sections. In retrospect, the cross should have been set up on the reciprocal manner with *CS* females crossed to *herzogRNAi3* males (Figure 4.2 B).

The impact of *herzog* knockdown on learning and short-term memory was then tested, and no significant difference between *herzog* knockdowns and controls was detected. These data show that *herzog* does not play a role in learning or short-term memory of courtship activity. Analysing memory by a different method, such as olfactory conditioning, would reveal whether these results are specific to courtship memory or if *herzog* is not required for other types of memory as well, as different neuronal pathways are required for the formation of different types of memory. It is possible that the Herzog may be required for long-term memory which was unable to be tested due to time constraints. The assay to measure long-term memory is like that used to measure short-term memory, using a longer training period of 5-7 hours followed by testing of memory after 24 hours, during which time the male is housed alone. There are examples of other proteins that are required for long-term memory but not learning and short-term memory, such as actin binding protein Moesin (Freymuth & Fitzsimons, 2017), HDAC4 (Fitzsimons et al., 2013), and Rpd3 (Fitzsimons & Scott, 2011). It is also possible that the knockdown does not result in low enough protein levels of Herzog to influence its function in the mushroom body.

4.8 Recent advances in Herzog research

Prior to embarking on this MSc project there had been no research into the role of Herzog in *Drosophila*, however during the course of this study, the first investigation was published (Nil et al., 2019). This study explored the role of *herzog* in embryos including an analysis of its phosphatase activity. It was shown that for Herzog to have an active phosphatase domain it was required to form an amyloid-like aggregation with other

Herzog monomers. Due to the role aggregation plays in various neurological disorders, it would be of interest to investigate whether *herzog* forms aggregates in the brain, and if so, under what conditions and in what subcellar areas of the neuron. Analysis of the confocal images generated in this study indicates no obvious aggregates of Herzog in the brain. An attempt at thioflavin T staining to observe the formation of any amyloid-like aggregates showed no staining and it is unclear whether this was due to the lack of amyloid formation or a technical issue with the staining. Due to SARS-CoV-2 related delays in research and shipping from international stock centres, it was not possible to import a fly line expressing a protein that forms amyloid as a positive control. Optimization of thioflavin T staining with the presence of correct controls would be of interest to determine whether *herzog* is forming aggregates in the *Drosophila* brain.

Further research into Herzog and whether it aggregates in the brain and under what circumstances would help to elucidate Herzog's function in the brain. The protein composition of the aggregates formed by Herzog in embryos is unknown as to whether it there are proteins in addition to Herzog present. The aggregation of Herzog is interesting as protein aggregation is often found to have a negative effect on neurons and it is a feature commonly observed in various neurodegenerative disorders. Alzheimer's disease is the result of the protein Amyloid aggregating to form plaques which in turn result in the degeneration of neurons, causing loss of memory and cognitive function. Huntington's disease is the result of a polyglutamine expansion within the huntingtin protein which causes the protein to be prone to aggregation. Huntingtin protein aggregates, also known as inclusion bodies, contain many proteins, including chaperons, and are insoluble (Finkbeiner, 2011). This is of interest as Herzog has previously been implicated in Huntington's disease (Weiss et al., 2012) and Alzheimer's disease via the interaction with tau (Blard et al., 2007).

Conclusion

We have found that Herzog is highly expressed within the *Drosophila* brain, especially within the mushroom body. No nuclear Herzog localisation was seen in the Kenyon cells however, it is present within in the cytoplasm. These contrasting expression patterns when compared with human CTDSP1 suggest that they may have diverged evolutionarily in regard to their phosphatase targets and functions. Mushroom body development did not show any defects when *herzog* was knocked down, however, *herzog* is required for normal eye development. The role of *herzog* in the eye is still unknown and requires further investigation to elucidate the mechanism. *herzog* is not required in learning and short-term memory, however, further investigation is required to determine whether it is needed for different types or phases of memory, such as olfactory or long-term memory. New research shows that in embryos *herzog* aggregates to activate its phosphatase domain, which is of interest in regard to *herzog* function, and may implicate *herzog* in neurological disorders involving protein aggregation. Further investigation is needed into whether *herzog* aggregates in the eye and/or brain, and whether this possible aggregation and therefore phosphatase activity is required for its function. If *herzog* aggregation and phosphatase activity is required then its phosphatase targets would be of interest to further elucidate its role.

5 References

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