OVEREXPRESSION OF MINIBRAIN AS A MODEL OF DOWN SYNDROME IN *Drosophila melanogaster*

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Overexpression of *minibrain* as a model of Down Syndrome in *Drosophila melanogaster*

by

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ABSTRACT

Down Syndrome (DS) occurs in about one in every 700 human live births, and includes cognitive impairment, learning and memory deficits, and reductions in neuronal number, volume, and density. One major DS candidate gene, *dual-specific tyrosine-regulated kinase 1a* (*dyrk1a*), encodes a protein that phosphorylates a variety of substrates. The *Drosophila melanogaster* homologue, *minibrain* (*mnb*), shares high sequence homology with human *dyrk1a* and both are important in neural development. Here directed *mnb* overexpression was accomplished in flies using the UAS-GAL4 system of gene expression. Overall eye development and bristle density were affected when *mnb* was overexpressed ubiquitously and in neural tissues. Climbing ability was poor in flies ubiquitously overexpressing *mnb*, whereas precocious loss of climbing ability was observed in flies with neural *mnb* overexpression. Increased *mnb* shortened the lifespan and delayed eclosion. Altered *mnb* expression also affected survival on starvation media, suggesting a role for *mnb* in cell survival. Taken together, these phenotypes suggest *mnb* overexpression models aspects of Down Syndrome in fruit flies.

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LIST OF ABBREVIATIONS

α-syn – alpha – synuclein

μm - micrometre

AD – Alzheimer disease

AgA – aging assay

APP – amyloid precursor protein

AS-C – achaete-scute complex

CI – confidence interval

CNS – central nervous system

da – daughterless gene

dILP – Drosophila insulin-like peptide

D. melanogaster – Drosophila melanogaster

dIR – Drosophila insulin-like receptor

dIRS – Drosophila insulin-like receptor substrate (*chico*)

DS – Down Syndrome

DSCR – Down Syndrome Critical Region

dyrk1a – dual-specific tyrosine-regulated kinase 1a

eIF2Bε - protein synthesis initiation factor 2B epsilon

EMS – Electron Microscopy Science

foxo – Forkhead box O transcription factor

Gal4 – yeast transcription factor GAL4

HD – Huntington disease

Hip1 – Huntingin-interacting protein 1

MB – mushroom body

HSA21 – human autosome 21 (*Homo sapiens* autosome 21)

IGF – insulin-like growth factor

InR – insulin receptor

IRS – Insulin receptor substrate

Mb – megabases

mC - microchaete

mL – milliliter

mm - millimetre

mnb – minibrain

n – number of flies

n/a – not applicable

NAD+ - nicotinamide adenine dinucleotide

NCBI – National Centre for Biotechnology Information

NFAT – nuclear factor of activated T-cells

NFATc – major isoform of NFAT transcription factors

NFB – also known as NK-κB; nuclear factor kappa-light-chain-enhancer of activated B cells

NFT – neurofibrillary tangles

PBS – phosphate buffer saline

PD – Parkinson disease

PI3K – phosphoinositide 3-kinase

PKB – protein kinase B, also known as akt

PTEN – phosphatase and tensin homologue

RCAN1 – regulator of protein phosphatase calcineurin 1

REST – RE1-silencing transcription factor

SEM – standard error of means

Shh – Sonic hedgehog

Sir2 – silent information regulator 2

SIRT1 – sirtuin 1; mammalian homologue of Sir2

T_{avg} – average temperature

UAS – upstream activating sequence

YAK1 – serine-threonine protein kinase found in *Saccharomyces cerevisiae*

Y-X-Y – tyrosine-X-tyrosine motif

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INTRODUCTION

Down Syndrome

Down Syndrome in Humans

Down Syndrome (DS), sometimes known as Trisomy 21, is the most common human genetic disease responsible for impaired cognitive development in humans and occurs with a frequency of one in 733 live births in the U.S.A., with similar frequency in Canada (Canfield *et al.* 2006; Canadian Down Syndrome Society 2009). Compounding this, there is a high risk of spontaneous abortion or miscarriage with DS fetuses (Buckley 2008; Park *et al.* 2009; Wiseman *et al.* 2009). Dr. John L. H. Down first described the condition in 1866 and later Lejeune and colleagues (1959) established it as a chromosomal disorder that is most generally linked to the occurrence of trisomy of human autosome 21 (HSA21). The phenotypic features associated with DS, in particular the severity of symptoms, can vary greatly. People with DS commonly have cognitive impairment and learning and memory deficits (Patterson 2009). They generally present with lower brain weights and reduced neuronal density, number and volume, which may account for some aspects of cognitive and learning difficulties (Wisniewski *et al.* 1984; Mann *et al.* 1987). The decrease in neuron density and number may be due to defects in neuronal differentiation, enhanced cell death (apoptosis) and/or decreased cell proliferation during central nervous system (CNS) development (Wisniewski *et al.* 1984; Tanzi 1996; Contestabile *et al.* 2007; Guidi *et al.* 2008). Other DS traits occur less frequently and often depend on the type and extent of trisomy. These include hypotonia or decreased muscle tone, decreased risk of solid tumours, high risk of developing leukemia, hypertension complications, and a higher incidence of congenital heart defects,

like atrioventricular septal defects, which can occur in about 44% of DS patients (Coyle *et al.* 1986; Freeman *et al.* 2008; Park *et al.* 2009). Ophthalmic disorders, including hyperopia and generally poor vision, are relatively common (reviewed in Creavin & Brown 2009). It is generally believed that these phenotypes may result from the altered expression of one or a number of genes located within HSA21.

Molecular Basis of DS and the Down Syndrome Critical Region

Approximately 95% of DS cases are due to whole-chromosome trisomy that is the result of meiotic or mitotic non-disjunction. Microtrisomy and single gene duplications are rare (together ~2% incidence), while partial trisomy, translocation due to abnormal meiosis involving a genomic region greater than 5 Mb (Antonarakis *et al.* 2004), occurs in about 3% of DS cases. While the chromosomal cause of DS has been determined, its molecular basis is still unclear. The principal hypothesis proposed is that an additional copy of one or more of the HSA21 genes leads to increased production of proteins and/or transcription factors, which may in turn influence gene expression, cell processes and interactions (Patterson & Costa 2005; Roper & Reeves 2006; Patterson 2009; Lyle *et al.* 2009; Wiseman *et al.* 2009). Several studies have reported partial trisomies of a specific HSA21 region, which could be the cause for most DS features (McCormick *et al.* 1989; Korenberg *et al.* 1994; Guimerá *et al.* 1996). The Down Syndrome Critical Region (DSCR) is a segment localized to the long arm of chromosome 21, with an estimated length between 3.8 to 6.5 Mb, containing between 25 and 30 putative genes (Rahmani *et al.* 1989; Delabar *et al.* 1993; Lyle *et al.* 2009). These genes, as stated above, have been the focus of most research into DS development. One of the best-studied candidate genes

located in this region is "*dual-specificity tyrosine-phosphorylation regulated kinase 1a*" (*dyrk1a*), which has been investigated in brain and neural development (Guimerá *et al.* 1996; Dowjat *et al.* 2007). The elevated expression of this gene and others in the DSCR may explain certain DS phenotypes.

Dual-specificity Tyrosine-phosphorylation Regulated Kinase 1A

The DS candidate gene *dyrk1a* encodes one of a family of proline-directed protein kinases capable of phosphorylating serine and threonine residues on other protein molecules, as well as auto-phosphorylating its own tyrosine residues (Kentrup *et al.* 1996; Becker *et al.* 1998; Himpel *et al.* 2000). Initially, the *dyrk1a* protein was identified as a novel kinase important in controlling nuclear function in rodent models (Kentrup *et al.* 1996) and the human gene homologue was isolated soon after (Shindoh *et al.* 1996; Song *et al.* 1996). These genes share high sequence similarity with the gene encoding the *YAK1* protein kinase found in the yeast *Saccharomyces cerevisiae*, a negative regulator of growth (Becker & Joost 1999, Zhang *et al.* 2001). Mutant YAK1 activity can also affect yeast-to-hyphal transition, preventing hyphae formation (Goyard *et al.* 2008). The kinase region in *dyrk1a* can be distinguished by the presence of several conserved and functionally necessary motifs, including the Y-X-Y motif of the activation loop in the catalytic domain (Becker *et al.* 1998; Becker & Joost 1999). The tyrosines in this loop are especially important in kinase function, as replacing these residues with phenylalanine almost completely suppresses *dyrk1a* activity (Kentrup *et al.* 1996). In both trisomic mice and human DS subjects, *dyrk1a* expression is increased 1.5-fold compared to controls, with high expression levels in the developing brain and other tissues such as the

spinal cord, retina, tongue, intestine and kidney (Song *et al.* 1996; Rahmani *et al.* 1998; Dowjat *et al.* 2007). These reports support the 'gene dosage' hypothesis of DS, which purports that the presence of an extra copy of this gene leads to increased expression in developing tissues and, eventually, to the typical DS phenotypes. The highly conserved nature of this kinase makes it a good focus for research in model systems.

Dyrk1a function and protein interactions

Phosphorylation is an important mechanism in many eukaryotic cellular processes, particularly regulation of gene expression via signal transduction. *Dyrk1a* has a variety of identified substrates within the cell, both in the nucleus and cytoplasm. The kinase has been found to regulate the NFAT (nuclear factor of activated T cells) family of transcription factors (Arron *et al.* 2006). NFATs are important in vertebrate development, particularly in heart development. Overexpression of *dyrk1a* and regulator of protein phosphatase calcineurin (RCAN1) can decrease endogenous NFATc protein levels by phosphorylating serine-proline residues in the regulatory region of NFATc, which is then exported from the nucleus (Arron *et al.* 2006; Gwack *et al.* 2006). *Dyrk1a* is important in the regulation of basic metabolic pathways, through phosphorylation of a variety of substrates, including cyclin L2, required during mitosis; *Foxo1*, important in insulin signaling pathway and apoptosis (Woods *et al.* 2001a); protein synthesis initiation factor 2B epsilon (eIF2Bε) (Woods *et al.* 2001b); glycogen synthase (Skurat & Dietrich 2004); and many endocytic proteins, like dynamin 1 and amphiphysin 1, these latter proteins suggesting that *dyrk1a* has a role in endocytic pathways in eukaryotic cells (Chen-Hwang

et al. 2002; Murakami *et al.* 2006). *Dyrk1a* thus seems to have several roles in the normal regulation of cell processes.

Overexpression of *dyrk1a* inhibits neural differentiation. As observed in mouse DS models, *dyrk1a* overexpression decreases RE1-silencing transcription factor (REST), which is required for maintaining pluripotency in developing neural cells and facilitating overall neural differentiation (Canzonetta *et al.* 2008). In the presence of additional *dyrk1a* protein in mice, trisomic granule cell precursors in the cerebellum have reduced mitogenic response to the morphogen *Sonic hedgehog* (*Shh*) (Roper & Reeves 2006; Roper *et al.* 2009). These cells are less likely to undergo mitosis, resulting in fewer neurons. Yabut and colleagues (2010) recently found that *dyrk1a* overexpression inhibits neural proliferation and promotes premature differentiation in the mouse cerebral cortex. Another neurogenic protein regulated by *dyrk1a* is Notch. Notch signaling is used to spatially and temporally regulate cell fate, proliferation and differentiation and apoptosis (reviewed in Bray 2006). Nuclear *dyrk1a* phosphorylates Notch within its ankyrin domain, thus reducing Notch transcriptional capacity (Fernandez-Martinez *et al.* 2009). The interaction with *dyrk1a* and other important substrates implicate the kinase's role not only in DS but also in neurodegenerative diseases.

Dyrk1a and neurodegenerative diseases

Examples of neurodegenerative disorders in which substrates and regulators of DS-linked *dyrk1a* have been implicated include Alzheimer Disease (AD), Parkinson Disease (PD), and Huntington Disease (HD). People with DS are at high risk of developing early-onset AD; by the age of 60, 50 to 70% of DS subjects develop AD-like

dementia (Holland *et al.* 2000). High levels of *dyrk1a* expression have been found in neurons of the neocortex, endorhinal cortex and hippocampus of AD subjects and in DS patients with AD-like pathology (Ferrer *et al.* 2005). AD is possibly linked to the trisomy of amyloid precursor protein (APP), also found on chromosome 21 in humans. *Dyrk1a* phosphorylates and activates APP, which consequently promotes ß–amyloid production (Grundke-Iqbal *et al.* 1986; Ryoo *et al.* 2008; Park *et al.* 2009). ß–amyloids are the proteins that compose the neurofibrillary tangles (NFTs) and amyloid plaques seen in both AD and early-onset AD associated with DS (Masters *et al.* 1985). *Dyrk1a* also hyperphosphorylates *Tau*, another protein involved in the formation of NFTs characteristic in AD (Grundke-Iqbal *et al.* 1986; Ryoo *et al.* 2007). Similar to the formation of amyloid plaques in AD, Lewy bodies formed in PD may be influenced by *dyrk1a* expression. The *dyrk1a* kinase phosphorylates α-Synuclein (α-syn), a major component of Lewy bodies. In fact, *dyrk1a* expression augments α-syn inclusion formation (Hashimoto *et al.* 2004; Kim *et al.* 2006). The development of some AD and PD characteristics may be influenced by *dyrk1a* expression.

In addition, there may be a connection between HD and DS. Although the mechanism has not been elucidated, both conditions share similar physical pathologies, including dementia and cognitive impairment (reviewed in Gusella & MacDonald 2000). *Dyrk1a* phosphorylates Huntington-interacting protein 1 (Hip1), which controls neuronal differentiation and cell death in neuroprogenitor cells in the hippocampus (Kang *et al.* 2005). Given *dyrk1a* involvement in the pathways discussed here, this kinase may have an important role in neuronal cell differentiation and cell death.

Minibrain is the Drosophila *dyrk1a* homologue

The Drosophila homologue of mammalian *dyrk1a* is *minibrain* (*mnb*) and was the first of the Dyrk kinase family to be discovered (Tejedor *et al.* 1995). Unlike mammalian *dyrk1a*, which is found on somatic chromosomes in mice and humans (16 and 21 respectively), *mnb* is located on the X chromosome in flies. The gene was identified by studying *mnb* mutations in fruit flies and is so named due to the observed 'small brain' phenotype (Tejedor *et al.* 1995). Throughout the catalytic region of *mnb*, there are highly conserved motifs, like the Y-X-Y activation loop, which indicates the presence of nucleotide binding and kinase activity. The *mnb* gene shares high nucleotide (65.5% to 75%) and amino acid (62.5% to 77.4%) sequence homology with exons of human and rodent *dyrk1a* (Shindoh *et al.* 1996; Figure 1). This high level of conservation suggests that *dyrk1a/mnb* may be associated with similar developmental processes in flies and mammals.

Learning and memory deficits associated with DS are linked to abnormal expression of *mnb*. Odour discrimination learning is poor in *mnb* mutant flies, as is visual pattern fixation, walking activity and speed (Tejedor *et al.* 1995). While *dyrk1a/mnb* has been implicated in these deficits, the exact mechanism is not yet known and the relationship between *mnb* and neural development may be indirect.

Down Syndrome etiology is closely linked with neurogenesis and neural differentiation. The mechanism by which *mnb* may influence neurogenesis is unknown. There are two waves of *mnb* expression in the developing fly, during embryonic

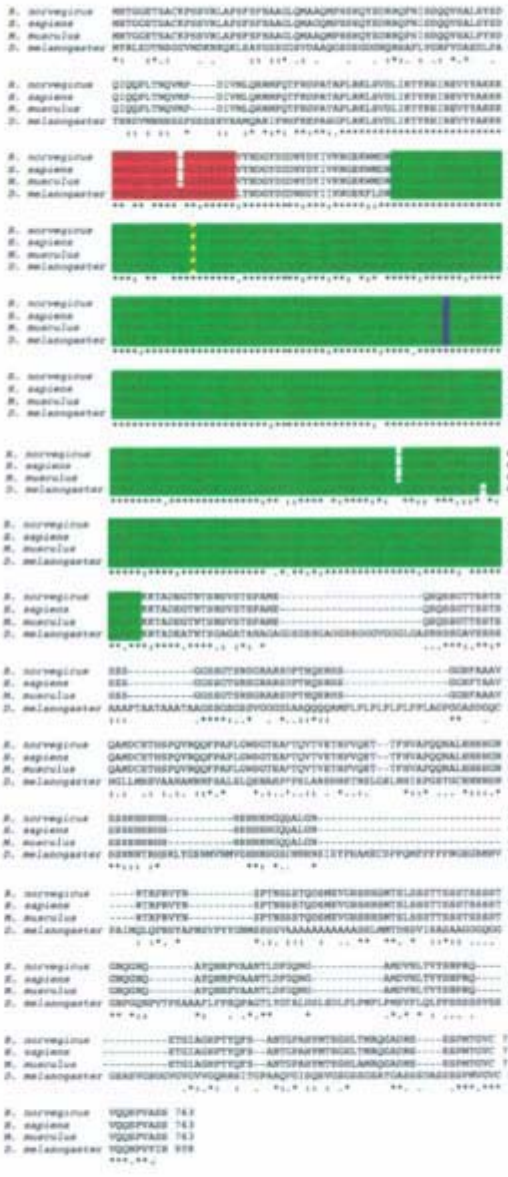

Figure 1 – The *minibrain* kinase is highly conserved. ClustalW multiple alignment of *Drosophila melanogaster mnb* with *dyrk1a* homologues of *Rattus norvegicus*, *Mus musculus* and *Homo sapiens*. Each kinase contains a serine/threonine-protein kinase domain (green), with conserved ATP binding sites (yellow) and activation sites (blue). A possible conserved sequence motif is shown in red.

neurogenesis and later in postembryonic neurogenesis in the 3rd instar larvae and pupae (Tejedor *et al.* 1995). It is during the latter of these that the adult nervous system develops, and *mnb* expression is important here in promoting proper neuron development. The *mnb* kinase is transiently expressed in neural progenitor cells from mitotic phases M to G1 possibly affecting the cell cycle (Hämmerle *et al.* 2002). This kinase influences cell differentiation through the phosphorylation of substrates that are important in cell signaling.

Drosophila as Model Organism

Model Organisms

Animal models are used extensively in genetic and biomedical research. By understanding the biological events in one organism, these discoveries can help shed light on similar pathways in other species. This is particularly important in research concerning human genetics and diseases, where experiments with human subjects may be neither practical nor ethical. Several examples of common eukaryote models used in research are *Rattus norvegicus* (rat), *Mus musculus* (mouse), *Drosophila melanogaster* (fruit fly), *Caenorhabditis elegans* (nematode) and *Saccharomyces cerevisiae* (Baker's yeast). The use of model organisms is possible due the shared ancestry and genetic similarity between species. The last common ancestor of humans and mice was about 75 million years ago and their genomes have a high level of conserved synteny, over 90% (Mouse Genome Sequencing Consortium 2002). Using model organisms has greatly increased our understanding of human disease.

Drosophila melanogaster as a model organism

The fruit fly is a powerful model for investigating human disease as around 75% of human disease genes have Drosophila homologues (Fortini *et al.* 2000; Bier 2005). Developmental processes and pathways have been conserved in the fly, the study of which can provide insight into human neurodegenerative and developmental disorders, including Parkinson, Huntington and Alzheimer diseases, as well as cardiac disease and cancer (reviewed in Bier 2005; Sang & Jackson 2005). In addition to highly conserved mechanisms, the fruit fly has several other features that make it a useful experimental subject. Flies are easy and inexpensive to maintain and have a rapid life cycle, developing from egg to adult in 10 days, producing large numbers of offspring thus increasing sample size (Bier 2005). The Drosophila genome has been fully sequenced and this knowledge coupled with recent advances in biotechnology, has allowed for the production of many transgenic fly lines (Greenspan 2004). The considerable similarity between human and fruit fly homologues provides a good platform for researching human diseases.

UAS-GAL4 System in Drosophila

One particularly useful advance in fly transgenics has been the development of the UAS-GAL4 expression system. The UAS-GAL4 system is a bipartite approach of directed or ectopic gene expression in flies (Duffy 2002). First designed by Brand and Perrimon (1993), the key to this system is the separation of driver, the yeast transcription factor *Gal4*, and reporter, the upstream activating sequence (UAS) in two distinct fly lines. The UAS is associated with a gene of interest (or target), which is silent due to the

absence of the *Gal4* protein required to activate transcription. In order to express the target gene, the two lines are crossed and transcription of the gene of interest will occur in those progeny containing both the driver and reporter (Phelps and Brand 1998; Duffy 2002; see Appendix 1). The UAS-GAL4 system is used in this study to direct expression of *minibrain* (*mnb*), a Down Syndrome candidate gene homologue found in Drosophila.

Development in *D. melanogaster*

Development and Growth

The growth of any organism is dependent on an intricate network of cell pathways. Organs and tissues tend to develop to a set size limited by a "normal" range for each species. Cell growth can involve the size of the individual cells or the number of cells within a tissue, each controlled by intra- and extracellular signals (Coelho & Leevers 2000; Day & Lawrence 2000). Controlled cell death (apoptosis) is important in determining the final shape and size of organs (Conlon & Raff 1999; Kango-Singh & Singh 2009). A balance between size and number is essential for normal development.

Nutrition is a major factor in proper growth and development of fruit flies. During the 3rd instar stage, the larva must reach the "minimal viable weight" in order for pupation to occur and to be able to survive through metamorphosis (Mirth & Riddiford 2007). Starvation after this point may delay pupation, but will not prevent it. Another significant event is reaching "critical weight", at which starvation will not delay metamorphosis (Mirth & Riddiford 2007). Interruptions in nutrient uptake and use can affect overall development in flies.

Drosophila melanogaster normally develop from egg to adult in about ten days. There is a period of about five days after hatching where the flies undergo three larval stages, called instars, where the larvae feed, grow and moult to the next stage until ready for pupation. Metamorphosis into the reproductive adult stage takes about four to five days, after which the adult fly emerges. Several factors can influence development, including genotype, animal density, temperature and nutrition (Edgar 2006). Altering one or more of these factors and observing the subsequent phenotypes may reveal underlying cellular processes important in development.

Nervous System

In *Drosophila melanogaster*, the presumptive CNS is specified at an early embryonic stage. The cell fates of proneural clusters located on developing imaginal discs are controlled via the *achaete-scute* complex (AS-C) (Bourouis *et al.* 1989). The AS-C is responsible for neuroblast differentiation and promotes Delta-Notch signaling in neuroectoderm cells (Villares & Cabrera 1987). Delta-Notch signaling is important in neural differentiation in that it promotes lateral inhibition of proneural cells. (Artvanis-Tsakonas *et al.* 1999; Lasky & Wu 2005). These cells essentially compete via neurogenic action to give rise to a single set of neural precursors. When Delta induces a Notch responding cell, the Notch cell will remain a progenitor cell, whereas the Delta cell will differentiate and become a neuronal precursor cell (Chitnis *et al.* 1995). These Delta cells move out of the ectoderm and divide asymmetrically, producing an apical cell (neural stem cell) and basal cell, a ganglion mother cell, which will differentiate into neurons

(Karcavich 2005). A complex cascade of intra- and intercellular signals controls these processes in order to produce the nervous system.

Compound Eye Formation

Closely related to neural development is the development of the Drosophila compound eye. Each eye is made of around 750 to 800 ommatidia, and each ommatidium in turn consists of eight photoreceptor neurons, four cone cells, two primary pigment cells, shared secondary pigment cells and mechanosensory bristles (Ready *et al.* 1976; Baker 2001). The eye develops from a single-layered epithelial sheet on the eye imaginal disc and while development begins in the embryo and continues through larval and pupal stages, specification and patterning of the adult eye only begins in the 3rd larval instar and pupa (reviewed in Bonini & Fortini 1999; Treisman & Heberlein 1998; Brachmann & Cagan 2003). During this stage, a morphogenic furrow sweeps posterior-to-anterior across the eye imaginal disc, activating proneural and patterning genes, for example *atonal*, *Notch*, and *scabrous* (Bonini and Fortini 1999; Baker 2001). The development of the ommatidia array in flies is controlled by a series of precise developmental events; the end result is an exact pattern with little individual variation between flies. It is this strict lattice structure and consistent developmental patterning that makes the Drosophila eye a good model for studying several developmental processes, including cell cycle regulation, cell proliferation and differentiation and neurogenesis (reviewed in Thomas & Wassarman 1999). Altered or abnormal gene expression can be detected by using biometrics to observe changes in the ommatidia array.

Mechanosensory Bristle Formation of the Dorsal Notum

Another useful, though not commonly used, model for investigating neurogenesis in flies is the development of bristles on the dorsal notum. Macrochaetae (large bristles) and microchaetae (small bristles) are associated with the peripheral nervous system, via mechanosensory neurons (Guo *et al.* 1996). Each bristle mechanosensory organ is composed of five different cells derived from precursor cells located in proneural clusters (Jan & Jan 1994; Gho *et al.* 1999). These cells include socket, shaft and sheath cells, a glial cell and a sensory neuron. Like most neurons, the development of this mechanosensory organ is controlled by the AS-C. In fact, loss-of-function and gain-of-function studies of *achaete* and *scute* have shown that these proneural genes are necessary and sufficient for bristle formation and organization (reviewed in Jan & Jan 1994). There are 26 macrochaetae set out in a regular pattern with thirteen on either side of the notum and between 200 and 250 microchaete arranged in 5 rows in the dorsocentral region of the notum (Hartenstein & Posakony 1989; Simpson *et al.* 1999). This consistent layout of macro- and microchaetae and their associated neurons present a reliable tool in investigating neurogenesis.

Goals of Research

The purpose of this project is to investigate the effects of directed *mnb* overexpression upon Drosophila biology in order to produce an easily studied model of Down Syndrome in fruit flies. The $mnb^{EY14320}$ transgenic line of Drosophila has a P-element inserted upstream from the endogenous *mnb* gene. This P-element contains GAL4-UAS enhancer, which allows the use of the UAS-GAL4 system to promote *mnb*

expression in specific tissues and cells. The main goal of this study is to determine the viability and effects of *mnb* overexpression upon neural development and behaviour in fruit flies. Electron micrographs of the eye and dorsal notum have been generated and analysed in order to observe any subtle phenotypic effects of abnormal neurodevelopment. Since DS subjects generally have decreased muscle tone and shortened life spans (Patterson 2009), flies were characterised using various bioassays to determine if *mnb* overexpression in flies can produce phenotypic features similar to DS. The Drosophila model lends itself to the observation of slight phenotypic alterations due to altered *mnb* expression.

MATERIALS AND METHODS

Fly stocks and crosses

All Drosophila stocks were obtained from the Bloomington Drosophila Stock Centre (University of Indiana, Bloomington, USA) except where otherwise noted. The *y* w^{67c23} $mnb^{EY14320}$ (Bellen *et al.* 2004) was created by the Drosophila Gene Disruption Project. See Appendix 2 for a schematic of inserted P-element, including a GAGA/GAL4-UAS enhancer. See Table 1 for list of genotypes, references and abbreviations for the experimental line, *y* w^{67c23} $mnb^{EY14320}$, the control line, *w; UAS-lacZ*$^{4-1-2}$ and the Gal4 driver lines, *da-GAL4*, *GMR-GAL4*^{12}, *y w; act-GAL4/CyO, y*$^{+}$, and *w; P[GawB]*$^{l(3)31-1}$*/TM6C*. Integral in its use as a control, *lacZ* expression is believed to be non-toxic and does not influence endogenous gene expression at any stage during fly development (Bier *et al.* 1984).

The mnb^{1} and mnb^{3}mutant lines (Tejedor *et al.* 1995) used to study longevity on starvation media were obtained from Martin Heisenberg (University of Wurzburg, Germany). The control line w^{1118} was received from Dr. Howard Lipshitz (University of Toronto, Canada). See Table 2 for list of genotypes, references and abbreviations.

Bioinformatic analysis

The sequence for the *Drosophila melanogaster* homologue of the serine/threonine-protein kinase *minibrain* (protein accession P49657) was found in the National Center for Biotechnology Information (NCBI). Homologues of *mnb* were identified using the tblastn algorithm (Altschul *et al.* 1990; Altschul *et al.* 1997) of NCBI, queried using the *D. melanogaster mnb* amino acid sequence, serine/threonine-protein

Table 1 – Genotypes, expression patterns, references and abbreviations of Drosophila stocks used to promote *mnb* expression in specific tissues.

Genotype	Expression pattern	Reference	Abbreviation	Balancer
Experimental line				
y w^{67c23} *mnb*^{EY14320}	---	Bellen *et al.* 2004	*mnb*^{EY14320}	---
Control line				
w; UAS-lacZ$^{1-2}$	---	Brand *et al.* 1994	*UAS-lacZ*	---
Driver lines				
da-GAL4 (III)	Ubiquitous	Wodarz *et al.* 1995	*da-GAL4*	---
GMR-GAL4^{12}	Eye	Freeman 1996	*GMR-GAL4*	---
y w; act-GAL4/CyO, y^{+}	Ubiquitous	Brand & Perrimon 1993	*act-GAL4*	*CyO*: curly wing (Curly)
w; P[GawB]$^{l(3)31-1}$*/TM6C*	Neurons and neuroblasts	Ito *et al.* 1997	*P[GawB]*$^{l(3)31-1}$	*TM6C*: tubby body (Tubby) and short bristles (Stubble)

Table 2 – Genotypes, references and abbreviations of Drosophila stocks used to analyse *mnb* mutant survival on amino-acid starvation media.

Genotype	Reference	Abbreviation
Control		
w^{1118}	Bingham *et al.* 1981	*w*^{1118}
Mutants		
mnb^{1}	Tejedor *et al.* 1995	*mnb*^{1}
mnb^{3}*/FM7C*	Tejedor *et al.* 1995	*mnb*^{3}
y w^{67c23} *mnb*^{EY14320}	Bellen *et al.* 2004	*mnb*^{EY14320}

kinase *mnb*. In order to show the sequence homology between *D. melanogaster* and mammalian *dyrk1a* homologues, a multiple alignment was constructed using the multialign program ClustalW available from the European Bioinformatics Institute (Larkin *et al.* 2007). Protein analysis of *dyrk1a/mnb* sequences in *D. melanogaster*, *Rattus norvergicus* (Q63470), *Mus musculus* (Q61214) and *Homo sapiens* (Q13627) were conducted through InterProScan (Zdobnov & Apweiler 2001).

Drosophila media and culture

In order to determine subtle neurodevelopmental effects of *mnb* overexpression, four to five virgin females of $mnb^{EY14320}$ were crossed with two to three males of each of the driver lines. Control crosses were set up using four to five virgin females each of *UAS-lacZ*, crossed to each of the drivers. Crosses were maintained on standard cornmeal-yeast-agar media at 25°C. Parental generation were transferred to fresh medium after two days, and again two days after that to increase number of progeny. Critical class males were determined as those not presenting with the balancer phenotypes *Tubby*, *Stubble,* and *Curly.*

Fly collection for SEMs

Fly crosses for each driver line were set up as described above in Drosophila culture. Between 50 and 80 critical class flies were aged on standard cornmeal-yeast-molasses-agar media for three days and placed in 1.5 mL microcentrifuge tubes and frozen at -70°C.

Biometric analysis of the eye

Critical class male flies were mounted on aluminum scanning electron microscope studs using double-sided sticky tape. Around 20 flies were placed on each stud, with left eyes facing up. Flies were desiccated overnight and gold-coated using Electron Microscopy Science (EMS) 500 Sputter Coater. Eyes were photographed using a Hitachi S-570 scanning electron microscope. Micrographs were taken at 150X magnification and analysed using ImageJ digital image analysis software (Abramoff *et al.* 2004). The number of ommatidia was counted in each image (n=10 per cross). The area (μm^2) of seven ommatidia (in a florette pattern) was determined for ten sets and the average area per ommatidia was calculated. Overall eye area was also measured. These data were exported into GraphPad Prism 5 (GraphPad Software, Inc.) and mean ± standard error of means (SEM) was plotted for each measured characteristic for each genotype. Statistical analyses were conducted using ANOVA followed by Newman-Keuls post-tests to determine significance between pairs. Significance was considered at $p < 0.05$.

Biometric analysis of the microchaete of the dorsal notum

Critical class male flies from *da-GAL4* and $P[GawB]^{l(3)31\text{-}1}$ crosses were mounted on aluminum scanning electron microscope studs using double-sided sticky tape. Around 15 flies were placed on each stud, ventral side down with legs removed and wings spread to the sides. Flies were desiccated overnight and gold coated using the EMS 500 Sputter Coater. Dorsal nota were photographed using a Hitachi S-570 scanning electron microscope. Micrographs were taken at 80X magnification and analysed using ImageJ digital image analysis software (Abramoff *et al.* 2004). The number of microchaete on

the dorsal notum was counted in each image (n= 25-30 per genotype). The total area (mm^2) of the dorsal notum was also measured in each case. Microchaete counts and total area were used to calculate the bristle density, expressed as number of microchaete per mm^2. These data were exported into GraphPad Prism 5 and mean ± SEM was plotted for each measured characteristic for each genotype. Statistical analyses were conducted using ANOVA followed by Newman-Keuls post-tests to determine significance between pairs. Significance was considered at $p < 0.05$.

Longevity of flies

Two aging assays were performed in this study, aging assay 1 (AgA1) and aging assay 2 (AgA2). AgA1 was done as a preliminary aging assay. Two hundred critical class males of each genotype were collected using gaseous carbon dioxide and kept on standard cornmeal-yeast-molasses-agar media at either room temperature ($T_{avg} = 20.5°C$) for AgA1 and 25°C for AgA2. Flies were maintained in non-crowded conditions of 1 to 20 flies per vial. Flies were transferred to fresh media every two days (Staveley *et al.* 1990) and scored for presence of dead adults. Survival data for AgA2 was transferred to the GraphPad Prism 5 program and were compared using ANOVA followed by Newman-Keuls post-tests. Significance was considered 95%, with $p < 0.05$.

Time to eclosion (development) of flies

Virgin females of $mnb^{EY14320}$ and *UAS-lacZ* and males of each driver line were collected and kept on standard cornmeal-yeast-molasses-agar media for three to four days before crossing. Each cross was set up as described above in Drosophila culture, using

three to four virgin females and two to three males. Flies were kept on the fresh food media for 6 hours before being transferred to fresh media and kept another 6 hours. This was repeated four times, with several vials per cross.

The number of eclosed adults for each cross was recorded and the percentage eclosion calculated using:

$$\% \text{ eclosed} = n/N * 100\%$$

where n is the number of eclosed adults for a cross on a given day, and N is the total number of F1 adults eclosed for that cross at the conclusion of culture development. Both male and female critical class flies were counted. Data was exported to and analysed in GraphPad Prism 5.

Locomotor ability of flies

Approximately two hundred critical class males of each cross were assayed for climbing ability according to the protocol described in Todd and Staveley (2004). Flies were assayed and maintained in groups of ten. Flies were kept on standard cornmeal-yeast-molasses-agar media at 25°C and assayed every 7 days. Climbing ability was determined using a glass tube apparatus, 30 cm in length, 1.5 cm in diameter, marked with five 2 cm sections with an additional buffer section at the top that limited interference between climbing flies. See Appendix 3 for a diagram of the climbing apparatus. Climbing ability was scored after 10 seconds based on which of the five marked sections had been reached. Flies were scored ten times per trial, and the climbing index calculated using:

$$\text{Climbing index} = \Sigma(nm)/N$$

Where n is the number of flies at a given section, m is the section number (1-5) and N is the total number of flies climbed in that trial. Data were exported to GraphPad Prism 5 (GraphPad Software, Inc.) and each climbing index was subtracted from five and a non-linear regression curve was fit to each set of data. Slopes of curves from each cross were compared with 95% confidence intervals.

A preliminary climbing trial was performed using flies from all five crosses (n = 30 flies per cross) and analysed for significant difference. Further climbing assays were not performed on flies overexpressing *mnb* in the eye (*GMR-GAL4*) due to no difference in climbing ability in the preliminary trial. The large-scale climbing assay described above was thus performed only on control flies and on those flies overexpressing *mnb* ubiquitously (*da-GAL4*, *act-GAL4*) and in the neurons ($P[GawB]^{l(3)31\text{-}1}$).

Longevity of flies on starvation media

In order to examine the effect of *mnb* overexpression on longevity of flies on starvation media, crosses for each genotype were set up as described above on standard food media. Roughly 200 critical class males of each genotype were collected and maintained in non-crowded conditions of one to twenty flies per vial. Adults were aged on fresh amino-acid starvation media, consisting of 5% sucrose in PBS and agar and were transferred to new media every two to four days and scored for the presence of dead adults.

Longevity studies of mutants lacking *mnb* function upon starvation media were carried out as well. See Table 2 for a list of genotypes, references and abbreviations. Roughly 200 males of each line, with the exception of $mnb^3/TM6C$ (n=129), were

collected and kept on fresh amino-acid starvation media and scored for survival as described above.

In both protocols, survival data was analysed in GraphPad Prism 5. Survival curves were compared using log-rank test, which compares actual and expected numbers of events (deaths) between survival curves at each observed event. Significance was considered 95%, with $p < 0.05$.

RESULTS

Biometric analyses of *mnb* overexpression

Investigation of *mnb* overexpression in the Drosophila eye

Neurogenesis and eye development is closely connected in flies and the development of each ommatidium and the arrangement of the ommatidia array are very tightly controlled (Thomas & Wassarman 1999). Any interruption in the developmental process can produce very distinct phenotypic changes, which can be examined using biometric analysis. Biometric analyses of scanning electron micrographs conducted on the eyes of flies overexpressing *mnb* in different expression patterns reveal alterations in eye development.

Ubiquitous overexpression of mnb affects ommatidia number and area

Eye analysis of ubiquitous *mnb* overexpression was conducted using the transgenes *da-GAL4* and *act-GAL4*, which drive ubiquitous expression at low and high levels respectively. Analysis of scanning electron micrographs of eyes ubiquitously overexpressing *mnb* show significant decreases in ommatidia number (Figures 2 and 3). When overexpressed through the activity of the ubiquitous *da-GAL4* transgene, the number of ommatidia is 712 ± 7 (n=10) compared to 745 ± 10 (n=10) in the control (Table 3). Under the control of *act-GAL4*, the ubiquitous overexpression of *mnb* results in a significant decrease in the number of ommatidia, 703 ± 7 (n=10) versus 726 ± 8 (n=10), and a significantly decreased ommatidia area with an average area of 198 ± 1 μm^2 compared to lacZ controls (Tables 3 and 6). Overexpression of *mnb* directed by these drivers has no significant effect on overall eye area (Figures 2 and 3). Significance values

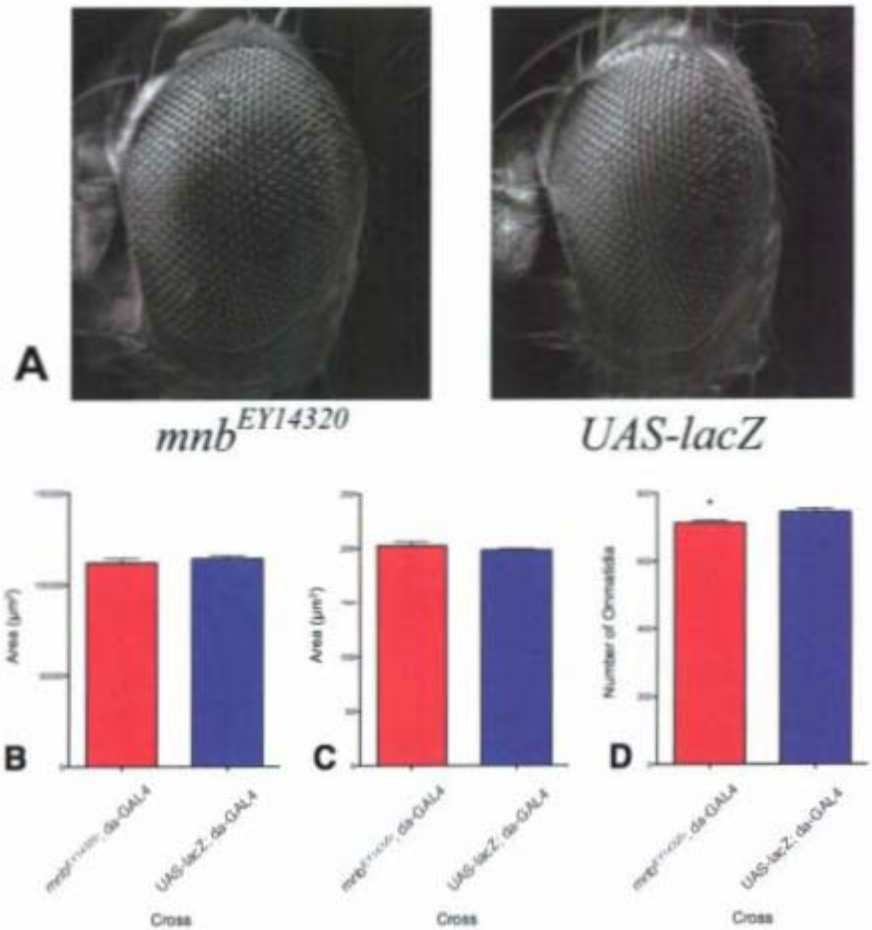

Figure 2 – Biometric analyses of eye development influenced by *mnb* overexpression under the control of the *da-GAL4* transgene. Directed low-level ubiquitous overexpression of *mnb* (n=10) has no effect on overall eye area or ommatidia area compared to *UAS-lacZ* controls (n=10). However, *mnb* overexpression using *da-GAL4* significantly decreases the number of ommatidia (n=10) compared to *UAS-lacZ* control (n=10). Scanning electron micrographs are shown in (A). Scale bar (white) represents 160 µm. Graphic representations of eye area, ommatidia area and number are shown in (B), (C) and (D) respectively. Values presented are mean ± SEM. "*" represents significant difference.

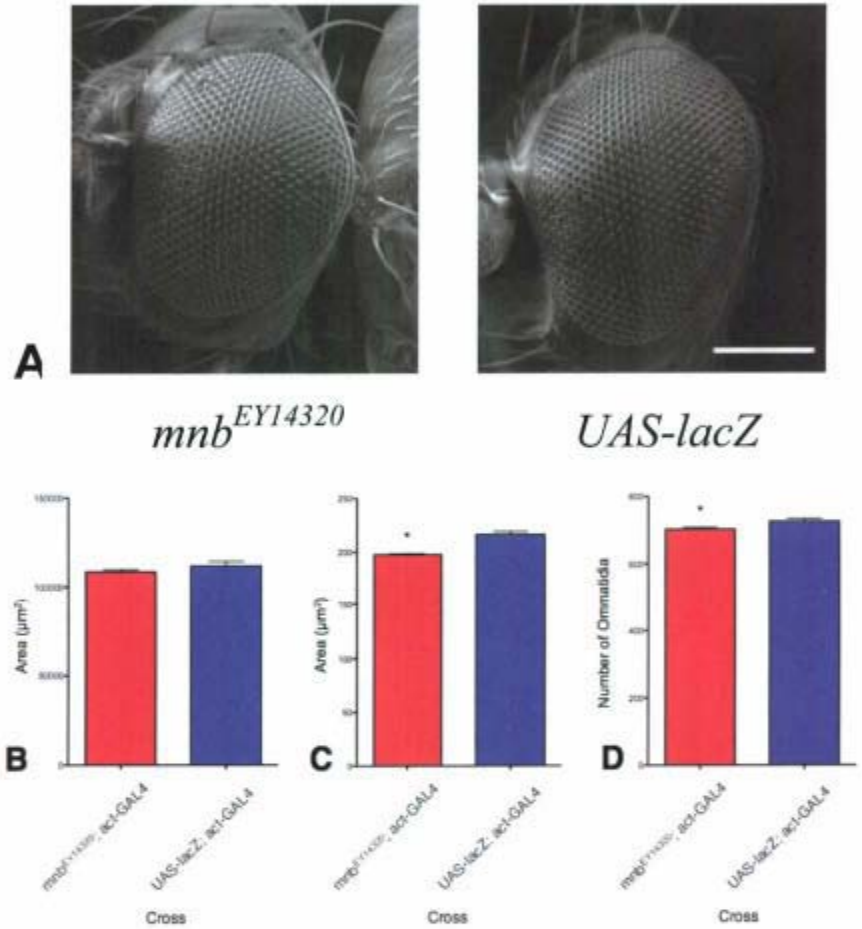

Figure 3 – Biometric analyses of eye development influenced by *mnb* overexpression under the control of the *act-GAL4* transgene. Directed ubiquitous overexpression of *mnb* (n=10) has no effect on overall eye area compared to *UAS-lacZ* control (n=10). However, *mnb* overexpression using *act-GAL4* (n=10) significantly decreases ommatidia area and number compared to *UAS-lacZ* control (n=10). Scanning electron micrographs are shown in (A). Scale bar (white) represents 160 µm. Graphic representations of eye area, ommatidia area and number are shown in (B), (C) and (D) respectively. Values presented are mean ± SEM. "*" represents significant difference.

Table 3 – Summary of overall eye area and ommatidia number and size in flies with ubiquitous overexpression of *mnb*. Values are expressed ± SEM.

Genotype	Eye area (μm^2)	Ommatidia area (μm^2)	Ommatidia #
Low-level			
UAS-lacZ; da-GAL4	114 234 ± 1532	198 ± 1	745 ± 10
$mnb^{EY14320}$; *da-GAL4*	111 966 ± 2400	203 ± 4	712 ± 7
High-level			
UAS-lacZ; act-GAL4	111 873 ± 2582	216 ± 3	726 ± 8
$mnb^{EY14320}$; *act-GAL4*	108 487 ± 1307	198 ± 1	703 ± 7

are summarized in Table 6. Ubiquitous overexpression of *mnb* affects ommatidia development, particularly via act-GAL4 driver expression, where not only number but also size is decreased.

Neural overexpression of mnb affects overall eye development

Overexpression of *mnb* in the neurons was achieved by using the driver *P[GawB]*$^{l(3)31-1}$ to promote *mnb* expression in the adult neurons and neuroblasts. Overexpression of *mnb* in the neurons and neuroblasts significantly affects overall eye development, producing flies with smaller eyes that have fewer and smaller ommatidia (Figures 4). With overexpression of *mnb* in the neurons and neuroblasts, eye area was 109, 357 ± 2195 μm^2, ommatidia area was 190 ± 3 μm^2 and ommatidia number was 691 ± 8, all reduced compared to the control (n=10) (Figure 4, Table 4). Significance values are summarized in Table 6. Neural overexpression of *mnb* affects eye development by decreasing growth and cell number in the ommatidia array.

Overexpression of mnb in the eye increases ommatidia area

GMR-GAL4 promotes expression behind the morphogenetic furrow during late eye development in flies. The transcription factor *foxo* is a putative target for *mnb* phosphorylation and studies of *foxo* expression in the Drosophila eye show a decrease of ommatidia area as well as the overall size of the eye (Kramer *et al.* 2003). Increased *mnb* expression in the eye using the *GMR-GAL4* driver has no effect on overall eye area or ommatidia number (Figure 5, Table 6). However, ommatidia area was slightly increased, 218 ± 2 μm^2 (n=10) compared to 221 ± 2 μm^2 (n=10) of the control (Table 5).

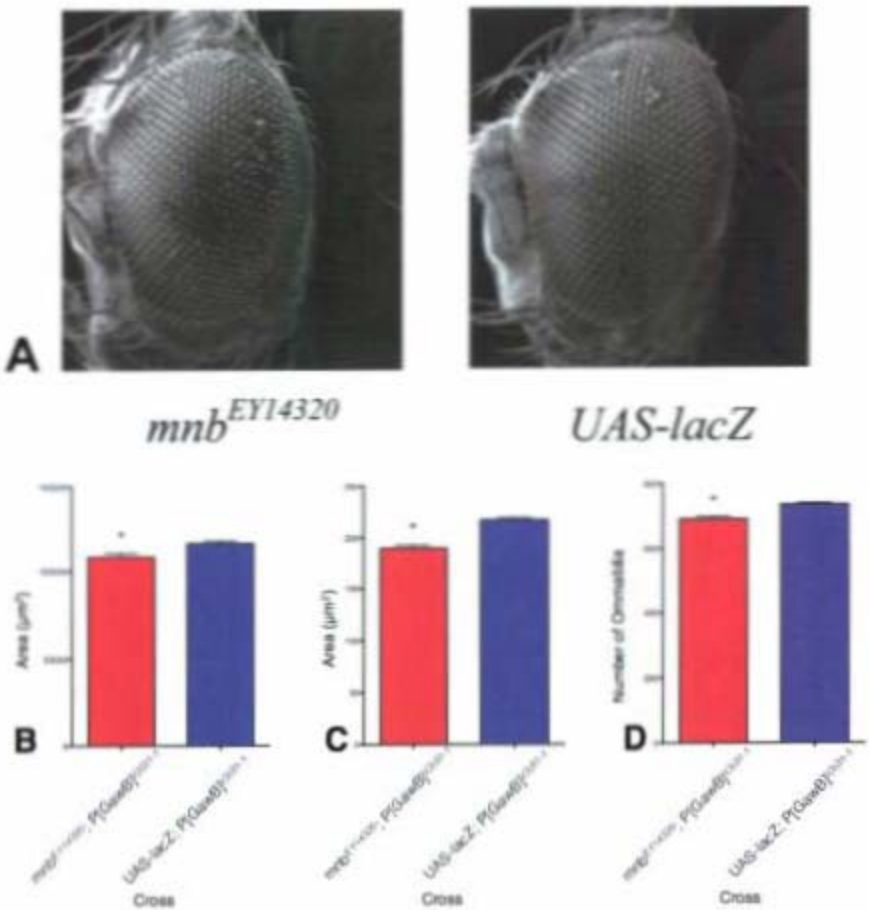

Figure 4 – Biometric analyses of eye development influenced by *mnb* overexpression under the control of the *PfGawB*$^{Eh31-1}$ transgene. Directed *mnb* overexpression in the neurons and neuroblasts (n=10) significantly decreases overall eye area, ommatidia area and ommatidia number compared to *UAS-lacZ* control (n=10). Scanning electron micrographs are shown in (A). Scale bar (white) represents 160 µm. Graphic representations of eye area, ommatidia area and number are shown in (B), (C) and (D) respectively. Values presented are mean ± SEM. "*" represents significant difference.

Table 4 – Summary of overall eye area and ommatidia number and size in flies with neural overexpression of *mnb*. Values are expressed ± SEM.

Genotype	Eye area (μm^2)	Ommatidia area (μm^2)	Ommatidia #
Neurons and neuroblasts			
UAS-lacZ; P[GawB]$^{elav-1}$	117 111 ± 1594	217 ± 2	736 ± 5
mnb^{EY14529}; P[GawB]$^{elav-1}$	109 357 ± 2195	190 ± 3	691 ± 8

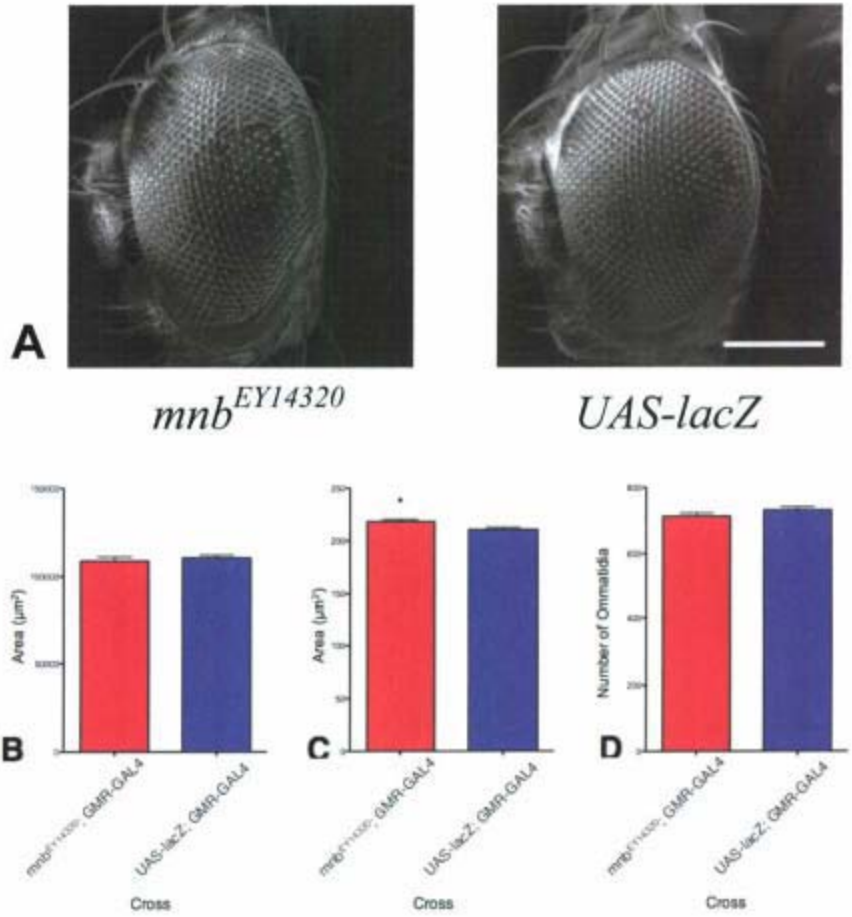

Figure 5 – Biometric analyses of eye development influenced by *mnb* overexpression under the control of the *GMR-GAL4* transgene. Directed *mnb* overexpression in the eye (n=10) significantly decreases ommatidia area but has no effect on overall eye area or ommatidia number compared to *UAS-lacZ* control (n=10). Scanning electron micrographs are shown in (A). Scale bar (white) represents 160 µm. Graphic representations of eye area, ommatidia area and number are shown in (B), (C) and (D) respectively. Values presented are mean ± SEM. "*" represents significant difference.

Table 5 – Summary of overall eye area and ommatidia number and size in flies overexpressing *mnb* in the eye. Values are expressed ± SEM.

Genotype	Eye area (μm^2)	Ommatidia area (μm^2)	Ommatidia #
UAS-lacZ; GMR-GAL4	110 469 ± 1723	211 ± 2	731 ± 10
mnb^{EY14320}; *GMR-GAL4*	108 792 ± 2338	218 ± 2	712 ± 10

Table 6 – Summary of biometric analyses on eyes from flies overexpressing *mnb* using five drivers. P-value determined from comparison to *UAS-lacZ* control. Arrows indicate increase (⇧) or decrease (⇩) in treatment.

Genotype	Eye area (μm^2)		Ommatidia area (μm^2)		Ommatidia #	
	P-value	Significant ?	P-value	Significant ?	P-value	Significant ?
Low-level ubiquitous						
UAS-lacZ; da-GAL4	n/a	n/a	n/a	n/a	n/a	n/a
mnb^{EY14320}; *da-GAL4*	0.4361	NO	0.3213	NO	0.0173	YES ⇩
High-level ubiquitous						
UAS-lacZ; act-GAL4	n/a	n/a	n/a	n/a	n/a	n/a
mnb^{EY14320}; *act-GAL4*	0.2572	NO	< 0.0001	YES ⇩	0.0433	YES ⇩
Neurons and neuroblasts						
UAS-lacZ; P[GawB]$^{elav-1}$	n/a	n/a	n/a	n/a	n/a	n/a
mnb^{EY14320}; *P[GawB]*$^{elav-1}$	0.0104	YES ⇩	< 0.0001	YES ⇩	0.0002	YES ⇩
Eye						
UAS-lacZ; GMR-GAL4	n/a	n/a	n/a	n/a	n/a	n/a
mnb^{EY14320}; *GMR-GAL4*	0.5708	NO	0.0174	YES ⇩	0.2027	NO

Investigation of *mnb* overexpression during development of the dorsal notum

The sensory bristles of the dorsal notum develop from a field of neuronal precursor cells, where each bristle is associated with a single sensory neuron. The density of bristles is influenced by Notch signaling during development (Tata & Hartley 1995; Ramain *et al.* 2001). Examination of bristle density can be a good indicator of neurogenesis. A recent study, which investigated the role of *Hip1* in neurogenesis (Moores *et al.* 2008), found that analysis of microchaete density is a sensitive assay that can assess subtle changes to cell signaling. In order to further study effects of excess *mnb* on neurogenesis, scanning electron micrographs were taken of the dorsal notums of flies with neural and ubiquitous overexpression of *mnb*. The density of microchaete, small bristles, on the dorsal notum was then measured as a gauge of sensory neuronal development.

Low-level ubiquitous overexpression of mnb affects neurogenesis in the dorsal notum

Given the effect of lower level ubiquitous *mnb* overexpression on eye development, as well as the results of several bioassays (see below), the density of bristles on the dorsal notum was examined to reveal subtle effects on neurogenesis. Overexpression of *mnb* using *da-GAL4* significantly decreases microchaete density (Figure 6). The density of $mnb^{EY14320}$; *da-GAL4* flies was 583 ± 8 mC/mm^2 (n=26) compared to the control with 667 ± 8 mC/mm^2 (n=29) (Table 7). The effects of ubiquitous expression using the *act-GAL4* driver will be assessed in a future study.

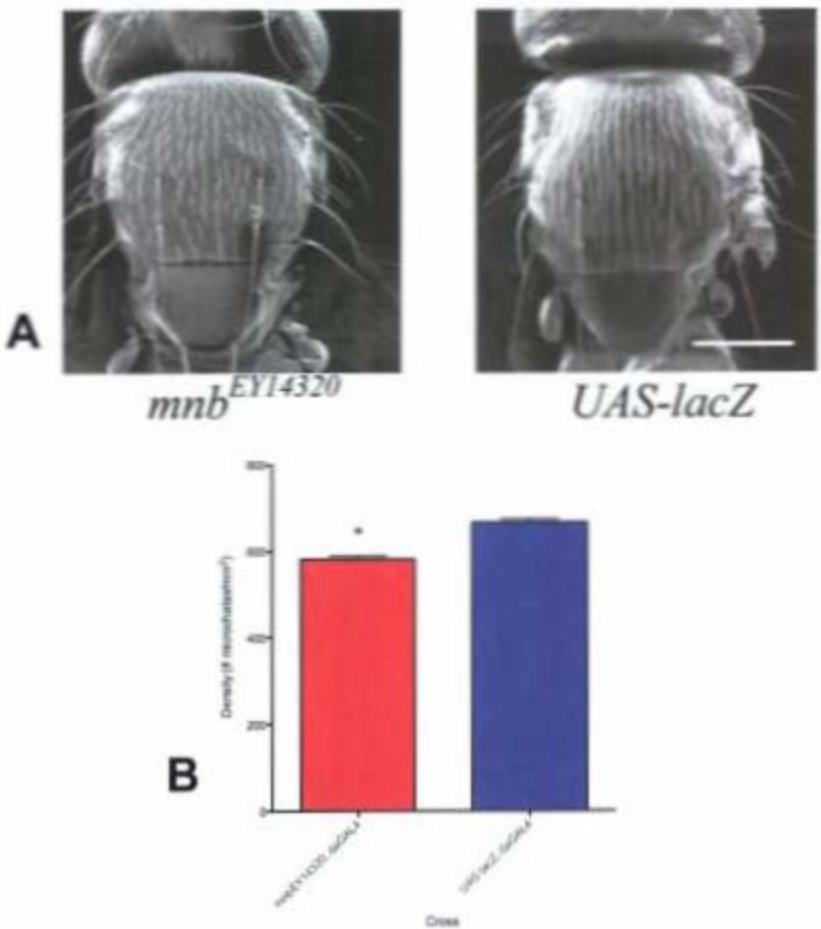

Figure 6 – Biometric analysis of bristle density: *mnb* vs *lacZ* expression under the control of the *da-GAL4* transgene. Low-level ubiquitous overexpression of *mnb* (n=26) significantly decreases microchaete density compared to *UAS-lacZ* control (n=29). Scanning electron micrographs are shown in (A). Scale bar (white) represents 0.30 mm. Graphic representation of microchaete density is shown in (B). Values presented are mean ± SEM. "*" represents significant difference.

Table 7 – Summary of microchaete density on dorsal nota of flies with ubiquitous or neural overexpression of *mnb*. Values are expressed ± SEM. mC = microchaete. Arrows indicate increase (⇧) or decrease (⇩) in treatment.

Genotype	Microchaete density (mC/mm^2)	P-value (compared with UAS-lacZ cross)	Signficant?
Low-level ubiquitous			
UAS-lacZ; da-GAL4	667 ± 8	n/a	n/a
mnb^{EY14320}; da-GAL4	583 ± 8	< 0.0001	YES ⇩
Neurons and neuroblasts			
UAS-lacZ; P{GawB}$^{elav3.3-1}$	646 ± 27	n/a	n/a
mnb^{EY14320}; P{GawB}$^{elav3.3-1}$	625 ± 44	0.0356	YES ⇩

Bristle density is affected by mnb overexpression during neural development

The *mnb* gene was overexpressed using the neural driver, $P[GawB]^{l(3)31\text{-}1}$ (Figure 7). Overexpression of *mnb* in the neurons and neuroblasts driven by the $P[GawB]^{l(3)31\text{-}1}$ transgene does slightly decrease mC density (Figure 7), with 625 ± 44 mC/mm^2 (n=29), however with lower significance than ubiquitous overexpression via *da-GAL4* (Table 7). The density of bristles on the dorsal notum is affected by neural overexpression of *mnb* in larvae.

Directed overexpression of *mnb* alters behaviour

Ubiquitous overexpression of mnb affects longevity, eclosion time and climbing ability

Ubiquitous overexpression of *mnb* was again completed using the transgenes *da-GAL4* and *act-GAL4*, driving low and high expression respectively. Several bioassays, including aging, developmental time and locomotory assays, were conducted to determine the effect of *mnb* overexpression in these patterns. In both cases, longevity of flies ubiquitously overexpressing *mnb* is decreased (Figures 8 and 9). The median life span of these flies is 10 days shorter in both crosses, while the maximum life span varied between 76 days ($mnb^{EY14320}$; *da-GAL4*, n=200) and 88 days ($mnb^{EY14320}$; *act-GAL4*, n=196). These life spans differed significantly compared to the controls (n=202, n=203) with p-values < 0.0001 (Table 8). Development time differed significantly when *mnb* was overexpressed with the *da-GAL4* driver (Figure 8). The majority of flies from this cross (~60%) eclosed one day later than the control (at 11 days versus 10) while flies with the *act-GAL4* driver eclosed the same time as the control cross, eleven days (Figure 9). Climbing was conducted over several weeks and analyses were stopped when 75% of the

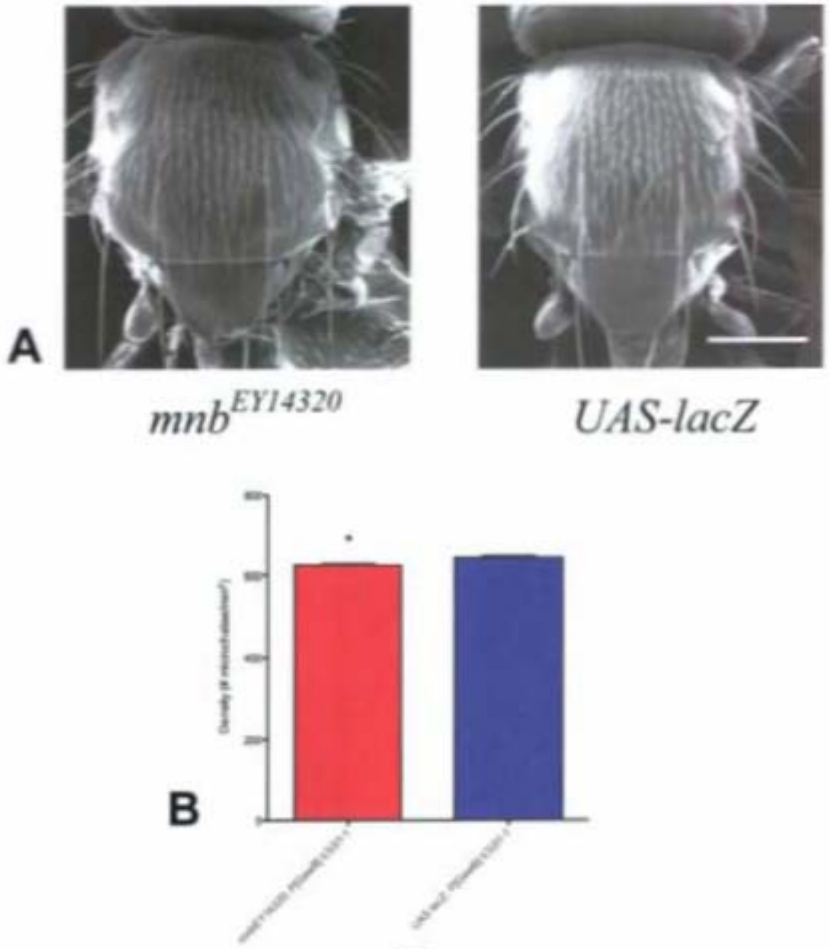

Figure 7 – Biometric analysis of bristle density: *mnb* vs *lacZ* expression under the control of the *PfGawB*$^{E1J1-1}$ transgene. Directed overexpression of *mnb* in the adult neurons and neuroblasts (n=29) significantly decreases microchaete density when compared to *UAS-lacZ* control (n=28). Scanning electron micrographs are shown in (A). Scale bar (white) represents 0.30 mm. Graphic representation of microchaete density is shown in (B). Values presented are mean ± SEM. "*" represents significant difference.

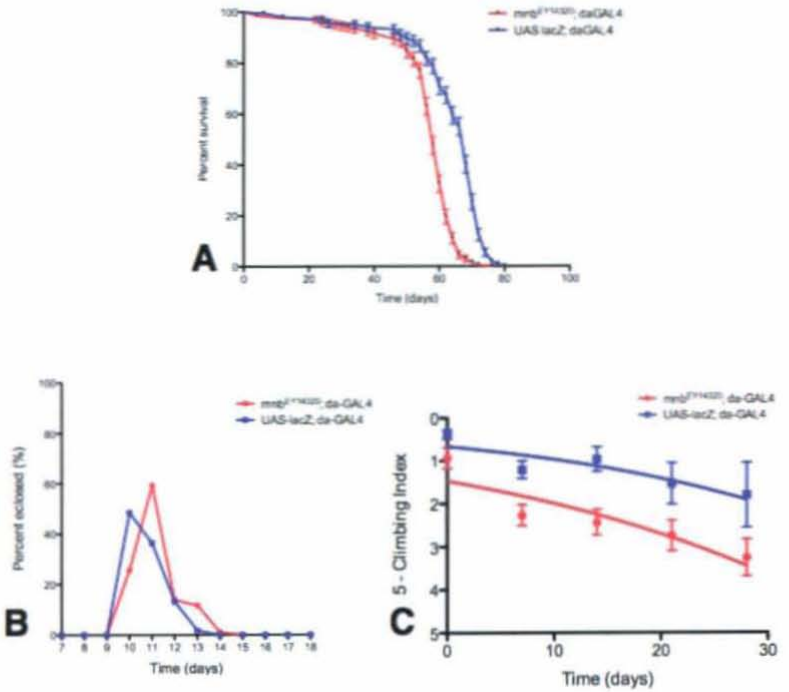

Figure 8 – Behavioural analyses of ubiquitous overexpression of *mnb* using the *da-GAL4* transgene. Directed low-level overexpression of *mnb* significantly decreases longevity, climbing ability and delays eclosion. (A) Longevity of flies overexpressing *mnb* (n=200) is significantly decreased compared to *UAS-lacZ* control (n=202). (B) Eclosion of adults is also delayed by 24 hours when overexpressing *mnb* (n=177) *UAS-lacZ; da-GAL4* n=155. (C) Flies overexpressing *mnb* (n=150) show decreased climbing ability, both initially and over time, compared to the *UAS-lacZ* control (n=150). Longevity is shown as percent survival ($P < 0.05$, determined by log-rank). Climbing ability was determined via non-linear curve fit (CI = 95%). Error bars = SEM.

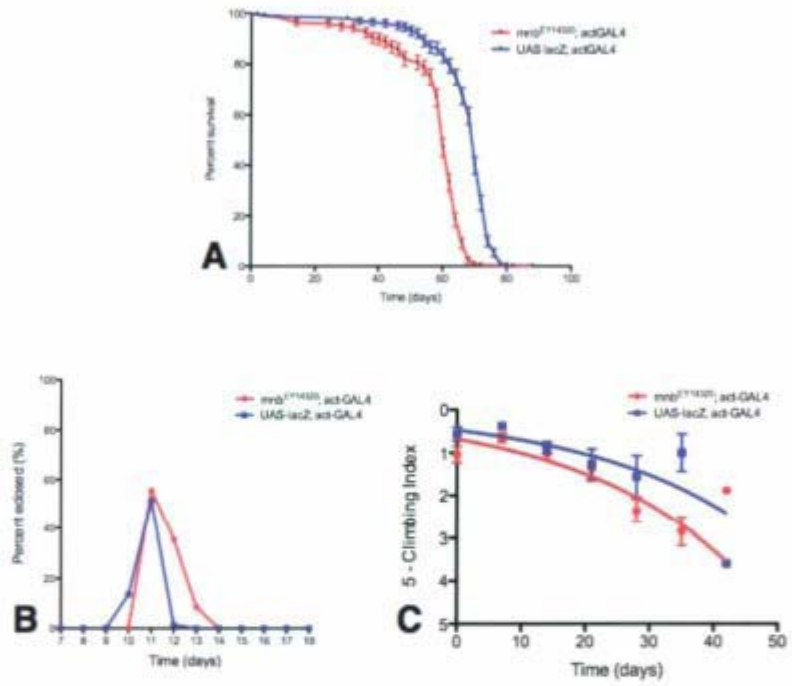

Figure 9 – Behavioural analyses of ubiquitous overexpression of *mnb* using the *act-GAL4* transgene. Directed higher-level ubiquitous overexpression of *mnb* decreases longevity on normal food media and causes slight loss of climbing ability over time, but has no effect on eclosion time. (A) Longevity of flies overexpressing *mnb* (n=196) is significantly decreased compared to the *UAS-lacZ* control (n=203). (B + C) Eclosion time and climbing ability of flies overexpressing *mnb* (n=116, n=150 respectively) are not significantly different from *UAS-lacZ* controls (n=33, n=150 respectively). Longevity is shown as percent survival ($P < 0.05$, determined by log-rank). Climbing ability was determined via non-linear curve fit (CI = 95%). Error bars = SEM.

Table 8 – Analysis of longevity of flies with ubiquitous overexpression of *mnb*. Survival curves analysed using log-rank test. Arrows indicate increase (⇑) or decrease (⇓) in treatment.

Genotype	Initial number of flies analysed (n)	Median survival day (50%)	Maximum Lifespan (day)	P-value (compared with *UAS-lacZ* cross)	Chi-square value (compared with *UAS-lacZ* cross)	Significant?
Low-level						
UAS-lacZ; da-GAL4	202	68	80	n/a	n/a	n/a
$mnb^{EY14320}$; *da-GAL4*	200	58	76	< 0.0001	87.79	YES ⇓
High-level						
UAS-lacZ; act-GAL4	203	70	82	n/a	n/a	n/a
$mnb^{EY14320}$; *act-GAL4*	196	60	88	< 0.0001	133.9	YES ⇓

initial number of flies had died. Overexpression of *mnb* using *da-GAL4* decreases climbing ability. The climbing index for $mnb^{EY14320}$; *da-GAL4* flies (n=150) starts lower than the control (n=150) and deteriorates over time (Figure 8). The climbing ability of $mnb^{EY14320}$; *act-GAL4* flies (n=150) however does not significantly differ from the control (n=150), though as the flies age, *mnb* overexpression seems to produce poorer climbing indices (Figure 9). Overall, *mnb* overexpression throughout the body can delay eclosion and decrease climbing ability, particularly at lower overexpression levels.

Neural overexpression of mnb affects longevity, eclosion time and climbing ability

The *mnb* gene plays an important role in post-embryonic neurogenesis in flies (Tejedor *et al.* 1995), and here we show that neural overexpression of *mnb* can affect fly development time and longevity. Neural overexpression was achieved using the $P[GawB]^{l(3)31\text{-}1}$ driver. Longevity studies of flies from these crosses show a decreased life span (Figures 10). The median life span is 62 days ($mnb^{EY14320}$; $P[GawB]^{l(3)31\text{-}1}$, n=207), which is significantly decreased compared to *UAS-lacZ* controls (n=216), with p-value < 0.0001 (Table 9). Maximum life span did not differ greatly from the control. Overexpression of *mnb* in the neurons and neuroblasts can also delay eclosion time. In the developmental delay analysis of $P[GawB]^{l(3)31\text{-}1}$-crossed flies, adults eclosed one day later than the control (Figure 10). Climbing assays were performed on flies from this cross and the control cross. Overexpression of *mnb* in the neurons and neuroblasts produces flies with a precocious loss of climbing ability (Figure 10).

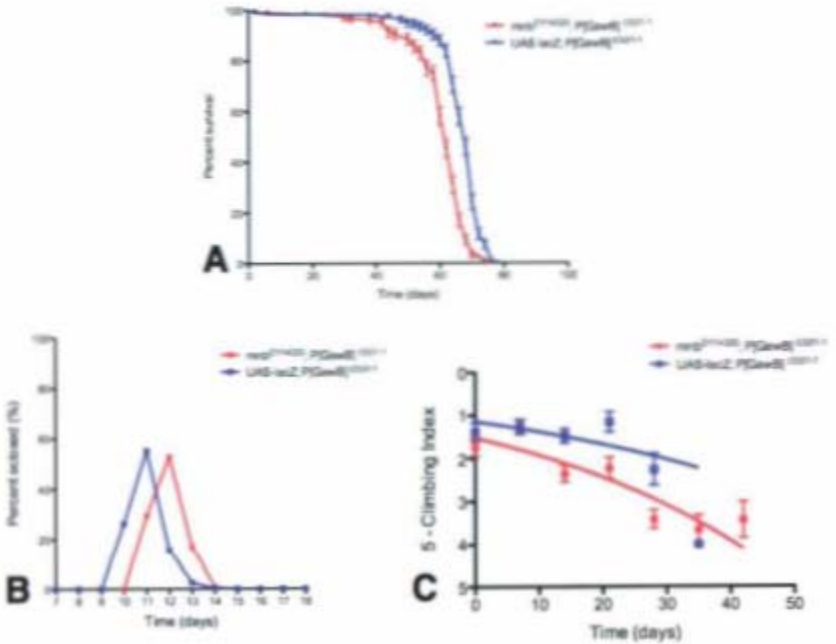

Figure 10 – Behavioural analyses of *mnb* overexpression primarily in adult neurons and neuroblasts using the *P{GawB}*$^{elav^{3}31-1}$ transgene. Directed overexpression of *mnb* in the neurons and neuroblasts decreases longevity, delays eclosion time, and causes poor climbing ability over time. (A) Longevity of flies overexpressing *mnb* (n=207) in the brain and neurons is significantly decreased compared to *UAS-lacZ* control (n=216). (B) Eclosion of adult flies overexpressing *mnb* (n=209) is delayed by 24 hours compared to the control (n=182). (C) Climbing ability is not significantly affected by *mnb* overexpression (n=150) compared to the control (n=150). Longevity is shown as percent survival ($P < 0.05$, determined by log-rank). Climbing ability was determined via non-linear curve fit (CI = 95%). Error bars = SEM.

Table 9 – Analysis of longevity of flies with neural overexpression of *mnb*. Survival curves analysed using log-rank test. Arrows indicate increase (⇑) or decrease (⇓) in treatment.

Genotype	Initial number of flies analysed (n)	Median survival day (50%)	Maximum Lifespan (day)	P-value (compared with *UAS-lacZ* cross)	Chi-square value (compared with *UAS-lacZ* cross)	Significant ?
Neurons and Neuroblasts						
UAS-lacZ; P[GawB]$^{l(3)31-1}$	216	68	78	n/a	n/a	n/a
mnb^{EY14320}; P[GawB]$^{l(3)31-1}$	207	62	78	< 0.0001	94.01	YES ⇓

Overexpression of mnb in the eye decreases longevity and delays eclosion time

Directed overexpression of *mnb* in the eye was accomplished using the *GMR-GAL4* driver. Longevity of flies overexpressing *mnb* in the eye (n=202) is slightly but significantly decreased compared to the control (n=203) (Figure 11). The median life span is shorter, 60 days compared to *UAS-lacZ; GMR-GAL4* with 68 days, and the maximum life span 74 to 80 days (Table 10). In addition, eclosion time is delayed by a day in $mnb^{EY14320}$; *GMR-GAL4* flies. These flies eclose at day 12, compared to day 11 for the control (Figure 11). Climbing studies show that the overexpression of *mnb* in the eye has no effect on climbing ability (Figure 11). Overall, *mnb* overexpression in the eye decreases life span and delays eclosion but has no affect on locomotor ability.

Effect of *mnb* overexpression on longevity under amino-acid deprived conditions

The insulin-signaling pathway is important in cell growth and survival and *mnb* has been shown to play a role in this pathway through the hyperphosphorylation of *foxo* that has been phosphorylated by *akt* (Woods *et al.* 2001a; Shingleton *et al.* 2005). As *foxo* mutant adults show sensitivity to amino-acid starvation and some *akt* mutant adults show resistance to amino-acid starvation (Kramer *et al.* 2008; Slade & Staveley, unpublished), a starvation assay was carried out on flies overexpressing *mnb*.

Low-level ubiquitous overexpression of mnb reduces survival on starvation media

Low-level ubiquitous *mnb* overexpression, achieved by using the *da-GAL4* driver, led to slightly reduced survival periods under amino-acid deprived conditions (Figure 12). The median life span was 18 days (n=222) compared to 20 days in the control

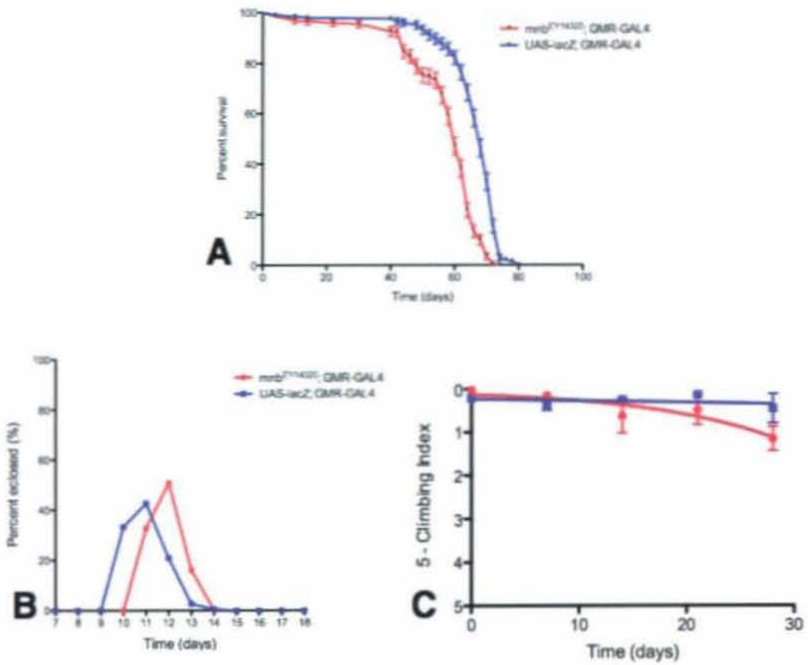

Figure 11 – Behavioural analyses of overexpression of *mnb* in the eye using the *GMR-GAL4* transgene. Directed overexpression of *mnb* in the eye decreases longevity and delays eclosion, but has no effect on climbing ability. (A) Longevity of flies overexpressing *mnb* (n=202) in the eye is significantly decreased compared to the *UAS-lacZ* control (n=203). (B) Eclosion of flies overexpressing *mnb* (n=180) in the eye is delayed by 24 hours compared to control (n=106). (C) Overexpression of *mnb* in the eye (n=150) has no effect on the climbing ability compared to *UAS-lacZ* (n=150). Longevity is shown as percent survival ($P < 0.05$, determined by log-rank). Climbing ability was determined via non-linear curve fit (CI = 95%). Error bars = SEM.

Table 10 – Analysis of longevity of flies with *mnb* overexpression in the eye. Survival curves analysed using log-rank test. Arrows indicate increase (⇑) or decrease (⇓) in treatment.

Genotype	Initial number of flies analysed (n)	Median survival day (50%)	Maximum Lifespan (day)	P-value (compared with *UAS-lacZ* cross)	Chi-square value (compared with *UAS-lacZ* cross)	Significant?
UAS-lacZ; GMR-GAL4	203	68	80	n/a	n/a	n/a
$mnb^{EY14320}$; *GMR-GAL4*	202	60	74	< 0.0001	98.21	YES ⇓

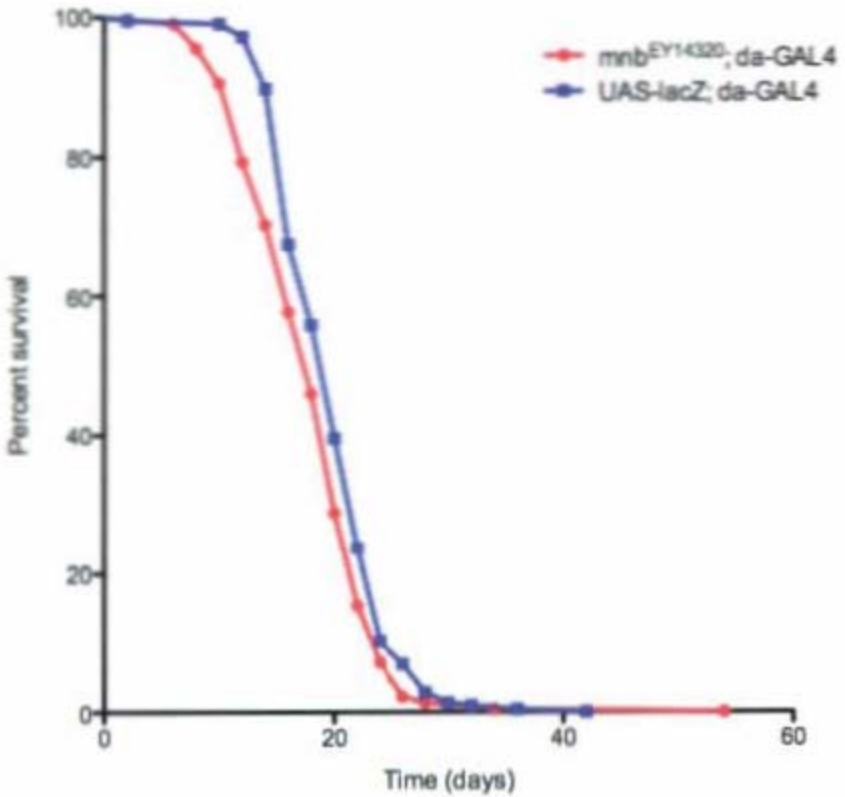

Figure 12 – Directed low-level ubiquitous overexpression of *mnb* decreased longevity of flies when under conditions of amino-acid starvation. Flies with ubiquitous overexpression of *mnb*, using *da-GAL4* (n=222) have slightly lower lifespans than *UAS-lacZ* control (n=215). Longevity is shown as percent survival ($P < 0.05$, determined by log-rank).

(n=215). Maximum survival (0%) occurred on day 54 in flies overexpressing *mnb* whereas control flies reached 0% survival at 42 days (Table 11). The decrease in life span is significant, p-value = 0.0018. Low-level overexpression of *mnb* throughout the body decreases survival on starvation media.

Neural overexpression of mnb decreases survival on starvation media

Overexpression of *mnb* in the neurons and neuroblasts decreased life span of flies on normal food media (Figure 10). Starvation studies performed on flies overexpressing *mnb* using the same neural driver showed that neural *mnb* overexpression significantly decreases survival under amino-acid deprived conditions (Figure 13). Flies with *mnb* overexpression in the adult neurons and neuroblasts (n=228) have a median life span of 16 days compared to 20 days for the controls (n=214) (Table 11). Flies from $mnb^{EY14320}$ crosses have higher maximum life span than the control. Overexpression of *mnb* throughout the body and in the neurons and neuroblasts significantly decreases survival on amino-acid deprived food.

Investigation of *mnb* mutant longevity on amino acid starvation media

Investigation into the survival of *mnb* mutants on starvation media has not been performed previously. Under normal conditions, *mnb* mutants show decreased longevity with the exception of $mnb^{EY14320}$, which does not differ from wild-type controls (Rotchford 2006). Given the decreased survival of flies overexpressing *mnb* under starvation conditions found above, a similar experiment was performed on the *mnb* mutant lines. The mutant lines used were: $mnb^{EY14320}$ (used to overexpress *mnb* in GAL4 crosses); mnb^{1}; and mnb^{3}. Survival of these lines was compared to that of w^{1118}, white

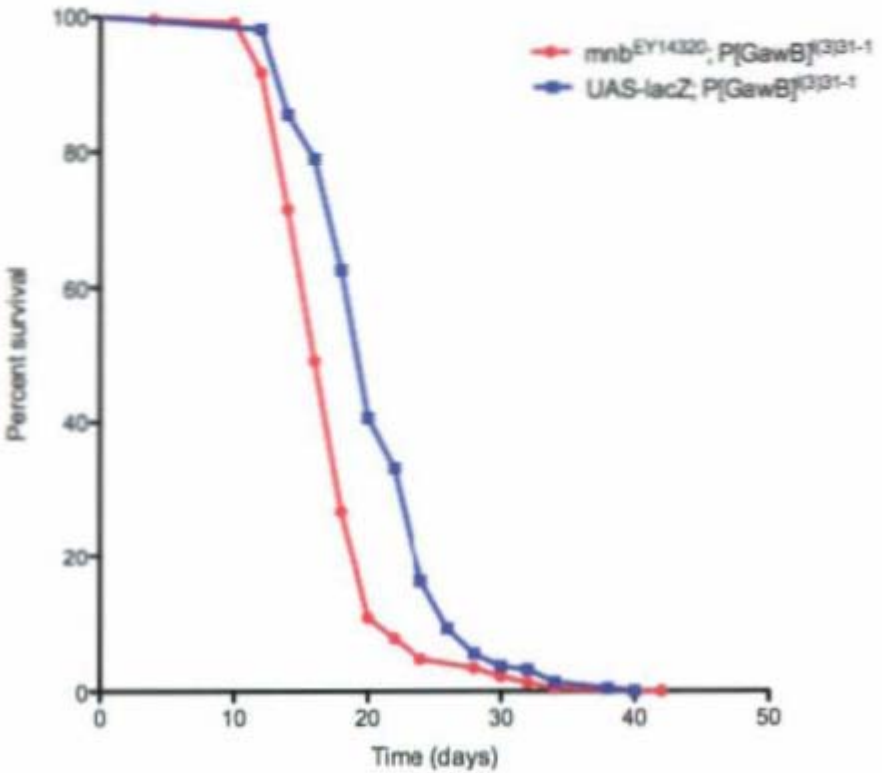

Figure 13 – Directed neural overexpression of *mnb* significantly decreases longevity of flies under conditions of amino-acid starvation. Directed neural overexpression of *mnb* using the *P[GawB]*$^{[3]31-1}$ transgene (n=219) significantly decreases the survival of flies kept on amino-acid starvation compared to *UAS-lacZ* controls (n=214). Longevity is shown as percent survival ($P < 0.05$, determined by log-rank).

Table 11 – Analysis of longevity of flies with ubiquitous or neural overexpression of *mnb* under conditions of amino-acid starvation. Survival curves analysed using log-rank test. Arrows indicate increase (⇑) or decrease (⇓) in treatment.

Genotype	Initial number of flies analysed (n)	Median survival day (50%)	Maximum Lifespan (day)	P-value (compared with *UAS-lacZ* cross)	Chi-square value (compared with *UAS-lacZ* cross)	Significant?
Low-level ubiquitous						
UAS-lacZ; da-GAL4	215	20	42	n/a	n/a	n/a
$mnb^{EY14320}$; *da-GAL4*	222	18	54	0.0018	14.19	YES ⇓
Neurons and neuroblasts						
UAS-lacZ; P{GawB}$^{l(3)31-1}$	214	20	40	n/a	n/a	n/a
$mnb^{EY14320}$; *P{GawB}$^{l(3)31-1}$*	228	16	42	< 0.0001	62.50	YES ⇓

eyed control flies. This control line (n=227) had a median life span of 14 days and maximum of 34 days. The transgenic line $mnb^{EY14320}$ (n=251) shows a median survival of 14 days and maximum life span of 58 days (Figure 14, Table 12). The mutation of *mnb* in $mnb^{EY14320}$ does not have a significant affect on survival ($p = 0.6110$) compared to the control.

Increasing mutant severity affects survival on amino-acid deficient media (Figure 14). The longevity of severe *mnb* mutant, mnb^{3} (n=129) is significantly decreased on starvation media. The median life span is 10 days and 0% survival occurs on day 24, resulting in significance with a p-value < 0.0001 compared to w^{1118} (Table 12). The less severe mutant mnb^{1} (n=201) shows an increase in longevity on starvation media (Figure 14). The median life span observed was 20 days and the maximum at 40 days. This is significantly higher than w^{1118} control ($p < 0.0001$).

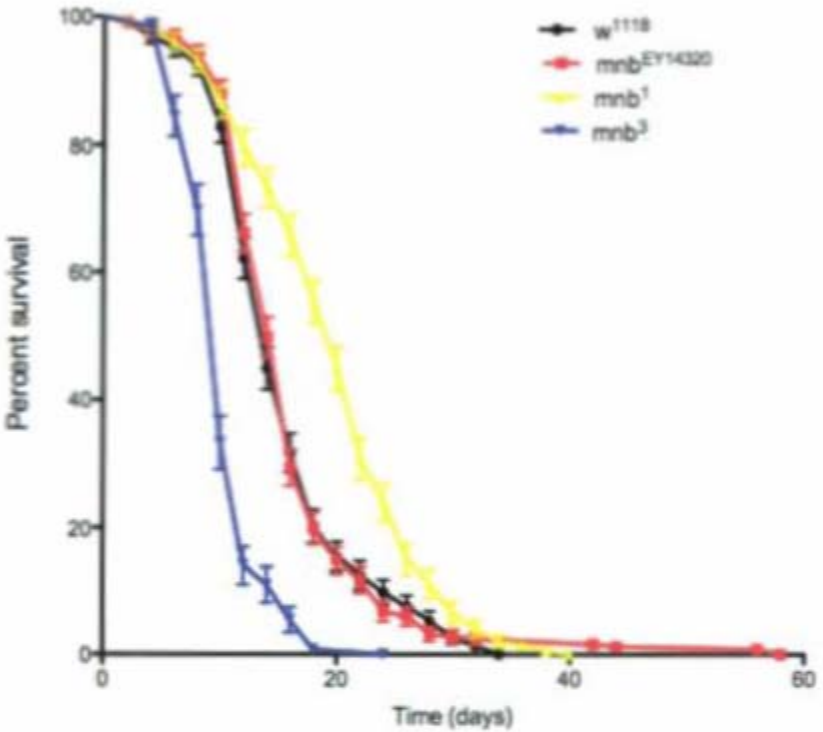

Figure 14 - Analysis of *mnb* mutant longevity under conditions of amino-acid starvation. The transgenic line $mnb^{EY14320}$ (n=251) has no significant difference in longevity on amino-acid deficient media compared to the w^{1118} control (n=227). Severe *mnb* mutant, mnb^{3} (n=129) has significantly decreased lifespan, whereas less severe mutant mnb^{1} (n=201) shows an increased longevity on starvation media compared to the control. Longevity is shown as percent survival ($P < 0.05$, determined by log-rank). Error bars = SEM.

Table 12 – Analysis of longevity of *mnb* mutant flies under conditions of amino-acid starvation. Survival curves analysed using log-rank test. Arrows indicate increase (⇧) or decrease (⇩) in treatment.

Genotype	Initial number of flies analysed (n)	Median survival day (50%)	Maximum Lifespan (day)	P-value (compared with w^{1118} cross)	Chi-square value (compared with w^{1118} cross)	Significant?
w^{1118}	227	14	34	n/a	n/a	n/a
$mnb^{EY14320}$	251	14	58	0.6110	0.5647	NO
mnb^{1}	201	20	40	< 0.0001	41.14	YES ⇧
mnb^{2}	129	10	24	< 0.0001	104.1	YES ⇩

DISCUSSION

Down Syndrome (DS) is one of the most common genetic causes of cognitive impairment. In addition to difficulties in mental development, patients with DS may have other symptoms, including poor locomotor skills, cognitive heart defects, gastrointestinal problems, and low brain weights resulting from fewer and smaller neurons (Park *et al.* 2009). While generally associated with whole or partial triplication of HSA21, recent research has focused on the expression of the DYRK protein kinase family. Within this family, *dual-specific tyrosine-regulated kinase 1a* (*dyrk1a*) is of great interest due to its localization in the adult DS brain, as well as its role in regulating growth and neural development (Guimerá *et al.* 1996; Fotaki *et al.* 2002). The Drosophila homologue, *minibrain* (*mnb*) is necessary for postembryonic neurogenesis in flies, mirroring one of established functions of *dyrk1a* (Tejedor *et al.* 1995). Overexpression studies performed in murine DS models show that *dyrk1a* upregulation can produce learning and memory deficits, motor abnormalities, altered neurogenesis and neurodevelopmental delay (Altafaj *et al.* 2001; Martinez de Lagrán *et al.* 2004; Branchi *et al.* 2004), whereas decreased expression in *mnb* mutant flies results in poor locomotion and poor odour-discrimination learning (Tejedor *et al.* 1995). In the current study, ectopic overexpression of *mnb* has been used to further investigate the effects of *mnb* on neural development, and to produce a model of DS in flies, where flies present with the DS-like phenotypes including decreased life span, poor locomotor ability and delayed development plus altered neuron number in the eye and dorsal notum.

Role of *mnb* in neurogenesis and neural development

The development of the Drosophila eye is closely linked with neurogenesis. Each eye unit or ommatidium is associated with several neural cells as well as with sensory bristles. The cells of the eye imaginal disc begin to differentiate during pupation, when the morphogenetic furrow crosses the eye disc, activating a series of highly regulated cell signals (Baker 2001). This tight control makes the eye an ideal model for the study of cell growth and survival, particularly neurogenesis. An early study of *mnb* mutant flies show that these flies have fewer and smaller neurons (Tejedor *et al.* 1995). Recent observations of *mnb* mutants reveal reduced ommatidia and bristle numbers to suggest that neurogenesis in the eye is impaired (Rotchford 2006). In the same study, directed overexpression of *mnb* in the eye suppresses the abnormal neurodevelopmental effects induced by the transcription factor *foxo*. Another study shows that *mnb* overexpression coupled with Huntingtin-interacting protein 1 (Hip1) in the eye can also suppress these effects (MacDonald 2008). In these studies, *mnb* overexpression suppresses reduced neurogenesis caused by abnormal *foxo* expression. The expression of *mnb* appears to be involved in the tightly regulated events that control development in fruit flies.

Overexpression of *mnb* affects the number and size of neurons in the developing eye. Low-level ubiquitous overexpression of *mnb* using the *da-GAL4* driver decreases ommatidia number and at higher expression levels via the *act-GAL4* driver, can decrease ommatidia size. This variation may simply be due to expression levels. The *act-GAL4* transgene has been characterised to drive expression at higher levels than *da-GAL4*, though the overexpression has yet to be quantified. Neural *mnb* overexpression produces

overall smaller eyes, with significantly smaller and fewer ommatidia compared to controls. These results suggest that directed overexpression of *mnb* in the neurons and neuroblasts inhibits neurogenesis in the eye. A recent study has shown that *dyrk1a* overexpression can inhibit proliferation of neural progenitor cells in mice, and cause them to differentiate early (Yabut *et al.* 2010). In flies, the *mnb* protein is asymmetrically localized to the differentiating rather than proliferating line of developing neural cells (Hämmerle *et al.* 2002). This may indicate that this gene product has a direct effect on neuronal differentiation, possibly taking the neuroepithelial cell out of the cell cycle and pushing it towards neuronal cell fate (Hämmerle *et al.* 2002; Hämmerle *et al.* 2003; Ohnuma & Harris 2003). The *GMR-GAL4* transgene drives expression during late eye development, posterior to the morphogenetic furrow (Yamada *et al.* 2003). Interestingly, directed overexpression of *mnb* under the control of *GMR-GAL4* does not affect total eye size or ommatidia number but does increase the size of individual ommatidia. It is likely that overexpression at such a late stage of development may not affect neural proliferation or differentiation, and instead only affect cell size.

The bristles of the dorsal notum develop from a field of neuronal precursor cells, where each bristle is associated with a single sensory neuron (Jan & Jan 1994). Like the ommatidia array, the density of microchaetae or small sensory bristles, is highly regulated and as such has been used as a good indicator of cell signalling and neurogenesis (MacDonald *et al.* 2008; Moores *et al.* 2008). The transmembrane receptor Notch is important in the regulation of cell fates, proliferation and death during development, including neural cell specification on the dorsal notum (Ramain *et al.* 2001). Neural

specification is accomplished through lateral inhibition and interaction between Notch and Delta. Flies with mutant Notch or Delta have an excess of neural precursors (Heitzler & Simpson 1991). When activated, Notch acts as a transcriptional regulator via an intracellular domain (NICD). Notch activity stops when this domain is phosphorylated (reviewed in Bray 2006; Fryer *et al.* 2004). A recent study shows that *Dyrk1a* can directly phosphorylate the NICD and down-regulate Notch signalling *in vivo* (Fernandez-Martinez *et al.* 2009). In Drosophila, microchaetae density is influenced by Notch signalling throughout development. However, directed *mnb* overexpression in the dorsal notum using *apterous-GAL4* and *pannier-GAL4* drivers has no significant effect on microchaetae density (MacDonald 2008). In contrast, the directed ubiquitous and neural overexpression of *mnb* investigated here can affect bristle density on the dorsal notum. Increased *mnb* expression throughout the body using the *da-GAL4* driver produces a lower microchaetae density compared to *mnb* overexpression in either larval or adult neurons. This may be due to the expression pattern of the *daughterless* (*da*) gene. The *da* transcription factor is important in neurodevelopment and is necessary for the expression of neural genes (Cadigan *et al.* 2002). Perhaps overexpressing *mnb* in the same spatio-temporal pattern as *da* during the development of the dorsal notum affects other cell signalling pathways necessary for proper neural proliferation.

The effect of neural *mnb* overexpression on bristle density seems to depend on when expression is driven. Overexpression later in development when driven in neurons and neuroblasts slightly but significantly decreases microchaetae density. Analyses of late stage embryos have shown that *mnb* is expressed solely in the CNS, becoming more

restricted as development continues (Tejedor *et al.* 1995). Therefore it is likely that the expression in this neural cross is driven more so in the CNS than in peripheral neurons such as those associated with microchaetae. This would account for the small change in bristle density.

Expression of mnb influences physiology and behaviour

One of the characteristics of DS is a shortened lifespan. The average age for persons with DS has changed drastically from 9 years in 1929 to about 55 years today (Glasson *et al.* 2002; Bittles & Glasson 2004; Barnhart & Connolly 2007). This is in part due to better health care and better quality of life for people with DS (Wiseman *et al.* 2009). However, the average lifespan of a person with DS remains significantly lower than the average in the general population, suggesting other yet unknown factors are at work. No data could be found on longevity in DS mice, as most studies with murine DS models focus on molecular and cognitive phenotypes. There is no current model for DS in Drosophila but altered *mnb* expression does affect longevity. Decreased expression in *mnb* mutants shortens the fly lifespan and decreases viability (Rotchford 2006). Altered *mnb* expression may play an indirect part in regulating lifespan.

The effect of increased *mnb* expression has not been investigated previously outside of our laboratory. The data presented in this report show that overexpression of *mnb* in the neurons, throughout the body or in the eye significantly shortens the lifespan of flies. The reason for this decrease is unclear. However, longevity and survival are commonly linked with the insulin-signalling pathway and associated downstream targets, including *akt* and the Forkhead box O transcription factor, *foxo* (Burgering & Medema

2003; Giannakou *et al.* 2004). The *akt* kinase can phosphorylate *mnb* and both *akt* and *mnb* phosphorylate and inactivate *foxo*, thereby inhibiting proapoptotic pathways downstream of *foxo*. The role of *mnb* in the insulin pathway may influence longevity.

In addition to shortened lifespan, motor dysfunction is a common trait in people with DS. Gross and fine motor control is initially poor in DS children, though improvement is seen at around one year of age (Bittles & Glasson 2004). Observations of locomotor ability and molecular work in the murine DS models (Ts65dn & TgDyrk1a) have linked *Dyrk1a* upregulation to delayed development of motor skills (Altafaj *et al.* 2001; Martinez de Lagrán *et al.* 2004). It is important to note that in these studies, this impairment was partially overcome with repeated training. These improvements in DS humans and mice are generally attributed to continued practice and to the development of compensatory mechanisms (Martinez de Lagrán *et al.* 2004). Abnormal *mnb* expression produces flies with poor locomotion. The mnb^{1} mutant flies have reduced walking activity and speed, as well as reduced negative geotaxis (Tejedor *et al.* 1995). Here we found that overexpression of *mnb* can decrease climbing ability when expressed at low ubiquitous levels. Precocious loss of locomotor ability was observed when *mnb* is overexpressed at higher ubiquitous levels and in the adult neurons and neuroblasts. No change was seen in flies overexpressing *mnb* in eye. Unlike mouse models, no improvement in locomotor ability was seen in any of these crosses.

While motor dysfunction in DS humans and mice is associated with hypotonia, this is most likely not the case in flies given the differences in muscle development between mammals and insects. Impaired climbing ability in flies (and perhaps in

mammals) may be a reflection of the role of *dyrk1a/mnb* in CNS development. *Dyrk1a* has been shown to inhibit neural cell proliferation in the developing mouse brain, and the optic lobes and central brain in *mnb* mutant flies are severely reduced with fewer and smaller neurons (Yabut *et al.* 2010; Tejedor *et al.* 1995). The mushroom bodies (MB) are dense networks of neurons in the brain of arthropods, including insects, best known for being involved in learning and memory. Interestingly, genetically or chemically ablating neurons in the MB results in initially increased followed by suppressed walking activity (Martin *et al.* 1998; Helfrich-Förster *et al.* 2002; Serway *et al.* 2009). Given the effect of mutated *mnb* on the CNS morphology in flies, it may be that neurodevelopment in the mushroom bodies is affected by increased *mnb* expression resulting in poor locomotor ability.

When discussing developmental delay and DS, researchers generally refer to either delays in neuromotor or learning development, rather than a delay in the time it takes to complete whole-organism development. In mammals, *dyrk1a* has a role in neurodevelopment and may be linked to neuromotor development (Yabut *et al.* 2010) but no studies in murine models have shown that *dyrk1a* expression affects development time. However, Heidenrich (1982) showed that *mnb* mutant flies need 10% more time to develop from egg to adult, and during eclosion some mutant flies have trouble leaving the pupal case, perhaps due to neural and motor deficits (Tejedor *et al.* 1995). Interestingly, observations made here show that flies overexpressing *mnb* directed at low ubiquitous levels, in the neurons and neuroblasts and in the eye need about 10% more time to develop from egg to adult, which normally takes about 10 days. While altered *mnb*

expression does seem to have an effect on development time, perhaps through pathways affecting organism growth like the insulin-signalling pathway or through neurodevelopment, the precise cause cannot be identified without further study. Flies with increased *mnb* expression in the larval neurons and at high ubiquitous levels had no change in development time. This could reflect the influence of the amount and/or spatio-temporal expression of *mnb* on development time.

Possible role for mnb in survival

The *mnb* gene may influence growth, development and ultimately survival through interactions with the insulin-signalling pathway, which is highly conserved between vertebrates and invertebrates (Brogiolo *et al.* 2001). Briefly, insulin and/or insulin-like growth factors (IGFs) bind to and activate the tyrosine kinase insulin receptors (InR) in the cell membrane. Activated InRs phosphorylate insulin/IGF receptor substrate (IRS) proteins, which then recruit and activate class 1A phosphoinositide-3-kinase (PI3K). These kinases aid in the synthesis of second messenger phosphoinositides, recruiting the *akt* kinase to the cell membrane to activate it. This activation is inhibited by the activity of the phosphatase and tensin homologue (PTEN). Activated *akt* initiates a series of downstream pathways, in particular the phosphorylation and subsequent inhibition of *foxo* transcription factors (reviewed in Lindsley 2010). In Drosophila, there are seven insulin-like peptides (dILPs) (Böhni *et al.* 1999; Brogiolo *et al.* 2001) that can bind to a single insulin-like receptor (dIR), and activate the insulin receptor substrate (dIRS) *chico*, initiating the insulin-signalling cascade via *akt* activity.

Insulin signalling influences a variety of processes, including metabolism, glucose uptake, longevity and cell division (Brogiolo *et al.* 2001; Barbeiri *et al.* 2003). Studies of mutant ILP, IR or IRS/*chico* genes in Drosophila indicate that they play an important role in organ size and cell number (reviewed in Weinkove & Leevers 2000; Shingleton *et al.* 2005). Mutations of the insulin-like receptor (InR) and IRS/*chico* can significantly extend the lifespan of flies (reviewed in Bartke 2001; Clancy *et al.* 2001; Tatar *et al.* 2001). It might be inferred that with decreased InR and IRS activity, *akt* kinase activity is decreased, which becomes important when considering a major downstream target of this pathway, *foxo*.

Forkhead box O (*foxo*) transcription factors were first identified as the protein products of proto-oncogenes. Three versions of *foxo* have been found in humans and mice (Sublett *et al.* 1995; Biggs *et al.* 2001), but there is only one homologue in Drosophila (Kramer *et al.* 2003). These transcription factors have a highly conserved DNA binding domain, three *akt* phosphorylation sites, as well as one *dyrk1a/mnb* phosphorylation site (Kramer *et al.* 2003). The *foxo* proteins are downregulated by *akt* phosphorylation, which sequesters them to the cytoplasm inhibiting their gene regulation function. These proteins play a role in survival and longevity. Overexpression of *foxo* in adult fly fat bodies, energy storage organs, has been shown to significantly increase the lifespan of female flies and reduce the synthesis of ILP2 from neurons (Giannakou *et al.* 2004; Hwangbo *et al.* 2004). Loss-of-function of *foxo* can decrease survival on amino acid starvation media, to suggest that *foxo* is particularly important in survival during periods of nutritional stress (Kramer *et al.* 2008). However, *foxo* overexpression can

phenocopy starvation in flies and result in developmental arrest of larvae and increased "wandering" away from food (Kramer *et al.* 2003). Under amino acid starvation conditions, *foxo* activity is increased (Kramer *et al.* 2008). This condition can be reversed when amino acids are re-introduced. Both *mnb* and *foxo* may interact in response to starvation.

To date, no studies have been performed examining starvation survival and *mnb* expression. In this study, overexpression of *mnb* decreases survival in amino acid-deprived conditions. Increased *mnb* in the neurons and neuroblasts and at low ubiquitous levels significantly shortens lifespan on starvation media. In general, *dyrk1a/mnb* is considered an anti-apoptotic kinase. A recent study shows that *Dyrk1a* can phosphorylate and activate SIRT1, promoting cell survival (Guo *et al.* 2010). SIRT1, a protein of the Sir2 (silent information regulator 2) family, is an NAD+ dependent protein deacetylase. It links metabolic status and gene regulation by coupling deacetylation of histones with a variety of transcription factors important in cell survival, including p53, E2F1, NFB and *foxo* (Luo *et al.* 2001; Nahle *et al.* 2002; Brunet *et al.* 2004; Yeung *et al.* 2004; Bishop & Guarente 2007). SIRT1 deacetylates *foxo*, which can promote anti-apoptotic activity. It is possible that the increased activity of *foxo* under nutritional stress may offset the anti-apoptotic activity of increased *mnb*. Alternatively, there may be a feedback mechanism triggered with upregulation of *mnb*, thereby inhibiting its activity. Further studies will be required to elucidate the cause.

Decreased *mnb* expression through *mnb* mutants has variable effects on survival and it seems the degree of mutation matters. Male homozygous flies were examined from

the following mutant lines: $mnb^{EY14320}$; mnb^{1}; and mnb^{3}. These lines vary in the severity of mutation of the *mnb* gene, with $mnb^{EY14320}$ being close to wild-type, mnb^{3} being the most severe and mnb^{1} lying somewhere in-between $mnb^{EY14320}$ and mnb^{3}. The $mnb^{EY14320}$ line does not alter longevity on normal food media compared to controls (Rotchford 2006) and the same occurs here during starvation stress. It is expected that the endogenous *mnb* gene would be mildly affected as the P-element is inserted upstream from it, therefore this line should be similar to wild-type in the absence of Gal4. The mnb^{1} flies show an increased lifespan on starvation media, whereas the survival of mnb^{3} flies is severely decreased. Both these lines show a decrease in longevity on normal food media (Rotchford 2006). The mutation in mnb^{1} flies seems to increase the anti-apoptotic role of *mnb*, promoting a positive response to starvation stress. The mutation likely reduces the activity of the kinase, influencing downstream targets. Similarly, select hypomorphic alleles of *akt* have increased lifespans on starvation media (Slade & Staveley unpublished). Both *akt* and *mnb* phosphorylate *foxo*; if their activity is decreased, there seems to be a shift towards cell survival, possibly via altered *foxo* regulation. The opposite may be true for flies bearing the stronger mutant mnb^{3} allele. Further study is required but it does seem that the severity of the mutation can alter kinase activity, affecting the regulation of downstream targets.

Conclusion

The examination of *mnb* overexpression presented here aids in understanding its role in physiology, both at cellular and whole-organism levels. The *mnb* gene seems to affect neurogenesis in the eye and dorsal notum, decreasing ommatidia number and size

and reducing microchaetae density, particularly when directed in the neurons, neuroblasts and low levels ubiquitously. Longevity is decreased in flies overexpressing *mnb*, perhaps as a result of the kinase's role in the insulin-signalling pathway, important in growth and survival. This may also cause the developmental delay with neural or ubiquitous *mnb* overexpression. Analyses of climbing ability reveal that overexpression could result in continuously poor locomotor ability, in the case of low-level ubiquitous expression, or precocious loss of climbing ability when overexpressed in the neurons or at high ubiquitous levels. Given the effect of *mnb* overexpression upon these behaviours and phenotypes, and the probable connection between insulin signaling and *mnb*, survival studies were performed, showing that ubiquitous and neural overexpression decrease survival under starvation conditions. However, analysis of *mnb* mutant lines reveal that the severity of mutation affects survival ability, likely through altered function in the kinase. The interactions between *mnb* and the components of the insulin-signaling pathway, such as *akt* and *foxo*, should be further investigated to elucidate the role of this kinase in growth and neurogenesis.

The *mnb* kinase examined here is a homologue of the DS candidate gene *dyrk1a*. *Dyrk1a/mnb* proteins have a role in neurogenesis and cell development and analysis of its overexpression here supports this. When *mnb* is overexpressed ubiquitously via *da-GAL4* and neurally using the $P[GawB]^{l(3)31\text{-}1}$ driver, several DS characteristics are phenocopied including decreased longevity, loss of locomotor ability, and delay of development time, which are supported by defects in neurogenesis observed in eye and dorsal notum analyses. These phenotypes suggest that we can model aspects of Down Syndrome in

fruit flies by overexpressing *mnb*. Additional analyses using microarray and Q-PCR of genes down-stream of *mnb* activity at critical developmental stages should be performed, in order to further explore the implications of *mnb* expression in the cellular pathways and development of DS.

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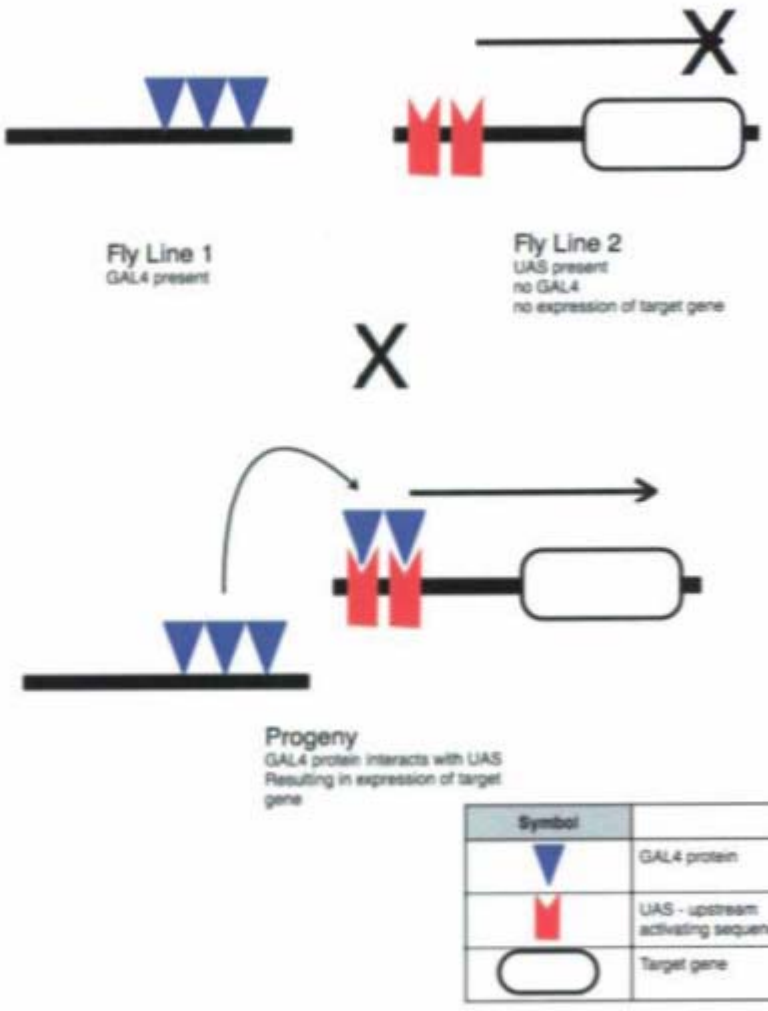
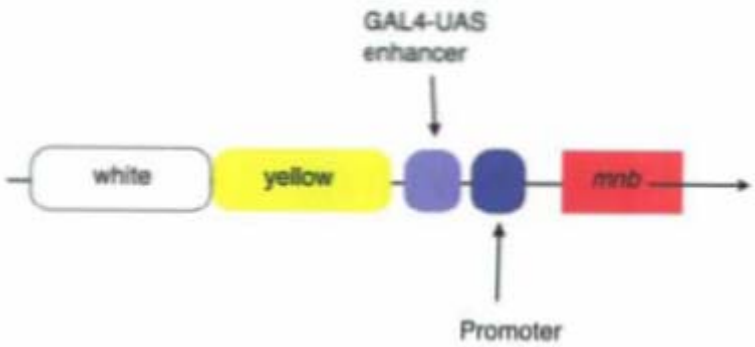
APPENDIX 1



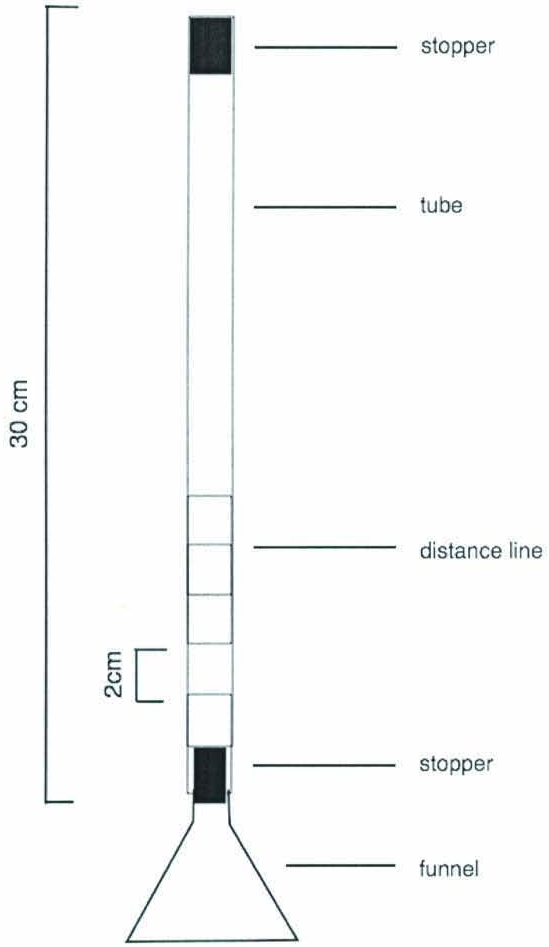
Schematic diagram of the GAL4-UAS system of ectopic gene expression used to direct overexpression of *mnb* (= Target gene).

APPENDIX 2



Schematic diagram of EY P-element insertion upstream of endogenous *mnb* gene. Adapted from Bellen *et al.* 2004.

APPENDIX 3



Schematic diagram of climbing apparatus used to measure locomotor ability in fruit flies. Adapted from Todd and Staveley (2004).

APPENDIX 4

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Expression of *GFP* can influence aging and climbing ability in Drosophila

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ABSTRACT. *Green fluorescent protein* (*GFP*) is widely used as a reporter transgene in a variety of organisms. Some of the advantages of using *GFP* include non-invasive visualization of biological events and/or tissues in live specimens and its benign nature. When *GFP* is expressed throughout the organism, in neurons and eyes, lifespan and climbing ability of flies are significantly decreased compared to similar crosses with a *lacZ* reporter. Also, *GFP* expression can have subtle effects on eye morphology, with neural and ubiquitous expression. Since *GAL4/UAS* expression of *GFP* can influence aging and climbing ability in the Drosophila system of directed gene expression, we found that the latter of these advantages, namely its harmless, non-toxic nature, can be conditional, depending upon the mode of expression and the biological endpoint. We suggest that caution should be used when using *GFP* to visualize cellular events, especially in long-term assays.

Keywords: *Drosophila melanogaster*, *Green fluorescent protein* (*GFP*), longevity, climbing

INTRODUCTION

The *green fluorescent protein* gene (*GFP*), originally isolated from the jellyfish *Aequorea victoria*, has been a vital tool in biological research, particularly for genetic screening and analyses of gene expression in *Drosophila melanogaster*. Initial studies used *GFP* included in P-element constructs, to facilitate localization of proteins and to visualize cell events, such as movement of ribonucleoprotein particles in Drosophila embryos (reviewed by Misteli and Spector 1997; Wang and Hazelrigg 1994). Another gene used in P-element

fusions to visualize cell and genomic components is *lacZ*, a gene from *E. coli*, which encodes ß–galactosidase (O'Kane and Gehring 1987). Creation of both the *UAS-GFP* (Yeh et al., 1995; Dickson, 1996) and the *UAS-lacZ* reporter lines (Manseau et al., 1998) presented novel ways of investigating gene expression in a variety of animals by using the *GAL4/UAS* system to direct expression in specific cell or tissue types (reviewed by Chalfie et al., 1994; Rosochacki and Matejczyk 2002; Zhan and Gong 2010). These transgenes have been used as controls to examine effects of gene expression.

Advantages of using the *GAL4/UAS* system with the *GFP* reporter includes being able to work with living cells with little or no need for sample preparation (reviewed by Misteli and Spector 1997; Duffy 2002). While either *GFP* or *lacZ* can be chosen as the reporter gene in certain assays, *GFP* allows researchers to visualize expression patterns non-invasively and continuously in live specimens, unlike when *lacZ*/X-Gal staining is used (Yeh et al., 1995; Phelps and Brand 1993). It is believed that both *GFP* and *lacZ* expression are non-toxic and do not influence endogenous gene expression at any stage during fly development (Bier et al., 1984; Chalfie et al., 1994). Despite this, some studies have suggested that *GFP* affects cell biology. Cells with *GFP*-containing constructs that localize *GFP* in the nucleus tend to be more damaged, possibly due to free-radical generation and damage to DNA (Misteli and Spector 1997). In analysis of zebrafish development, expression of *GFP* is delayed and a longer recovery period is required before target genes are activated (Zhan and Gong 2010). The subtle consequences of *GFP* expression are of considerable interest to a wide range of biologists.

In addition to these effects of *GFP*, *GAL4* has been shown to affect cell biology in flies. Effects on ommatidia development have been observed and it was established that these altered phenotypes are caused by cell death in the eye (Freeman 1996; Kramer and Staveley 2003). In addition, *elav-GAL4* reduces longevity (Todd and Staveley, unpublished). Examination of transgenes used for driving reporter expression is another route for research.

We find that *GFP* expression promoted by select *GAL4* transgenes can have adverse effects upon morphology and behavior in flies, in particular lifespan and climbing ability, when compared to similar population of flies expressing the *lacZ* transgene.

MATERIAL AND METHODS

Fly stocks and crosses

All *Drosophila* stocks were obtained from the Bloomington Drosophila Stock Centre (University of Indiana, Bloomington). The two responder fly lines used were *w; UAS-lacZ*$^{4-1-2}$ (Brand et al., 1994) and *UAS-GFP* (Dickson, 1996), each crossed with one of the following *Gal4* transgenes, *da-GAL4* (Wodarz et al., 1995), *GMR-GAL4*^{12} (Freeman, 1996), *y w; act-GAL4/CyO, y*$^{+}$ (Brand & Perrimon, 1993) and *w; P[GawB]*$^{3(3)31-1}$*/TM6C* (Ito et al., 1997).

Drosophila media and culture

Crosses were set up using four to five virgin females each of *UAS-lacZ* and *UAS-GFP*, mated to each of the drivers (*da-GAL4*, *GMR-GAL4*, *act-GAL4/CyO*, y^+ and $P[GawB]^{l(3)31-1}$/*TM6C*). All flies were maintained on standard cornmeal-yeast-agar media at 25°C. Parental generation was transferred to new food after two days, and again two days after to increase the number of progeny. Critical class males were determined as those not presenting with the balancer marker phenotypes of *Tubby (TM6C)*, *Stubble (TM6C)*, and *Curly (CyO)*.

Scanning Electron Microscopy

Critical class male flies were isolated under CO_2 and aged on standard cornmeal-yeast-molasses-agar media for three days, placed in 1.5 mL microcentrifuge tubes and frozen at -80°C.

These animals were mounted on aluminum scanning electron microscope studs using double-sided sticky tape, with left eye facing up for eye SEMs and ventral side down with legs removed and wings spread to the sides for dorsal notum SEMs. Progeny that inherit the transgenes *da-GAL4* and $P[GawB]^{l(3)31-1}$ underwent dorsal notum analysis. The mounted flies were desiccated overnight and gold-coated using Electron Microscopy Science (EMS) 500 Sputter Coater. Eyes were photographed at 150X magnification and dorsal nota were photographed at 80X magnification, using a Hitachi S-570 scanning electron microscope.

Micrographs were analyzed using ImageJ digital image analysis software (Abramoff et al., 2004). For the eye SEMs, the number of ommatidia was counted in each image (n=10 per cross). The area (μm^2) of seven ommatidia (in a florette pattern) was determined for 10 sets, and the average area per ommatidium was calculated. Finally, overall eye area was measured. For dorsal notum analysis, the number of microchaetae on the dorsal notum was counted in each image (n= 25 to 30 counts per genotype). The total area (mm^2) of the dorsal notum was also measured. Microchaetae counts and total area were used to calculate the bristle density, expressed as number of microchaetae per mm^2. All scanning electron micrograph (SEM) data were exported into GraphPad Prism 5 and mean ± standard error of means was plotted for each characteristic for each genotype. Statistical analyses were conducted using ANOVA, followed by Newman-Keuls post-tests to determine significance between pairs. Significance was considered at $p < 0.05$.

Longevity Assay

Two hundred critical class males of each genotype were collected using gaseous carbon dioxide and kept on standard cornmeal-yeast-molasses-agar media at 25°C. Flies were maintained in non-crowded conditions of 1 to 20 flies per vial. Flies were transferred to fresh media every two days (Staveley et al., 1990) and scored for presence of dead adults. Survival data was transferred to the GraphPad Prism 5 program and were compared using ANOVA, followed by Newman-Keuls post-tests. Significance was considered 95%, with $p < 0.05$.

Time to eclosion (developmental assay)

Virgin females *UAS-lacZ* and *UAS-GFP* and males of each driver line were collected and aged on standard cornmeal-yeast-molasses-agar media for three to four days before mating. Each cross was set up as described above in Drosophila culture, using three to four virgin females and two to three males. Flies were kept on the fresh food media for six hours before being transferred to fresh media and kept another six hours. This was repeated four times, with several vials per cross.

The number of eclosed adults for each mating was recorded and the percentage eclosion calculated using:

% eclosed = n/N * 100%

where n is the number of eclosed adults for a cross on a given day, and N is the total number of adults eclosed. Both male and female critical class flies were counted. Data was exported to and analyzed in GraphPad Prism 5.

Climbing assay

Approximately 200 critical class males of each cross were assayed for climbing ability according to a protocol developed in our laboratory (Todd and Staveley, 2004). Flies were assayed and maintained in groups of 10. Flies were kept on standard cornmeal-yeast-molasses-agar media at 25°C and assayed every seven days. Climbing ability was determined using a glass tube apparatus, 30 cm in length, 1.5 cm in diameter, marked with five 2 cm sections with a buffer section at the top to limit interference between climbing flies. Climbing ability was scored after 10 seconds based on which of the five marked sections had been reached. Flies were scored 10 times per trial, and the climbing index calculated using:

Climbing index = $\sum(nm)/N$

Where n is the number of flies at a given section, m is the section number (1-5) and N is the total number of flies that climbed in that trial. Data were exported to GraphPad Prism 5 (GraphPad Software, Inc.) and each climbing index was subtracted from five and a non-linear regression curve was fit to each set of data. Slopes of curves were compared with 95% confidence intervals.

RESULTS

Biometric analyses

The development of ommatidia in the fly eye and microchaetae on the dorsal notum are two tightly controlled processes. Any interruption in these can produce phenotypic changes, which can be examined using biometric analysis. Scanning electron micrographs of the eyes and dorsal nota of flies expressing *lacZ* and *GFP* in different patterns revealed alterations in development.

Ubiquitous expression of *GFP* affects ommatidia size and microchaetae density

Examination of ubiquitous expression of *lacZ* and *GFP* was conducted using the transgenes *da-GAL4* and *act-GAL4*, which have been characterized to drive expression at low and high levels respectively. Analyses of eye SEMs show that *GFP* expression does not greatly affect overall eye development. When directed in the pattern of *da-GAL4*, ommatidia number (747 ± 4) and eye area (115154 ± 1714 μm^2) were not unlike that seen for *lacZ*-expressing flies (745 ± 10; 114234 ± 2532 μm^2) (Figure 1A). Ommatidia area was significantly larger in *UAS-GFP; da-GAL4* flies (216.6 ± 2.62 μm^2) compared to *UAS-lacZ; da-GAL4* (198.5 ± 1.17 μm^2) (Figure 1A (iv); Table 1). When directed by the *act-GAL4* driver, *lacZ* and *GFP* expression did not affect eye area or ommatidia number or size (Figure 1B; Table 1). Ubiquitous expression of these two reporters had no significant effect on eye development. However, in the dorsal notum, low-level ubiquitous expression did affect microchaetae density. The density of small bristles in *UAS-GFP; da-GAL4* flies was significantly less (583.6 ± 10.9 mm^2) than *UAS-lacZ; da-GAL4* flies (667.1 ± 7.96 mm^2) (Figure 2A; Table 2). The expression of *lacZ* and *GFP* genes had variable effects on the morphology of flies, as observed in the eye and dorsal notum.

Neural *GFP* expression affects ommatidia number and microchaetae density

Analyses of neural expression of *lacZ* and *GFP* were accomplished by using $P\{GawB\}^{elav-1}$, which drives gene expression in the neurons and neuroblasts. Expression of *GFP* via this driver did not greatly affect eye morphology compared to *lacZ*-expressing flies. The ommatidia number in *UAS-GFP;* $P\{GawB\}^{elav-1}$ flies (714 ± 7) was significantly less than in *UAS- lacZ;* $P\{GawB\}^{elav-1}$ flies (736 ± 5) (Figure 1C (v)); however, ommatidia and eye areas were not different (Figure 1C (iii – iv). Microchaetae density varied with neural expression of *GFP* compared to *lacZ*. Expression via the $P\{GawB\}^{elav-1}$ driver produced a higher microchaetae density in *GFP*-expressing flies (671.0 ± 5.70 mm^2) versus *lacZ*-expressing flies (646.5 ± 5.18 mm^2) (Figure 2B; Table 2). Neural driver coupled with *GFP* expression altered some aspects of morphology compared to *lacZ* expression.

***GFP* expression in the eye increases overall eye area**

Directed expression of *lacZ* and *GFP* was done using the *GMR-GAL4* driver. This driver pushes gene expression posterior to the morphogenetic furrow, post-cell determination (Baker 2001). *GFP* expression did not affect ommatidia number or size but did significantly increase overall eye area (111935 ± 1854 mm^2) compared to *lacZ* (110469 ± 1723 mm^2) (Figure 1D, Table 1). *GFP* expression seemed to affect eye development slightly when expression was driven in the eye, specifically.

Behavioral Analyses

Behavioral analyses can show wide-ranging effects of gene expression. We performed a few assays that measured various aspects of development, including longevity, development time and climbing ability. We find that *GFP* expression significantly alters fly behavior compared to *lacZ* counterparts.

Ubiquitous *GFP* expression affects longevity and climbing ability and can delay development time

Ubiquitous expression of *GFP* was again completed using the transgenes *da-GAL4* and *act-GAL4*, driving low and high expression respectively. In both cases, longevity of *UAS-GFP* flies was decreased (Figure 3A and 3B). The median life span of flies from these crosses was 58 days, 10 – 12 days shorter than *lacZ* flies, and the maximum life span varied from 78 (*UAS-GFP; da-GAL4*) to 82 days (*UAS-GFP/act-GAL4*) (Table 3). Though flies with the *da-GAL4* driver eclosed at the same time as the control (Figure 4A), development time differed significantly when *GFP* was expressed with the *act-GAL4* driver (Figure 4B). About 50% of flies from this cross eclosed one day earlier than the *lacZ* flies (at 10 days versus 11). Climbing tests were conducted over several weeks and analyses were stopped when 75% of the initial number of flies had died. Expression of *UAS-GFP* using *act-GAL4* decreases climbing ability over time. The climbing indices for *UAS-GFP; da-GAL4* flies remain similar to *UAS- lacZ; da-GAL4* flies (Figure 5A and 5B). The climbing ability of *UAS-GFP/act-GAL4* flies, however, was significantly lower than that of the *lacZ* control (Figure 5B). Overall, *GFP* expression throughout the organism had variable effects on eclosion time and climbing ability, and severely decreased longevity.

Expression of *GFP* in the brain and neurons affects longevity and climbing ability

We found that neural *GFP* expression can affect longevity and locomotor ability in flies. Neural expression was achieved using the $P[GawB]^{l(3)31-1}$ driver. Longevity studies of these flies showed significantly decreased life span in *GFP*-expressing flies (Figure 3C). The median life span was 58 days for *UAS-GFP*; $P[GawB]^{l(3)31-1}$ flies compared to *UAS-lacZ*; $P[GawB]^{l(3)31-1}$ flies with 68 days (Table 3). Maximum life spans for *GFP* and *lacZ* crosses did not differ significantly. Neural *GFP* expression did not affect eclosion time as *GFP* flies eclosed on the same day as *lacZ* flies – 12 days (Figure 4C). Expression of *GFP* produced flies with poor climbing ability when expressed in the neurons and neuroblasts (Figure 5C). Clearly, the directed neural expression of *GFP* affected the behavior of flies when compared to controls expressing *UAS-lacZ*.

***GFP* expression in the eye affects longevity, development time and climbing ability**

Directed *GFP* expression in the eye was accomplished using the *GMR-GAL4* driver. Longevity of *UAS-GFP*; *GMR-GAL4* flies was significantly decreased compared to the control (Figure 3D). The median life span was shorter, 54 days compared to *UAS-lacZ; GMR-GAL4* with 68 days, and the maximum life span 70 to 80 days (Table 3). Development time of *UAS-GFP; GMR-GAL4* flies was shorter by one day (10 days) compared to the control (11 days) (Figure 4D). *GFP* expression in the eye also produced poor climbing ability (Figure 5D). *GFP* expression in the eye significantly affected behavior in these fruit flies.

DISCUSSION

The *GFP* gene is commonly used as a marker that allows visualization of tissues and biological events during development. The use of *GFP* has expanded into other species, including mice and zebrafish (Nowotschin et al., 2009; Zhan and Gong 2010). Siegmund and Korge (2001) used *GFP* expression to visualize the ring gland in the Drosophila brain.

UAS-GFP-bearing flies have been used in various assays, as it is believed that *GFP* expression has no physiological effect in flies. We demonstrated that this is not always the case. While *GFP* expression is not shown to affect physical characteristics significantly, such as in the eye and dorsal notum, *GFP* expression can affect physiological phenotypes.

Scanning electron micrograph analyses of the eye did not reveal many significant differences when compared to the *lacZ*-expressing flies. Slight variations in eye area, ommatidia area or count were observed with some of the trangenes. However, the nature of these differences seems minor, suggesting there is no serious effect of *GFP* expression on development or neurogenesis in the eye. Examination of bristle density can be a good indicator of neurogenesis. A recent study, which investigated the role of *Hip1* in neurogenesis (Moores et al., 2008), found that analysis of microchaetae density is a sensitive assay that can assess subtle changes to cell signaling. Here we found that *GFP* expression under the influence of *da-GAL4* and $P[GawB]^{l(3)31-1}$ transgenes can affect bristle density, indicating that *GFP* may influence the cell signaling patterns important in bristle development and neurogenesis. These SEM data indicate that use of *GFP* in assays with certain transgenes may need to be carefully evaluated to ensure that *GFP* expression has not affected events during development, producing altered phenotypes.

Expression of *GFP* may only slightly affect neuronal development and eye and dorsal notum morphology. However, its expression significantly decreases longevity and climbing ability when directed in neural tissues and throughout the organism. The influence upon longevity may greatly influence continual developmental experiments, by introducing bias. Expression of *GFP* affects the condition of the flies over the long-term, resulting in poor climbing ability. This suggests that analyses of long-term locomotor assays (reviewed by Iliadi and Boulianne, 2010) or muscle development in flies may be affected when using *GFP* as a marker (Soler et al., 2004). If *GFP* influences fly health enough over time to not only decrease longevity but also locomotor (climbing) ability, its use in long-term development assays may be problematic, when expression is directed with the drivers that we used here.

Perhaps controlling the initiation of *GFP* expression, as with heat-shock promoters, would reduce the possible effects on development, allowing analysis in longitudinal assays (Grover et al., 2008). Caution should be taken when *GFP* is used in long-term experiments with any drivers.

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Figure Legends

Figure 1 – Biometric analyses of eye development influenced by *GFP* expression under the control of various transgenes. Scanning electron micrographs of left eyes from male flies. Scale bar (white) represents 160 µm. Genotypes are as follows: A (i) – *UAS-lacZ/da-GAL4*. A (ii) – *UAS-GFP/da-GAL4*. B (i) – *UAS-lacZ/act-GAL4*. B (ii) – *UAS-GFP/act-GAL4*. C (i) – *UAS-lacZ;P[GawB]*$^{l(3)31\text{-}1}$. C (ii) – *UAS-GFP; P[GawB]*$^{l(3)31\text{-}1}$. D (i) – *UAS-lacZ/GMR-GAL4*. D (ii) – *UAS-GFP/GMR-GAL4*.
Graphic representations of SEM eye analyses from *da-GAL4* flies (A iii – v), *act-GAL4* flies (B iii – v), *P[GawB]*$^{l(3)31\text{-}1}$ flies (C iii – v), and *GMR-GAL4* flies (D iii – v). *UAS-lacZ* flies are presented in blue and *UAS-GFP* flies are presented in green. Number of eyes analyzed from each cross (n = 10). Graphic representations of eye area, ommatidia area and number are shown in (iii), (iv) and (v) respectively. Values are mean ± SEM. "*" represents significant difference calculated by Newman-Keuls post-tests.

Figure 2 – Biometric analysis of bristle density: *GFP* vs *lacZ* expression under the control of the ubiquitous transgene *da-GAL4* and the neural transgene *P[GawB]*$^{l(3)31\text{-}1}$.
A (i – iii): Ubiquitous *GFP* expression (n=27) significantly decreases microchaetae density compared to the expression of *lacZ* (n=29). B (i – iii): Neural *GFP* expression under *P[GawB]*$^{l(3)31\text{-}1}$ (n=30) significantly increases microchaetae density compared to *lacZ* (n=29). *UAS-lacZ* flies are presented in blue and *UAS-GFP* is presented in green. Scanning electron micrographs are shown in A (i) and (ii) and B (i) and (ii). Scale bar (white) represents 0.30 mm. Graphic representation of microchaetae density is shown in (C). Values are mean ± SEM. "*" represents significant difference by Newman-Keuls post-tests. Genotypes are as follows: A (i) – *UAS-lacZ/da-GAL4*. A (ii) – *UAS-GFP/da-GAL4*. B (i) – *UAS-lacZ;P[GawB]*$^{l(3)31\text{-}1}$. B (ii) – *UAS-GFP; P[GawB]*$^{l(3)31\text{-}1}$.

Figure 3 –Behavioral analysis: Effect of *GFP* expression throughout the organism, in the neurons and in the eye upon longevity. *GFP* expression significantly decreases lifespan in flies. Graphic representations of longevity represented: ubiquitous expression - *da-GAL4* (A) and *act-GAL4* (B) drivers; neural expression – *P[GawB]*$^{l(3)31\text{-}1}$(C); and eye expression – *GMR-GAL4* (D). Longevity is shown as percent survival ($P < 0.05$, determined by log-rank). Error bars = SEM.

Figure 4 – Behavioral analysis: Effect of *GFP* expression throughout the organism, in the neurons and in the eye upon development (eclosion) time. *GFP* expression significantly does not have a significant effect on eclosion time in flies. Graphic representations of eclosion time (development time) represented: ubiquitous expression - *da-GAL4* (A) and *act-GAL4* (B) drivers; neural expression – $P[GawB]^{l(3)31\text{-}1}$ (C); and eye expression – *GMR-GAL4* (D). Percentage of total flies eclosed each day shown. Error bars = SEM.

Figure 5 - Behavioral analysis: Effect of *GFP* expression throughout the organism, in the neurons and in the eye upon climbing ability. *GFP* expression produces flies with significantly decreased climbing ability when driven by the ubiquitous transgene *act-GAL4*, the neural transgene $P[GawB]^{l(3)31\text{-}1}$ and eye-specific transgene *GMR-GAL4*. Graphic representations of longevity represented: ubiquitous expression - *da-GAL4* (A) and *act-GAL4* (B) drivers; neural expression – $P[GawB]^{l(3)31\text{-}1}$ (C); and eye expression – *GMR-GAL4* (D). Climbing ability was determined via non-linear curve fit (CI = 95%). Error bars = SEM.

Table 1 – Summary of eye scanning electron micrograph analyses: effect *GFP* vs *lacZ* expression upon morphology. Values are expressed ± SEM. Arrows indicate increase (⇧) or decrease (⇩) in treatment, and significant is denoted by P-value. Unless otherwise noted, there is no significant difference.

Genotype	Eye area (μm^2)	Ommatidia area (μm^2)	Ommatidia #
Low-level ubiquitous			
UAS-GFP; da-GAL4	115 154 ± 1714	217 ± 3 *** ⇧	748 ± 4
UAS-lacZ; da-GAL4	111 966 ± 2400	198 ± 1	745 ± 10
High-level			
UAS-GFP; act-GAL4	111 078 ± 1716	217 ± 2	739 ± 5
UAS-lacZ; act-GAL4	111 873 ± 2582	216 ± 3	726 ± 8
Neurons and neuroblasts			
UAS-GFP; P[GawB]$^{elav-1}$	113 069 ± 1579	213 ± 2	715 ± 7 * ⇩
UAS-lacZ; P[GawB]$^{elav-1}$	117 111 ± 1594	217 ± 2	736 ± 5
Eye			
UAS-GFP; GMR-GAL4	111 935 ± 1854 ** ⇧	216 ± 2	718 ± 4
UAS-lacZ; GMR-GAL4	110 469 ± 1723	211 ± 2	731 ± 10

Table 2 – Summary of microchaete density on dorsal nota of flies with ubiquitous or neural *GFP* expression. Values are expressed ± SEM. mC = microchaete. Arrows indicate increase (⇑) or decrease (⇓) in treatment.

Genotype	Microchaete density (mC/mm^2)	P-value (compared with UAS-lacZ cross)	Significant?
Low-level ubiquitous			
UAS-GFP; da-GAL4	584 ± 11	< 0.0001	YES ⇓
UAS-lacZ; da-GAL4	667 ± 8		
Neurons and neuroblasts			
UAS-GFP; P[GawB]$^{l(3)31-1}$	671 ± 6	< 0.0001	YES ⇑
UAS-lacZ; P[GawB]$^{l(3)31-1}$	646 ± 5		

Table 3 – Analysis of longevity of flies with directed expression of *GFP* compared to *lacZ*. Survival curves analysed using log-rank test. Arrows indicate increase (⇑) or decrease (⇓) in treatment.

Genotype	Initial number of flies analysed (n)	Median survival day (50%)	Maximum Lifespan (day)	P-value (compared with *UAS-lacZ* cross)	Significant?
Low-level					
UAS-GFP; da-GAL4	184	58	78	< 0.0001	YES ⇓
UAS-lacZ; da-GAL4	202	68	80		
High-level					
UAS-GFP; act-GAL4	156	58	82	< 0.0001	YES ⇓
UAS-lacZ; act-GAL4	203	70	82		
Neurons and Neuroblasts					
UAS-GFP; P[GawB]$^{l(3)31-1}$	223	58	70	< 0.0001	YES ⇓
UAS-lacZ; P[GawB]$^{l(3)31-1}$	216	68	78		
Eye					
UAS-GFP; GMR-GAL4	205	54	70	< 0.0001	YES ⇓
UAS-lacZ; GMR-GAL4	203	68	80		

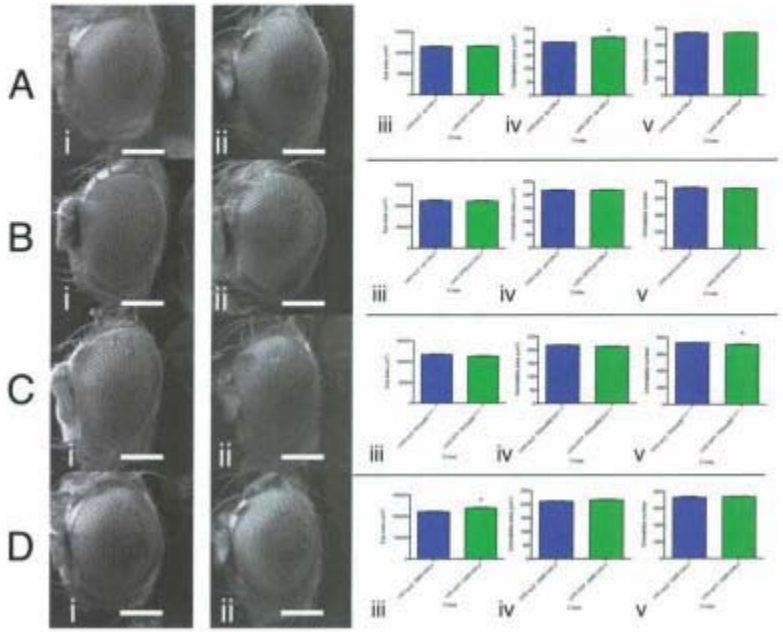

Figure 1 – Biometric analyses of eye development influenced by *GFP* expression under the control of various transgenes.

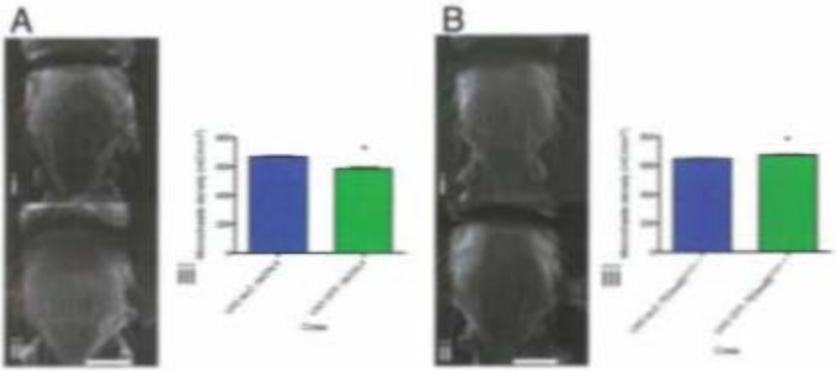

Figure 2 – Biometric analysis of bristle density: *GFP* vs *lacZ* expression under the control of the ubiquitous transgene *da-GAL4* and the neural transgene *PfGawBf*$^{E3103-1}$.

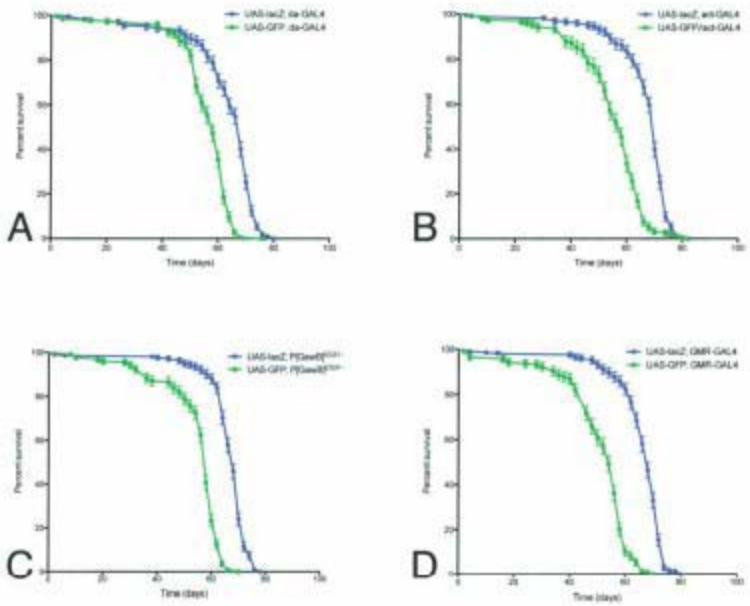

Figure 3 – Behavioural analysis: Effect of *GFP* expression throughout the organism, in the neurons and in the eye upon longevity.

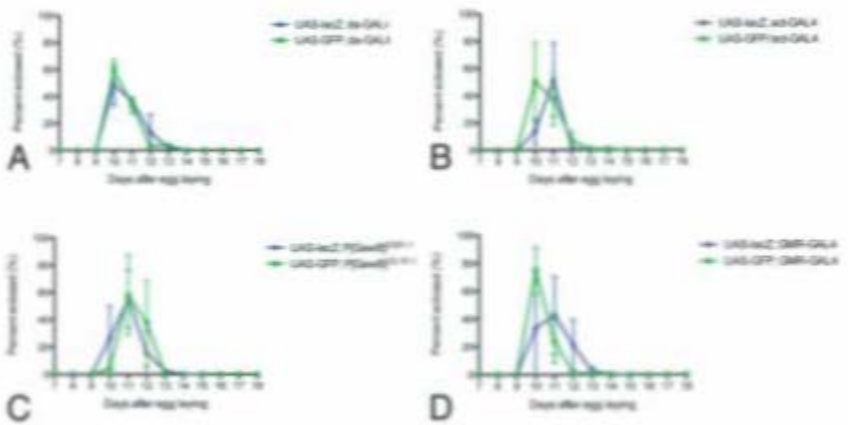

Figure 4 – Behavioural analysis: Effect of *GFP* expression throughout the organism, in the neurons and in the eye upon development (eclosion) time.

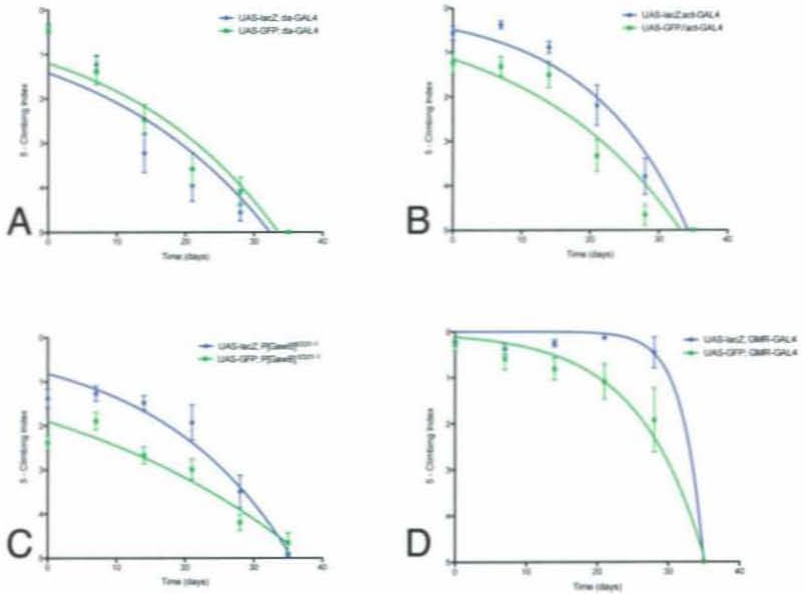

Figure 5 – Behavioural analysis: Effect of *GFP* expression throughout the organism, in the neurons and in the eye upon climbing ability.