

UNIVERSITY OF BIRMINGHAM

School of Biosciences

Project 1

ANALYSIS OF EMBRYONIC LETHAL ABNORMAL VISION SYSTEM FUNCTION IN NEURONAL DEVELOPMENT

&

Project 2

FUNCTION OF *DROSOPHILA* NEUROTROPHIN IN DENDRITIC DEVELOPMENT

By

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A combined research thesis submitted to the University of Birmingham as part of the requirement for the degree of Master of Research (MRes) in Molecular and Cellular Biology

2013/14

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Summary

This thesis submitted to the University of Birmingham consists of two research projects.

The **first research project** carried under **Dr. Matthias Soller** focused on identifying the effect the ectopic expression of embryonic lethal abnormal vision system (Elav) have on the differentiating neuronal precursor cells i.e. eye primodium and neuroblasts during early stages of neurogenesis in *Drosophila*.

&

The **second research project** carried under **Dr. Alicia Hidalgo** focused on identifying the effect of ectopic expression of *Drosophila* neurotrophins and Toll receptors on the structure of dendrites in the adult *Drosophila* brain. In addition, the project also looked into the dendrite arborisation of circuitry and sensory neurons in *Drosophila* larvae.

To the backbone of my life

My parents

&

To all the wonderful souls across the globe

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By

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Dr. Matthias Soller



As part of the requirement of degree of MRes in Molecular and Cellular Biology

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Abstract

Cells generate numerous mRNAs from a single gene via alternative splicing, which increase the proteome and level of post-transcriptional expression. Post-transcriptional gene expression by RNA binding proteins (RBPs) is important for generating organismal complexity in eukaryotes. Brain is an organ where alternative mRNA processing is abundant, with RBPs being key regulators of alternative splicing and polyadenylation. Elav (embryonic lethal abnormal vision system) is a RBP that is expressed only in differentiated neurons in the brain, where it is involved in alternative splicing, polyadenylation, mRNA stability and translation. In here, we wanted to see what effect does the expression of Elav during early stages of neurogenesis have on the differentiating neuronal precursor cells i.e. eye primordium and neuroblasts. Therefore, we tested ectopic Elav expression in the developing eyes using various drivers in an *in-vivo Drosophila* tumour eye model *via* the GAL4/UAS expression system. In addition, we also ectopically expressed Elav in various neuroblast, neuron and glial drivers and via immunostaining observed variations in the optic lobes and the ventral nerve cord of the larval brain.

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Abbreviations

AA	Amino acid
APA	Alternative splicing and polyadenylation
Ase	Asense
Brat	Brain tumor
BSA	Bovine serum albumin
Chr	Chromosome
CyO	Curly of Oster
Dl	Delta
Df	Deficiency
Edr	Eye disc reduced
ELAV	Embryonic lethal abnormal vision system
ELAVL	Embryonic lethal abnormal vision system like proteins
Ey	Eyeless
GOF	Gain of function
GFP	Green fluorescent protein
GMC	Ganglion mother cell
Grh	Grainyhead
HA	Human influenza hemagglutinin
Insc	Inscuteable
LOF	Loss of function
mAB	Monoclonal antibody
mRNA	Mature ribonucleic acid
NLS	Nuclear localization sequence
PBS	Phosphate buffered saline
PEM	Paraneoplastic encephalomyelitis
Pros	Prospero
Rbf	Retinoblastoma

RRM	RNA Recognition motif
Sb	Stubble
SCLC	Small cell lung cancer
Ser	Serrate
SN	Sensory neuropathy
UAS	Upstream activation sequence
UTR	Untranslated region
VNC	Ventral nerve cord
Wt	Wildtype
Wor	Worniu
YFP	Yellow fluorescent protein

Chapter One

INTRODUCTION

1.1 General Overview

Regulation of the level of alternative splicing, polyadenylation, as well as mRNA transport, stability and degradation in the cell has been crucial for the flow of information from transcriptome to proteome. Considered as the initial step in information processing, post-transcriptional regulation (PTR) determines the fate of transcripts that become part of the proteome within a cell. With the capability to change their regulatory mechanism to the environmental condition and manipulating the proteome, the post-transcriptional regulation mechanism has been of great relevance in the function of neurons, where protein's response to cellular and sub-cellular condition is important for the overall activity of the body (Simone and Keene, 2013). But to understand the finer details of PTR (and RNA binding proteins) mechanism in the nervous system, and to provide a basic idea on a protein's role inside the neurons is an area that is still under exploration. This research is one such work that shall focus on understanding the activity of a neuronal maker protein, i.e. embryonic larvae abnormal vision (Elav), during nervous system development.

1.2 Neural Development

Being a complex organ, the nervous system has been designed in such a way that majority of the neurons and energy is contributed for its constant maintenance, function and coordination (Skeath and Thor, 2003). Over the years, to understand the nervous system, researchers have been using vertebrate and invertebrate model organisms like zebra fish, *Caenorhabditis elegans* and mouse to provide information on processes like neuron birth, survival and death. In this aspect, one such robust invertebrate model that has provided us with better understanding on the development and maintenance of the nervous system is the diptera, *Drosophila* (Skeath and thor, 2003). The development of the nervous system (i.e. central (CNS) and peripheral nervous system (PNS)) in *Drosophila* begins quite early in embryogenesis. Like in humans, the birth of the neuron in *Drosophila* begins with the asymmetric division of the progenitor cells and precise regulation of various proteins at different timeline to help in proper differentiation and attainment of their designated developmental fate.

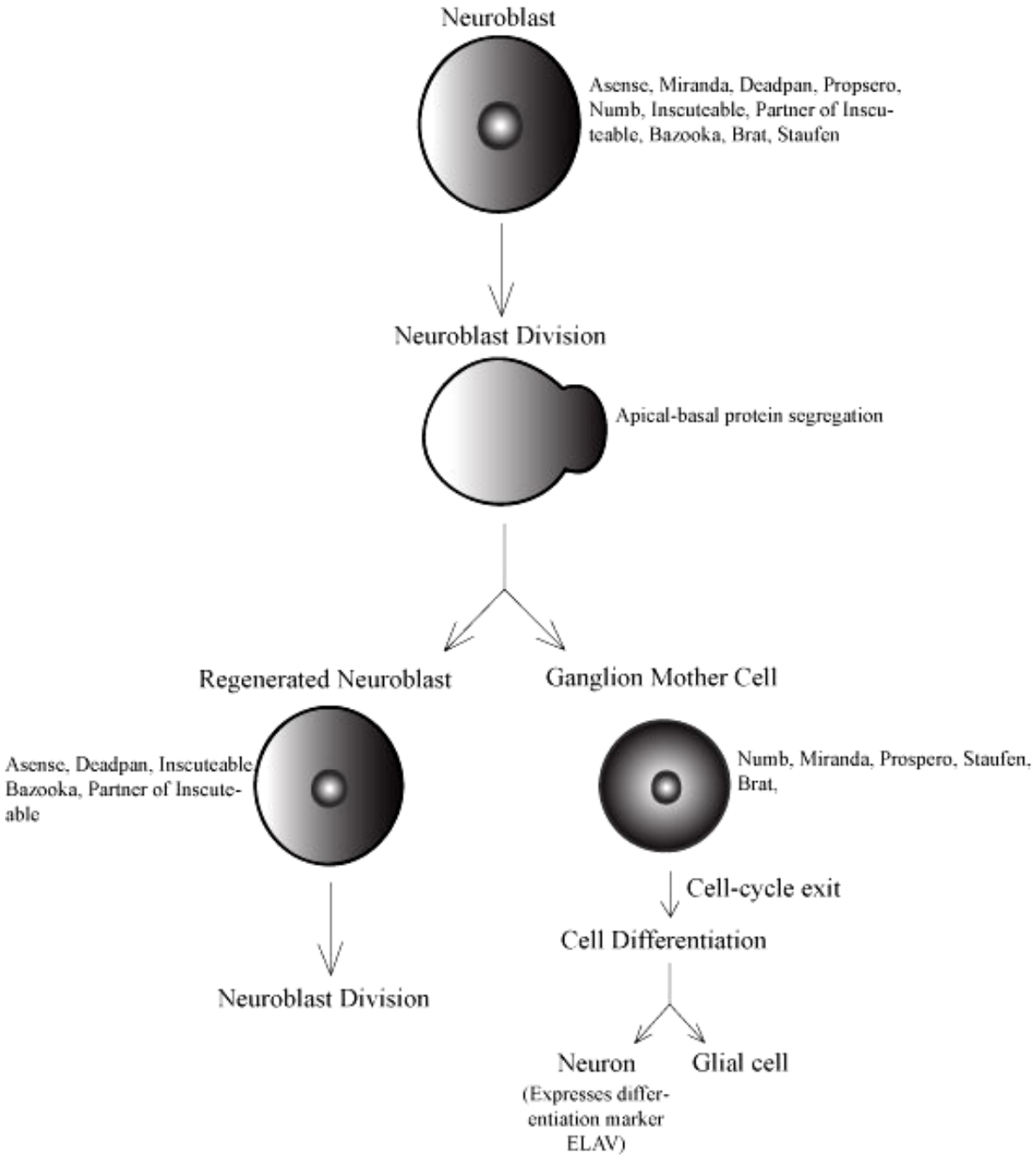


Figure 1: Asymmetrical division of neuroblast in *Drosophila*. In the dividing neuroblast, the cytokinesis of the neuroblast results in the formation of a regenerated neuroblast and a smaller ganglion mother cell. In the regenerated neuroblast, which has the self-renewal capacity, the Propero is held back in the cytoplasm by the numb. On contrary, in the ganglion mother cell, the Propero enters the nucleus and represses key cell cycle genes, thereby resulting in the division of the ganglion mother cell for one final time to give rise to two cells i.e. neurons (expressing differentiation marker Elav) or glia cells.

The process of neurogenesis in *Drosophila* begins with the differentiation of neuroepithelial cells from the ectoderm (i.e one of the three primary germ cell layers) to form a neural plate/sheet. During embryo neurogenic period, the neural plate/sheet undergoes systemic division to form neural equivalence groups. The ongoing cell-cell based interaction amongst the cells in the neural equivalence group leads one cell from each group to proceed towards developing into a neuroblast (NB) (Bhat., 1999). As the rest of the members in the equivalence group attain an epidermal cell fate, the NB (i.e expressing Prospero, Numb and Miranda proteins, with Prospero kept at cytoplasm by Numb protein) enlarges itself and undergoes a segregation process that results in the development of 30 NBs with an invariant pattern. (Skeath and Thor., 2003; Doe., 2008)

Each of the generated NBs (Fig. 1.1), which are presumed to have an identity (i.e. expressing transcription factor like Achaete-Scute, Huckebein, Runt, Msh and Seven-up) divides in an asymmetric fashion to produce the ganglion mother cell (GMC) and a regenerated NB. At this time in the GMC, the Prospero that was previously held back by the protein Numb in the cytoplasm enters into the nucleus and represses the cell-cycle genes. This in turn leads the GMC to undergo one final asymmetric mitotic cell division to produce two cells namely neurons (inter or motor neurons) and/or glia. (Berger *et.al.*, 2007; Doe., 2008)

1.3 Neuroblasts:

The neuroblast fate of a cell in the neural sheet equivalence group has been identified to be determined by the cell-cell based interactions carried out within the group. But however recent research investigations (Doe, 2008) have revealed that along with these interactions, the manipulative expression of the apical-basal proteins at different time frame has led the neuroblasts to be categorization into two different types:

Type I Neuroblast:

In the developing lobes, the type I neuroblast is differentiated by the presence of Deadpan (Dpn) & Asense (Ase) in the nucleus and prospero in the cytoplasm (Bayraktar *et.al.* 2010). The type I neuroblasts (90 in number) through asymmetric division gives rise to a small ganglion mother cell having the basal marker proteins Miranda, Prospero and Brain tumour (Brat), and a regenerated neuroblast (NB) having the apical marker proteins Asense, Deadpan and Inscuteable (Bello *et.al*, 2006; Lee *et.al*, 2006; Bayraktar *et.al.* 2010). The small ganglion

mother cell undergoes one final division to produce two cells, which either attains a neuronal fate or glial fate (Robinow and White, 1988), while the regenerated neuroblast continues to divide further.

Type II neuroblast:

In the developing lobes, the type II NBs is large cells characterised by the presence of Deadpan and absence of Asense/Prospero (Bayraktar *et.al.* 2010). Being different from type I, type II neuroblastst (8 in number) generally gives rise to an intermediate neural progenitor cell (INPs) (between NBs and GMCs) and a ganglion mother cell or regenerated NB. Each INPs divides further to give rise to another INP and GMC, which in turn produces a pair of neurons expressing the differentiation marker Elav (Robinow and White, 1988; Bello *et.al*, 2008). It has been observed that at the neurogenic period in the larval stage (i.e. after embryonic quiescence), it is the extended INPs proliferation that contribute to 90% of neurons in the adult *Drosophila* brain (Bayraktar *et.al.* 2010).

1.4 Post-Transcriptional Regulation:

A neuron's integrity in the brain depends on its ability to modulate signals from various parts of the body and to program the cellular responses to the ongoing changes (Pascale *et.al.*, 2008). The cellular processes in neurons strongly depend on regulation, modification, transcription and metabolism of the mature ribonucleic acid (mRNA), so that neuronal functions are carried out without any interruptions (Lunde *et.al*., 2007). One such activity that is considered important in mRNA formation is post-transcriptional regulation (PTR). Post-transcriptional regulation is a fundamental mechanism that involves the processing of pre-mRNA in the nucleus to give rise to active mRNA, which can later be functionally expressed at the right place at the right time via translation anywhere in the cell (Krause *et.al*., 1994; Lunde *et.al*., 2007).

1.4.1 PTR Mechanism:

The post-transcriptional regulation process begins with the association of RBPs (like Brat, Elav, Smaug, Vasa, Nanos) to the heterogeneous nuclear RNAs (hnRNAs) (Dreyfuss *et.al.*, 2002; Di Liegro *et.al*. 2013). After 3'end cleavage, the assembled nascent hnRNAs attaches to the poly(A) tail, which leads to events like transcription termination and assembly of the

spliceosome complex (Wahle *et.al*., 1991). The spliceosome complex (consisting of 5 small nuclear RNPs (snRNP)) acts on the pre-mRNA in a specific manner that selectively removes the introns. Excision of the introns are followed by capping of the 5'end, joining of the exons, polyadenylating the 3'end and mRNA transportation to the cytoplasm by the export proteins (Di Giammartino *et.al,* 2011). During the processing of the mRNA, the interaction of the spliceosome is important for determining which sequence(s) needs to be present in the mRNA or act as checkpoints before being transported to the cytoplasm (Di Liegro *et.al*. 2013). Once in the cytoplasm, the mRNA is acted upon by the ribosomes that translate the mRNA to the desired protein. However, at times if there is a termination codon present upstream to the first exon, the mRNA undergoes degradation in the cytoplasm via non-sense mediated decay (NMD) pathway (Maquat, 1995). It has been observed that it is the modulating ability of the *trans*-acting factors (i.e U1and U2 small nuclear RNPs) present in the system that helps in effective translation of any mRNA produced from the genetic code in the nucleus (Wahle, *et.al*., 1991).

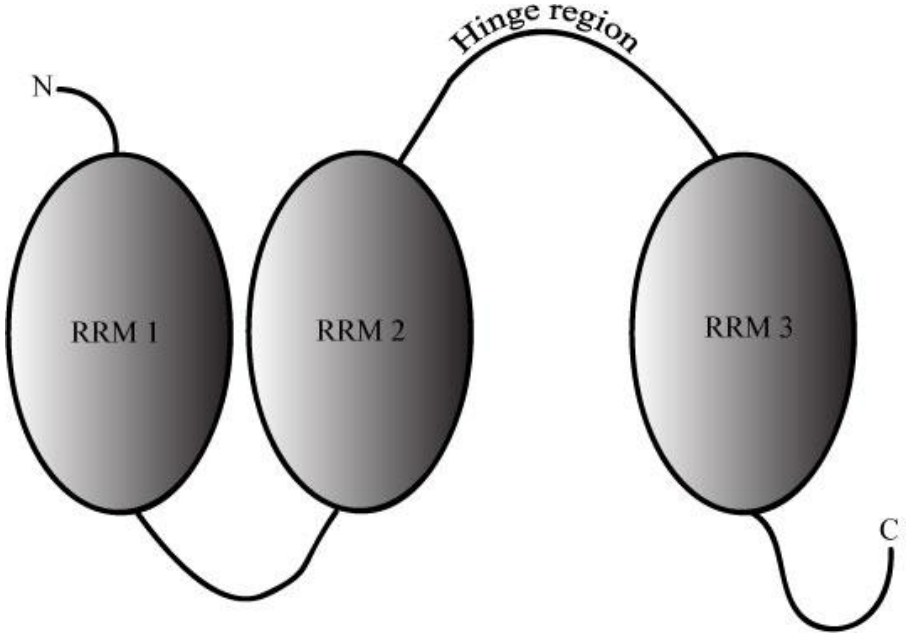


Figure 1.2: RNA binding protein (RBP) with motifs: One-dimensional representation of RNA binding protein Elav with its RNA recognition motifs (RRM). Model is shown to have three RRM with a hinge region separating the second RRM from the third RRM.

1.5 RNA Binding proteins:

The processing of mRNA has been observed to strongly depend on the *trans*-acting factors. RNA binding proteins (RBPs) (Fig. 1.2) is one such factor that tag from the formation of pre-

mRNA in the nucleus to transport of mRNA to the translation site in the cytoplasm (Lunde *et.al*., 2007). The characteristic nature of RBPs is the ability to bind in a sequence-dependent manner. The RBP binds to the secondary structures (i.e. hair-pin stem loop) present in the 3' (and at times also in 5') untranslated regions (UTR) in the mRNA with the help of RNA-binding domain (RBD) or RNA recognition motif (RRM) via inter or intra molecular cooperatively (Darnell, 2013). The binding of RBP to the unspliced or spliced mRNA may be in the intronic, coding or in the non-coding alternatively spliced variants. For the RRMs of the RBPs to bind to the RNA, the RBPs position themselves in such a way that they can structurally change their RRMs based on the alterations (i.e. unfolding) the mRNA undergoes after getting in contact with RBPs. (Di Liegro *et.al*., 2013; Mauger *et.al.*, 2013)

Different RBPs present in the system have different number of RRMs present in them (Fig. 1.2) and forms a unique secondary structure with the mRNA. In the brain, unique RBPs specific to the nervous system have been found to be involved in the development of neuroblasts/neurons. One such RBP that has is multipurpose and endowed to neuronal program from differentiation to maintenance is the RBP protein embryonic lethal abnormal vision (Elav) (Colombrita *et.al*., 2013).

1.6 Elav/Hu RNA Binding Protein:

Deriving its name from embryonic lethality and malformation in the vision & nervous system caused by deletion of the gene, Elav is a RBP with three different RNA recognition motifs (RRM) (Campos *et.al*., 1985; Robinow *et.al*., 1988). Being pan-neurally expressed and homologous (70-85%) to the human form of protein i.e. Hu (a.k.a. Elav-like proteins (ElavL)), the Elav/Hu family has been majorly involved in post transcriptional gene regulation (Yao *et.al*, 1993). Ranging their activity from alternative splicing, editing, polyadenylation and export, in the nucleus, to regulating stability, localization, translation and degradation, in the cytoplasm, the Elav/Hu family has been a predominant member in the development and survival of photoreceptor cells, optic lobes and the neurons in the brain (Pascal *et.*al., 2008; Darnell, 2013; Colombrita *et.al*., 2013). Consisting of Elav, found in neurons (Fne) and RNA-binding protein 9 (Rbp-9) in *Drosophila*, and HuA/HuR (ElavL1), HuB (ElavL2), HuC (ElavL3) and HuD (ElavL4) in humans, each of the Elav/Hu family member are characteristic in recognizing AU-rich mRNA sequences (Fig. 1.3). The beta-sheet (β-sheet) of the RBP forms a cleft that results in specific targeting and expression of mRNAs

(Zhang *et.al*., 2005). However variations in the amino acid sequences has the effect of providing each family member (i.e. Elav, Rbp-9 and Fne) with different localization and functional property (Table 1.1)

Name	Species	Developmental expression	Tissue Distribution	Localzation	Secondary structure with mRNA	Activity
Elav	*Drosophila*	Differentiated neurons	Central and peripheral nervous system	Predominantly Nucleus. Subcellular localization also occurs	Four stranded beta-sheet against two alpha helices ($\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$) that binds to AU-rich sequence and has the ability to oligomerise with the bound mRNA	Alternative splicing, polyadenylation, regulation and stability
Rbp-9	*Drosophila*	Wandering late 3rd-instar larvae	Central nervous system, testes and ovaries	Cytoplasm/Nucleus		Splicing and negative regulation of mRNA stability in gonads
Fne	*Drosophila*	Embryogenesis: birth of neurons	Central and peripheral nervous system	Cytoplasm		Regulation, polyadenylation and mRNA stability

Table 1.1: Expression, distribution and localization of Elav/Hu family members**.**

In *Drosophila*, (i) Elav is specific to the nervous system as a neuronal differentiation marker and is expressed during all developmental stages in the neurons, where it predominantly localizes to the nucleus and is involved in alternative splicing, polyadenylation, regulation and stability, (campos *et.al*., 1988, Robinow *et.al.*, 1988; *Yannoni* and White, 1999, Colombrita *et.al*, 2013) (ii) Rbp-9 is expressed in gonads and nervous system, during the 3rd instar stage, where it localizes in cytoplasm and nucleus, respectively, and is functionally involved in splicing and negative regulation of mRNA stability in the gonads (Kim and Baker 1993; Colombrita *et.al*, 2013), and (iii) Fne is also specific to the nervous system, where it is expressed during late neurogenesis and localizes to the cytoplasm and is involved in regulation, polyadenylation and mRNA stability (Samson and Chalvet, 2003; Colombrita *et.al*, 2013).

In humans, (i) HuD is functionally similar to Elav and is expressed only in the nervous system, but in late neurula stage the HuD localizes to cytoplasm (ii) HuB like Rbp-9 is expressed in the gonads and nervous system, but localizes to the cytoplasm in the gonads and nervous system, upon expression during gastrulation. (iii) HuC is expressed during late

gastrula stage, and like HuD is expressed specifically in the nervous system and localizes to the cytoplasm, and (iv) HuA/HuR is expressed in many cells, and is different from other family members by being expressed at all stages during cell development and are found to localize to the nucleus. All the family members of the Elav/Hu human counterpart are involved in providing functions like splicing, polyadenylation, positive/negative regulation of stability to mRNA and translation enhancement. (Kasashima *et.al*., 1999; Kasashima *et.al*., 2002; Pascale *et.al*., 2008; Colombrita *et.al*, 2013)

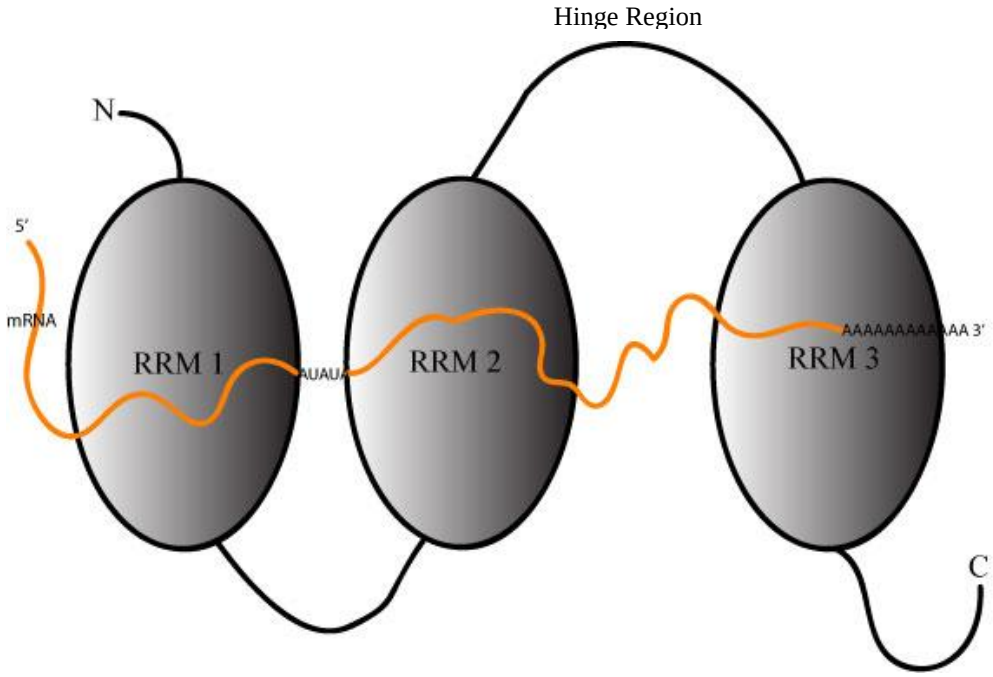


Figure 1.3: **One-dimensional representation of Elav binding to an mRNA**. The adaptation is based on Elav as a model and therefore the representation is not exactly scaled. Elav has been identified to bind and act on three vital genes i.e. *ewg*, *Nrg*, *arm* in the neurons and they are found to recognises the mRNAs through the presence of AU rich motifs in the mRNAs. (Adapted from Soller and White, 2004)

1.7 ELAV/Hu Structure:

Being the first identified member of RRM containing proteins, the Elav/Hu is 40 KDa in size with an alanine/glutamine rich amino-terminal followed by three RNA recognizing domains of approximately 90 AA each. In Elav/Hu family, the third RRM is separated from the first two RRMs by a hinge region of approximately 54-80 amino acids (Fig. 1.3). Although comparison of RRMs between the family members shows significant identity, but there has been considerable divergence within the hinge region of the family members. This has been

the potential modulator for the nuclear localization of some of the proteins (i.e. Elav) in the family (Soller and White, 2004; Soller *et.al* 2010).

In ELAV/Hu, it is the first two RRMs present in the N-terminal region that interact with the AU-rich sequence in the mRNA to form the cleft (Fig. 1.3). The binding nature of the third RRM present in the c-terminal is not clearly known. But, it is the affinity towards the poly(A) tail of mRNAs (shorting of poly-A tail over a period of time leads to degradation of the mRNA by enzymes) and the hinge sequence variations present in Elav/Hu, (i.e. octapeptide motif in the region 333-374 of their 483 amino acid sequence) that provides the Elav/Hu with the dominance to contribute to the mRNA stability, nuclear localization, and auto regulation its own mRNA. (Pascale *et.al.*, 2008; Colombrita *et.al*., 2013)

1.8 Elav/Hu protein in disease:

Even though, Elav/Hu protein has been considered critical for the development and maintenance of the neurons, however ectopic expression of Elav/Hu and their subsequent interactions with other proteins have been found to provide negative effects. One such rare autoimmune associated syndrome caused due to the ectopic expression of Elav/Hu in the cell is paraneoplastic encephalomyelitis/sensory neuropathy (PEM/SN) syndrome. Paraneoplastic syndrome is an overlapping neurological or multifocal central nervous system inflammatory disorder occurring due to the autoimmune reactivity to the neuronal protein, Elav/Hu. The disorder causes inflammatory neuraxis, neuronal death and gliosis (Hinman and Lou, 2008). A malignant cancer type that has been found to result in PEM syndrome condition in patients is the endocrine system lung cancer variant, small cell lung cancer (SCLC). In here, the cancer cells present in lungs ectopically express Elav/Hu protein, which causes the body to mediate an immune response against the protein and result in an autoimmune/inflammatory response towards the neural cells present in the brain.

Considered to be rapidly metastatic, resistant to cytotoxic treatment, and cause high mortality amongst patients, the SCLC rarely results in PEM/SN condition in the patients (Dalmau *et.*al., 1992; Tsou *et.al.*, 2009). PEM/SN is very remotely associated to be a result of metastasis or tumour formation in the brain. Majority of patients with SCLC associated PEM/SN have been found to show symptoms like dementia, sensory neuropathy and myelopathy, and die because of these neurological conditions rather than of cancer.

PEM/SN mechanism:

The autoimmune response and neurodegeneration in the PEM/SN SCLC patients has been observed to be due to the expression of anti-Hu, which is found in high titre in the serum and cerebrospinal fluid of the patients (Dalmau *et.*al., 1992). Biomarker specificity analysis has reported abnormal expression of the neural protein Hu in all SCLC patients with or without PEM/SN, but the reason as to why only few patients show an immune response against the Hu antigens, considering the facts the tumours switches off the expression of major histocompatability (MHC) is still not clearly understood (Szabo, *et.al,*, 1992, Tsou *et.al.*, 2009).

However, theoretical models have been outlined by researchers to give an overview of the mechanism, taking into account similar neoplastic syndrome like Lambert Eaton myasthenic syndrome, where autoimmune response causes neuromuscular failure due to calcium flux inhibition. Accordingly the PEM/SN model states, Hu being a neuron specific protein is not available during the establishment of immune tolerance due to the absence of MHC in the neurons. But during the ectopic expression of Hu antigen (with altered protein sequence) in the tumour, a misdirected immune response is triggered by the system. The generated anti-Hu antibody now available in the lymphocytes, apart from reacting with the onconeural form of Hu present in the region of tumour also infiltrates the neural system and reacts with the regularly expressed Hu antigens in the neurons and thereby causes neuronal function inhibition.

Even though expression of the autoantibodies against Elav/Hu has been observed in patients, but the exploitation on using ELAV/Hu proteins to induce suppression of the autoantibodies has been thought of and carried out only in mice, where encouraging results have paved way for the development of tumour-vaccination strategies based on Elav/Hu (Tsou *et.al*., 2009). Using this new strategy clinical research has now focused on targeting other forms of solid cancer tumours like breast, ovarian and colorectal (suggested involvement of HuR) and neuroblastoma (childhood tumour derived from defect in sympathetic ganglia development).

1.9 Ectopic Expression of ELAV in early neurogenesis:

Research publications over the last decade have commonly pointed out the case that aberrant protein expression is the reason for a cell to turn cancerous. To support this it has been

observed in the *Drosophila* system that loss of function of Brat or Miranda or Prospero or Scribble or Numb or Earmuff, during the embryonic neurogenesis stage, gives rise to tumour formation in the brain (Lee *et.al.*, 2006). As research has always focused on investigating tumour formation through mutational changes in the proteins, this raised a question as to whether a neuronal protein's ectopic expression at a wrong place at a wrong time (like the occurrence of ectopic expression of Elav/Hu by the tumour cells in the lungs of the patients with SCLC that results in PEM/SN disorder), during early stage of neurogenesis, can give rise to tumours like those observed with the loss of function of Brat. This initial thought made us choose Elav for the ectopic expression analysis, as Elav is a protein that is strongly expressed only in neurons, homologous to human protein Hu and is not expressed anywhere else during neurogenesis. To test this hypothesis, the research shall look into ectopically expressing Elav in the developing *Drosophila* system using GAL4/UAS and observe if Elav's expression affects the differentiation of neuronal precursor cells i.e. eye primordium and neuroblast.

Chapter Two

MATERIALS AND METHODS

2.1. Materials:

The *Drosophila* stocks and fly food were provided by Dr. Matthias Soller's lab (Table 2.1). The Corning 3524 Costar 24 well polystyrene cell culture cluster plate was from Sigma-Aldrich®, bovine serum albumin (BSA) was from Sigma life sciences, Phosphate buffered saline and Triton®X-100 was from Sigma. Goat anti-rat IgG Alexa Fluro® 488 and 647, anti-mouse ELAV, anti-rabbit Miranda, 4', 6-diamidino-2-phenylindole (DAPI) were from Invitrogen™. The Vectashield mounting solution was from Vector laboratories. The dissection fluorescent microscope Leica MZ FLIII was from Leica, imaging fluorescent microscope Axioplan 2 attached with Axiocam Hrc camera was from Carl Zeiss and the TCS SP scanning confocal microscope was from leica.

2.2 Drosophila Maintenance:

2.2.1 Drosophila Food Composition:

Plastic or glass vials with standard agar food medium of 10ml containing 8.5% sugar, 6% cornmeal, 2.5% nipagin and 1% agar in water was supplemented with dried live yeast, and were plugged with water absorbent cotton.

2.2.2 Stock Maintenance:

The stocks were maintained either at 25°C or 18°C, on the basis of their requirement for experiments. The stocks maintained at 25°C were flipped to new vials on a weekly basis, while those maintained at 18°C were flipped at three or four week time interval.

2.3. Drosophila genotype cross setup and generation of new genotypes:

2.3.1 Experimental genetic-cross setup and maintenance:

The experimental crosses were setup by collecting virgin females from one genotype (e.g. UAS-gene A) and crossing them with the males of another genotype (e.g. gene B-GAL4). The crosses were maintained at 25°C with 70% humidity. The crosses were regularly flipped into new vials on every 3rd day to provide better conditions for egg laying and to avoid overcrowding of larvae.

2.3.2 Generation of double genotype stock:

2.3.2.1 Generation of the genotype Elav^{C155}/FM7;; UAS Rbf/TM3Serrate with GFP genotype:

To generate the desired genotype (Fig. 2.1), homozygous Elav^{C155} (X chromosome) genotype virgin female (G0) flies were crossed with the male flies of balancer genotype (G0) carrying first multiple 7 bar eyed (FM7 Bar) on the 1st chromosome and third multiple 3 serrate tagged with green fluorescent protein (TM3Ser with GFP) on the 3rd chromosome. The homozygous UAS Rbf (3rd Chromosome) male flies (G0) were crossed with virgin female flies of the balancer genotype (G0) having FM7 Bar on the 1st chromosome and third multiple 3 stubble tagged with GFP (TM3Sb with GFP) on the 3rd chromosome. From the progenies (F1) of the above two G0 crosses, Elav^{C155} virgin females having bar eyes and serrate wings (F1) expressing GFP were crossed with UAS Rbf males having bar eyes and stubble bristles (F1) expressing GFP. From the progeny (F2) between the F1 flies, the males and virgin females having bar eyes and serrate wings expressing GFP were selected and were used for building up the double genotype flies i.e. flies carrying Elav^{C155} in the first chromosome and UAS Rbf on the third chromosome.

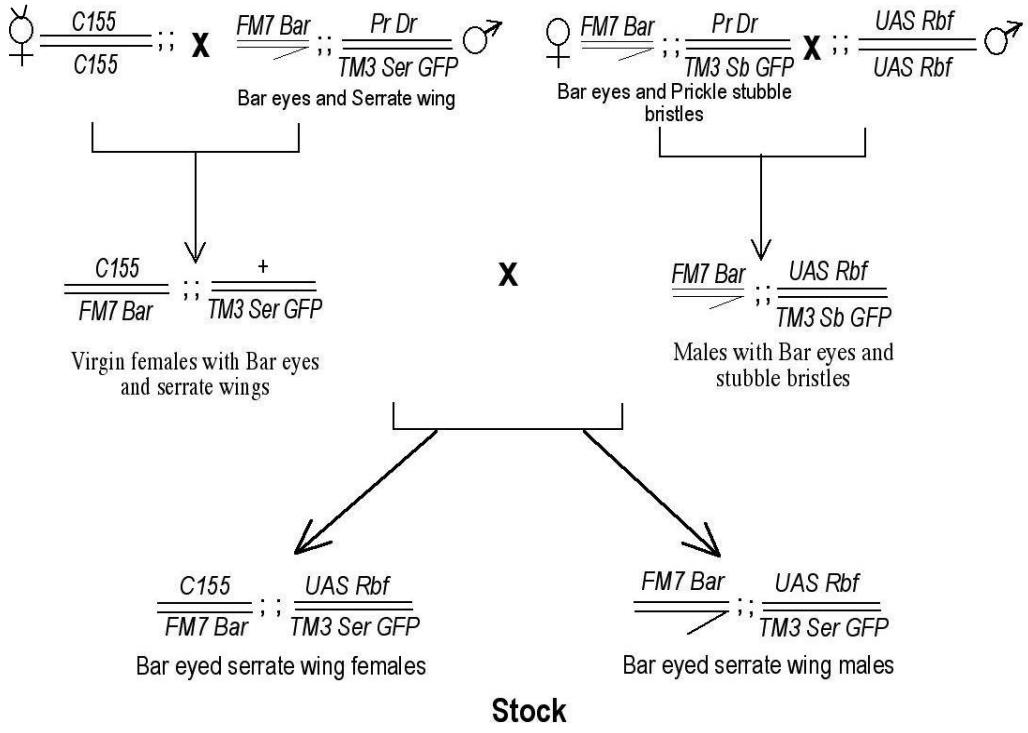


Figure 2.1: Illustrative representation of crossing patter between Elav^{C155} and UAS Rbf genotype flies that was carried out to generate the genotype Elav^{C155}/FM7;; UAS Rbf/TM3Serrate with GFP.

2.3.2.2 Generation of the genotype Elav^{C155}/FM7;; UAS Brat76/TM3 Serrate with GFP:

To generate the desired double stock with Elav^{C155} in the first chromosome and UAS brat76 on the third chromosome, the crossing pattern as mentioned before was followed for this genotype generation too (Fig. 2.2). But instead of UAS Rbf males in the G0, the UAS brat76 genotype males were crossed with the virgin females of the balancer genotype FM7 Bar ;; TM3 Sb tagged with GFP.

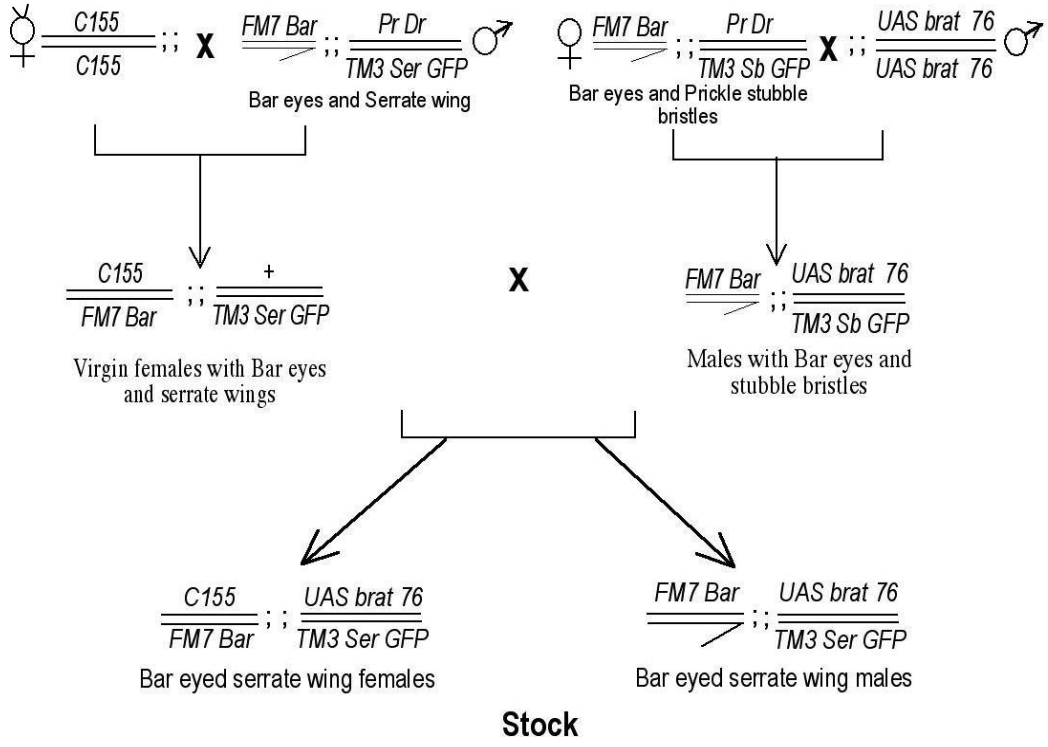


Figure 2.2: Illustrative representation of the crossing patter between genotypes Elav^{C155} and UAS Brat76 that was carried out to generate the genotype Elav^{C155}/FM7;; UAS Brat/TM3Ser with GFP.

2.3.2.3 Generation of ;UAS Rbf RNAi/Cyo GFP; UAS ELAV$^{NLS\ HA}$/TM3 Ser GFP genotype:

The generation of the desired genotype (Fig.2.3) was carried out by crossing homozygous virgin female flies (G0) of UAS Rbf RNAi with the male flies (G0) of the balancer genotype having second multiple 6 rough eyes (SM6 Roi) on the second chromosome and third multiple 3 serrate with GFP (TM3 Ser with GFP) on the third chromosome, and by crossing the virgin female flies of the balancer genotype (G0) having curly of oster with GFP (CyO with GFP); TM3 stubble with GFP (TM3 Sb with GFP) with the homozygous male flies (G0)

of UAS Elav^{NLS}. From the progeny (F1) between these two separate G0 crosses, the virgin females (F1) of UAS Rbf RNAi having rough eyes and serrate wings expressing GFP were crossed with the males (F1) of UAS Elav$^{NLS\ HA}$ having curly wings and stubble bristles expressing GFP. In the progeny (F2) between the F1 cross, the virgin females and males having curly and serrate wings expressing GFP were selected and were used for building up the double stock flies carrying UAS Rbf RNAi in the second chromosome and UAS Elav^{NLS} in the third chromosome.

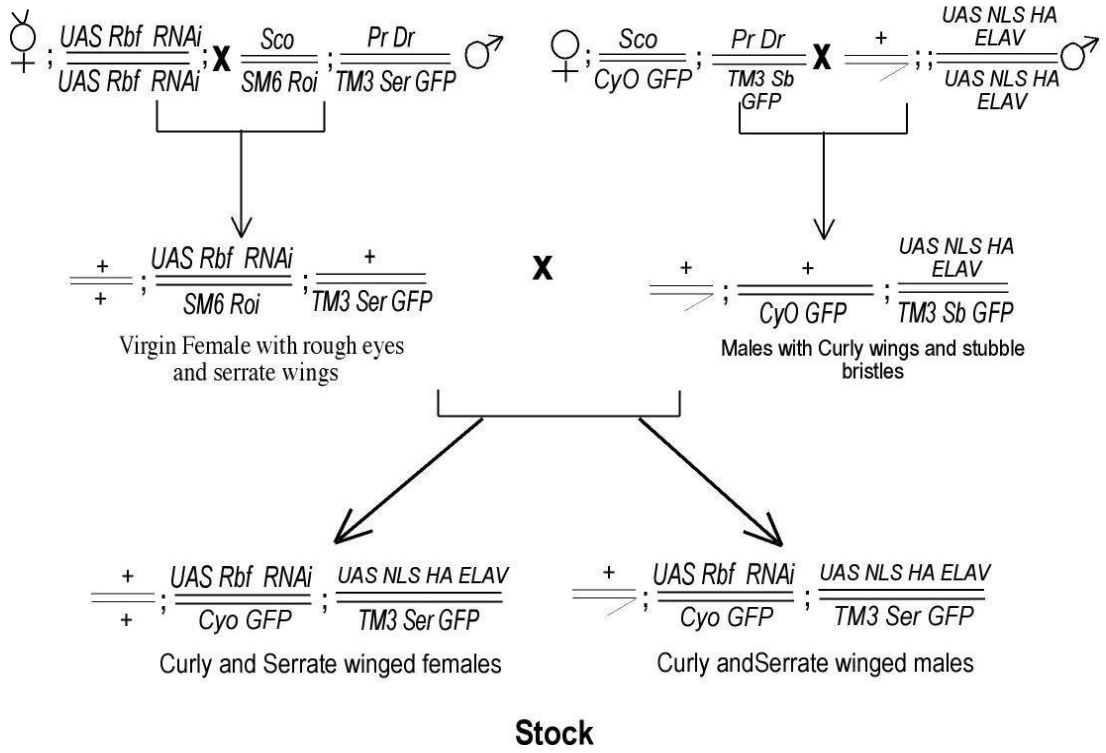


Figure 2.3: Illustrative representation of the crossing patter between genotypes UAS Rbf RNAi and UAS Elav$^{NLS\ HA}$ that was carried out to generate the genotype ;UAS Rbf RNAi/CyO GFP;UAS Elav$^{NLS\ HA}$ /TM3Ser with GFP.

2.4 Larval Collection:

The experimental crosses maintained at 25°C were occasionally checked for larvae development, after the eggs hatched. For larval brain analysis, wandering third-instar larvae were used for the purpose of dissection. In case of identifying or segregating larvae expressing fluorescent protein (i.e. target gene or balancer gene tagged with fluorescent protein), the larvae was viewed under a dissecting fluorescent microscope and the desired larvae was selected based on the fluorescence expression in their system. For larvae expressing the GAL4 the expression of GFP was identified to be strong in the salivary gland.

In the case of larvae carrying the balancer tagged with GFP, the expression of the GFP was found to be present near the abdomen and in the posterior spiracles.

S.No.	Genotype	Referred to as	Chromosome (Chr)	Origin
1	;;UAS NLS ELAV	$ELAV^{NLS}$	3	Lab stock
2	$ELAV^{C155}$ GAL4;;	C155 or $ELAV^{C155}$	X	Lab stock
3	;Inscuteable^{1407} GAL4;	$Insc^{1407}$	2	Bloomington
4	;Inscuteable^{1407} GAL4 brat^{11}/CyO;	$Insc^{1407}$GAL4 brat^{11}	2	Bloomington
5	;Eyeless GAL4 UAS Delta/CyO;	EyelessGAL4-UAS Delta	2	Bloomington
6	;Eyeful Eyeless GAL4 UAS Delta/CyO;	Eyeful	2	Bloomington
7	;;OK107 GAL4 Eyeless	Ey^{OK107} GAL4	3	Bloomington
8	; Worniu GAL4/CyO;	Worniu GAL4	2	Bloomington
9	;; UAS ELAV-13	UAS $ELAV^{-13}$	3	Lab stock
10	; His YFP/CyO; Prospero GAL4/TM6B	Prospero GAL4	2 & 3	Lab stock
11	;UAS Rbf RNAi;	UAS Rbf RNAi	2	Bloomington
12	; UAS Rbf ;	UAS Rbf (2nd Chr)	2	Bloomington
13	;;UAS brat76	UAS brat	3	Bloomington
14	;;UAS Rbf	UAS Rbf (3rd Chr)	3	Bloomington
15	Castor GAL4 GFP/TM3 Sb	Castor GAL4	3	Bloomington
16	;;UAS $ELAV^{N26-N9}$	$ELAV^{N26-N9}$	3	Lab stock
17	;; Repo GAL4/ TM3 Sb	Repo GAL4	3	Bloomington
18	;UAS $ELAV^{Q2}$;	$ELAV^{Q2}$	2	Lab stock
19	; SpgGAL4 UAS Dicer2 / SM6 Roi;	Moody GAL4	2	Lab stock
20	;;$ELAV^{edr}$	$ELAV^{edr}$	3	Lab stock
21	UAS Brat RNAi/CyOTM3 Ser	UAS Brat RNAi	2	Lab stock
22	YFP/CyO; UAS NLS ELAV/ TM3 Ser	YFP; $ELAV^{NLS}$	2 & 3	Lab stock
23	;;UAS $ELAV^{Q3}$	$ELAV^{Q3}$	3	Lab stock
24	;; UAS ELAV OH	$ELAV^{OH}$	3	Lab stock
25	;Inscuteable^{1407} GAL4 brat^{K}/CyO;	$Insc^{1407}$ GAL4 brat^{K}	2	Lab stock
26	;; UAS $ELAV^{N9}$	$ELAV^{N9}$	2	Lab stock
27	Df brat/CyO ; UAS NLS ELAV/ TM3ser GFP	Df brat; UAS $ELAV^{NLS}$	2 & 3	Lab stock
28	;; UAS NLS ELAV OH	$ELAV^{NLSOH}$	3	Lab stock
29	YW^{+};;	Wild type	X	Lab stock
30	;UAS YFP;	UAS YFP	3	Lab stock
31	;; UAS NLS ELAV -13	UAS $ELAV^{NLS-13}$	3	Lab stock
32	;;UAS $ELAV^{wt}$	$ELAV^{wt}$	3	Lab stock
33	; Grainyhead GAL4 UAS mCD8GFP/CyO;	Grainyhead GAL4	2	Lab stock
34	$ELAV^{C155}$ GAL4/ FM7;;UAS brat/TM3 Ser GFP	ELAVC155 GAL4; UAS brat	X & 3	Generated for this project
35	$ELAV^{C155}$ GAL4/ FM7;;UAS Rbf/TM3 Ser GFP	ELAVC155 GAL4; UAS Rbf	X & 3	Generated for this project
36	;UAS Rbf RNAi/CyO; UAS $ELAV^{NLS}$/TM3 Serrate-GFP	UAS Rbf RNAi/CyO; UAS ELAVNLS	2 & 3	Generated for this project

Table 2.1: List of genotypes used in the experiments.

2.5 Dissection of larvae:

Dissection of larvae was carried out in phosphate buffered saline (PBS) solution. Using forceps, the larvae was ripped open and the fat bodies and other developing tissues surrounding the brain were removed carefully. This was performed to reduce interference by other tissues during immunostaining. The dissected larval brain was left attached to the mouth hook after dissection to have better visibility during washing process.

2.6 Fixing and immunostaining of larval brains:

The dissected larval brain was fixed by placing it in 4% Para-formaldehyde for a period of 30-45 minutes. The fixed brain was then washed thrice for 15 minutes in Phosphate buffered saline containing BSA (1%) and Triton-X (0.1%) (PBT). After washing thrice and draining the PBT thoroughly, the primary antibody (Anti-HA 1:20) diluted in PBT and 10% normal goat serum (to avoid non-specific binding) was added to the sample and incubated overnight at 4°C. The overnight incubation was followed by removal of primary antibody and washing the larval brain thrice with PBT for 15 minutes. The secondary antibody (Rat-anti HA 1:250) diluted in PBT with 10% normal goat serum was then added to the sample and incubated in dark at room temperature for 4 hrs. After the incubation period, the sample was washed thrice with PBT for 15 minutes and was finally suspended in PBS. However, in the case of multiple antibody immunostaining the primary antibody mixture consisted of mouse anti-Elav (1:20) and rabbit anti-Miranda (1:20)

2.7. Fixation of fluorescent protein expressing larval brain samples:

In case of larvae expressing fluorescent protein, the dissected brain was fixed in 4% Para-formaldehyde for a period of 15-20 minutes. After fixing, the brain sample was initially provided a quick wash with PBT and then followed by three 15 minute washes with PBT.

2.8 Mounting of larval brains:

The immunostained or fluorescent protein expressing larval brains were carefully dissected from the mouth hook attached via the eye disc in PBS solution. The brain was later placed on a clean glass slide containing Vectashield, with the dorsal side facing upward. After positioning the brain, cover slip was used to cover the sample and nail varnish was applied on all four sides to seal the cover slip. To avoid the brain from getting squashed, the larval mouth

hook and cuticle were placed alongside the brain to provide support and to reduce squashing. The mounted slide was stored at 4°C in a glass slide holder.

2.9 Fluorescence microscope imaging and processing:

All the mounted slides containing larval brains (stain/fluorescent protein expression) were observed using an Zeiss Axiocam fluorescent microscope or Leica TCS SP scanning confocal microscope. Imaging was carried out under different spectrum range/filters based on the use of fluorescent protein/dye used in the experiment. The data was processed using Adobe Photoshop, Freehand MX Multimedia and Image J.

2.10 Scoring of over-proliferation in the eyes of *Drosophila* model 'Eyeful':

The experimental setup for observation of the over-proliferation in the eyes was carried out by expressing different UAS reporters with the genotype 'Eyeful' EyelessGal4 UAS-Delta. The *Drosophila* model 'Eyeful' has over-expression of delta, *lola* and *psq* in the photoreceptors which causes them to have tumour growth in the retina (Bossuyt *et.al*, 2009), and it is this advantage of an *in-vivo* eye tumour model that made us chose 'Eyeful' *Drosophila* for the purpose of investigation. Observations for changes in the eyes were made by scoring the eyes of the progenies using the scoring system shown below (Table.2.1). In here, the $Elav^{edr}$ ($Elav^{eye\text{-}disc\ reduced}$) corresponds to an *elav* transgene insert which helps it to encode normal Elav expression in the neurons, but result in reduced Elav expression in the photoreceptor cells (Koushika *et.al*, 1996). The reduced expression of Elav causes the eyes of the *Drosophila* to be smaller than the normal eyes of the flies. Analysis of the observed result was carried out by plotting scatter plot using Graph pad Prism®.

Eye getting smaller	
Score	**Eye condition**
0	Wild type
-1	Elav edr like (up to 25% smaller)
-2	Elav edr like + out growth
-3	Medium (between 25-75% smaller)
-4	Medium + out growth
-5	Small (more than 75% smaller)
-6	Small + out growth
-7	No eye
-8	Incomplete transformation into other tissue

-9	Complete transformation into other tissue

Eye getting bigger	
Score	**Eye condition**
0	Wild type
1	Big (Eyeless Gal4 UAS Delta Overexpression)
2	Wild type with folded surface
3	Eyeless Gal4 UAS Delta with folded surface
4	Bigger than Eyeless Gal4 UAS Delta
5	Bigger than Eyeless Gal4 UAS Delta with folded surface
6	Wild type without growth
7	Eyeless Gal4 UAS Delta with outgrowth
8	Bigger than Eyeless Gal4 UAS Delta without growth

Table 2.2: Scoring pattern used for evaluating the changes observed in the eyes of *Drosophila* Eyeful model flies.

Chapter Three

RESULTS

Elav promotes over-proliferation in eye tumour model:

3.1 Effect of Elav function in *Drosophila* model with tumorous eyes:

To investigate if loss of function (LOF) or gain of function (GOF) of ELAV has an effect on the *Drosophila* model "Eyeful", a fly model that has over-expression of *delta*, *lola* and *psq* in the photoreceptor cells which leads them to have tumour growth in the retina (Bossuyt *et.al*, 2009), we expressed $Elav^{edr}$ and UAS $Elav^{NLS}$ in Eyeful flies. In here, the $Elav^{edr}$ ($Elav^{eye\text{-}disc\ reduced}$) corresponds to an *elav* transgene insert which helps in encoding normal Elav expression in the neurons, but have reduced Elav expression in the photoreceptor cells (Koushika *et.al*, 1996). $Elav^{NLS}$ corresponds to the addition of NLS (nuclear localization sequence) to Elav that helps in shuttling back the sub-cellular distributed Elav back to the nucleus thereby increasing the functional activity of ELAV in the nucleus. The eyes of each progeny expressing the GAL4/UAS were individually scored to analyse the effect of Elav. Eyes showing excessive over-proliferation of cells in the retina or development of eyes in any other part of the fly i.e. thorax or abdomen regions were considered metastatic. For the purpose of comparison, wild type and 'Eyeful' *Drosophila* flies were used as controls.

In the controls (Fig. 3.1A and B), the wild type flies had no tumour growth in their eyes, while 'Eyeful' flies on the other hand showed tumour development in their eyes, which was visibly seen as folds in the retina. It was also observed that 7.5% of 'Eyeful' flies had metastatic outgrowth from the retina.

Over-expression of Elav by expressing $Elav^{NLS}$ (Fig. 3.1D) in the 'Eyeful' flies showed an increase in the tumour formation, by either increasing the eye size or the number of folds in the retina. In addition, it was also found that 33.3% of these flies had outgrowths from the retina. However, in contrast, loss of function of Elav by $Elav^{edr}$ (Fig. 3.1C) expression showed a proportionate decrease in the size of the eye and tumorous folds in the retina. It was also observed that with $Elav^{edr}$ 5% of the flies had a drastic reduction of tumours in the retina that made them to have eyes that resembled the characteristic feature of a normal retina.

The above data suggests that Elav as a functional protein in the developing photoreceptors of the *Drosophila* 'Eyeful' model has the capability to induce excessive cellular proliferation upon over-expression. However, to test the effect of ectopic Elav expression in a non-tumorous system, ectopic Elav expression was carried out separately in *Drosophila* genotype

with normal eyes, for which the results are provided in the later part of the report. Although the loss of function of Elav in the tumour cells has not provided a conclusive data, but it can be assumed that reduced expression of Elav might have led the tumour cells to exit their cell-cycle. This might be the reason due to which the flies had reduced tumour growth in their retina. Considering the data obtained as preliminary, further investigation needs to be carried out in the future to increase the number of flies (N should be at least >= 50 individual samples for a continuous data with a natural distribution but not normal) being analysed and to provide relevant statistical analysis (Kurtosis (for normality) and Levene's test and one-way Anova (for P value significance)).

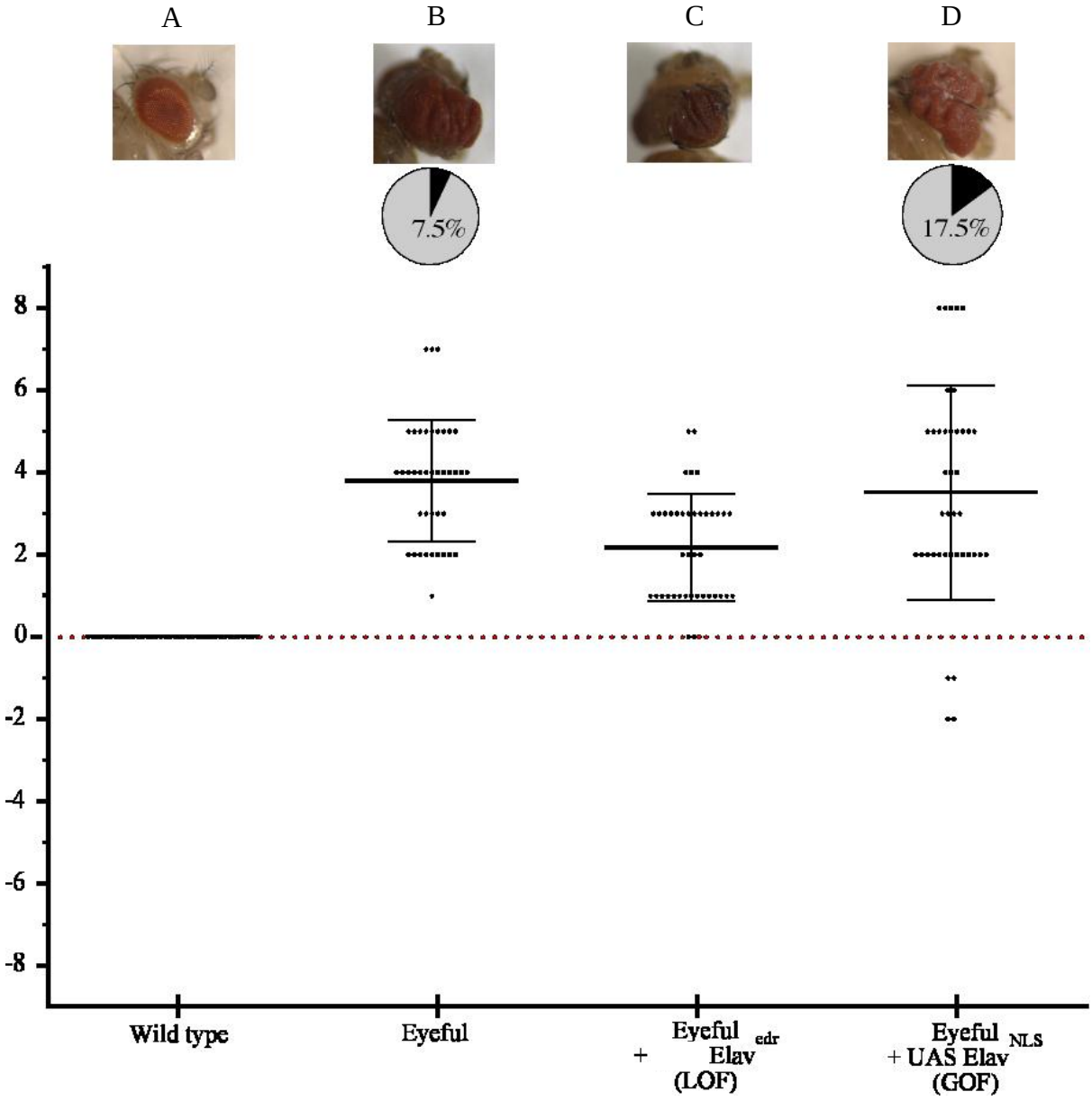


Figure 3.1: Quantitative and qualitative representation of the loss and gain of function of Elav. The total number of eyes taken into consideration was 40 (n=40), with the eye scoring being carried out as described in the table shown in materials and methods. Each individual point in the scatter plot corresponds to an eye. The pie chart indicates the incidence of metastasis, and any value above 6 was considered metastatic, with the percentage being calculated against the total number of flies taken into consideration. In the graph, wild type is A, Eyeful is B, Eyeful + $Elav^{edr}$ is C that represents loss of function (LOF) of Elav and Eyeful + UAS $Elav^{NLS}$ is D that represents gain of function (GOF) of Elav. The images on the top of the graph are a representation of the eyes of each genotype.

3.2 Role of Elav localization and insertion in *Drosophila* model with tumorous eyes:

To identify if localization of Elav or region of insertions of Elav has an effect on the tumorous eyes of the *Drosophila* 'Eyeful' model, we expressed different localization forms of Elav i.e. UAS $Elav^{wt}$, UAS $Elav^{NLS}$, UAS $Elav^{NLS\ OH}$, and different Elav insertions forms i.e. UAS $ELAV^{Q2}$, UAS $ELAV^{Q3}$, UAS $Elav^{N26\text{-}N9}$ and UAS $Elav^{N9}$ in the *Drosophila* Eyeful model. In here, $Elav^{wt}$ corresponds to wild-type pattern of distribution of Elav i.e. Elav localizes to the nucleus and sub-nuclear regions (Colombrita *et.al.,* 2013). $Elav^{NLS}$ corresponds to $Elav^{wt}$ added with a nuclearlocalization sequence. $Elav^{NLSOH}$ correspond to Elav that is predominantly cytoplasmic in localization due to deletion of an octapeptide sequence in the hinge region. $Elav^{Q2}$, $Elav^{Q3}$, $Elav^{N26\text{-}N9}$ and $Elav^{N9}$ corresponds to different insertion and location of Elav, with $Elav^{Q2}$ and $Elav^{Q3}$ corresponding to Elav inserted into chromosomes 2 and 3and $Elav^{N26\text{-}N9}$ and $Elav^{N9}$ corresponding to differentially located Elav in the 3rd chromosome. Eyes of the progenies were scored as previously described, with the Eyeful and wild type flies used as controls (3.2A and B).

On analysing the data, it was observed that the adult fly eyes showed variations in tumour intensity for nuclear and cytoplasmic localization of Elav. $Elav^{NLS}$ expression (Fig. 3.2C) increased the tumour growth in the progenies, with about 43.3% flies having excessive outgrowth in the retina. However, the percentage of flies expressing outgrowth is different from Fig 3.1D because of the difference in number of flies taken into consideration i.e. for 3.1D the number of eyes taken into consideration was 40 and in 3.2.C the number of eyes taken into consideration is 30. Expression of $Elav^{wt}$ (Fig. 3.2D) in Eyeful flies resulted in both extremes i.e. drastic reduction in the eye size with reduced folds and increased eye size with metastatic tumour growth in the retina. However, in the case of $ELAV^{NLSOH}$ expression (Fig. 3.2E), the flies seemed to have a reduction in the tumour formation in their eyes. This was observed as reduced eye size with no metastatic outgrowths or increase in the number of folds in the retina, which is found to be more suppressive in effect when compared with $Elav^{edr}$ (Fig 3.1 C).

On analysing the data for different Elav insertions, the expression of $Elav^{N26\text{-}N9}$ and $Elav^{N9}$ in Eyeful flies was found to be lethal, with all the flies expressing the respective GAL4/UAS found to be dead inside their pupated cases with relatively smaller eyes than the normal eye size. The expression of both $Elav^{Q2}$ and $Elav^{Q3}$ (Fig. 3.2F and G) more or less showed similar

effect on the Eyeful flies, with 80-90% showing a reduction in tumour formation in the retina and the eye size. It was also observed from the data that 13.3% of the flies expressing $Elav^{Q3}$ had excessive outgrowth in their retina. But considering the quantity of eyes (n=30) taken into consideration the number seems to be lesser than the minimal range required (n>=50) for performing a proper statistical analysis (Kurtosis (for normality) and Levene's test and one-way Anova (for P value significance)).

Overall analysis of both these data shows that different localization and insertions of Elav in the chromosome does have varying effects on the over-proliferating nature of the tumorous cells, with the nuclear localization Elav being found to be more inclined towards aiding in tumour over-proliferation and the Elav insertions forms $Elav^{N26\text{-}N9}$ and $Elav^{N9}$ being lethal to *Drosophila*. But further analysis is required to provide a strong statistical analysis on the current data that has been obtained.

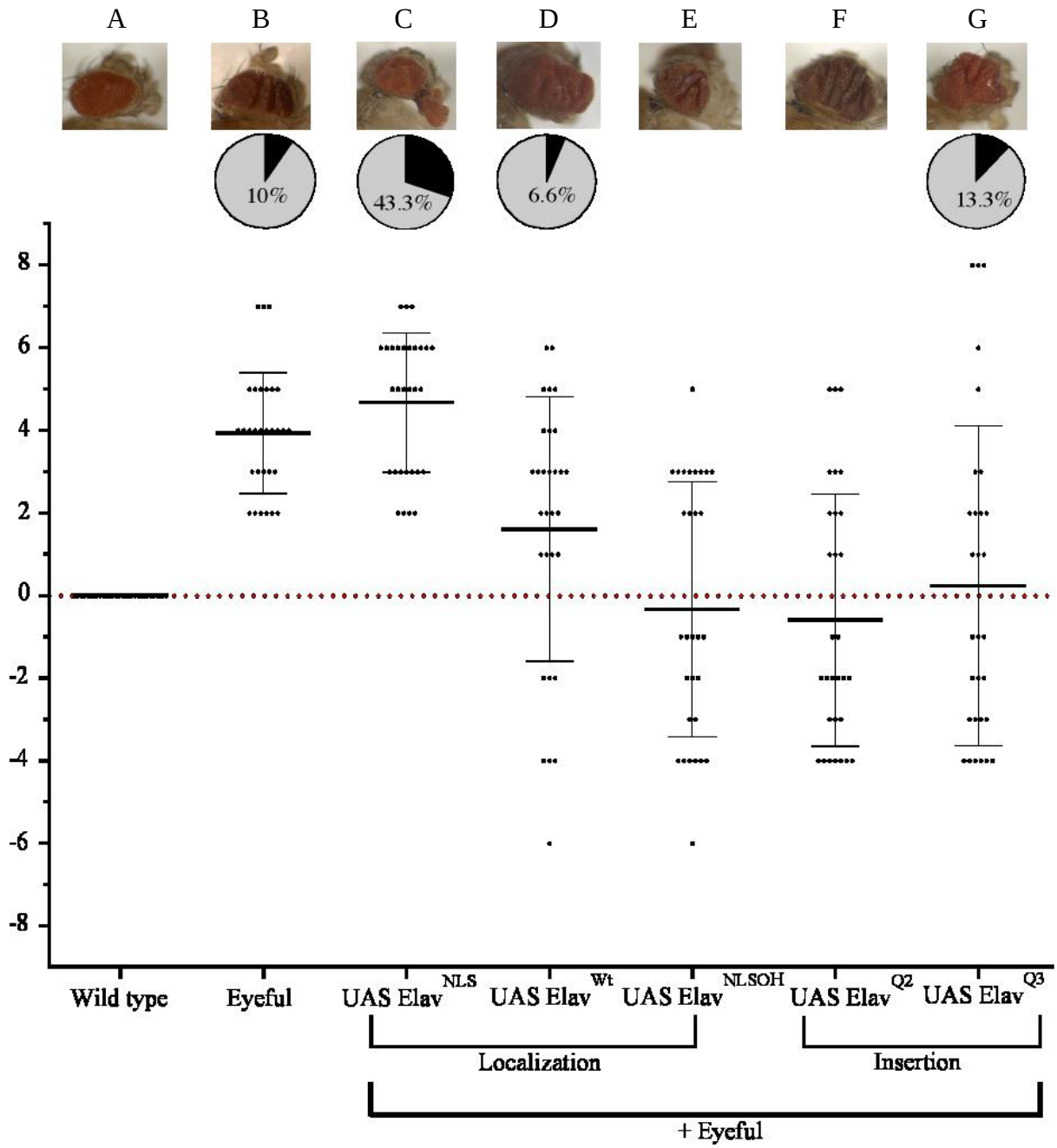


Figure 3.2: Quantitative and qualitative representation of the effect of different ELAV localization and insertions. The total number of flies taken into consideration was 30 (n=30), with the scoring being carried out as described in the table shown in materials and methods. Each individual point corresponds to an eye. The pie chart shows the incidence of metastasis, and any value above 6 was considered as metastasis, with the percentage being calculated against the total number of flies taken into consideration. In here, wild type is A, Eyeful is B, Eyeful + UAS ELAV^{NLS} is C, Eyeful + UAS Elav^{wt} is D, Eyeful + UAS Elav^{NLSOH} is E, Eyeful + UAS Elav^{Q2} is F and Eyeful + UAS Elav^{Q3} is G. Wild type and Eyeful were considered as controls, while UAS Elav^{NLS}, UAS Elav^{wt} and UAS Elav^{NLSOH} were considered for localization, and Elav^{Q2}, Elav^{Q3}, Elav $^{N26\text{-}N9}$ (data not show) and Elav^{N9} (data not shown) were considered for insertion effect. The images on the top of the graph represent the eyes of the individual genotypes.

3.3 Functional activity of tumour suppressors in *Drosophila* model with tumorous eyes:

To identify if functional loss or gain of tumour suppressors has an effect on the tumourous eyes of the 'Eyeful' flies, we expressed UAS Brat RNAi and UAS Rbf RNAi, for the loss of function (LOF) and UAS Brat, UAS Rbf (2^{nd} Chromosome) and UAS Rbf (3^{rd} Chromosome) for the gain of function (GOF) in the Eyeful flies. The scoring was carried out as described previously, with the wild type and Eyeful flies being used as controls (Fig. 3.3A and B).

Analysis of the data showed that knockdown of Brat and Rbf has different effects in the eyes of the adult flies. The loss of Brat (Fig. 3.3C) resulted in an increase in over-proliferation in the retina of the Eyeful flies. On comparing the data with the Eyeful control, it can be clearly seen that the loss of function of Brat seems to have more effect on the differentiating photoreceptor cells, as 22.5% of the flies with Brat knockdown showed metastatic outgrowth compared to the 7.5% flies of Eyeful control. However, loss of function of Rbf (Fig. 3.3D) showed an interesting result that was contrary to loss of Brat. Rbf loss resulted in reduction of tumour growth in the retina of the flies with 10% of the flies having no tumourous folds in their retina and having eyes resembling to that of a wild type.

Observation of the data for the ectopic expression, i.e. gain of expression of tumour suppressors in the eyeful model showed a reduction in the tumour folds in the retina. Even though both the tumour suppressors i.e. Brat and Rbf, reduced tumour development in the eyes, but the ability to act and inhibit over-proliferation was found to be more prominent by the expression of Rbf (Fig. 3.3F and G) than by Brat (Fig. 3.3E). It could also be seen from the data that irrespective of the placement of Rbf in the chromosomes, their expression seems to help in reducing the tumour growth i.e. 42.2% of flies expressing the 3^{rd} Chromosome Rbf and 30% of flies expressing the 2^{nd} Chromosome Rbf having eyes resembling to that of a wild type i.e. no tumourous folds in their retina.

Overall analysis of the loss and gain of function of tumour suppressors clearly shows that Rbf expression is important for the development of the photoreceptors and that Rbf expression in tumourous eyes can reduce the over-proliferation of the photoreceptor cells and help the eye size to reduce. But however, further analysis and an increase in the number of flies analysed is required to provide a strong statistical analysis on the reduction of eye size, as for now it can

only be assumed that the reduced eye size may be due to the tumourous cells exiting their cell-cycle.

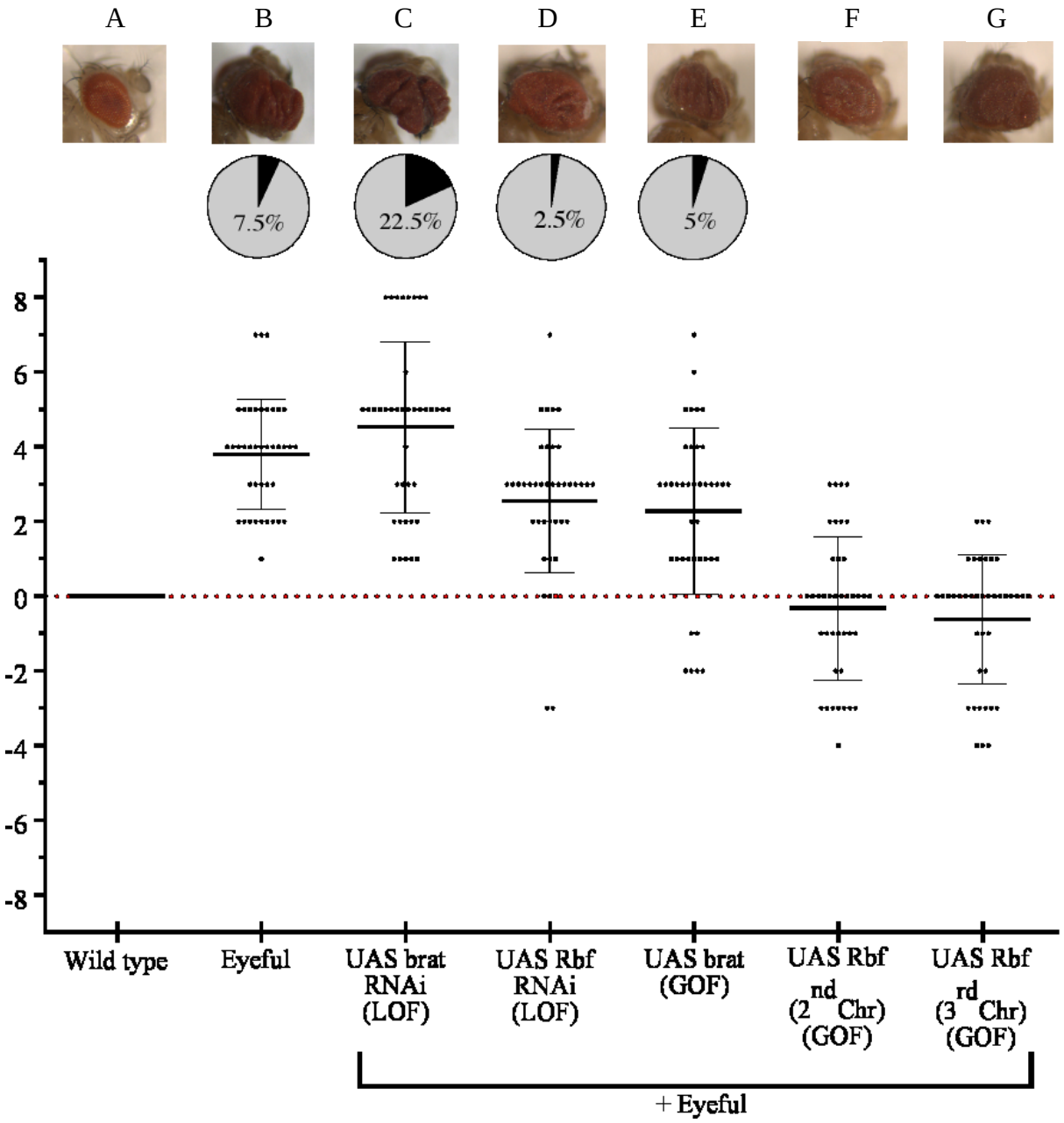


Figure 3.3: Quantitative and qualitative representation of loss and gain of function of tumor suppressors. The total number of flies taken into consideration was 40 (n=40), with the scoring being carried out as described in the table shown in materials and methods. Each individual point corresponds to an eye. The pie chart shows the incidence of metastasis, and any value above 6 was considered as metastasis, with the percentage being calculated against the total number of flies taken into consideration. In here, wildtype is A, Eyeful is B, Eyeful + UAS Brat RNAi is C, Eyeful + UAS Rbf RNAi is D, Eyeful + UAS Brat is E, Eyeful +UAS Rbf (2^{nd} Chr) is F and Eyeful + UAS Rbf (3^{rd} Chr) is G. Wild type and Eyeful were considered as controls, while RNAi lines were considered for loss of function (LOF) and the other genotypes are considered for gain of function (GOF). The images on the top of the graph represent the eyes of the individual genotypes.

3.4 Ectopic Elav expression does induce tumour growth in *Drosophila* with normal eyes:

To check if over-expression of Elav in flies with normal eyes could result in tumour growth in the developing photoreceptor cells, we expressed UAS $Elav^{NLS}$ in genotypes Eyeless GAL4 and Eyeless GAL4-UAS Delta. In here, $Elav^{NLS}$ was used instead of $Elav^{wt}$ for the ectopic expression analysis because $Elav^{NLS}$ has the nuclear localization sequence attached to it, which helps in transporting even the sub-cellular Elav to the nucleus and thereby increasing the activity of Elav. For comparison, the wild type, Eyeless Gal4 and Eyeless GAL4-UAS Delta were used as controls (Fig. 3.4A, B and C). It should be noted in here that in the Eyeless GAL4-UAS Delta genotype the over-expression of Delta results in the flies to have bigger eyes than normal wild type eye. It should also be noted that this genotype is different from 'Eyeful' Eyeless GAL4-UAS Delta because in 'Eyeful' flies apart from Delta over-expression of *lola* and *psq* also occurs, which is the reason for the flies to have over-proliferation in the retina.

Analysis of the data showed that ectopic expression of Elav in the developing eyes does alters the fate of the photoreceptor cells. It can observed that $Elav^{NLS}$ expression in Eyeless GAL4 flies (Fig. 3.4D) resulted in over-proliferation of the photoreceptor cells, which was clearly seen as tumourous folds in the retina, with 3.3% flies having a metastatic outgrowth from the retina. In addition to this, it was also observed that 20% of the progeny flies had a reduction in their eye size, but in those 20% too all the flies had tumourous folds in their retina.

However, data obtained for Eyeless GAL-UAS Delta with $Elav^{NLS}$ ectopic expression (Fig. 3.4E) showed a difference from Eyeless GAL4. In Eyeless GAL-UAS Delta with $Elav^{NLS}$ ectopic expression, 50% flies showed a reduction and 50% showed an over-growth in the eye size. But as Delta was found to already result in the in bigger eyes, the activity of Elav expression in here seems can be assumed to be more towards inhibiting the cell from evading apoptosis and leading them to undergo cell-cycle exit.

As the data seems to be only preliminary, it can only be assumed that Elav when ectopically expressed in a developing system shall result in over-proliferation, and when expressed alongside in a system already over-expressing Delta they shall counteract the over-proliferation activity and lead the cells to undergo cell-cycle exit. But however, to conclusively prove the above mentioned statement further investigation and statistical and

molecular analysis needs to be carried out to understand the significance of the interaction that undergoes between Elav and Delta.

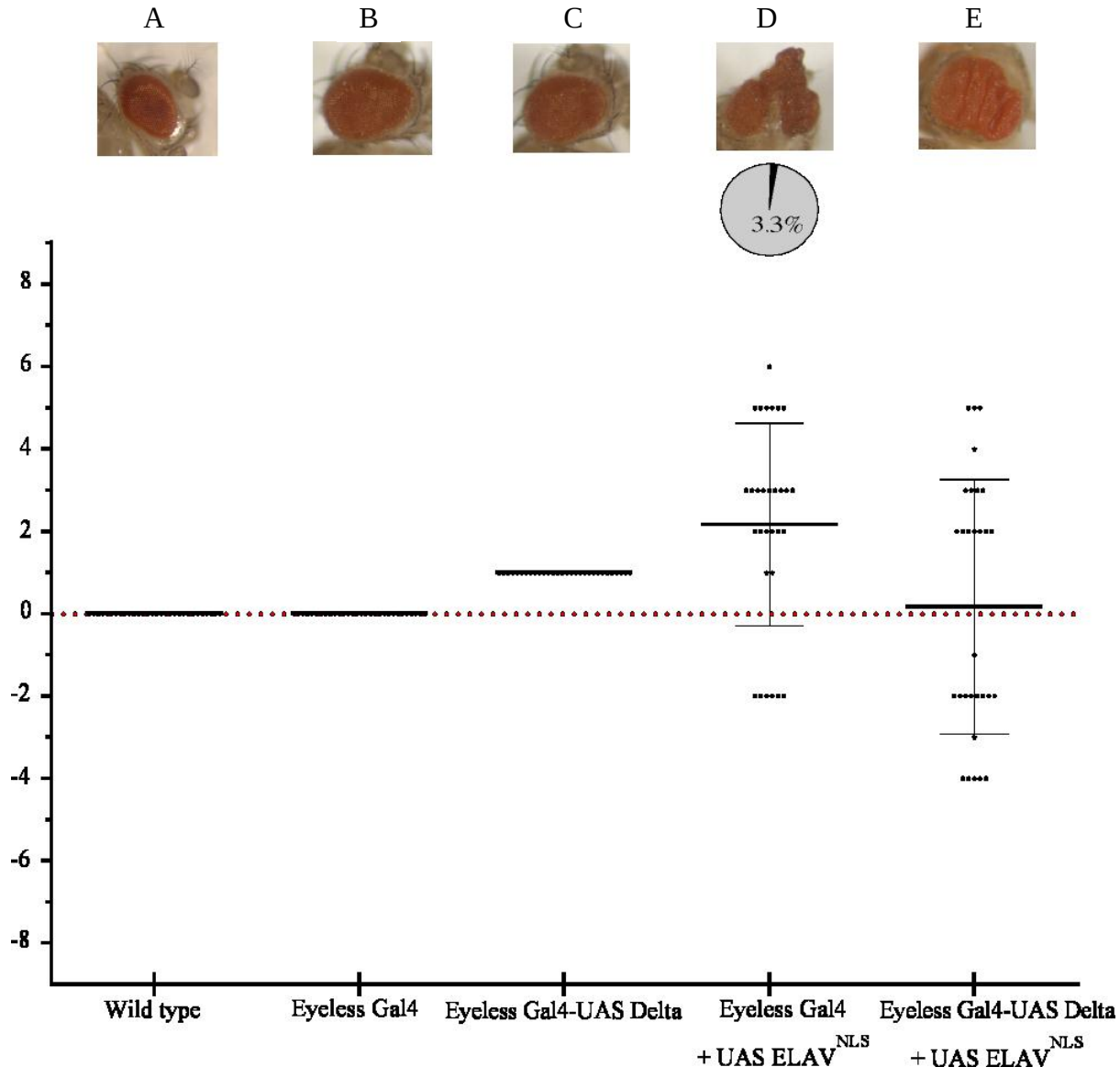


Figure 3.4: Quantitative and qualitative representation of tumour development ectopic ELAV expression in wild type *Drosophila* flies. The total number of flies taken into consideration was 30 (n=30), with the eye scoring being carried out as shown in table 1 in materials and methods. Individual points on the graph correspond to an eye. The pie chart shows the incidence of metastasis, and any value above 6 is considered metastasis, with the percentage being calculated against the total number of flies taken into consideration. In here, wild type is A, Eyeless GAL4 is B, EyelessGAL4 + UAS Delta is C, Eyeless GAL4 + UAS $Elav^{NLS}$ is D and Eyeless GAL4-UAS Delta + UAS $Elav^{NLS}$ is E. The images on the top of the graph represent the eye of each genotype.

Ectopic expression of Elav in neuroblast can induce tumorous development

3.5 Elav^{NLS} expression induces over-proliferation in the neuroblast and neuronal Gal4 drivers:

As ectopic expression of Elav seemed to induce over-proliferation in the developing photoreceptor cells, we wanted to check if ectopic Elav expression results in over-proliferation of the neuroblast or neuron or glia in the developing brain of *Drosophila*. To observe the effect that Elav may have during various stages of neurogenesis, we used the GAL4/UAS targeted expression system to ectopically express Elav in the differentiating neuroblasts and differentiated neurons and glial cells. To identify the effect of ectopic Elav expression, the neuroblast drivers- Inscuteable^{1407}GAL4, Asense GAL4, Prospero GAL4, Grainyhead GAL4, Worniu GAL4, Castor GAL4, Eyeless^{OK107}GAL4 and WorniuGAL4 AsenseGAL80, neuronal drivers – C309, Elav^{C155} and Eg3, and glial drivers – Repo GAL4 and Moody Gal4 were expressed with ;UAS GFP/Cyo;UAS Elav^{NLS}/ UAS Elav^{NLS}. UAS YFP with the above mentioned GAL4 drivers were used as controls (Fig. 3.5A and C).

Analysis of the data showed that Elav^{NLS} when ectopically expressed with neuroblast driver Inscuteable^{1407} GAL4 (Fig. 3.5B) and neuronal driver Elav^{C155} GAL4 (Fig. 3.5D) gave rise to tumours in the ventral nerve cord region in the *Drosophila* larval brain, which was clearly visible as outgrowths when compared to the controls (Fig. 3.5 A and C). Given the significance of the observation, repeats of Elav^{NLS} expression with Inscuteable^{1407} GAL4 and Elav^{C155} GAL4 drivers showed recurring over-proliferation in the VNC, with one in every 2 larvae showing an over-proliferation in the VNC of ectopic Elav^{NLS} expressing Insc^{1407}GAL4 and one in every 3 larvae showing an over-proliferation in the VNC of ectopic Elav^{NLS} expressing Elav^{C155} GAL4. Additional observation also showed that the range and location (Data in Appendix: Section VI and VII) of the outgrowth differed within and between Insc^{1407} and Elav^{C155} GAL4 lines. However, Elav^{NLS} when expressed with other neuronal/neuron/glial GAL4 drivers very rarely showed tumour growth either in the optic lobes or the ventral nerve cord regions of the larvae brain (Data in Appendix: Section VIII), except for neuronal drivers C309 and Eg3 and Glial driver Repo GAL4, where the expression of Elav^{NLS} was found to be embryo or larvae lethal.

These results indicated that expression of Elav during early stages of neuroblast differentiation or in the differentiated post-mitotic neurons can result in cellular over-proliferation and lead to tumourous outgrowths in the VNC.

Figure 3.5

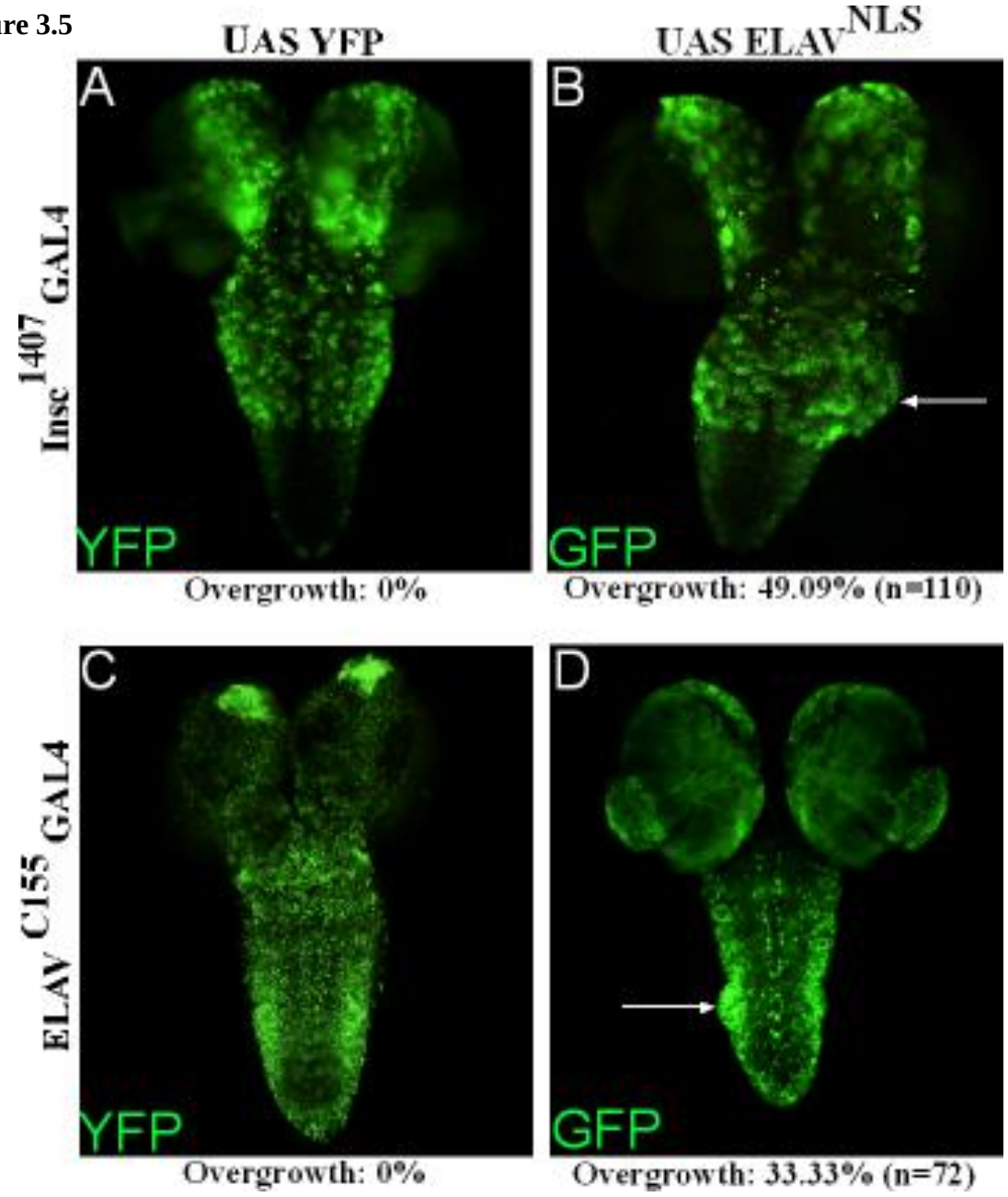


Table: 3.1

Driver (GAL4)	Reporter	Temperature	Number of brains	Number of brains with tumour	Percentage	Presence in the VNC	Average Size of Tumour
$Insc^{1407}$	UAS $ELAV^{NLS}$	25°C	110	54	49.09	Anterior	Medium
$ELAV^{C155}$			72	24	33.33	Anterior	Medium

Figure 3.5: $Elav^{NLS}$ expression showing over-proliferation in the ventral nerve cord region with neuroblast driver $Inscuteable^{1407}$ GAL4 and neuronal driver $Elav^{C155}$ GAL4. (A) and (C) shows the expression pattern of $Inscuteable^{1407}$ and $Elav^{C155}$ GAL4 drivers with UAS YFP, which are used as controls. (B) shows expression of UAS GFP/Cyo; UAS $Elav^{NLS}$ /UAS $Elav^{NLS}$ in $Insc^{1407}$ GAL4 with tumourous growth in the anterior side of the ventral nerve cord indicated by arrow. (D) shows expression of UAS GFP/Cyo; UAS $Elav^{NLS}$ /UAS $Elav^{NLS}$ in $Elav^{C155}$ GAL4 shows tumourous growth in the posterior side of the ventral nerve cord indicated by arrow.

Table 3.1: Shows the value, presence and size of tumour(s) observed in the neuroblast driver $Inscuteable^{1407}$ and neuronal driver $Elav^{C155}$ GAL4, when expressed with ectopic $Elav^{NLS}$. Elaborate table showing the scoring for the range and location of tumour in the VNC for all individual samples that showed tumour growth has been attached to the Appendix as section VI and VII.

3.6 Role of Elav localization in tumour development:

Although it became clear that ectopic expression of Elav during neurogenesis results in over-proliferation of neuroblasts/neurons in the VNC, but it was unknown if difference in localization of Elav had any effect on inducing tumours at the same proficiency. To investigate this, the neuroblast GAL4 driver Inscuteable^{1407}, which showed the highest tumour frequency, was chosen for the analysis and was expressed with UAS Elav^{wt} (nucleo-cytoplasmic in localization), UAS GFP/Cyo; UAS Elav^{NLS} /UAS Elav^{NLS} (nucleo-cytoplasmic localization attached with nuclear localization sequence), UAS Elav$^{NLS\ OH}$ (predominantly cytoplasmic in localization attached with nuclear localization sequence) and UAS Elav^{OH} (predominantly cytoplasmic in localization) individually. In here, Elav^{OH} is predominantly cytoplasmic in localization because of the deletion of sequence in the hinge region present between RRM2 and RRM3, and therefore attachment of nuclear localization sequence in Elav^{NLSOH} helps in the transport of the cytoplasmic Elav to the nucleus. Insc^{1407}GAL4 driving YFP expression was used as control (Fig. 3.6A). Staining was carried out with mAB HA, as all the UAS Elav forms have HA tag in their sequence.

It was observed from the results that irrespective of the localization of Elav in the cells, they resulted in over-proliferation in the VNC, which was visible either as tumourous outgrowth or internal overgrowth. However, the tumour outgrowth in the VNC differed between the type of Elav localization. Insc^{1407} progenies expressing Elav^{wt} (Fig. 3.6B) showed tumour outgrowths in the VNC region that was slight bigger to that observed in Insc^{1407}expressing ectopic Elav^{NLS} (Fig. 3.6C). In addition to this, localization of Elav in the cytoplasm through expression of Elav^{OH} (Fig. 3.2D) and Elav $^{NLS\ OH}$ (Fig. 3.2E) in Insc^{1407} GAL4 also resulted in tumour outgrowth. Although, cytoplasmic Elav resulted in tumour growth, but difference between Elav^{OH} and Elav$^{NLS\ OH}$ was quite obvious with Elav^{OH} having tumour outgrowth slightly bigger than that observed with Elav$^{NLS\ OH}$.

Even though the outcome of the results clearly demonstrates that different localization of Elav has different effect on tumour formation at the early stages of neurogenesis i.e. with nuclear Elav localization being more active and frequent in inducing tumour growths, but however further investigation and replication of the results, as number of larval brains analysed were low ($n<3$), needs to be carried out in the future to understand why nuclear Elav is more effective in inducing over-proliferation.

Figure 3.6

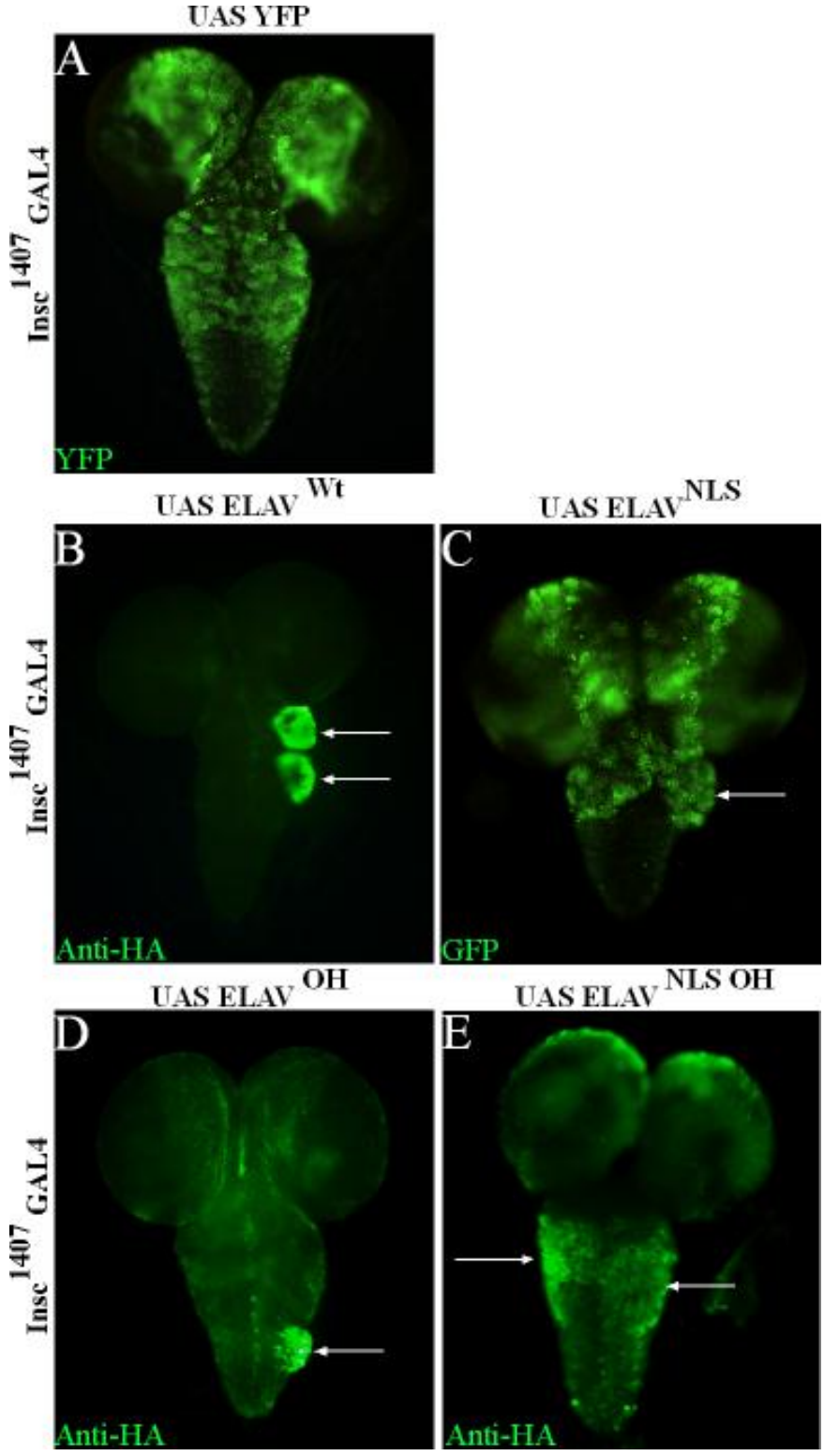

UAS YFP
A
Insc1407 GAL4
YFP
UAS ELAV Wt
B
Insc1407 GAL4
Anti-HA
UAS ELAV NLS
C
GFP
UAS ELAV OH
D
Insc1407 GAL4
Anti-HA
UAS ELAV NLS OH
E
Anti-HA

Figure 3.6: Elav localization in the development of tumour (n<3): (A) shows the expression pattern of Inscuteable in the larval VNC with UAS YFP, which was used as control. (B) and (C) shows the expression of UAS $Elav^{wt}$ and UAS $Elav^{NLS}$, which are nuclear in localization, with $Insc^{1407}$ GAL4. The difference in staining pattern between 3.6B and C is due to the staining pattern, as 3.6B is stained with Anti-HA, while in 3.6C $insc^{1407}$ is driving the expression of GFP and $Elav^{NLS}$ (D) and (E) shows the expression of UAS $Elav^{OH}$ and UAS $Elav^{NLS\ OH}$, which are cytoplasmic in localization, with $Insc^{1407}$ GAL4. The arrows represent the region of tumour outgrowth, which was observed to be more intense and frequent with the nuclear Elav than with cytoplasmic Elav.

3.7 Does the ectopic Elav induced tumour express endogenous Elav?

To determine if tumours induced by ectopic Elav express endogenous Elav in them, we expressed UAS Elav^{NLS} (nucleo-cytoplasmic in localization attached with nuclear localization sequence), UAS Elav^{-13} (nucleo-cytoplasmic in localization missing 13 amino acid antibody recognising epitope in the hinge region) and UAS Elav^{NLS-13} (nucleo-cytoplasmic in localization attached with nuclear localization sequence missing 13 amino acid antibody recognising epitope in the hinge region) with the neuroblast driver Inscuteable^{1407}GAL4. Larval brains expressing the GAL4/UAS were stained for anti-Miranda and anti-Elav to label the neuroblast and endogenous Elav, respectively. Insc^{1407} GAL4 on its own was used as control.

The results clearly showed a difference in the developing larvae brain upon expressing ectopic Elav. As observed previously, ectopic Elav expression in Insc^{1407} GAL4 resulted in tumour development in the larvae VNC with the different forms of Elav. The control, Insc^{1407} (Fig 3.7 A, B and C), shows the pattern of expression of endogenous Elav in the dorsal and ventral region of the larvae VNC. Upon individually analysing the scans of the three different Elav forms, it became clear that the tumours formed due to ectopic Elav expression did express endogenous Elav, where cells underwent over-proliferation. This is because immunostaining of the brain for miranda and Elav labels only the neuroblasts and the endogenous Elav in the larvae brain, respectively. As ectopic Elav^{NLS}, Elav^{-13}and Elav^{NLS-13} is devoid of a 13 AA antibody recognizing epitope, the anti-Elav immunostaining resulted in the identification of only the endogenous Elav, which in this case was seen to be expressed by the over-proliferating cells in the VNC region.

In the case of Elav^{-13} (Fig 3.7 D, E and F) and ELAV^{NLS} (Fig 3.7 G, H and I) expressing larval brains the tumour was visibly seen as an outgrowth in the VNC region and upon staining with anti-Elav it only labelled the endogenous Elav that didn't have the 13AA epitope missing in the hinge region. However, this was quite different in ELAV^{NLS-13} expressing larval brains (Fig 3.7 J,K and L), where the over-proliferation of the cells were seen to be present more towards the thoracic region in the VNC. This can be observed by comparing the dorsal scan image of Elav$^{NLS\ -13}$ (Fig 3.7 K) with the control image (Fig 3.7 B), where endogenous Elav expression seems to be much denser than the normal expression pattern of Elav.

Even though these data show that over-proliferating cells express endogenous Elav, but however further investigation needs to be carried out by staining the larval brains with anti-HA that shall label the ectopic Elav. The reason as to why anti-HA needs to be used is because the ectopic Elav are tagged with HA in their c-terminal. If all three staining are to be carried out together, then a conclusive data can be obtained on the endogenous Elav expression by the over-proliferating cells.

Figure 3.7

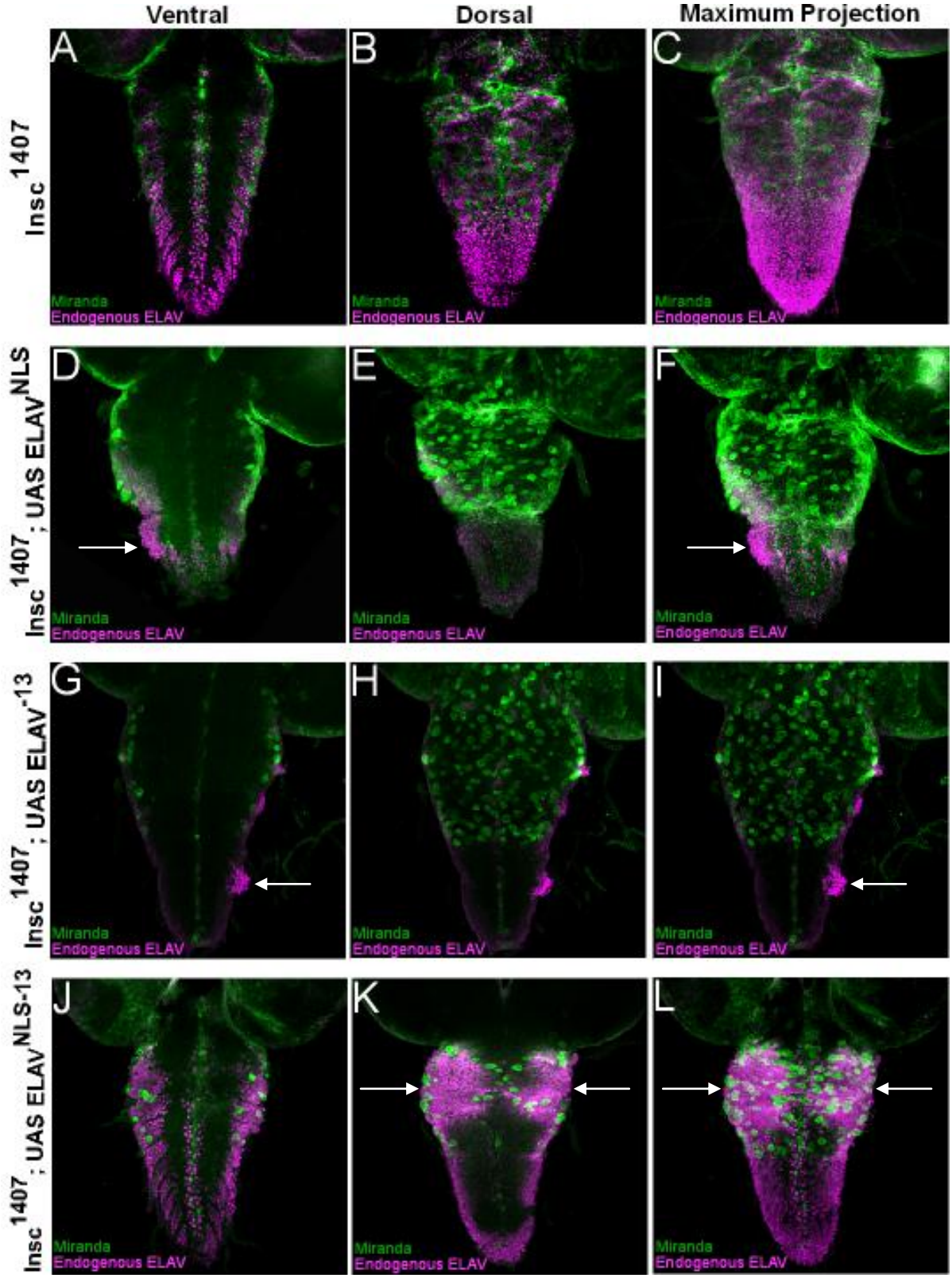

Ventral
Dorsal
Maximum Projection
Insc1407
Insc1407; UAS ELAVNLS
Insc1407; UAS ELAV-13
Insc1407; UAS ELAVNLS-13
A
B
C
D
E
F
G
H
I
J
K
L
Miranda
Endogenous ELAV

Figure 3.7: Endogenous Elav is expressed in the over-proliferating tumour cells. A, B and C shows the expression pattern of neuroblast (anti-Miranda - Green) and endogenous Elav (anti-Elav -Magenta) in Inscuteable^{1407}. D, G and J shows the ventral pattern of Inscuteable^{1407}GAL4 with ectopic Elav^{NLS}, Elav^{-13} and Elav^{NLS-13}, where the over-proliferating cells are found to express endogenous Elav (anti-Elav - Magenta). E, H and K shows the expression pattern of the neuroblast in the dorsal region of the ventral nerve cord in the larval brain, with K showing over-proliferation of the cells in Inscuteable^{1407} expressing ectopic Elav^{NLS-13} F, I and L shows the maximum projection of larval VNC (dorsal and ventral), which shows the presence of endogenous Elav expression in the over-proliferating region in the VNC.

3.8 Is the brat tumour suppressor important for Elav induced tumour formation?

To determine if Elav can induce tumour growth in a mutated tumour suppressor background, we chose to test ectopic Elav expression in Brat mutants, as it has been shown by Hilgers *et.al.* (2007) that Brat 3' UTR is regulated in a dose-dependent manner by Elav. To analyse this, we expressed ;Insc^{1407}GAL4;Brat^{11} with UAS Elav^{NLS} and UAS YFP, and ;Insc^{1407}GAL4; Brat^{K} with Df Brat and ;Df Brat; UAS Elav^{NLS}. In here, Brat^{11} and Brat^{K} are null alleles of Brat in the 3rd chromosome and Df Brat corresponds to deficiency encompassing Brat present in the 2nd chromosome. The larval brains expressing the GAL4/UAS were immunostained either with mAb HA (as UAS Elav^{NLS} has HA tag in its sequence) or mAb Miranda.

Immunostaining showed the homozygous brat mutants controls (Fig. 3.8 C) to have enlarged optic lobes, which is typical of cellular over-proliferation in the central brain due to loss of Brat function. It should be noted in here, that not all homozygous Brat mutants showed an over-proliferation in the optic lobes or VNC, as the effect of Brat mutation differs between larvae. When Elav^{NLS} was ectopically expressed in Insc^{1407}GAL4 with null mutant allele Brat^{11}, the tumour was found to be similar (Fig 3.8 B) to that of control (Fig 3.8 A). But however in the homozygous Brat mutants (Fig 3.8 D) this was quite different, as there was a reduction in tumour growth in both the VNC and optic lobe region of the brain, when compared to the control (Fig 3.8 C). In the homozygous Brat mutants, as Elav seem to have no effect in inducing tumour outgrowths; it can only be assumed that Elav interacts with null form of Brat via the 3' UTR and down-regulates its negative activity in the cell.

The data indicates that Elav interaction with Brat tumour suppressor during the early stages of neurogenesis is important for the fate of the precursor cells. But however, the reason as to why the homozygous Brat mutants had a reduction in their optic lobe size is quite unclear. Considering the above data as preliminary, further investigation and repetition of the results need to be carried out in the future. This should be carried out to not only increase the number of larval brains analysed (current analysis only involved the analysis of larval brain with $n<3$), but to also provide a better analysis that can quantify and provide a value on the percentage of reduction of over-proliferation in the optic lobes and the VNC of the larval brains.

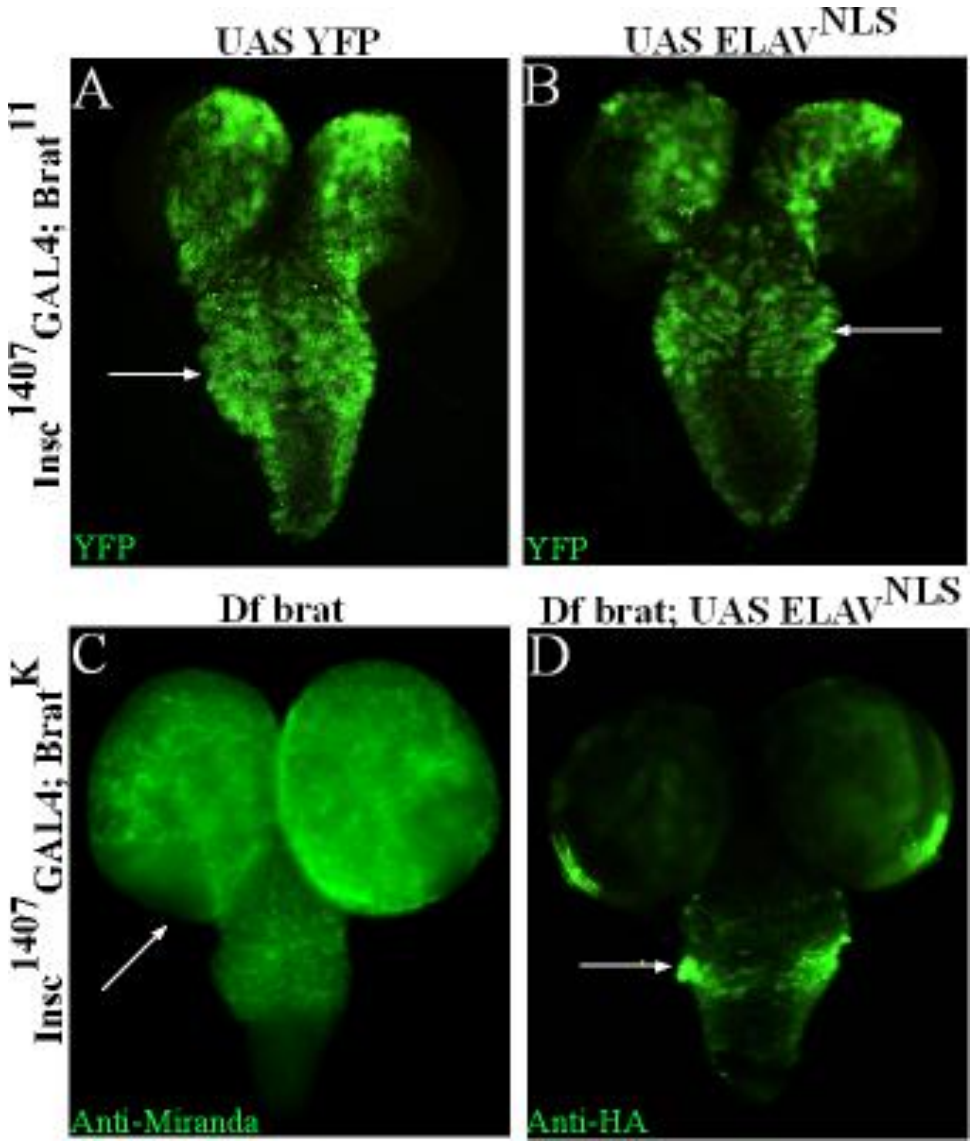


Figure 3.8: Effect of Elav on brat mutants (n<=3): (A) and (C) are the controls that shows that expression pattern of null brat mutant $Insc^{1407}Brat^{11}$ GAL4 and $Insc^{1407}Brat^{K}$ GAL4 with UAS YFP and Df Brat, respectively, where homozygous Brat mutants results in enlarged optic lobes (C - indicated by arrow). (B) shows the effect that UAS $Elav^{NLS}$ has on the Brat loss of function $Insc^{1407}$;$Brat^{11}$ GAL4, which seem to have resulted in over-proliferation in the ventral nerve cord (indicated by the arrows) similar to the control. (D) shows the homozygous Brat mutant effect i.e. Df brat; UAS $Elav^{NLS}$ with $Insc^{1407}brat^{K}$ GAL4 driver, which on a whole seems to have resulted in reduction in the tumourous outgrowths both in the optic lobes and VNC (indicated by the arrow).

3.9 Ectopic tumour suppressor expression in Elav induced tumours in differentiated neurons

To analyse the effect ectopically expressed tumour suppressors have on Elav induced tumours in the differentiated neurons, we generated genotypes $Elav^{C155}$GAL4;;UAS Brat and $Elav^{C155}$GAL4;;UAS Rbf and expressed them with UAS $Elav^{NLS}$. As the generated flies have balancers coupled with GFP, larvae expressing appropriate GAL4/UAS expression were identified by choosing those that didn't express GFP. UAS YFP and UAS $Elav^{NLS}$ with $Elav^{C155}$ were used as controls (Fig. 3.9A and B). Immunostaining of the larval brains were carried out with Anti-HA.

Results showed that expression of the tumour suppressor, Brat, had limited effect, when compared to the expression of Rbf in the larval brains. Even though tumour growth was present in both the genotypes due to the $Elav^{NLS}$ expression, the propensity to reduce over-proliferation and tumour growth was found to be more effective in the brains where Rbf (Fig. 3.9 E and F) was expressed than when Brat (Fig. 3.9 C and D) was expressed. The data also shows the brain to have a proportionate increase in the size of the optic lobes and that the cluster of cell present in the central brain region in the optic lobes of the control (Fig. 3.9 A) seemed to have partially disappeared when $Elav^{NLS}$ is expressed (Fig 3.9 B) and totally disappeared when $Elav^{NLS}$ is expressed alongside with brat (Fig 3.9 C and D) or Rbf (Fig 3.9 E and F). This disappearance of the cells in the optic lobes can be assumed to be due to cells undergoing differentiation/proliferation and may have been distributed over the lobes, which might have contributed to the increase in size of the lobes.

Observation of the overall result provides us with an answer that irrespective of the tumour suppressors i.e. brat or Rbf, when they are expressed in an Elav induced tumour system, their expression seems to induce tumour reduction, with the activity of Rbf being far more effective than brat. But however, this can't be considered as a conclusive data, because of the number of brains taken into account ($n<=3$). So, to provide a strong conclusive data further investigation is required to understand the fate of the cluster of cells present in the optic lobe region. In addition to this, analysis also needs to be done to identify the effect of ectopic tumour suppressor expression in $Elav^{NLS}$ expressing $Inscuteable^{1407}$ GAL4 system.

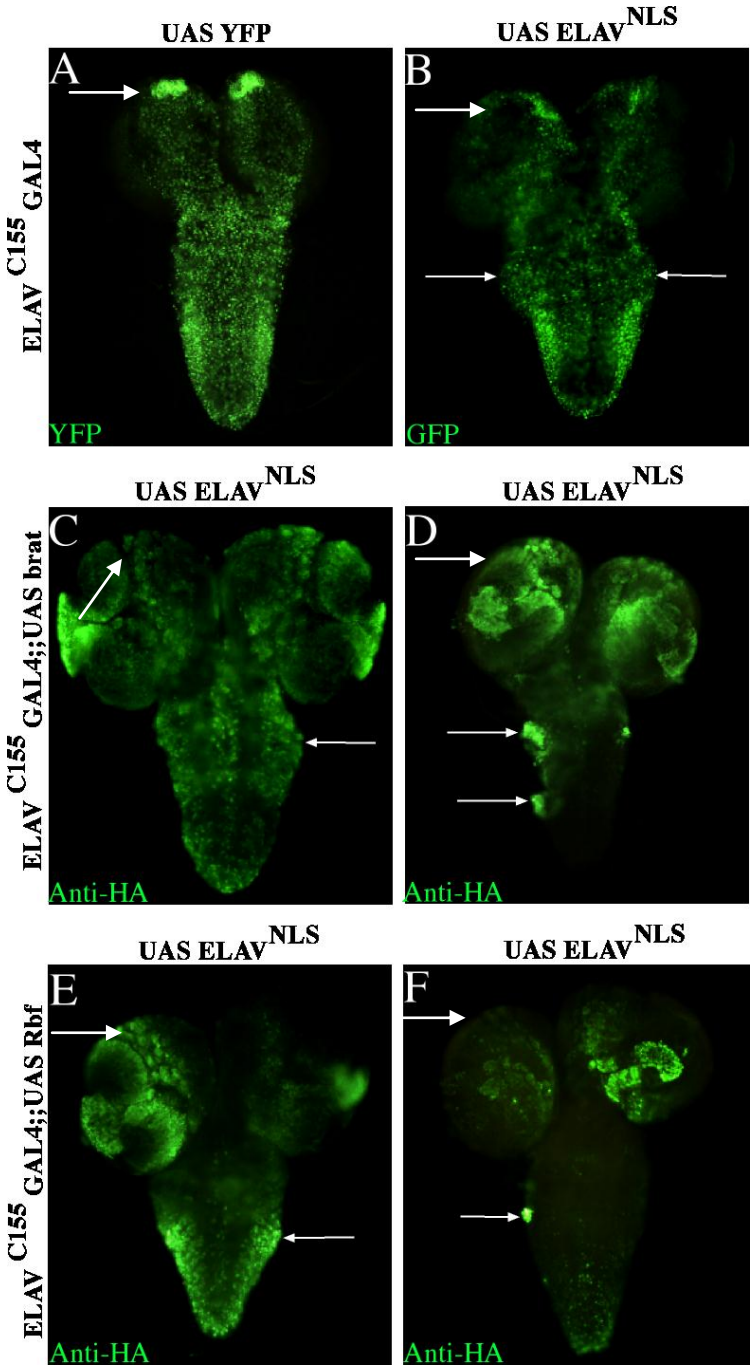


Figure 3.9: Tumour suppressor effect on Elav induced tumour in neurons. (A) shows the expression pattern of Elav^{C155} in the larvae brain when expressed with YFP. (B) shows expression of Elav^{NLS} results in formation of tumourous outgrowth in the ventral nerve cord, which is indicated by arrows. In here, (A) and (B) are used as controls for comparison. (C) and (D) shows the effect of ectopic tumour suppressor brat expression in Elav^{C155}-UAS Elav^{NLS} system, where they seem to have limited activity, as tumour outgrowths are still clearly visible in the VNC region, which are indicated by arrows. (E) and (F) shows the effect of tumour suppressor Rbf, a form of Retinoblastoma, expression in the Elav^{C155}-UAS Elav^{NLS} system, where they seem to have a positive effect, as they have reduced the tumour expression to a minimal level, which is indicated by arrows.

Chapter Four

DISCUSSION and CONCLUSION

Elav is a marker for the differentiated neurons in the developing *Drosophila* brain. Elav's expression, during developmental stages, is in such a way that Elav is expressed strongly only after neurons are born from the ganglion mother cell (Berger *et.al*, 2007). This unique expression of Elav made us want to test what effect it has on early neurogenesis and how different would the differentiating eye-primordium and neuroblast react to Elav expression. For this purpose, we used the GAL4/UAS targeted expression system in *Drosophila* to ectopically express Elav in the developing photoreceptor neurons, differentiating neuroblasts and differentiated neurons and glial cells.

4.1 Ectopic Expression of Elav causes excessive over-proliferation in the eyes of *Drosophila* model 'Eyeful':

To test the effect of ectopic Elav on developing photoreceptor neurons, we used a *Drosophila* model with tumourous eyes called 'Eyeful'. The Eyeful fly model has over-expression of *delta*, *lola* and *psq* in the photoreceptors which causes them to have tumour growth in the retina (Bossuyt *et.al*, 2009). It is this advantage of an *in-vivo* eye tumour model that made us chose 'Eyeful' *Drosophila* for Elav expression in early stages of eye development.

The data we obtained showed that gain of function of Elav i.e. expression of $Elav^{NLS}$ resulted in excessive over-proliferation, which was seen as an increase in the number of flies showing metastatic outgrowth in the retina. This increase in over-proliferating tumour cells may be due to the reason that ectopic expression of Elav during developmental stages might have disrupted the mitotic division and apical-basal protein distribution in an otherwise normally dividing photoreceptor neurons, which in turn could have led them to become self-renewal and tumourous in nature.

Another interesting result obtained from the analysis was the reduction of tumour growth in the retina of Eyeful flies due to $Elav^{edr}$ (loss of function of Elav). In general, $Elav^{edr}$ ($Elav^{eye\text{-}disc\ reduced}$) has an *elav* transgene insert which helps them to encode normal Elav expression in the neurons, but have reduced Elav expression in the photoreceptor cells (Koushika *et.al*, 1996). On correlating this to the result, it can be assumed that Elav indeed has a hidden role in the development of tumour and that the interaction(s) or mechanism they play with other RNAs may be the reason for the imbalance in tumourous photoreceptor cells.

But in both the cases i.e. GOF and LOF, the activity of Elav inside the tumourous system is only an assumption and thus considering the above obtained data as preliminary evidence further investigation needs to be carried out in the future to look into the molecular changes and interactions that actually occur in the over-proliferating cells, upon Elav expression.

4.2 Elav localization plays an important role in cellular over-proliferation in the eyes of *Drosophila* model 'Eyeful':

The analysis of the cytoplasmic and nuclear localization of Elav in the photoreceptors of Eyeful flies showed Elav localizing in the nucleus to be more effective in inducing tumour growth than Elav localizing in the cytoplasm.

The data of nuclear Elav i.e. $Elav^{wt}$ and $Elav^{NLS}$ with Eyeful showed variations between them on the basis of the ability to induce tumours i.e. $Elav^{NLS}$ being more effective in inducing metastatic tumour progression than $Elav^{wt}$. The reason as to why this variation might have occurred is because, $Elav^{wt}$ apart from being majorly localized in the nucleus are also distributed sub-cellular (Colombrita *et.al.*, 2013). But in $Elav^{NLS}$ the addition of NLS (nuclear localization sequence) helps in shuttling back the sub-cellular distributed Elav back to the nucleus thereby increasing the functional activity of Elav in the nucleus. It may be due to this variation that within the Elav nuclear localization genotypes there was a difference in the range of tumour inducing tendency.

However, $Elav^{NLSOH}$ (cytoplasmic in localization due to the deletion of sequences in the hinge region) which was shuttled back from the cytoplasm to the nucleus through the NLS attachment was not able to produce an effect similar to nuclear Elav. This was seen by 50% of the flies having a reduction in the eye size and tumourous folds in the retina. This observation brings out the point that the activity of Elav in the nucleus, where it is involved in alternative splicing and polyadenylation (APA) of mRNA, is far more effective in inducing tumour than the Elav present in the cytoplasm, where it is involved in promoting mRNA stability and translation of mRNA. As evidences have shown alternative splicing and polyadenylation to be important for proper gene expression during development (Di Giammartino *et.al.*, 2011) and oncogenic transformation (Mayr and Bartel, 2009), it can be that as Elav is involved in regulated APA-mediated 3'extensions in a dose-dependent manner (Hilgers *et.al.* 2012) their ectopic expression in the tumourous environment might have led them to act upon the

mRNAs excessively and cause imbalance in the system, which could have caused the cells to excessively over-proliferate.

In addition to this, varying Elav insertion in the chromosome ($Elav^{N26\text{-}N9}$ and $Elav^{N9}$ & $Elav^{Q2}$ and $Elav^{Q3}$) also had different effect on the *Drosophila*, with $Elav^{N26\text{-}N9}$ and $Elav^{N9}$ being lethal to *Drosophila* than $Elav^{Q2}$ and $Elav^{Q3}$. Even though the effect was different, the data showed that different insertion of Elav in different chromosomes does play a role in the tumour formation and have an effect on the viability of the *Drosophila*. But the reason as to why insertion of Elav at different sites in the chromosomes has different effect on the viability of the *Drosophila* is not clearly known and a proper explanation can't be arrived with this data.

From the results, it can be clearly seen that the site of insertion of Elav in the *Drosophila* genome and the localization of Elav in the system does have a major role in the activity and the fate of the over-proliferating cell.

4.3 Ectopic tumour suppressor expression does reduce tumours in the *Drosophila* model 'Eyeful':

In general, tumours have inactivation of tumour suppressor genes in them due to genetic or epigenetic changes, and it is this gene suppression that gives them the ability to over-proliferate (Dalmau *et.*al, 1992; Ferres-Marco *et.al*, 2006). In the Eyeful flies, the tumours formed in the retina have been found to have down-regulation of the Rbf gene, due to their hypermethylated state and the collaborative activity of epigenetic silencers and Notch (Ferres-Marco *et.al*, 2006). So in order to understand the effect of tumour suppressors, we carried out RNAi knockdown and ectopic expression of tumour suppressor Brat and Rbf in the Eyeful flies.

The RNAi knockdown of Brat and Rbf tumour suppressors showed difference in activity between them on the tumourous eyes of the Eyeful flies. While in Brat knockdown (Brat RNAi), the tumour development increased, but in Rbf knockdown (Rbf RNAi) it resulted in the reduction of tumourous folds in the eyes. The result has hinted that Brat is essential for the cell to divide in a controlled manner. It also shows that even in the over-proliferating tumour cells complete knockdown of Brat can leads to excessive tumour proliferation, which can be seen as increase in the tumorous folds in the eye. However, knockdown of Rbf, a form of

retinoblastoma protein, resulted in reduction of tumour progression that seems to be quite interesting. The reason may be that, as the Rbf function is disrupted due to its hypermethylation state in the 'Eyeful' flies (Ferres-Marco *et.al*, 2006), the restriction of formation of hypermethylated Rbf by introducing Rbf RNAi might have resulted in the cells to undergo an unknown mechanism that could have caused them to exit their cell-cycle and lead to the reduction of the tumourous folds in the retina. However, to understand the effect Brat and Rbf RNAi knockdown has on the expression of Elav in the Eyeful flies molecular analysis needs to be carried out in the future to clearly point out the effect RNAi knockdown has on normal Elav expression in the developing eye primordium.

Another area we looked into was the ectopic expression of Brat and Rbf (with different chromosome insertion sites) in the 'Eyeful' flies. The ectopic expression of Brat had minimal effect on reducing the tumour formation, but ectopic expression of Rbf (2^{nd} or 3^{rd} Chromosome insertion) showed a considerable reduction in the tumour formation in the eyes of the Eyeful flies. This result helps us assume that Brat may be essential only during early cellular differentiation/ proliferation, but Rbf is the protein that may be required in the cell for their survival after differentiation/proliferation. But however, further investigation is required in here to provide a conclusive statement on the importance of Brat and Rbf in the over-proliferating system.

4.4 Ectopic expression of Elav can cause Eyeless GAL4 *Drosophila* flies with normal eyes to have eyes with tumorous over-proliferation:

To check if ectopic expression of Elav in *Drosophila* with normal eyes could result in tumour growth, we expressed $Elav^{NLS}$ in Eyeless GAL4 and Eyeless GAL4-UAS Delta flies. The reason as to why ectopic Elav expression was carried out in the eye primordium is due to the fact that apart from neurons the Elav protein is expressed in photoreceptor cells. In both, the expression resulted in the photoreceptors to over-proliferate and form tumourous folds in the retina of the eyes and to also have a reduction in the size of the eyes. As Eyeless helps in determining the fate of the cells in the eyes, it can be seen that ectopic expression of Elav has resulted in the cells to become over-proliferative in nature. In here, the reason that can be assumed is that Elav's activity of alternative splicing and polyadenylation could have disturbed the distribution levels of the proteins in the cells and might have rendered the daughter cells to become self-renewal and tumourous in nature.

In addition to this, when $Elav^{NLS}$ was expressed in Eyeless GAL4-UAS Delta genotype (flies with sensitized eyes due to Delta over-expression) about 50% of the progeny showed reduction in the eye size. The only assumption that can be made in here is that Elav could have acted alone or paired with other transcription factor(s) and could have down-regulated the activity of Delta in the cells, thereby making the cells to lose their tumourous nature and undergo cell-cycle exit. But however, further studies needs to be carried out to understand if there is an interaction between Delta and Elav to provide a reason for the reduction in the eye size of EyelessGAL4-UAS Delta flies expressing $Elav^{NLS}$.

4.5 Ectopic Elav expression induces neuroblasts in the larvae VNC to over-proliferate:

As ectopic expression of Elav in the developing photoreceptor neurons resulted in the cells to be over-proliferative, we wanted to test if ectopic Elav expression has any effect on the differentiating neuroblasts, during early neurogenesis, or differentiated neurons or glial cells in the larval brain.

Our data demonstrated that Elav expression during early stages of neurogenesis does result in the formation of tumours in the ventral nerve cord of the brain of the developing larvae. Even though various drivers like Asense GAL4, Castor GAL4, Prospero GAL4, Scabrous GAL4, $Eyeless^{OK107}$ GAL4 and Grainyhead GAL4 rarely showed tumour formation in the VNC, but we observed tumour occurrences to be more frequent only with the neuroblast driver $Inscuteable^{1407}$ GAL4 and neuronal driver $Elav^{C155}$ GAL4.

The reason as to why tumour development might have occurred in the neuroblast driver $Inscteable^{1407}$ GAL4 is because inscuteable is a protein that is expressed in neuroblast undergoing asymmetric cell division. During mitotic division, inscuteable is responsible for (i) activity of apical-basal orientation of the spindle fibres (ii) perpendicular division of the neuroblast from the surface and (iii) distribution of Brat, Numb, Prospero and Miranda to the basal daughter cell, i.e. GMC (Kraut *et.al* 1996). On correlating these points together, it can be assumed that Elav expression at the time of Inscuteable the dividing neuroblast might have resulted in the disruption of the apical-basal spindle fibre distribution. This could have led to the misorientation of important cell-cycle markers like Brat and Prospero, which may have led the neuroblast to undergo symmetric division rather than an asymmetric division and cause both the daughter cells to attain a neuroblast fate and result in over-proliferation in the

VNC. The reason as to why the above mentioned could be a possibility is because Betschinger *et.al* (2006) had previously reported asymmetric segregation of Brat to result in the differentiating daughter cells to act as neuroblast than be oriented as GMC.

Another result observed was the development of tumours with the neuronal driver $Elav^{C155}$ GAL4. Neurons are cells formed from the basal GMC upon final differentiation and it is only at this point Elav starts to become strongly expressed in them for the very first time. But our analysis shows that if Elav is expressed in excess in the neurons through $Elav^{C155}$ GAL4 they do result in formation of tumours in the VNC of the larval brain. Although the reason as to why this occurred in neurons (i.e. a cell that normally expresses Elav, has exited cell cycle and doesn't differentiate) requires further investigation, but the only explanation that can be presented is that the presence of excessive Elav might have changed the protein balance within the system. This in turn could have suppressed the function of tumour suppressor like Brat, which might have led the neurons to become self-renewal and result in tumour formation in the VNC.

4.6 Elav localization does change the intensity of tumour development in the VNC:

Preliminary data between nuclear and cytoplasmic localization forms of Elav showed the nuclear localization form of Elav ($Elav^{wt}$ and $Elav^{NLS}$) (Fig 3.6 B and C) to have an increase in tumour growth in the ventral nerve cord than the cytoplasmic localization form of Elav ($Elav^{OH}$ and $Elav^{NLS\ 0H}$) (Fig 3.6 D and E). The ability of the nuclear localization form of Elav to induce increased over-proliferation may be due to their active nature in the nucleus where they are involved in transcriptional mechanisms like alternative splicing and polyadenylation. However, as cytoplasmic Elav ($Elav^{OH}$ and $Elav^{NLSOH}$) used in here is inactive, but their ability to still induce tumour growth was quite unexpected. The reason as to why the cytoplasmic Elav may be functionally less active is not because of the mutational deletion of the octapeptide in the hinge region, which was used to produce $Elav^{OH}$ and $Elav^{NLSOH}$, but it's due to their localization to the cytoplasm (Yannoni and White, 1999). In this case, the ability of $Elav^{OH}$ and $Elav^{NLSOH}$ to induce tumour growth might have been because of the fact that even through Elav is cytoplasmically localized, there are still tracer amount of Elav that is nuclear bound (Yannoni and White, 1999). It may be the residual function of this nuclear Elav that could have caused the neuroblasts to undergo over-proliferation and result in tumour growth. But however this is just an assumption in here with regards to a previously published

data (Yannoni and White, 1999). Therefore in the future further investigation needs to be carried out in this regard by staining the larval brains with anti-HA to find out if Elav is present in the nucleus to confirm if over-proliferation in $Elav^{OH}$ and $Elav^{NLSOH}$ expressing larval brain is actually due to tracer nuclear Elav or due to any other unknown factor.

From the observation it can be understood that nuclear Elav are more active in the cells than its counterpart i.e. cytoplasmic Elav and that imbalance in the differentiating neuroblast can lead them to over-proliferate during early stages of neurogenesis. But to arrive to a concluding remark, as stated before, further investigation needs to be carried out.

4.7 Tumours formed due to Ectopic Elav do express endogenous Elav:

As we wanted to find out if the tumours observed in the neuroblast driver $Inscuteable^{1407}$ GAL4 expressed endogenous Elav, we expressed $Elav^{-13}$ and $Elav^{NLS-13}$ with $Inscuteable^{1407}$ and immunostained the brains with anti-Miranda and anti-Elav. The data showed the presence of endogenous Elav in the tumours induced by ectopic expression of Elav in the neuroblasts. The reason as to why it can be said that endogenous Elav expression was found in the tumours was because (i) $Elav^{-13}$ and $Elav^{NLS\ -13}$ has a deletion of 13 AA epitope between RRM1 and RRM2, which is the region that is normally recognized by the mAbs raised against endogenous Elav (Yannoni and White, 1999), (ii) mAbs are very specific and are never found to be cross reactive with any other protein(s), as they are normally raised against a target protein and (iii) the expression of endogenous Elav was found to be present exactly in the region of tumour growth or region where over-proliferation occurred inside the VNC. All these points when brought together helped us assume that the tumour cells did express endogenous ELAV in them.

The data we generated has now raised a new question as to whether the tumour cells undergoing over-proliferation are neuroblasts that give rise to neurons that express Elav or is it the differentiated neurons that undergoes over-proliferation through self-renewal that expresses Elav. As a clear view on this is not available, this would require further investigation in the future by tagging the cell with specific antibodies that could differentiate dividing cells from non-dividing cells, and the endogenous Elav (Anti-Elav) from the ectopically expressed Elav (Anti-HA). In addition, the investigation should also analyse the effect of ectopic expression of $Elav^{-13}$ and $Elav^{NLS-13}$ with $Elav^{C155}$ so as to answer the

question as to whether the tumour arises from the same cells or different cell type upon ectopically expressing Elav with Inscuteable^{1407} and Elav^{C155} GAL4.

4.8 Elav induces tumour formation in the VNC region of Brat mutant phenotypes:

Analysis of the data that tested if Elav can induce tumour in Brat loss of function mutants showed promising results that helped in assuming the type of neuroblast that might have undergone over-proliferation. The data showed that UAS Elav^{NLS} when expressed with ;Inscuteable^{1407} GAL4;Brat^{11} or ;Df Brat; Elav^{NLS} when expressed with ;Inscuteable^{1407} GAL4;Brat^{K} resulted in an increase in the formation of tumour in the ventral nerve cord region.

It has been previously reported (Betschinger *et.al*, 2006) that in a genotype with loss of Brat function (i.e. Brat^{11} or Brat^{K}), the Brat is not asymmetrically segregated along with Prospero to one of the daughter cells produced from neuroblast during mitosis. This causes both the daughter cells to gain self-renewal property and become tumourous in growth. Although Brat is found in all parts of the embryo and larval nervous system, but mutation of Brat resulted in over-proliferation of the cells only in the central brain region, but not in the ventral nerve cord region of the larval brain. This activity has been mainly associated with region specific cell-cycle control difference or an unknown mechanism(s) actively suppressing the Brat mutant phenotype in the VNC (Bello *et.al* 2006).In addition, it has also been shown (Neumuller and Knoblich, 2009; Saini and Reichert, 2012)that the medial posterior surface of the central brain in the lobes is a region which hosts type II neuroblast that helps in increase of brain size during later part of the *Drosophila* development, and rest of the central brain region and the ventral nerve cord hosts type I neuroblast. On comparing the results we obtained from the Brat mutants with these previously published data, it can be assumed that the type of neuroblast undergoing over-proliferation may be the type I neuroblast. Even though this is an assumption, further analysis needs to be carried out in the future via immunostaining to differentiate the type I from type II neuroblast, and this can be carried out by staining with anti-Deadpan and anti-Asense. Anti-Deadpan helps differentiate the neuroblast from the ganglion mother cells, and the anti-Asense helps differentiate the type I from type II neuroblast, as type I neuroblast have Asense in the nucleus, while type II neuroblast has it missing from the nucleus. If further investigation is to be carried out with the above mentioned staining, it can pointed out and confirmed that it is indeed the type I neuroblast that

fails to segregate properly and gives rise to tumour growth in the ventral nerve cord region of the larval brain.

4.9 Rbf as a tumour suppressor actively reduces over-proliferation in differentiated neurons:

To analyse the effect of ectopic tumour suppressor expression in the differentiated neurons that underwent over-proliferation due to over-expression of Elav, we expressed Brat and Rbf with the neuronal driver $Elav^{C155}$ GAL4. The preliminary data we obtained showed the activity of Rbf to be more efficient in reducing over-proliferation in the VNC region than Brat (Fig 3.9). Brat is a protein that is expressed during early differentiation or proliferation stages. Brat's activity is more important during early stage of neurogenesis and it may be due to this reason that they were not effective in reducing over-proliferation of the neurons. But however on contrary, Rbf seems to be prominently expressed only after proliferation and are crucial role for cell survival. It is this difference in activity between Brat and Rbf that may have resulted in the tumourous cells expressing ectopic Rbf to be more efficient in exiting their cell-cycle. Apart from this it has also been reported (Ferres-Marco *et.al*, 2006) that in tumourous cells the Rbf is in a hypermethylated form, which is a mutant form of Rbf that is inactive inside the cell. Therefore ectopic expression of normal Rbf in the tumour cells might have interacted differently and could have led the cells to exit their cell cycle either via apoptosis or an unknown mechanism. However, this is not a conclusive statement and is just an assumption from the data obtained. Therefore, to confirm this further investigation needs to be carried out to provide sufficient evidence that can confirm the above mentioned statement with suitable statistical proof, as in the preliminary data the number of brains observed were of minimal quantity ($n<=3$). On a whole, this analysis has shown that even though tumour suppressors are present in the cell, they are indeed expressed at different timeframe and have difference range of activity in the cell i.e. Brat as a tumour suppressor is important during early stages of neurogenesis and Rbf as a tumour suppressor is important for the survival/stability of the cell. In addition to the data generated, in the possible future an analysis also needs to be carried out to look at the activity of ectopic tumour suppressor expression in $Inscuteable^{1407}$ GAL4 expressing ectopic Elav.

CONCLUSION:

Our analysis on expressing ectopic Elav during early stages of neurogenesis clearly showed Elav to have an effect on the differentiating neuroblasts / photoreceptors neurons, during early neurogenesis. The only possible assumption that can be assumed for the formation of tumour in the eyes of the adult and in the VNC of the larval brains is that Elav's mis-expression might have caused disturbance in the apical/basal protein distribution in the neuroblast and this could have led the neuroblast to give rise to daughter cells that are capable of self-renewal and becoming over-proliferative. The reason for this assumption is because during normal neuroblast division, the asymmetric division results in proper segregation of the apical/basal protein between the dividing cells so that one becomes a neuroblast and another becomes the ganglion mother cell (Kraut *et.al* 1996). If and only if this process is disturbed, the division leads to improper segregation of important cell-cycle proteins like Brat, Prospero, numb and Miranda that result in both the daughter cells attaining a neuroblast fate (Betschinger *et.al*, 2006). As the current data obtained has only led us to an assumption, further analysis needs to be carried out in the future in order to confirm if it is really due to apical/basal protein disruption or is some other factor, to find out the cell-cycle protein that has been mis-interacting with Elav and to find the mechanism or interaction that leads to the formation of tumours in the eyes and in the VNC of the larval brain. In addition to the above, we also found that in the neuroblast and eye primordium, the nuclear localization form of Elav was more active in causing tumour formation, and that even tracer amount of nuclear Elav is sufficient to make the cells tumourous. Observation with Brat mutants showed the tumourous outgrowths in the VNC may be due to type I neuroblast over-proliferation and that the over-proliferating cells were capable of expressing endogenous Elav, but however staining with anti-Asense and anti-Dpn is required in future to confirm the type of neuroblast undergoing over-proliferation. Apart from this, it was also observed that tumour outgrowths were able to be reduced by ectopically expressing tumour suppressor Rbf, thereby hinting that Rbf as a tumour suppressor is more effective than brat in over-proliferating cell. However in all these cases the mechanism and interaction that is involved in causing the over-proliferation is unknown and it is a question that can be answered only through further investigation.

APPENDIX:

Section I: Scoring table of the Eyeful *Drosophila* model expressing ectopic $Elav^{NLS}$ and $Elav^{edr}$ for gain and loss of function of Elav.

Wild type (N=40)	Eyeful (N=40)	Eyeful + UAS $Elav^{edr}$ (LOF) (N=40)	Eyeful + UAS $Elav^{NLS}$ (GOF) (N=40)
0	4	1	3
0	2	0	2
0	4	1	-2
0	2	1	5
0	4	1	2
0	5	3	2
0	5	2	5
0	2	3	-1
0	3	1	2
0	3	3	2
0	4	3	8
0	5	3	4
0	5	2	5
0	4	3	8
0	2	0	3
0	3	3	5
0	4	1	5
0	2	1	3
0	2	1	2
0	3	1	-2
0	4	1	5
0	2	1	2
0	2	5	2
0	1	3	5
0	4	3	-1
0	4	3	2
0	5	1	2
0	4	1	8
0	3	3	4
0	5	1	2
0	5	4	4
0	5	5	5
0	7	4	6
0	5	3	8
0	2	3	2
0	4	1	6
0	7	4	3
0	4	2	8
0	4	2	2
0	7	3	5

Section II: Scoring of Eyeful *Drosophila* flies expressing tumour suppressors Brat and Rbf for the loss (LOF) and gain of function (GOF).

Wild type (N=40)	Eyeful (N=40)	Eyeful + UAS brat (GOF) (N=40)	Eyeful + UAS brat RNAi (LOF) (N=40)	Eyeful + UAS Rb2 RNAi (LOF) (N=40)	Eyeful + UAS Rb (2nd Chr) (GOF) (N=40)	Eyeful + UAS Rb (3rd Chr) (GOF) (N=40)
0	4	3	3	1	0	0
0	2	1	0	5	0	1
0	4	3	2	0	3	1
0	2	1	5	3	0	-4
0	4	3	3	4	0	0
0	5	3	1	2	1	0
0	5	4	0	1	0	-3
0	2	-1	5	0	-3	0
0	3	1	2	2	-3	0
0	3	3	3	-1	0	-2
0	4	3	0	2	0	-4
0	5	5	1	5	-1	0
0	5	1	3	5	0	-1
0	4	2	3	1	0	0
0	2	5	3	-2	-3	0
0	3	1	3	-2	-3	1
0	4	3	2	0	3	0
0	2	5	4	-1	-1	0
0	2	4	3	5	-1	-3
0	3	2	3	5	0	0
0	4	5	3	2	2	1
0	2	3	4	1	2	1
0	2	4	3	5	3	2
0	1	-2	3	2	1	-3
0	4	3	4	1	2	0
0	4	1	3	4	3	0
0	5	-2	5	5	-1	-3
0	4	-2	3	2	-3	0
0	3	3	2	2	-1	-3
0	5	3	7	5	-1	-2
0	5	3	5	-1	0	-4
0	5	-2	-3	0	-2	2
0	7	6	4	2	2	-1
0	5	1	2	-2	0	0
0	2	1	3	2	-3	0
0	4	1	2	0	-1	2
0	7	4	2	5	-4	0
0	4	7	-3	5	-3	-1
0	4	3	3	2	-2	-3
0	7	-1	1	8	1	1

Section III: Scoring table of Eyeful *Drosophila* flies expressing different forms of nuclear, cytoplasmic and chromosome insertion sites of Elav.

Wild type (N=30)	Eyeful (N=30)	Eyeful + UAS $Elav^{wt}$ (N=30)	Eyeful + UAS $Elav^{NLS}$ (N=30)	Eyeful + UAS $Elav^{NLS\ OH}$ (N=30)	Eyeful + UAS $Elav^{Q\ 2}$ (N=30)	Eyeful + UAS $Elav^{Q\ 3}$ (N=30)
0	4	6	3	3	-2	2
0	2	6	-4	-4	2	-4
0	4	3	4	3	1	-3
0	2	3	5	-3	-2	8
0	4	5	4	3	-4	-4
0	5	3	3	2	3	-2
0	5	2	3	2	5	-3
0	2	6	-4	-2	5	-1
0	3	2	2	-4	-3	2
0	3	7	5	3	-4	-2
0	4	6	1	-3	-2	-3
0	5	5	4	-4	5	1
0	5	6	1	-4	-4	-1
0	4	7	2	3	-4	5
0	2	5	2	2	-4	-4
0	3	6	2	5	2	6
0	4	7	6	2	3	-4
0	2	3	-2	-6	-4	3
0	2	3	-4	3	1	-3
0	3	6	3	-2	2	1
0	4	6	1	-1	-2	2
0	2	3	3	-4	-3	2
0	2	2	1	-4	-3	-2
0	1	3	3	3	-2	8
0	4	6	6	-1	-4	-4
0	4	2	-2	-1	-2	-4
0	5	5	-2	-2	1	3
0	4	6	5	3	3	1
0	3	5	3	-1	-1	-1
0	5	5	-6	-1	-1	8

Section IV: Scoring table of Eyeless GAL4 and Eyeless GAL4-UAS Delta *Drosophila* flies expressing ectopic Elav.

Wild type (N=30)	Eyeful (N=30)	Eyeless GAL4 (N=30)	Eyeless GAL4 UAS Delta (N=30)	Eyeless GAL4 + UAS $Elav^{NLS}$ (N=30)	Eyeless GAL4-UAS Delta + UAS $Elav^{NLS}$ (N=30)
0	4	0	1	-2	-2
0	2	0	1	2	-1
0	4	0	1	-2	-4
0	2	0	1	-2	-3
0	4	0	1	-2	2
0	5	0	1	-2	2
0	5	0	1	-2	2
0	2	0	1	3	3
0	3	0	1	1	2
0	3	0	1	2	2
0	4	0	1	1	-4
0	5	0	1	3	5
0	5	0	1	3	4
0	4	0	1	3	3
0	2	0	1	2	-2
0	3	0	1	6	-4
0	4	0	1	2	-2
0	2	0	1	3	5
0	2	0	1	2	-2
0	3	0	1	3	-2
0	4	0	1	5	2
0	2	0	1	5	-2
0	2	0	1	3	3
0	1	0	1	2	-2
0	4	0	1	5	2
0	4	0	1	5	3
0	5	0	1	5	-4
0	4	0	1	3	5
0	3	0	1	5	-2
0	5	0	1	3	-4

Section V: Scoring table of the different drivers that showed tumour growth in the VNC region when $Elav^{NLS}$ was ectopically expressed.

Driver (GAL4)	Reporter	Temperature	Number of brains	Number of brains with tumour	Percentage	Presence in the VNC	Average Size of Tumour
$Insc^{1407}$	UAS $ELAV^{NLS}$	25°C	110	54	49.09	Anterior	Medium
$ELAV^{C155}$			72	24	33.33	Anterior	Medium
Scabrous			54	1	1.85	Anterior	Small
Grainyhead			39	1	2.56	Anterior	Small
Asense			40	1	2.50	Posterior	Small
Castor			32	1	3.13	Posterior	Medium
Prospero			8	1	12.50	Anterior	Medium
Worniu			58	1	1.72	Posterior	Small

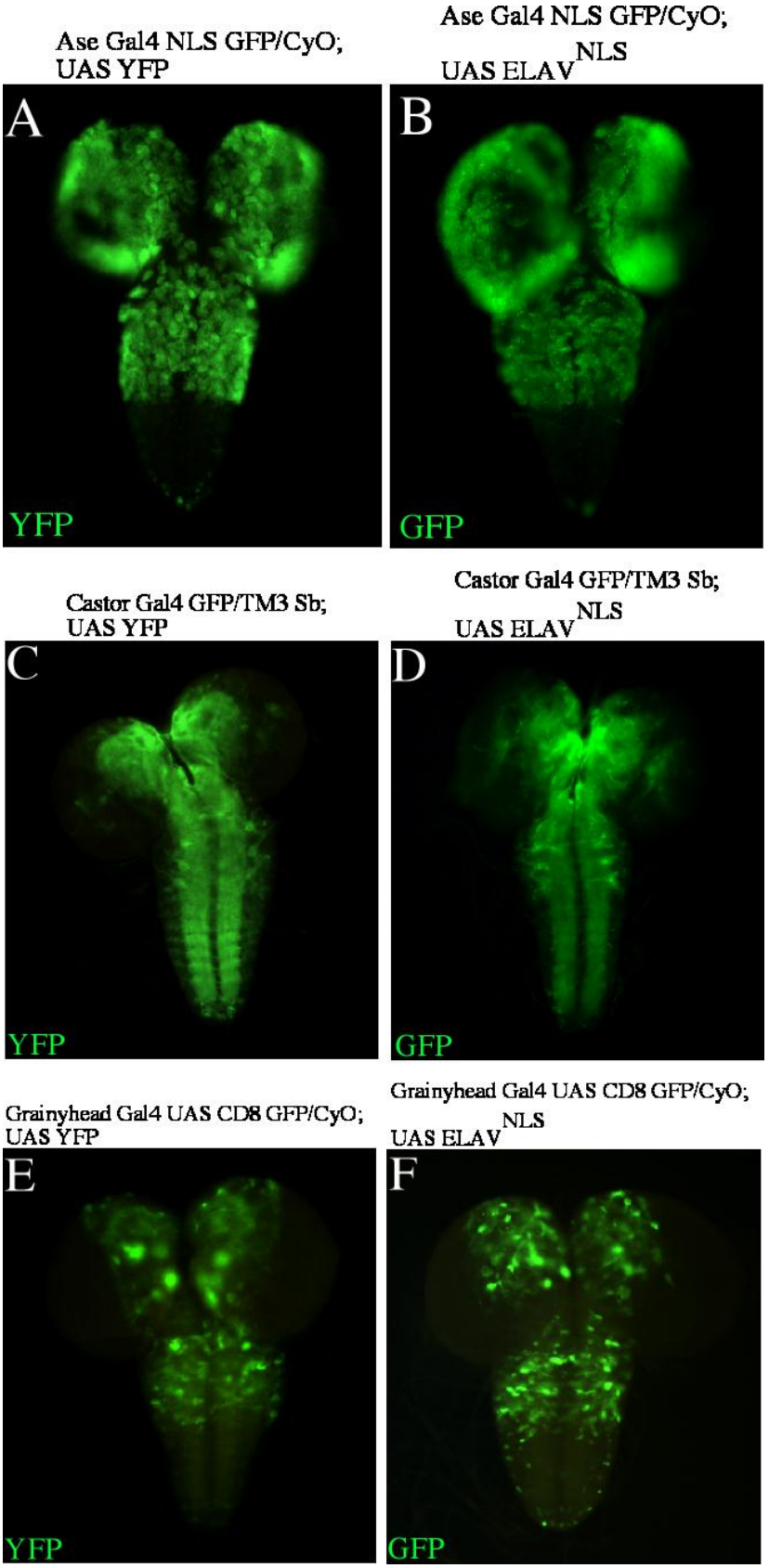
Section VI: table showing the tumour position, number and size observed in the VNC of Inscuteable[1407] GAL4 larvae expressing ectopic Elav[NLS].

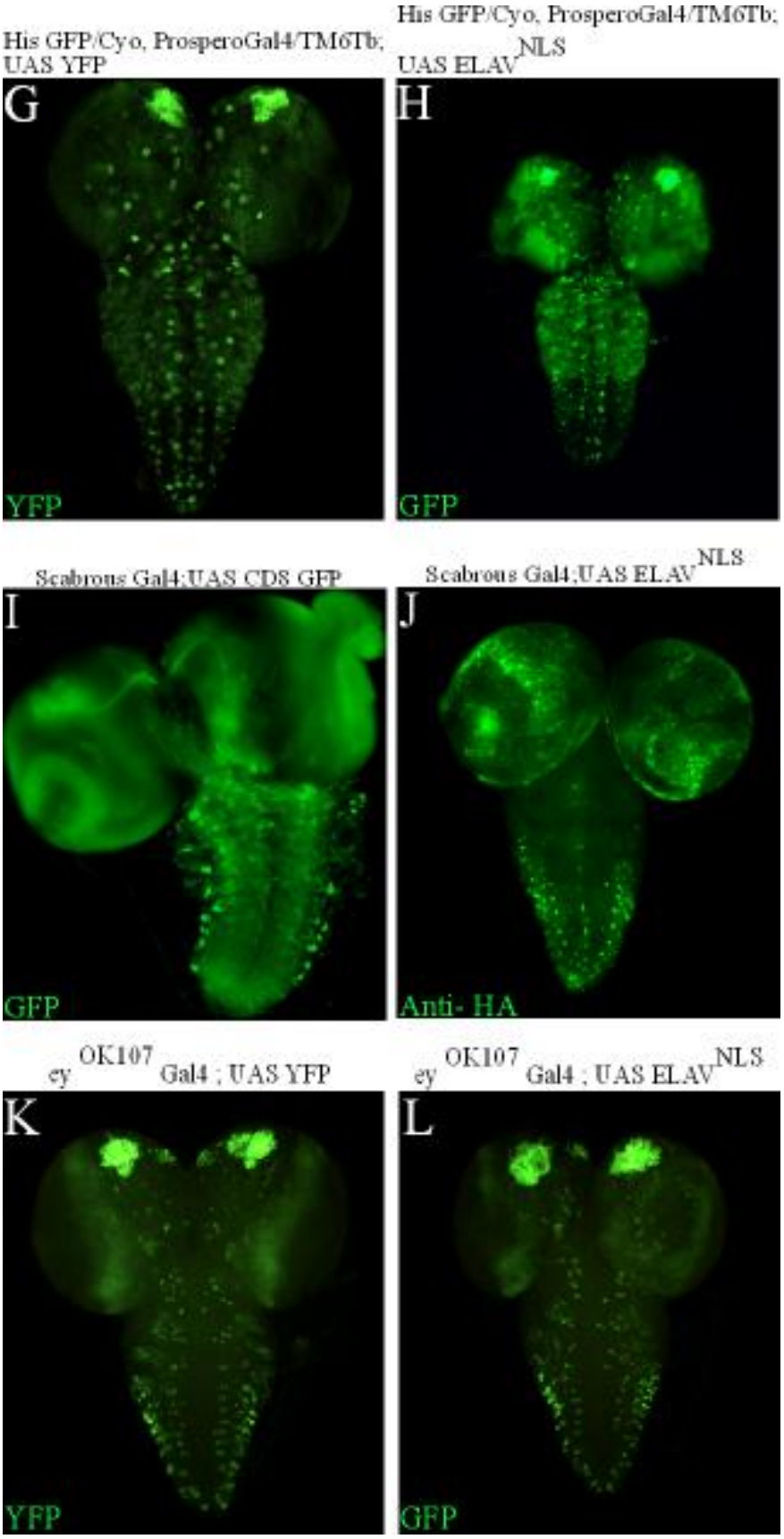
Inscuteable[1407] GAL4x UAS ELAV[NLS]							
Brain number	**Tumour Position**	**Number of Tumour(s)**	**Size**	**Number**	**Tumour Position**	**Number of Tumour(s)**	**Size**
1	Anterior right	1	Large	**26**	Anterior right	2	Medium
2	Anterior left	1	Small	**27**	Anterior right	1	Medium
3	Optic lobe size	1	Medium	**28**	Optic lobe size	1	Medium
4	Anterior right	1	Large	**29**	Anterior right	1	Medium
5	Anterior left	1	Small	**30**	Anterior right	1	Small
6	Anterior right	1	Large	**31**	Posterior right	1	Medium
7	Optic lobe size increase and posterior left	2	Optic lobe - Medium VNC - Small	**32**	Anterior right	2	Small
8	Optic lobe size increase and Anterior left and right	2	Optic lobe - Medium VNC - Medium	**33**	Anterior left	1	Medium
9	Anterior left	1	Small	**34**	Posterior right	1	Medium
10	Anterior right	1	Medium	**35**	Anterior left	1	Medium
11	Anterior right	2	Medium	**36**	Optic lobe size	1	Medium
12	Anterior right	1	Large	**37**	Optic lobe size	1	Medium
13	Optic lobe size	1	Small	**38**	Anterior right	2	Large
14	Optic lobe size	1	Medium	**39**	Anterior right	1	Medium
15	Anterior right	2	Large	**40**	Anterior right	1	small
16	Anterior right	1	Large	**41**	Anterior right	1	Large
17	Anterior left	1	Small	**42**	Anterior right	2	Medium
18	Anterior right	2	Medium	**43**	Anterior right	1	Medium
19	Anterior right	1	Medium	**44**	Anterior right	1	Small
20	Anterior right	1	Small	**45**	Anterior left	1	Small
21	Anterior right	2	Medium	**46**	Posterior left	1	Medium
22	Anterior right	1	Large	**47**	Anterior right	2	Medium
23	Optic lobe size	1	Large	**48**	Anterior right	1	Large
24	Anterior left	1	Large	**49**	Posterior right	1	Small
25	Anterior left	1	Medium				

Section VII: table showing the tumour position, number and size observed in the VNC of $ELAV^{C155}$ GAL4 larvae expressing ectopic $Elav^{NLS}$.

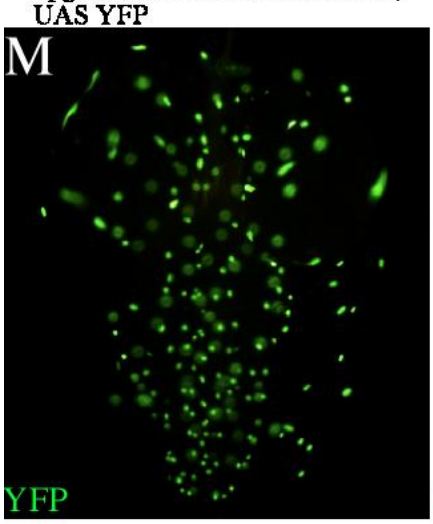
$ELAV^{C155}$ x UAS $ELAV^{NLS}$			
Brain number	**Tumour Position**	**Number of Tumour(s)**	**Size**
1	Posterior left	1	Small
2	Anterior right	2	Small
3	Anterior left	1	Medium
4	Anterior right	1	Small
5	Anterior right	1	Medium
6	Anterior left	1	Medium
7	Anterior left	1	Small
8	Anterior right	1	Large
9	Anterior left and right	2	Medium
10	Posterior right	1	Medium
11	Anterior right	2	Small
12	Anterior left	1	Medium
13	Posterior right	1	Medium
14	Anterior right	1	Large
15	Posterior left	1	Medium
16	Anterior left and right	1	Medium
17	Optic lobe size	1	Small
18	Anterior right and Posterior right	2	Small
19	Posterior right	1	Medium
20	Anterior left	1	Large
21	Anterior right	1	Small
22	Optic lobe size	1	Medium
23	Anterior right and Posterior right	2	Medium
24	Posterior right	1	Small

Section VIII: Images showing the expression pattern of drivers Asense GAL4, Castor GAL4, Grainyhead GAL4, Prospero GAL4, Scabrous GAL4, Eyeless^{OK107} GAL4, Worniu GAL4, WorniuGAL4AsenseGAL80 and Moody with YFP and Elav^{NLS}.

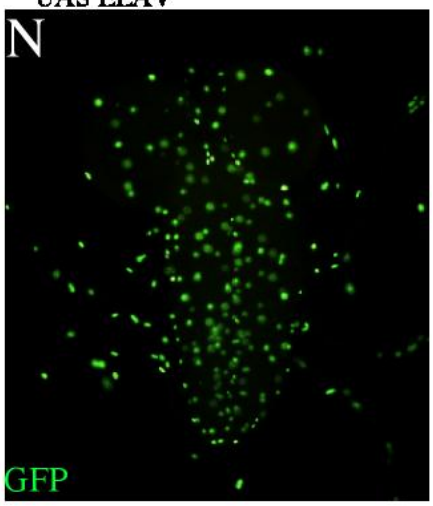



His GFP/Cyo, ProsperoGal4/TM6Tb;
UAS YFP
His GFP/Cyo, ProsperoGal4/TM6Tb;
UAS ELAV NLS
G
H
YFP
GFP
Scabrous Gal4;UAS CDS GFP
Scabrous Gal4;UAS ELAV NLS
I
J
GFP
Anti- HA
ey OK107 Gal4 ; UAS YFP
ey OK107 Gal4 ; UAS ELAV NLS
K
L
YFP
GFP

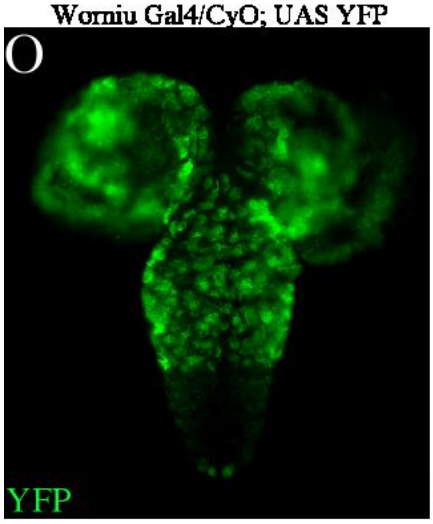
Spg Gal4 UAS Dicer2/SM6 Roi;
UAS YFP



Spg Gal4 UAS Dicer2/SM6 Roi;
UAS ELAV^{NLS}



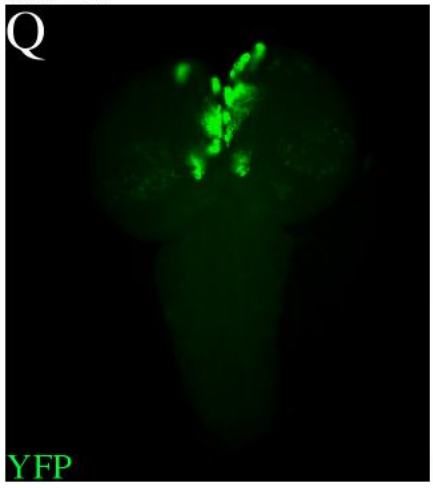
Worniu Gal4/CyO; UAS YFP



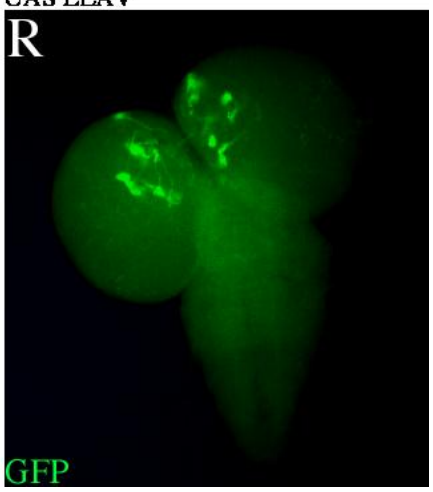
Worniu Gal4/CyO; UAS ELAV^{NLS}



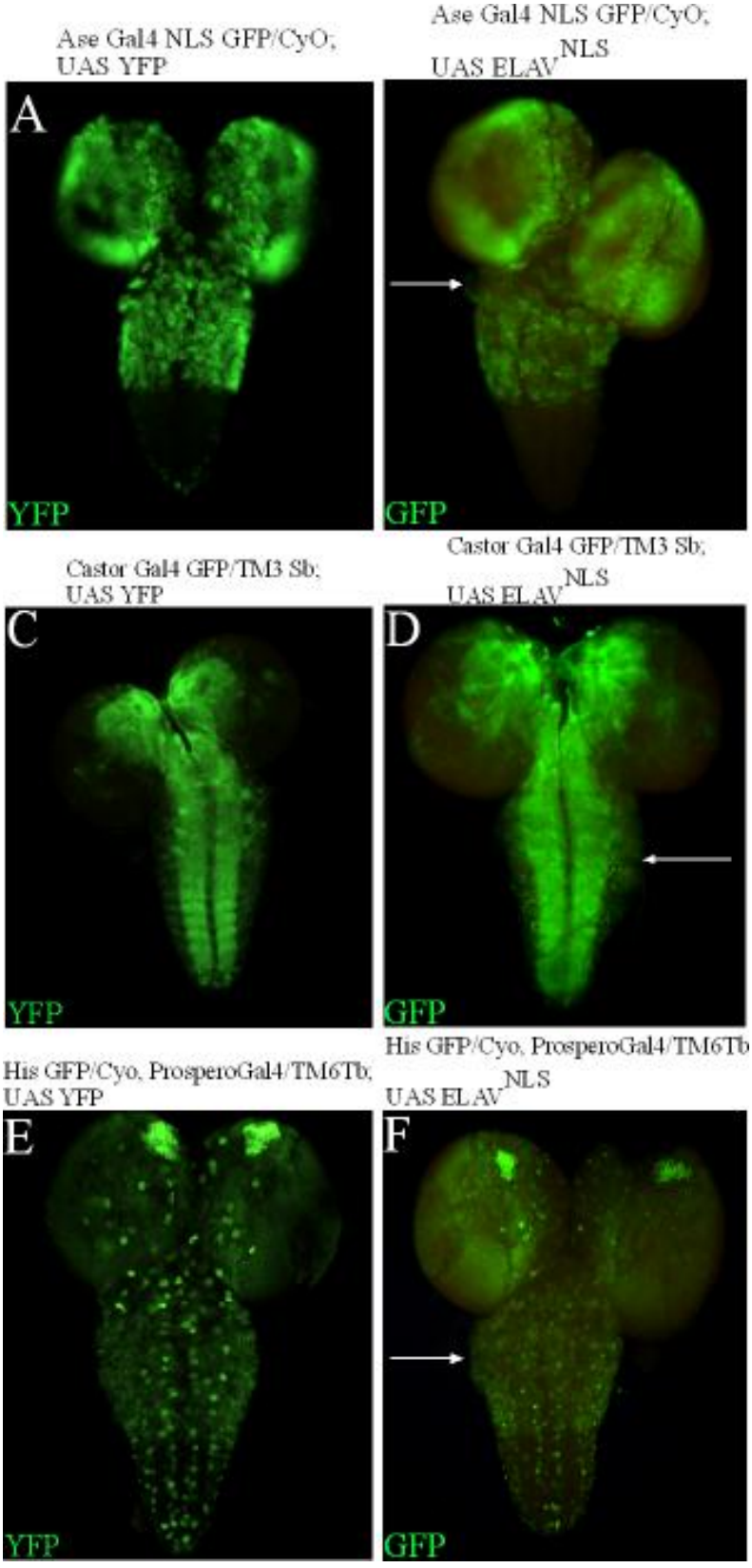
UAS CD8GFP,WorGal4AseGal80/CyO;
UAS YFP

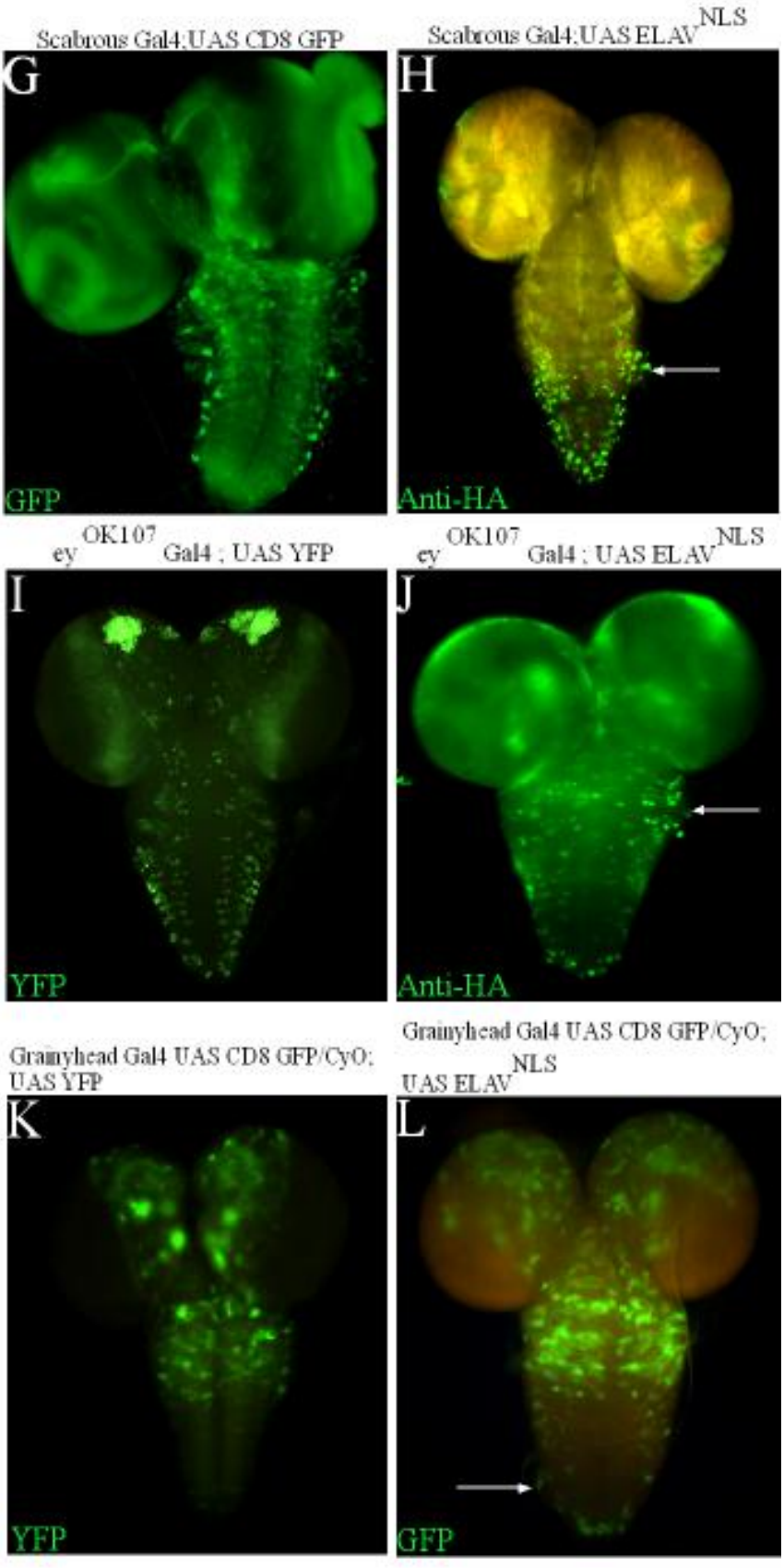


UAS CD8GFP,WorGal4AseGal80/CyO;
UAS ELAV^{NLS}



Section IX: Image showing the rarely observed tumour outgrowth in driver lines Asense GAL4, Castor GAL4, Prospero GAL4, Grainyhead GAL4, Scabrous GAL4 and Eyeless^{OK107} GAL4 upon expressing Elav^{NLS}.




Scabrous Gal4;UAS CD8 GFP
G
GFP
Scabrous Gal4;UAS ELAV NLS
H
Anti-HA
ey OK107 Gal4 ; UAS YFP
I
YFP
ey OK107 Gal4 ; UAS ELAV NLS
J
Anti-HA
Grainyhead Gal4 UAS CD8 GFP/CyO; UAS YFP
K
YFP
Grainyhead Gal4 UAS CD8 GFP/CyO; UAS ELAV NLS
L
GFP

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FUNCTION OF *DROSOPHILA* NEUROTROPHIN IN DENDRITIC DEVELOPMENT

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Abstract

Neurotrophin and Toll receptors play key roles in birth, survival and death of neurons in the brain. In neurons, dendrite morphology and structure of the dendritic tree is important for proper function and connectivity between neuronal circuits. Many proteins have been involved in shaping up the synaptic connections and shapes of the dendrites, but our understanding on dendrite remodelling, arborisation and circuitry is still limited. In here, we look into how ectopic expression of *Drosophila* neurotrophins and Toll receptors affect the structure and dendrite morphology of lobula plate tangential cell (LPTC) neurons and ellipsoid body (EB) associated R-neurons in the adult *Drosophila* brain using immunostaining and confocal microscopy. In addition, we also look into identifying suitable GAL4 lines for central (CNS) and peripheral nervous system (PNS) that could characterise circuitry and sensory neuron dendrite arborisation. To visualize the dendrite arborisation of CNS interneurons and PNS DAIV neurons, we used mCD8GFP and DenMark to observe the dendrite branching pattern. Analysis through ImageJ showed DNTs and Toll receptors to have a potential effect on the structural organisation of dendrites in adult *Drosophila* brain, while in the case of circuitry and sensory neurons the orientation of dendrite arborisation was found to be distinct for different neurons.

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Abbreviations

Act-Spz	Activated Spatzle
BDNF	Brain derived neurotrophic factor
Chr	Chromosome
CNS	Central nervous system
Cha	Cholinergic
CK	Cysknot
CyO	Curly of Oster
DA	Dendritic arborisation
Df	Deficiency
DNT1	*Drosophila* neurotrophin 1
DNT2	*Drosophila* neurotrophin 2
EB	Ellipsoid body
EL	Eve lateral
FL	Full length
GFP	Green fluorescent protein
HS	Horizontal system
ISN	Intersegmental nerve
LPTC	Lobula plate tangential cells
mAB	Monoclonal antibody
NGF	Nerve growth factor
NT	Neurotrophin
NTR	Neurotrophin receptor
PNS	Peripheral nervous system
PPK	Pick-pocket 1
SN	Segmental nerve
Spz	Spatzle
TLR	Toll like receptors

TNFR	Tumour necrosis factor receptor superfamily
Trk	Tyrosine kinase receptor
UAS	Upstream activation sequence
VNC	Ventral nerve cord
VS	Vertical system
Wt	Wild type

CHAPTER 1

INTRODUCTION

Introduction:

In the brain, neurons or nerve cells receive, process and transmit information through electro-chemical signalling process. With the brain being made up of over billion neurons, the structure of a neuron is made up of (i) a cell body called soma, which contains the cell nucleus, (ii) long branching filaments called dendrites, which branches out from the cell body, (iii) a single long filament called axon, which may be several times longer than the soma, and (iv) an axon terminal, which are structures containing neurotransmitters that lies at the end of axon. In the brain, as neurons are in close proximity to each other, transfer of information between the neurons occurs via dendrite/axon terminal structures, where the signal transmission occurs through specialized connections called synapses. During synapses, the neuron sending the signal is called the pre-synaptic neuron and the neuron receiving the signal is called the post-synaptic neuron.

1.1 Neuronal Signalling

Neurons depend on interconnected signal transfer (i.e. synapse) for their coordinated functioning. The signalling mechanism in neurons begins with the transfer of charged ion particles through the protein membrane barrier into the pre-synaptic neuron. The resulting change in concentration of ions and the shift of membrane potential inside the pre-synaptic neuron (resting potential -70 millivolts) causes large electrical signal generation, which is called as action potential. The generated signal is then transferred along the pre-synaptic neuron's axon and is made to reach the axon terminal. The arrival of signal results in the migration of vesicles containing neurotransmitter to the membrane and the release of neurotransmitter into the synaptic cleft via exocytosis. The released neurotransmitters bind to specific receptor(s) present on the membrane surface of the dendrites of the post-synaptic neuron and results in opening of the ion-channels, which causes an influx of ions to enter into the post-synaptic neuron. This signalling process is called as chemical neurotransmission. Apart from chemical neurotransmission, electrical neurotransmission also exists between neurons, where physical connection between the neurons through gap junction helps in the transfer of electrical impulses between one another (Stufflebeam, 2008).

1.2 Vertebrate Neurotrophins

Being the core component of the central nervous system, neuron or nerve cell has a distinct feature that differentiates them from other cells. The neurons upon birth cease to proliferate and rather increase itself in size and remain to live longer. This question as to how neurons survive and how targeted branching and connections gets established between neurons after they are born was gradually answered by the discovery of neurotrophins.

Neurotrophins (NT) are dimeric polypeptide proteins that activate specific receptors via ligand/receptor binding mechanism. Neurotrophins have been found to be present in both vertebrates and invertebrates. The secretion of neurotrophins in the neurons has been found to play a vital role in the neuron differentiation and survival, axon and dendrite remodelling, synapse plasticity, cytoskeleton assembly, membrane trafficking and long-term potential (i.e. memory and learning) (Huang and Reichardt, 2001).

In the vertebrates, nerve growth factor (NGF) (discovered in 1954), brain derived neurotrophic factor (BDNF) (discovered in 1982), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (discovered in 1991) together form the neurotrophin (NT) family. Neurotrophin-4 and neurotrophin-5 are similar proteins that are expressed in different species, i.e. *Xenopus laevis* and mammals, respectively (Huang and Reichardt, 2001). Apart from the above, two additional neurotrophins, neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7), that is not present in any major class of tetrapods are also identified in fish (Gotz *et.al*., 1994 and Nilssion *et.al.*, 1998).

The genes that code for neurotrophins are present in different chromosomes, i.e NGF in chromosome 1p, NT-3 in chromosome 12p, BDNF in chromosome 11p, and NT-4/5 in chromosome 19q (Hallbook, 1999). Comparison of these neurotrophin gene sequences and structures suggested homology between them and shows evolution from a common neurotrophin ancestral gene (Lewin and Barde, 1996). It was reported by Urfer *et.al*. (1994) that NT-3 might be the ancestor gene, as replacement of seven specific amino acids in the NT-3 sequence has been found to result in a neurotrophin that has the functional similarity to that of NGF, BDNF and NT-3. However, some research states duplication to be the reason for the formation of the neurotrophin family, with NGF and NT-3 evolving from the duplication of one intermediate ancestor gene and BDNF and NT-4/5 evolving from another.

1.3 Neurotrophin structure

All neurotrophins are characteristically similar in molecular weight, primary structure and isoelectric points, and are found to exist as two non-covalent sub-units with six conserved cysteine residues that are bound together by three inter-chain disulfide bonds (Mowla *et.al*., 2001). In the neurons, neurotrophins are basically secreted as pre-pro-neurotrophins, which contain the signal peptide, pro-domain and cysteine knot domain (Fig. 1). In order to produce an active mature form of NT, the process begins in the endoplasmic reticulum with the cleavage of the signal peptide. The resulting proform undergoes two fate (i) intracellular enzymatic cleavage by furin or by pro-convertases i.e. PC1, PC2, PACE4, PC5/6 at the dibasic amino-acid cleavage site to release stable mature proteins or (ii) they are released into the extracellular environment as a proform, where depending on the availability of proteases they are cleaved for use (Chao., 2003; Schweigreiter, 2006). It has also been observed that the pro-domain regions in the neurotrophins are important for sorting them inside the neurons for enzymatic cleavage and for providing proper folding (Schweigreiter., 2006).

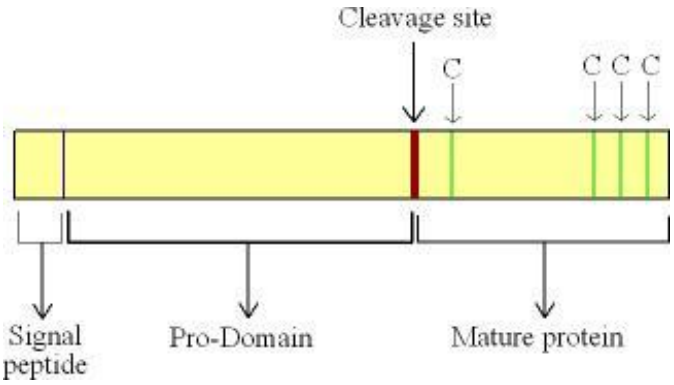


Figure 1: Schematic representation of a neurotrophin with the signal peptide, pro-domain and mature protein domain region. The 'C' represents the conserved cysteine residues in the mature protein region that forms the cysteine knot motif. (Reproduced from Schweigreiter, R., 2006).

1.4 Vertebrate neurotrophin receptors

The vertebrate neurotrophins, as a ligand, are found to bind three different classes of transmembrane neurotrophin receptor proteins with varying affinity. The first identified transmembrane neurotrophin receptors was p75 neurotrophin receptor ($p75^{NTR}$). Belonging to the tumour necrosis factor receptor superfamily (TNFR) with an intracellular death domain, the negatively charged $p75^{NTR}$ binds pro and mature form of all neurotrophins with low affinity by targeting the basic amino acids in the neurotrophin dimers with their leucine rich extracellular domain (Roux and Barker, 2002). The second transmembrane receptor class that

was found to interact with neurotrophins were the tyrosine kinase receptor (Trk) family, which consists of TrkA, TrkB and TrkC. Like $p75^{NTR}$, the Trk receptor family has two cysteine rich clusters with three leucine repeats in their extracellular domain that helps in ligand binding. Being coded by three different *trk* genes, Snider W.D. (1994) showed evidence that tyrosine kinase receptors are crucial in mediating neurotrophin effects in the central nervous system (CNS) and peripheral nervous system (PNS). As different Trk receptors bind with different affinity, it was found that NGF binds with highest affinity to TrkA, BDNF and NT4/5 binds with stronger affinity to TrkB and NT3 binds with strong affinity to TrkC and with a weak affinity to TrkA and TrkB (Barbacid, 1994). Apart from these two, the most recent and the third class of receptor that was found to bind the neurotrophins was the Vsp10-domain transmembrane receptor family member, Sortilin. Sortilin was previously found to bind neurotensin, but it has now been found to also bind the pro- or unprocessed forms of NGF and BDNF (Teng *et.al.*, 2005).

1.5 Importance of neurotrophin receptors in neuron survival and death

In the neurons, the binding of the neurotrophins to their respective receptor (NTRs) has been found to result in agonistic and antagonistic effects i.e. neuronal survival and programmed cell death (apoptosis), respectively.

1.5.1 Neuron survival:

One of the active receptor that has been found to be responsible for neuron survival in the CNS and PNS is the Trk superfamily. The binding of mature neurotrophin to Trk receptor superfamily are found helpful in the development of neurons in prenatal and postnatal stages. NGF binding to TrkA has been implicated in cholingergic neuron survival, and BDNF binding to TrkB has been deemed vital for cortical neuron maintenance (Schweigreiter, 2006). The signalling mechanism involved in these processes involves the binding of neurotrophins that result in dimerization and autophosphorylation of the receptor that causes conformational changes and docking of an adaptor protein like Shc and PLC-gamma. This coupling causes the receptor to interact with intracellular signalling molecules (Ras-Erk and PI-3K-Akt) and result in the neutralization of apoptosis-inducing agents like Fas ligand (Huang and Reichardt, 2003). In addition to Trk superfamily, binding of $p75^{NTR}$ to the neurotrophins either alone or together with Trk receptors has been shown to activate NF-κβ, a potent pro-survival factor

that has been found to regulate survival of neurons during embryonic development. (Hamanoue *et.al*, 1999).

1.5.2 Neuronal death

Apart from helping in survival, the $p75^{NTR}$ in combination with sortilin has been found to promote apoptosis in the neurons (Teng *et.al.*, 2005). By binding pro-neurotrophins effectively, $p75^{NTR}$-sortilin receptor complex triggers apoptosis on the basis of concentration of unprocessed form of neurotrophins. At present, the mechanism underlying death signal transduction from the $p75^{NTR}$-sortilin receptor complex is not known, but mechanism involving the binding of cytoplasmic interactors to $p75^{NTR}$ that propagates a NT-dependent death signal has been roughly detailed. The mechanism of the process is such that the binding of the cytoplasmic interactors trigger the activation of JNK that causes mobilization of pro-apoptotic proteins (i.e. Bcl-2 family) and apoptosis of the cell. In addition to the above, members of the TNFR superfamily i.e. Fas, has also been observed to be involved in $p75^{NTR}$ mediated cell death by the action of death domain region present in the cytoplasmic region of $p75^{NTR}$ (Frade *et.al.*, 1996; Schweigreiter, 2006).

1.6 Importance of neurotrophins in mammalian system:

To establish a balanced environment in the nervous system, the neurotrophins are released in sufficient levels in the CNS and PNS based on an activity-dependent manner. The three neurotrophins, NGF, BDNF and NT3 are regulated in mammals on the basis of epileptic activity and electrical stimulation (Choa, 2003). Researches performed on the effect of neurotrophin in mice models have clearly shown the importance these three neurotrophins have on the nervous system. Mice that lack neurotrophin expression completely in the brain are found to die few weeks after their birth (Choa, 2003). The heterozygous mice for NGF are 50% viable and showed neuronal loss in the PNS and reduction in retention and memory (Chen, K. *et.al*, 1997). In heterozygous BDNF mice, abnormal neuronal function was observed in the cortex, forebrain, and hippocampus, which result in the loss of neurons in the PNS, elevated dopamine levels and loss of long-term potential and mechanosensitivity (Bartolett *et.al.*, 2002). Heterozygous NT3 mice apart from showing loss of neurons in the PNS, also results in impairment of mechanoreceptors and amygdale kindling activity (Emfors

et.al., 1994). In addition to the above, homozygous BDNF mice have shown alteration in the plasticity, which causes impairment in memory tasks and spatial memory (Choa, 2003).

1.7 *Drosophila* as a model to understand the brain

The ability to use model organisms in research has helped us understand neurotrophins in detail. Neurotrophins expressed in various organisms are homologous to mammalian neurotrophins, but at different ranges. It is the capability to manipulate the neurotrophins in the model organisms with minimal redundancies for observing changes in neurons that has made them easy targets for studying and understanding the nervous system.

Drosophila for long has been considered as a powerful invertebrate model for studying nervous system development for its (i) conserved developmental and neurological pathways, (ii) small genome size and (iii) 60% homologous similarity it shares with human genes (Rubin, 1988). The feasibility to disrupt or misexpress or over-express protein and to perform large-scale genetic screening in *Drosophila* has also rendered them as a perfect genetic test tube to understand the activities of genes and proteins involved in any genetic pathway(s) (Reiter and Bier, 2002).

Drosophila larval central nervous system (CNS)

The larvae CNS in *Drosophila* consist of the anterior central brain and the ventral nerve cord. With the first appearance of neuroblast in stage 8 of embryogenesis, neurons and glia are generated from the ganglion mother cell by stage 12, with the extension of axons and interneuron projection completed within stage 14-17. By the end of stage 17 in embryogenesis, the VNC condenses into a thick and short structure, with a neuropile (consisting of neurons and glial cells) arranged in the fashion of a ladder that has longitudinal axons running on either side cross-connected by the commissural axons. (Campos-prtega and Hartenstein, 1985; Therianos *et.al.*, 1995).

Drosophila larval peripheral nervous system (PNS)

The larvae PNS consists of sensory neurons that are spread throughout the epidermis, which helps in responding to various chemical, mechanical and thermal stimuli by transforming the stimuli into neuronal activity. In *Drosophila* embryos, the differentiation of the sensory neurons begins at stage 14 and by the end of stage 16 most of the sensory neurons and motor

neurons meet at their targets to form segmental (SN) and intersegmental (ISN) nerves. In the PNS, each hemisegment consists of neurons that are differentiated into dorsal, ventral and lateral clusters, with each being further classified into external sensory, chordotonal, bipolar dendritic and multiple dendritic neurons on the basis of dendrite morphogenesis. Based on the dendritic arborisation, the neurons have also been classified into Dendritic arborisation (DA) neurons class I to IV, with arborisation pattern increasing from I to IV and the DA IV neurons being the highly branched. In the DA neurons, the dendrites tend to not cross-over each other, but however in the case of Class III and IV the similar adjacent class neuron's dendrites tend to overlap each other because of their homotypic repulsion behaviour, which is not seen in Class I or II (Grueber *et.al*, 2003). The reason as to why *Drosophila* PNS is being studied for dendrite branching is mainly because of their accessibility, large size, characteristic pattern that helps in easy comparison and robust stereotypical development. (Campos-prtega and Hartenstein, 1985; Jan and Jan, 1993).

1.8 *Drosophila* Neurotrophin

Drosophila has been utilized in studies involving behavioural changes, biological rhythms and neuronal path finding for understanding the regulated expression, localization and modification of genes/proteins in the brain. For a long period of time *Drosophila* was considered to be devoid of neurotrophins and that their neuronal network was imagined to be hard-wired in terms of connectivity. This led to the assumption that neurotrophins are endogenous only to vertebrate system and stereotyped in the fly model, until this view was changed when Spatzle was identified.

Spatzle (Spz), a disulfide-linked dimer, was identified in *Drosophila*, as a member of new family of neurotrophin-like signalling molecule that helped in the dorsal-ventral patterning in the *Drosophila* embryos (Belvin and Anderson, 1996). But no sooner after identifying Spz, its striking similarity to horseshoe crab coagulogen subdued its consideration as a potential neurotrophin (Mizuguchi *et.al*, 1998).

In 2001, Parker *et.al* found five additional *Drosophila* homologues of Spatzle i.e. Spz2-6, and the sequence alignment of Spatzle with mammalian NGF showed homology to amino acids involved in binding. Even though, this clearly pointed out Spatzle's capability to form salt bridges and hydrogen bonds with their receptor (Weber *et.al*, 2007),but it was not until the

discovery of two new members of the *Drosophila* neurotrophin family that spatzle was rightfully considered as a neurotrophin molecule.

With the notion of NTs existence in flies being uncertain, a simple BLAST search based on sequenced genome via computational analysis by Venter *et.al*. (2001) affirmed neurotrophins absence in flies. However what was found to be a miss in the BLAST search was the structural conservation the proteins had in them despite their sequence divergence (Zhu *et.al*. 2008). But, Zhu *et.al* (2008) using TBLASTN and PSI-BLAST on the *Drosophila* genome identified a potential candidate (CG18318). This candidate was able to identify BDNF presence in various species. So they isolated the full length cDNA and subjected the protein sequence of the cDNA to FUGUE, which was a program that helped in identifying proteins that retained their structural conservation through evolution. By comparing the protein sequence with HOMSTRAD protein database, FUGUE successfully identified the human NTs as the probable homologs and this led to naming of the protein as *Drosophila* neurotrophin 1 (DNT1). PSI-BLAST of Spz paralogs on *Drosophila* genome revealed DNT1 as Spz2 (Spatzle 2), and FUGUE also identified DNT1 to be closely related to Spz and Spz5, and recognized them to be close to the neurotrophins present in humans (Zhu *et.al*, 2008).

As structural considerations, expression pattern in the embryonic CNS midline, and loss of function analysis through mutant alleles showed Spz5 to have neurotrophic characteristics, Spz5 was later renamed and referred to as *Drosophila* neurotrophin 2 (DNT2) (Zhu *et.al*, 2008). With the completion of the *Drosophila* neurotrophin family, DNT1 (Spz2), DNT2 (Spz5) and Spz, it was concluded that the vertebrate and invertebrate neurotrophins must have evolved from a common ancestral gene. The evolutionary changes has reduced the overall genetic sequence homology, but still seemed to have retained the structural similarity and homology in the cysknot region. *Drosophila* neurotrophin like the vertebrate neurotrophins are secreted as pro-neurotrophin, which contains the pro-domain between the signal peptide and the cysknot domain (contains conserved cystine residues). To be released as a stable mature protein the pro-neurotrophin undergoes enzymatic cleavage to remove the pro-domain. In *Drosophila*, the neurotrophins are involved in neuron survival, plasticity, axon and dendrite guidance, targeting and connectivity.

1.9 *Drosophila* neurotrophin receptors

Of the *Drosophila* neurotrophin receptors, the first to be identified through genetic screening was the Toll-1(Toll) receptor. Accounting for a total of nine i.e. Toll-1 to Toll9, the Tolls and Toll-like receptors (TLRs) together form the Toll receptor superfamily in *Drosophila* (McIlroy *et.al*, 2013). Being the first member to be identified, the Toll-1 receptor has been found to be important in *Drosophila* for dorsoventral polarity patterning. Null mutation in the Toll-1 genes has been found to be embryonic lethal (Gay and Gangloff, 2007). Apart from aiding in development, the Toll-1 receptor has also been found to be involved in *Drosophila* innate immunity (Tauszig *et.al*, 2000).

With similarity in molecular structure, all the Toll receptors are mainly composed of leucine-rich repeat ectodomain and cysteine-rich flanking motifs (Gay and Gangloff, 2007). However, one exception is the Toll-9 receptor. Toll-9 seems to be more similar to the mammalian Tolls by having only one cysteine-rich motif in between the transmembrane and leucine-rich repeats (Valanne *et.al*, 2011). *Drosophila* Tolls like the mammalian IL-1Rs have also been found to be active in downstream events via interaction with adaptor molecules by sharing a cytosolic homology domain called the Toll/IL-1R domain (TIR domain) (Imler and Hoffmann, 2001).

Even though *Drosophila* Tolls have been speculated to be involved in immune response like the mammalian TLRs, but the roles of Toll2-9 has been largely unresolved except for a few functional confirmations. Toll-5 has been shown to induce *drosomycin and metchnikowin* expression that lead to dorsal-dependent transcription similar to Toll receptor (Tauszig *et.al*., 2000). Toll-6 and Toll-7 have been shown to function upstream of the NF-kB pathway, and bind DNT2 (Spz5) and DNT1 (Spz2), respectively, where they are involved in neuron development and survival process, normal locomotion and motor axon targeting (McIlroy, *et.al*, 2013). Toll-9 has been reported in activating *drosomycin* expression by involving components of the Toll signalling pathway (Betterncourt *et.al.*, 2004).

1.10 Importance of *Drosophila* neurotrophin and dendrite branching

In vertebrates the functional characteristics of the neurotrophins are to maintain survival of neurons and enable targeting and connectivity. In a related manner in the invertebrate the *Drosophila* neurotrophins i.e. DNT1, DNT2 and Spatzle have been found to play similar roles

in *Drosophila* development. DNT1 is observed to be expressed in the midline (intermediate target of interneurons) of CNS during stage 17 in the embryo, larvae optic lobe lamina and adult optic lobes and central brain. Zhu *et.al* (2008) has shown that DNT1 nonautonomously promotes neuron survival in the *Drosophila* CNS and enables motor neuron targeting. Spatzle, in the *Drosophila* embryo has been found to be expressed in the CNS midline and lateral muscles and has been shown to be important for neuron survival and to be important for proper targeting of the SNa motor axons (Zhu *et.al.* 2008). DNT2, which is spz5, in *Drosophila* embryo has been found to be expressed in the CNS midline, muscles, PNS and in the epidermis. DNT2 has been found to be important for neuron survival and is found to have complementary function with DNT1 to control the survival of neurons in the brain (Zhu *et.al.*, 2008). In adults, flies mutant for Spz or DNT1 and DNT2 have been found to have locomotion defects that is quite similar to those observed in neurotrophin mutant mice models (Schwartz *et.al*, 1997; Chao, 2003), and it has also been observed that *Drosophila* neurotrophin triple mutants are embryo lethal in *Drosophila* (Zhu *et.al.*, 2008).

Drosophila as an invertebrate model organism has helped us in studying dendrite morphology and arborisation via genetic and cellular analysis in the central and peripheral nervous system. Studies on *Drosophila* have shown important cues on dendrite remodelling, branching, targeting and functions. Kim and Chiba (2004) showed directed dendrites outgrowth by cell-surface receptor molecules previously known as axon guidance molecules. Dimitrovo *et.al* (2008) and Boyle *et.al* (2006) showed ligand-receptor axon guidance molecules Robo-Slit and semaphoring-neuropilin to play major roles in axon development and in dendrite position, orientation and synaptic connectivity for transmission. Biochemical and genetic studies on model organisms have shown dendritic tree to be the most complex part of the neuron with wider arborisation morphologies ranging from single axon-fibre to planar form. Research till date has focused more towards understanding neuron birth and axonal branching, but our knowledge on dendrite branching and arborisation is limited. Even though in the vertebrates neurotrophins have been found to be essential for survival of neurons and enabling them to attain connectivity and targeting, but neurotrophin role in dendrite branching hasn't been looked in detail considering the evidence that many genes are involved in structuring the complexity of the dendrite. Researchers have shown that dendritic growth of a neuron begins with territory targeting, branching and attainment of specific architecture before establishing the dendrite attains complete maturity. Even though many proteins have been studied for their

specific activity on the structure and branching of dendrites, but the activity of neurotrophin and toll receptors on dendrite growth and branching is limited.

In this aspect, to provide an initial insight on the effect of neurotrophin and Toll receptors on the dendrite arborisation and morphology, we thought of ectopically expressing the *Drosophila* neurotrophins and Toll receptors in the adult flies to observe their effect(s) on the dendrite branching and arborisation.

To carry out the study, the driver line lobula plate tangential cells neurons GAL4 shall be used to characteristically analyse the effect of ectopic neurotrophin expression in the adult flies.

Drosophila Lobula plate tangential cells:

The lobula plate tangential cells or LPTCs are a group of six vertical system (VS) and three horizontal system (HS) neuron that is present in the posterior region of the third optic neuropile (lobula plate) (Scott *et.al,* 2002). In the adult *Drosophila*, the VS and HS neurons combine together to form a large complex dendrite arborisation, which has been found to be helpful in genetic analysis of dendrite morphogenesis. Scott *et.al* (2002) and Raghu *et.al* (2009) have specifically used the LPTC driver lines and MARCM labelling to provide high resolution imaging of each individual VS and HS neuron, their axon and dendritic tree, pre and post-synaptic markers and dendritic spines. In addition, as the position and dendrite size of the LPTCs are consistent between different fly models (e.g. *calliphora* and *Drosophila*), they have been largely used in locomotion studies, as the cells in the lobula plate has been observed to encode output visual signals to the motor neurons in the thoracic ganglia via descending neurons (Borst and Haag, 2002).

Drosophila Ellipsoid body

The ellipsoid body (EB) is one of the four interconnected substructure in the central complex in *Drosophila* central brain. The EB is made up of two circular neuropile, which forms the shape of a concentric ring. Being involved in multiple forms of learning and visual pattern memory, the EB is connected to a subset neuron called the R neurons (R2/R4m neurons), which has been found to be involved in short-term olfactory memory. The EB lies posterior to the fan-shaped body and receive its neuron terminals from the protocerebral bridge, and shares the dendrites with the fan-shaped body (Hanesch *et.al.*, 1989; Yufeng *et.al.*, 2009).

Apart from studying LPTC dendrite arborisation, this research work also use the UAS DenMark reporter line to identify:

(i) Suitable GAL4 candidate from HB9 GAL4 (*HB9* expressing ventral motorneurons and interneurons), D42 GAL4 (Toll 6 receptor expressing interneurons), RRK GAL4-EVE GAL4 (*Eve* expressing dorsal motorneurons), EL GAL4 (*Eve*-positive lateral interneuron) and CHA GAL4 (any neuron capable of acetylcholine secretion), that has its interneuron dendrite branching in the mechanosensory domain of larvae ventral nerve cord.

This is in connection with the observation of the *Drosophila* Spatzle neurotrophin in the mechanosensory domain (Unpublished work) by Mei Ann Lim, a PhD student in Hidalgo lab. The identification of a suitable GAL4 line shall be helpful in the future to provide further details on the activity of Spatzle in the mechanosensory domain.

(ii) To characterise dendrite arborisation of DAIV neuron in the peripheral nervous system using PPK GAL4 (Pickpocket 1 – subunit of epithelial sodium channel family that plays an important role in rhythmic locomotion control).

AIM OF THE PROJECT

1. To study the effect ectopic expression of *Drosophila* neurotrophin and Toll receptors have on the structure and arborisation of dendrites of the lobula plate tangential cell neurons and ellipsoid body associated R-neurons using the driver line LPTC GAL4. This investigation is carried out to understand the role of neurotrophins and Toll receptors in the branching and morphology of dendrites in the brain. As researches till date have focused more towards axon branching and targeting, a preliminary investigation on the effect of neurotrophin activity on dendrite branching can help in bridging the knowledge gap.

2. To identify a suitable circuitry neuron from the GAL4 lines HB9 GAL4, RRKGAL4-EveGAL4, Cha GAL4, D42 GAL4 and EL GAL4 that may have its interneuron dendrite branching in the mechanosensory domain of the ventral nerve cord neuropile. This investigation is carried out as identification of a suitable GAL4 line shall be helpful in proceeding PhD student Ann's project further towards answering the question as to where Spz is secreted i.e. is it from the mechanosensory domain to the PNS or is it from the PNS into the mechanosensory domain?

3. To visualize the characteristic dendrite branching of PPK GAL4, i.e. pickpocket1 expressing neuron in the peripheral nervous system using mCD8GFP and DenMark, to get insight on the DAIV neuron dendrite branching in the epidermis. This investigation is carried out to identify and see if in the future PPK GAL4 and PNS DA IV neurons can be used as a potential candidate for studying the effect of neurotrophins and Toll receptors in the developing *Drosophila* PNS.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Drosophila genotypes were obtained from Dr. Alicia Hidalgo's lab and Bloomington (Table 1). The HLA Terasaki multiwell plates and Coring 3524 Costar 24 well polystyrene cell culture plates were from Sigma-Aldrich®. Phosphate buffered saline, sodium chloride, potassium chloride, magnesium chloride, calcium chloride, sucrose, HEPES, formaldehyde and Triton®X-100 were from Sigma. Primary antibodies rabbit anti-DsRed (1:100) was from Clontech, rabbit anti-GFP (1:500) was from molecular probes and mouse anti-BP102 (1:250) was from Iowa hybridoma bank. Secondary antibodies anti-mouse Alexa 488, anti-mouse Alexa 546, anti-rabbit Alexa 488, anti-rabbit Alexa 546 and anti-rabbit Alexa 660 were from molecular probes. The vectashield mounting solution was from Vector laboratories. The dissecting epifluorescence microscope and TCS SP scanning confocal microscope were from Leica, and the LSM 710 confocal scanning microscope was from Zeiss.

2.2. *Drosophila* Maintenance

2.2.1 Houston food composition:

Plastic vials or glass bottles with 10ml or 30ml standard 1.1% agar medium containing 8.5% sugar, 6% cornmeal, 2.5% nipagin and 1.1% agar was supplemented with water and 3.2% dried yeast. The agar medium, upon solidification was sprinkled with dried yeast, if required, and were plugged with water absorbent cotton.

2.2.2 High-yeast food composition:

Plastic vials with 10ml standard 1% agar medium containing 2.5% nipagin, 10% cornmeal and 8.8% dextrose was supplemented with water and 2.35% dried yeast. Upon solidification the vials were plugged with water absorbent cotton, and if required was supplemented with additional dried yeast.

2.2.3 Maintenance of stocks:

Genotypes required for experimentation were maintained at 25°C, while a backup stock was maintained at 18°C. Stocks maintained at 25°C were flipped on a weekly basis into new vials, while stocks at 18°C were flipped every second week. New born virgin females obtained from genotypes, upon collection, were kept at 18°C until required for setting up the genetic crosses.

S.No.	Genotype	Referred to as	Chromosome (Chr)	Origin
	GAL4			
1	DB331 LPTC GAL4/LPTC GAL4;;10xUASmCD8RFP/10xUASmCD8RFP	LPTC GAL4	X and 3	Lab stock
2	;;RRK GAL4 - EVE GAL4/RRK GAL4 - EVE GAL4	RRKGAL4-EVEGAL4	3	Lab stock
3	;;HB9 GAL4/TM6B	HB9 GAL4	3	Lab stock
4	;;D42 GAL4/TM6B [Toll6 GAL4]	D42 GAL4	3	Lab stock
5	W; B19 Cha GAL4/B19 Cha GAL4;	CHA GAL4	2	Lab stock
6	W; PPKGAL4/PPKGAL4;	PPK GAL4	2	Bloomington
7	;;EL GAL4 / EL GAL4	EL GAL4	3	Bloomington
	UAS			
8	W;+/CyoB;UAS Spz FL HA^{REGALV}/MKRS	UAS Spz FL	3	Lab stock
9	W;IF/CyoB;UAS Spz^2 FL HA^{REGALV}/MKRS	UAS Spz^2 FL	3	Lab stock
10	W; UAS DNT1 cDNA3 (FL) (o)2231L/UAS DNT1 cDNA3 (FL) (o)2231L;	UAS DNT1 FL	2	Lab stock
11	W;; UAS DNT2 FL 80c/UAS DNT2 FL 80c	UAS DNT2 FL	3	Lab stock
12	;;UAS DNT2 CK 6B-9/UAS DNT2 CK 6B-9	UAS DNT2 CK	3	Lab stock
13	; UAS DNT1 CK 31^+ (3B) 6303B 6T1/CyO;	UAS DNT2 CK	2	Lab stock
14	;UAS Act Spz (14)/CyO;	UAS Act Spz	2	Lab stock
15	;;UAS $Toll^{10b}$ /UAS $Toll^{10b}$	UAS $Toll^{10b}$	3	Lab stock
16	;UAS $Toll6^{CY}$/Cyo;	UAS $Toll6^{CY}$	2	Lab stock
17	;UAS DenMark /UAS DenMark;	UAS DenMark	2	Lab stock
18	;UAS mCD8GFP/UAS mCD8GFP;	UASmCD8GFP	2	Lab stock
19	W;;10xUASmyr::tomato/10xUASmyr::tomato	UAS myr::tomato	3	Lab stock

Table 2.1: List of the genotypes used in the experiments.

2.3 Genetic cross setup and maintenance

Experimental crosses required for analysis were setup by crossing virgin females of the driver genotype, i.e. geneA-GAL4 with the males of reporter genotype i.e. UAS-gene B or vice-versa (genetic protocols shown below). The experimental crosses were maintained at 70% humidity in 25°C, with flipping crosses into new vials carried out every 2nd day. Regular flipping was carried out to provide better condition for the flies to lay more eggs and to avoid larvae overcrowding.

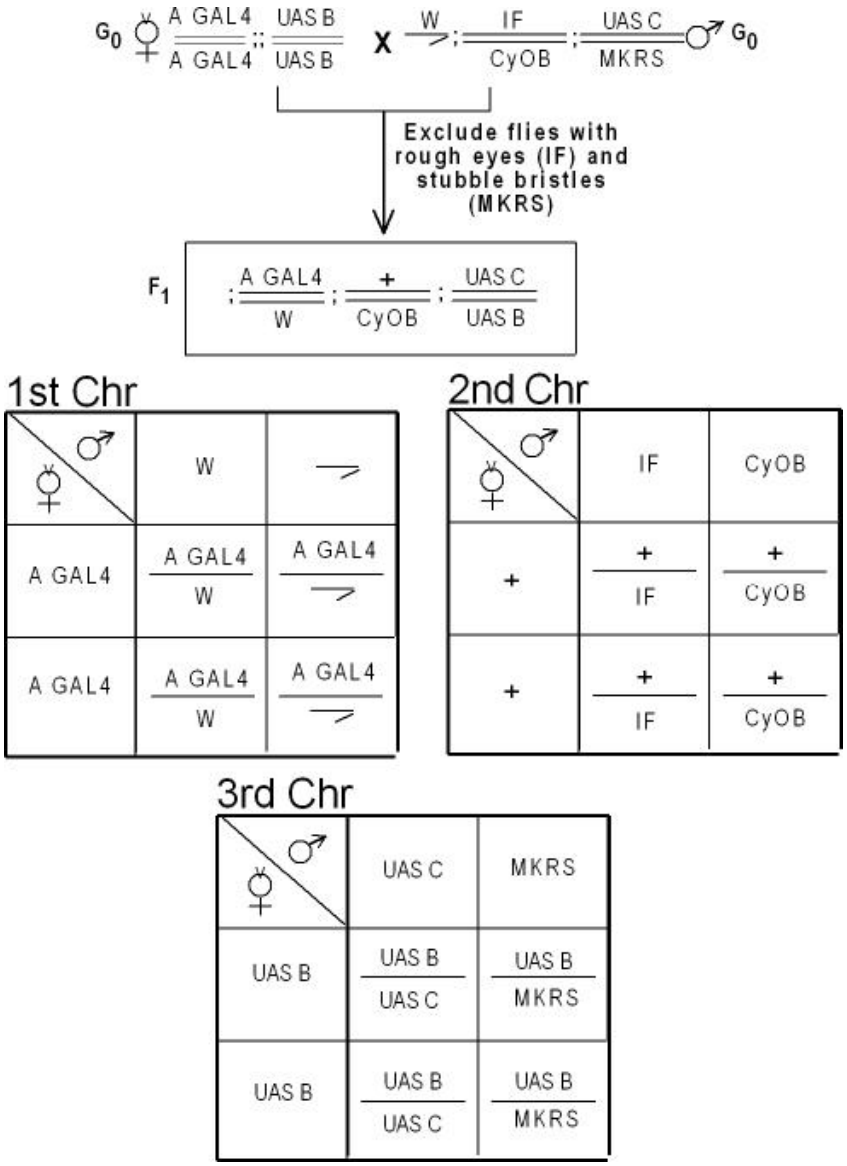


Fig 2.1 Genetic protocol for X and III chromosome (with balancer): Illustrative example showing the Punnett table outcomes that is obtained when a GAL4 driver is crossed with an

UAS reporter. In here, the example shows the virgin females homozygous for 'A GAL4' on the X and 'UAS B' on the 3rd Chr are crossed with males carrying 'UAS C' on the 3rd over MKRS (Stubble bristle) and IF (rough eyes) over CyOB (curly wings) on the 2nd chr. The segregation table is shown for the 1st, 2nd and 3rd chromosome, where 'W' corresponds to the white gene. Even though different combination is possible between the three chromosomes, but the genotype to be selected from the F1 progenies should have A GAL4 on the X chromosome and UAS B and UAS C on the 3rd chromosome with Curly wings.

2.4 Experimental larva and adult fly selection for brain dissection

Once the eggs hatched, the experimental crosses maintained at 25°C were checked occasionally for development of larvae. For larvae dissection, wandering third instar larvae were chosen and washed in water before placing them in PBS for dissection of either central or peripheral nervous system. In case of adult brain analysis, progenies obtained from the experimental cross were segregated by the expression of gene of interest. Female flies were particularly chosen for dissection because of the presence of LPTC GAL4 on the X chromosome.

2.4.1 Larval brain dissection

Larvae dissection was carried out by transferring larvae from water to cold phosphate buffered saline solution (PBS), and using forceps, fat bodies and other developing tissues were carefully removed after the larva was ripped open. The larva brain was then fixed in 4% paraformaldehyde in 0.1% PEM for an hour at room temperature.

2.4.2 Adult brain dissection

The flies were first anesthetized on ice and were place in ethanol for 30 seconds to remove the wax coat from their body. The anesthetized flies were then transferred to cold PBS, and dissection was carried out by carefully removing the head tissues and retina. Dissection was carried out for a period of 20 minutes. The trachea attached to the brain were removed carefully without tearing the optic lobes or the central brain, and the brain was then fixed in 4% paraformaldehyde in 0.1% PEM for a period of one hour at room temperature.

2.4.3 Larval peripheral nervous system dissection

Larvae flat preparation for PNS dissection was carried out in low Ca^{2+} saline solution (128mM NaCl, 2mM KCl, 4mM $MgCl_2$, 0.1mM $CaCl_2$, 35.5mM Sucrose and 5mM HEPES (pH 7.2)) in a magnetic dissection chamber. With the larvae spiracles facing upwards, centre pins were placed to anterior and posterior ends and the larvae was stretched a little to make dissection easier. Using a dissection scissors, a small cut was made to the posterior end and keeping the lower blade below the cuticle, a smooth cut was made from the posterior to anterior side along the middle. Upon opening the larvae, the internal organs were removed and the side pins were used to stretch the larvae cuticle to form it as a flat sheet. After removing the organs, saline was aspirated and was replaced with 4% paraformadelhye in 0.1% PEM with pins stills attached. After fixing for 5 minutes, the pins were removed and the cuticle was incubated again with 4% paraformadelhye in 0.1% PEM for 15 minutes at room temperature.

2.5 Immunostaining of *Drosophila* larval brains

After the incubation period, fixative was removed and samples were quickly rinsed twice with phosphate buffered saline containing triton x-100 (0.5%) (PBT). The quick rinse was followed by washing the samples three times with PBT at room temperature with 15 minute intervals. After the final wash, the samples were blocked with PBT in 10% normal goat serum for an hour at room temperature. The blocking was followed by placing the samples in terasaki plates with primary antibody solution (rabbit anti-DsRed (1:100)) and incubating overnight at 4°C. The incubation was followed by washing the samples with PBT 5x at room temperature with a 10 minute interval. After washing, secondary antibody (goat anti-rabbit Alexa 546 (1:250) in PBT was added to the sample and incubated over night at 4°C. In case of double staining, the same procedure as stated prior is carried out by washing the samples with PBT and then adding primary (mouse anti-BP102 (1:250)) and secondary (1:250) antibodies in a sequential manner with the same incubation period. After completion of staining, the samples were washed thrice with PBT and twice with PBS before mounting them.

2.6 Immunostaining of *Drosophila* adult brains

For adult brain staining, the procedure was similar to larvae brain immunostaining, however, the incubation period is quite longer, with primary antibody (Rabbit anti-DsRed (1:100)) incubation being carried out for 2-3 days at 4°C and the secondary antibody incubation (Goat anti-Rabbit A488 (1:250)) being carried out for 1-2 days at 4°C.

2.7 Immunostaining of *Drosophila* peripheral nervous system

For PNS staining, the procedure and incubation was similar to larvae staining, with primary antibody being either rabbit anti-GFP (1:500) or rabbit anti-DsRed (1:100) and the secondary antibody being goat anti-rabbit A488.

2.8 Mounting of samples

The immunostained larvae brain, adult brain or PNS cuticle samples after final wash with PBS were mounted in glass slide with bridges using Vectashield. The samples were placed with the dorsal side facing upwards in case of larval/adult brains while for PNS the cuticle was placed with the interior facing upwards. The mounted samples were covered with thin cover slips and were sealed with nail polish. The slides were then stored at 4°C.

2.9 Imaging and processing of samples

The mounted samples were imaged using either the Leica TCS SP or Zeiss LSM 710 scanning confocal microscope, with 25x or 40x oil lenses, 1x zoom, speed 400 HZ, expansion 3, 0.5µm section and 512x512 or 1024x1024 resolutions. The scanned images and the stacks were processed using Image J, Adobe Photoshop and Adobe Illustrator.

CHAPTER 3

RESULTS

Ectopic expression of *Drosophila* neurotrophins in adult *Drosophila* central nervous system (CNS)

3.1 Structural patterning of lobula plate tangential cell neurons and ellipsoid body associated R-neurons

To identify the expression pattern of the lobula plate tangential cell (LPTC) neurons and the R-neurons associated with EB, we analysed brains of flies of the driver line LPTC GAL4/FM7; +/+; 10xUASmCD8RFP/TM3 Serrate, which has expression of RFP in the LPTCs and EB associated R-neurons. In here, female flies were chosen for analysis because their body size is being bigger than male flies, which tends to make the dissection easier. Another reason for choosing the female flies was due to the presence of the first multiple 7 balancer. As females have two X chromosomes, even if they carry the FM7 balancer they can still be used for the purpose of dissection, but however this is not possible for male flies which carries one X and one Y chromosome.

Due to low fluorescence emission of endogenous RFP in the adult brain, primary staining with DsRed (i.e. targets RFP) and secondary staining with Alexa 488 was carried out to enhance the labelling of dendrite arborisation and structure of neurons and axons. The *Drosophila* brain expressing the GAL4/UAS showed presence of different layers in the optic lobes, which could be differentiated as medulla (M), lobula plate (LP) and lobula (L). However, the ability to segregate the vertical system (VS) and horizontal system (HS) neurons in the lobule region seemed to be quite difficult because they appeared as a single massive dendrite. The six VS cells and the three HS cells were organized in a serially overlapped fashion with their axons extending to the periesophagal region. The extending axons seemed to bifurcate into the central brain region, which can be clearly differentiated upon moving from the dorsal to ventral region of the *Drosophila* brain. The VS somas seem to be present in the lobula plate region, and the HS somas consisting of the HSN, HSE and HSS, even though not distinguishable, was seen to be distributed anterior to the VS cells in the lobula plate region.

In addition to LPTC neurons, the staining outlined the ellipsoid body (EB) with their associated R-neurons. The EB, present in the central brain region, was easily noticed as a structure with two concentric rings with the outer neuropile ring being less dense than the inner neuropile ring (Fig. 3.1C). The R-neurons cell bodies were found to be located as a

cluster of cells with the dendrites present on the lateral protuberance. Axons of the associated R-neurons were observed to be running through the centre of the EB structure on the periphery of the inner concentric region.

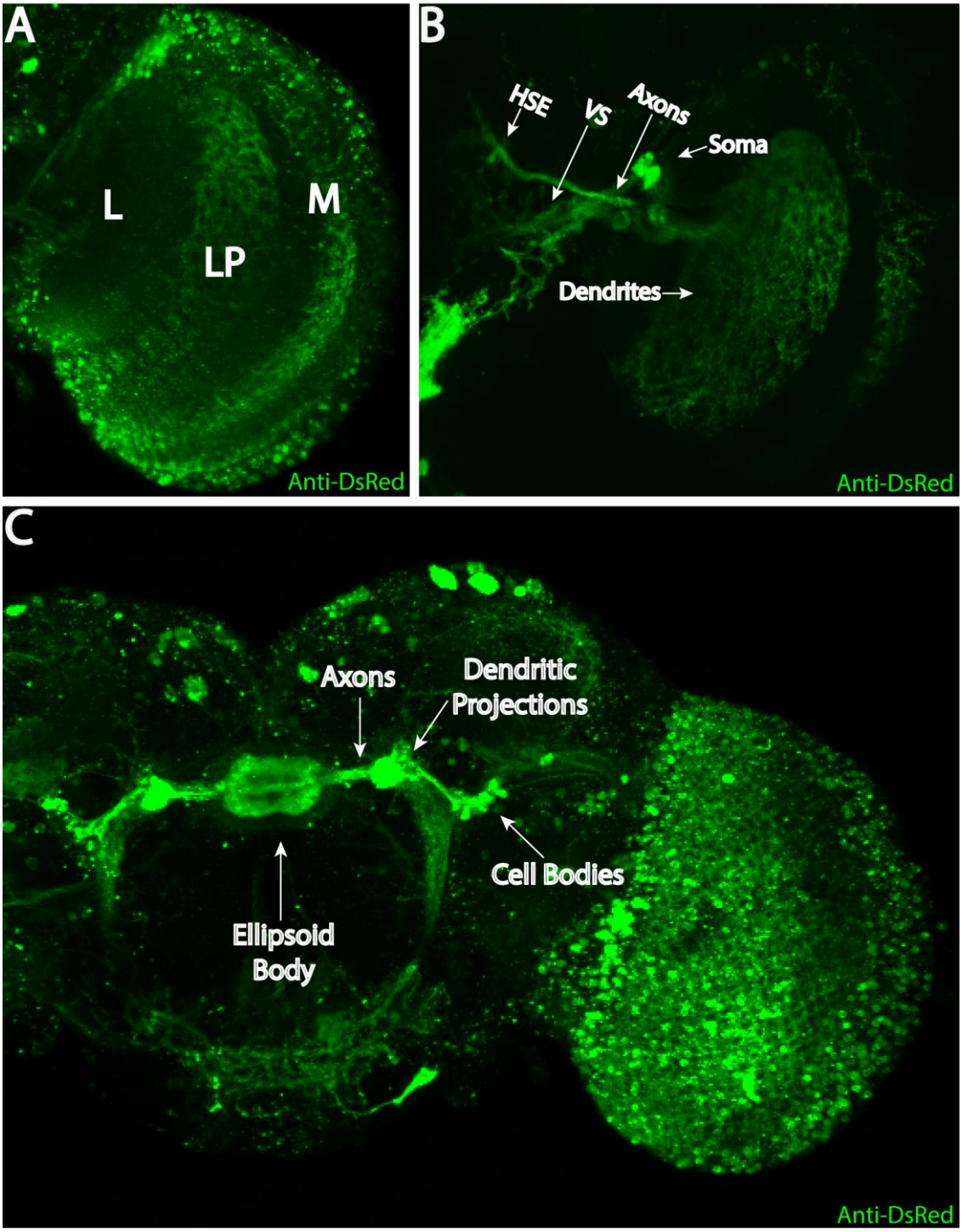


Figure 3.1: Expression pattern of the driver line LPTC-GAL4 showing LPTC neurons and ellipsoid body associated R-neurons. (A) Shows the segmentation in the optic lobe region, which is seen as three region i.e. the outer medulla (M), the centre lobula plate (LP) and inner lobule (L). (B) shows the LPTC neuron compartmentalization and structural feature, which includes the massive dendrite in the optic lobe, the adjoining soma and the elongating axons that gives rise to branches, i.e. VS and HSE. (C) shows the ellipsoid body as two concentric ring in the central brain region, with the R-neuron consisting of the axon, dendrite projection and cell bodies running through the centre of the EB's concentric ring structure.

3.2 Ectopic expression of *Drosophila* neurotrophin 1 (DNT1) in the adult *Drosophila* CNS

To observe the effect of DNT1on adult *Drosophila* brain, we ectopically expressed full length DNT1 (UAS DNT1 Full Length) and activated form of DNT1 (UAS DNT1 CystineKnot) with the driver line LPTC GAL4/FM7; +/+; 10xUASmCD8RFP/TM3 Serrate, using the GAL4/UAS targeted expression system. In here, full length DNT1 are basically pre-pro-neurotrophins, which contain the signal peptide, pro-domain and cysteine knot domain (Fig. 1.1) and the cysknot DNT1 are the active or mature form of the neurotrophin that is devoid of the signalling peptide (Fig.1.1). Here, expression of UAS mCD8RFP by the driver line LPTC GAL4 was used as control.

Results showed difference between the flies expressing DNT1 FL and control, but not with flies expressing DNT1 CK. On comparing the morphology of the ellipsoid body, the flies expressing DNT1 FL (Fig 3.2B) and DNT1 CK (Fig 3.2C) seemed to have a no differences i.e. the ellipsoid body structure (presence of two distinguishable concentric neuropile rings) was similar to control (Fig 3.2A). But however in the DNT1 FL progenies the cell bodies of the EB associated R-neuron were quite different from control and DNT1 CK. In DNT1 FL, the R neurons seemed to be more scattered than being seen as a cluster in the anterior side of the brain. In addition to this, the axons of the cell bodies of the R-neurons were thinner and less densely packed (Fig 3.2B indicated by arrow). However, in both the cases i.e. less densely packed (DNT1 FL) or normal (DNT1 CK), the axons of the R-neurons seems to pass through the inner concentric ring of the EB.

On visually comparing the LPTC neuron's dendrite arborisation in the optic lobes, the structure of the soma cells and the dendrite branching in the lobule plate region seemed to be similar between the control (Fig 3.2D) and progenies expressing DNT1 FL (Fig 3.2E) & DNT1 CK (Fig 3.2F). The VS and HS axons branching into the ventro-lateral protocerebrum region also seemed to be quite unchanged between the control and progenies expressing DNT1 FL and DNT1 CK.

Even though the overall observation showed the DNT1 FL to have an effect on the EB associated R-neuron, but apart from this there was no other visible changes present in the EB or the LPTC neurons. However in the above cases, the observations are only through visual analysis of the processed image. In order to clearly identify the molecular effects of DNT1 on

the dendrite branching and volume, axons and soma numbers, further analysis through modelling software (i.e. Amira) and use of MARCM clones is required in the future to identify the possible changes in individual VS and HS cell dendrite branching to have a strong evidence that can point to any possible change that might have occurred due to the ectopic expression of DNT1 FL or CK.

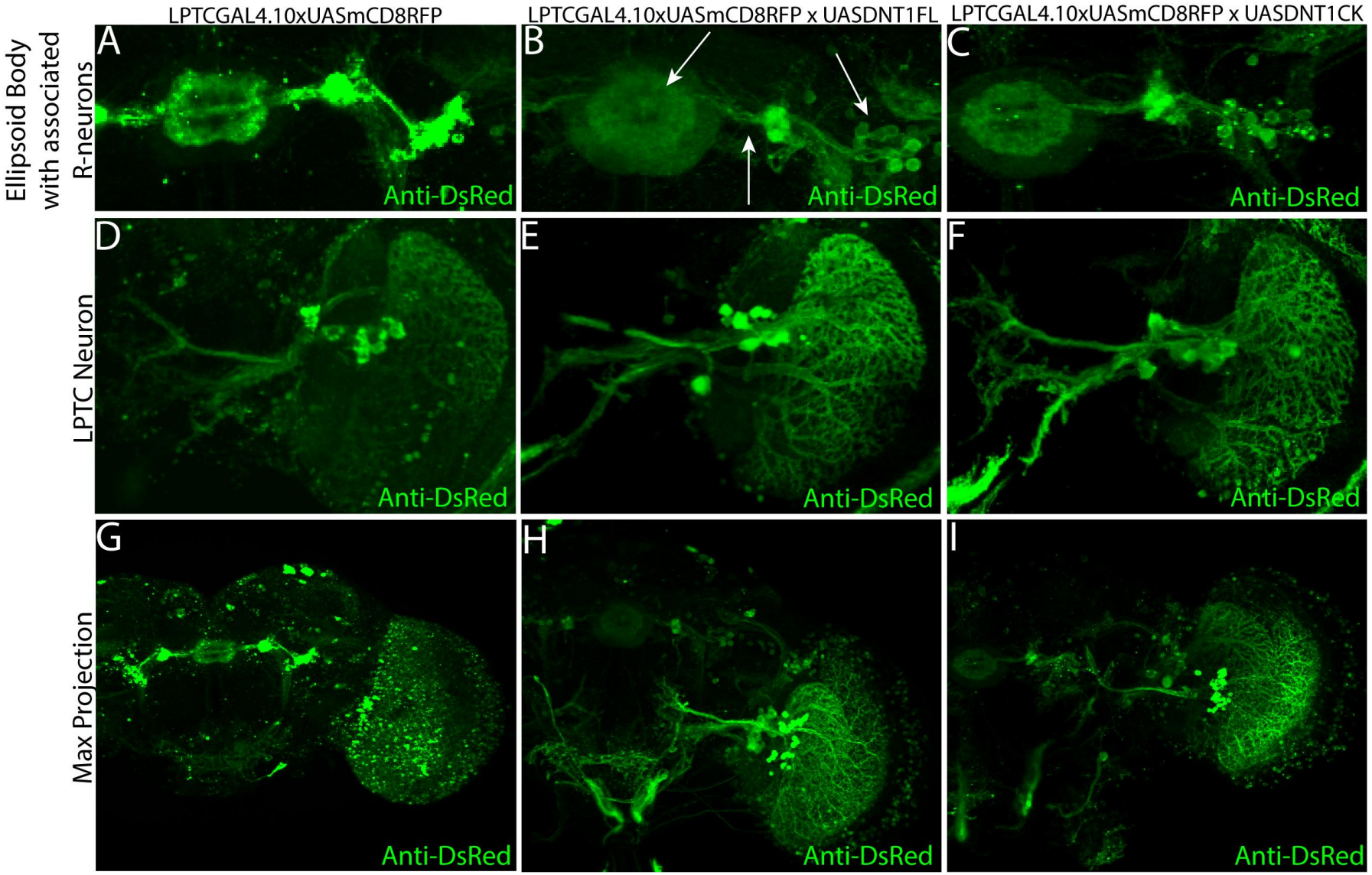

LPTCGAL4.10xUASmCD8RFP
LPTCGAL4.10xUASmCD8RFP x UASDNT1FL
LPTCGAL4.10xUASmCD8RFP x UASDNT1CK
Ellipsoid Body with associated R-neurons
LPTC Neuron
Max Projection
A
B
C
D
E
F
G
H
I
Anti-DsRed

Figure 3.2: Expression pattern of DNT1 full length and Cysknot with the driver line LPTC GAL4. (A) and (D) shows the architecture of Ellipsoid body and LPTC neuron in the control, with (G) representing the overall projection of the adult *Drosophila* control brain. (B) and (C) representing the ellipsoid body pattern in DNT1 FL and DNT1 CK, respectively, doesn't show any difference when compared to the control except for the defasiculation of axon and disturbance in the soma cell cluster indicated by arrows in DNT1 FL expressing brains. (E) and (F) representing the LPTC neuron's massive dendrite of DNT1 FL and DNT1 CK expressing flies, respectively, doesn't show any difference between them or with the control, which can also be clearly seen in the overall projection (H) and (I) for the adult *Drosophila* brain.

Driver	Responding line	Number of brains analyzed	Penetrance		Expressivity [Phenotypes]						
			Brains with Phenotype	Percentage	EB and associated R-neurons				LPTC Neuron		
					Axon defasiculation	EB Structure defect	Soma clustering disturbance	Reduced dendrite arborisation	Axon defasiculation	Soma clustering disturbance	LPTC dendrite arborisation
LPTC GAL4;;10x UAS mCD8RFP (Control)		5	0	0	0	0	0	0	0	0	0
LPTC GAL4;;10x UAS mCD8RFP	UAS DNT1 FL	5	3	60	4	0	3	0	0	0	0
	UAS DNT1 CK	5	0	0	0	0	0	0	0	0	0

Table 3.1: Penetrance and expressivity of UAS DNT1 FL and UAS DNT1 CK with the driver line LPTC GAL4. Here, the number of flies taken into consideration was 5 (n=5). The control and UAS DNT1 CK expressing flies showed no structural defects, but flies expressing UAS DNT1 FL showed structural changes on the axons and soma cell cluster of the R-neurons

3.3 Ectopic expression of *Drosophila* neurotrophin 2 (DNT 2) in the adult *Drosophila* CNS

To observe the effect the *Drosophila* Neurotrophin 2 has on the structural organisation of the LPTC neurons and on ellipsoid body associated R neurons, we ectopically expressed full length DNT2 (UAS DNT2 FL) and activated form of DNT2 (UAS DNT2 CK) with the driver line LPTC GAL4/FM7; +/+; 10xUASmCD8RFP/TM3 Serrate. In here, full length DNT2 are basically pre-pro-neurotrophins, which contain the signal peptide, pro-domain and cysteine knot domain (Fig. 1.1) and the cysknot DNT2 are the active or mature form of neurotrophin that is devoid of the signalling peptide (Fig.1.1). In here, the driver line LPTC GAL4 expressing UAS mCD8RFP was used as controls.

Visualisation of the LPTC neurons or EB or the R neurons was not possible in flies expressing either DNT2 FL or DNT2 CK. The structure of the neurons in the adult brain wasn't able to be scanned using scanning using confocal microscopy because of high intensity RFP expression by the cells present on the surface of the central brain and the photoreceptor cells in the optic lobe region. The interference of RFP was mainly due to the PhiC31-based integration system that was used to insert UAS DNT2 FL and DNT2 CK in the *Drosophila* genome. The PhiC31-based integration system developed by Konard Basler (Bischof *et.al.*, 2007) has *attP* sites mapped into the *Drosophila* genome at specific sections in the chromosomes that helps in the perfect insertion of transgene at any desired location. The construct of the docking site [M{3xP3-RFP*attP*}] is designed in such a way that the inbuilt RFP is not removable and is expressed in the progenies as part of their genome.

Data has not been provided for the DNT2 FL or DNT2 CK because of the above mentioned hardship. But however, if UAS DNT2 FL and UAS DNT2 CK flies are to be generated using *P*-element based insertion system, observation of results focusing on the effect of DNT2 FL and DNT2 CK is a possibility in the future.

3.4 Ectopic expression of *Drosophila* Spatzle (Spz) in adult *Drosophila* CNS

To observe the effect *Drosophila* Spatzle has on ellipsoid body (EB) with its R-neurons and the lobula plate tangential cell (LPTC) neurons, we ectopically expressed Spatzle full length (UAS Spz FL) and activated Spatzle (UAS Act Spz) in LPTC GAL4/LPTCGAL4; +/+; 10xUASmCD8RFP/10xUASmCD8RFP, using the GAL4/UAS targeted expression system. In here, full length Spz are basically pre-pro-neurotrophins, which contain the signal peptide, pro-domain and cysteine knot domain (Fig. 1.1) and the activated or cysknot form of Spz are the active or mature form of the neurotrophin that is devoid of the signal peptide (Fig.1.1). The LPTC GAL4 progenies expressing UAS mCD8RFP were used as control.

On analysing the data, a comparison wasn't able to be made with the control (Fig 3.3A) for the progenies expressing Spz FL. This was due to the strong interference by the endogenous fluorescence of the photoreceptor cells in the optic lobe region and in the central brain region. This interference was due to RFP expression by the PhiC31-based integration system that was used in the transgenesis of the UAS Spz FL gene into the *Drosophila* genome. (Fig 3.3H, indicated by arrows).

However, data for the expression of activated form of Spatzle (Act-Spz) did show an effect on the structural organisation of the neurons in the *Drosophila* adult brain. Upon looking at the ellipsoid body, ectopic expression of Act-Spz seemed to have resulted in the fusion of the two concentric ring structures to a single ring structure with a visible hole in the centre (Fig. 3.3C indicated by arrow) of the inner concentric neuropile ring of the ellipsoid body. Also, the R-neurons axons seemed to be not projecting into the EB's inner ring structure and are rather found to be more defasiculated than being tightly packed as a coiled structure. The R-neuron dendrites and cell bodies also seemed to be disorganised and scattered in the anterior region (Fig 3.3C, indicated by arrow), when compared to the control. In LPTC neurons, Act-Spz expression seems to have not caused any disruption in the soma cell cluster, but on the other hand the VS axons projecting into the ventro-lateral protocerebrum region seems to be defasiculated (Fig. 3.3 F, indicated by arrow). The massive dendrites in the optic lobe region that forms from the VS and HS cells also seemed to be different from the control, with the projection of the dendrites being more branched in the anterior and posterior region of the optic lobe.

The data clearly shows activated Spatzle over-expression to result in structural deformation of the EB, EB associated R-neurons and the LPTC neurons. But however the effect of Spz FL

on the LPTC neurons or EB associated R-neurons weas not able to be obtained because of the interference by the transgenesis system. This interference can be rectified either by creating a new genotype using *P*-element mediated germ-line transformation or making a driver line LPTC GAL4 with UAS mCD8GFP rather, which has a different emission spectrum.

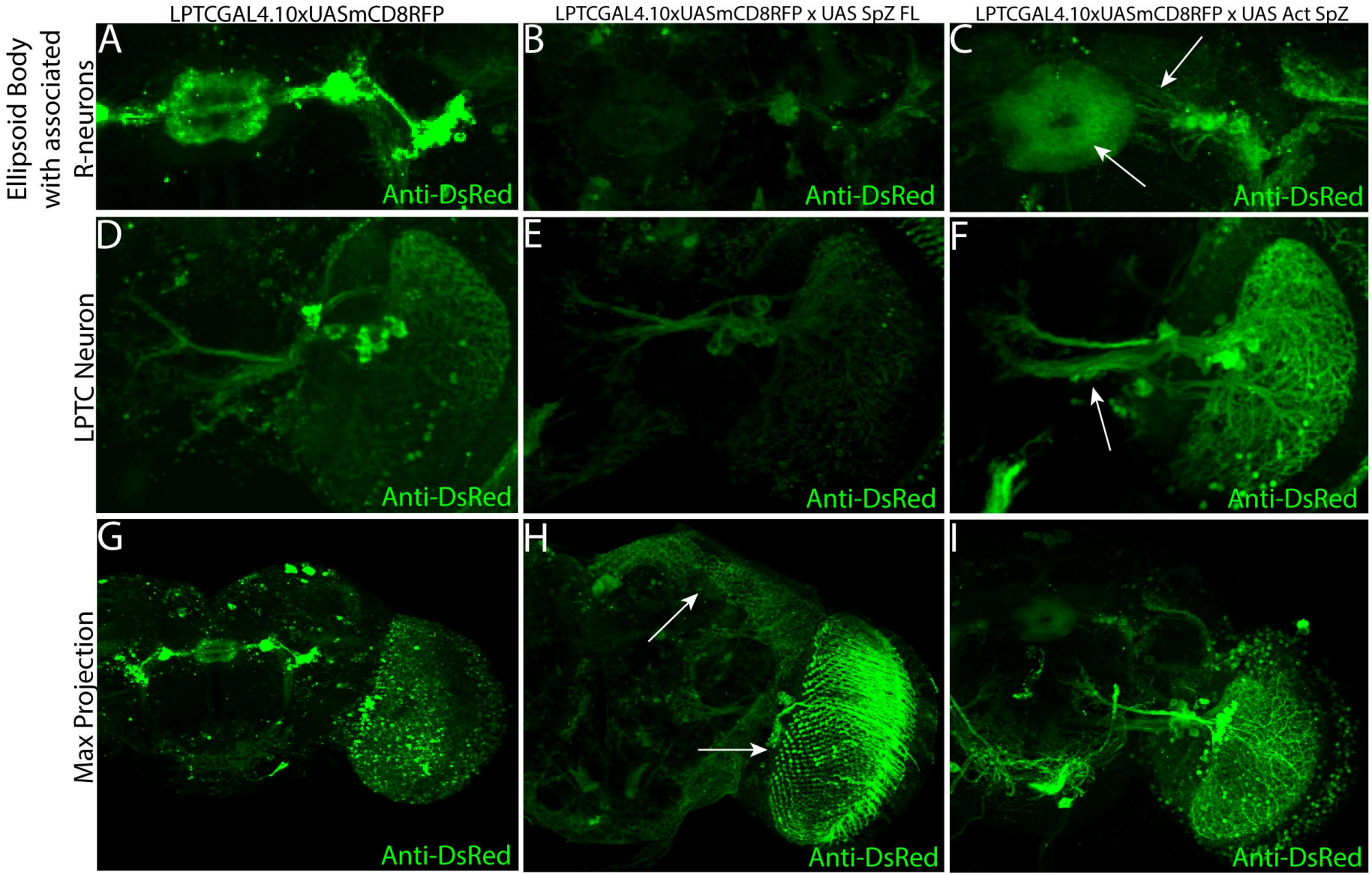

LPTCGAL4.10xUASmCD8RFP
LPTCGAL4.10xUASmCD8RFP x UAS SpZ FL
LPTCGAL4.10xUASmCD8RFP x UAS Act SpZ
Ellipsoid Body with associated R-neurons
LPTC Neuron
Max Projection
A
B
C
D
E
F
G
H
I
Anti-DsRed

Figure 3.3: Expression pattern of UAS Spz FL and UAS act Spz with the driver line LPTC GAL4. (A) and (D) shows the Ellipsoid body, its associated R-neuron and LPTC neuron in the control, with (G) representing the overall projection of the adult *Drosophila* control brain. (B) and (C) represents the ellipsoid body and R-neuron pattern of SpZ FL and Act Spz expressing progenies, respectively, with a comparable difference seen only with the Act Spz, which is the fusion of EB concentric rings and defasiculation of the R-neuron axon and soma cell cluster disturbance that are indicated by arrow. (E) and (F) represents the LPTC neuron's massive dendrite in Spz FL and Act Spz expressing progenies, respectively, with a difference seen only in the Act Spz, where the VS axon seems to be defasiculated. (H) shows the overall projection of a *Drosophila* adult brain expressing Spz FL with interference by the cells in the central brain region and optic lobe region due to PhiC31 integrase system (indicated by arrow) and (I) shows the overall projection for the adult *Drosophila* brain expressing Act Spz.

Driver	Responding line	Number of brains analyzed	Penetrance		Expressivity [Phenotypes]						
			Brains with Phenotype	Percentage	EB and associated R-neurons				LPTC Neuron		
					Axon defasiculation	EB Structure defect	Soma clustering disturbance	Reduced dendrite arborisation	Axon defasiculation	Soma clustering disturbance	LPTC dendrite arborisation
LPTC GAL4;;10x UAS mCD8RFP (Control)		5	0	0	0	0	0	0	0	0	0
LPTC GAL4;;10x UAS mCD8RFP	UAS Spz FL	5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	UAS Act Spz	5	4	80	4	4	4	Unknown	4	0	3

Table 3.2: Penetrance and expressivity of UAS Spz FL and UAS act SpZ with the driver line LPTC GAL4. In here, the number of flies taken into consideration was 5 (n=5). The control flies doesn't have any structural defects, but flies expressing UAS Act Spz did show structural changes in the EB, R-neurons and in the axons of the LPTC neurons. As further analysis is required on the dendrites of the R-neurons they have been labelled unknown. For UAS Spz FL expressing flies, as analysis was not possible due to PhiC31-based integrase system interference they have been labelled N/A i.e. not available.

Ectopic expression of *Drosophila* Toll receptors in adult *Drosophila* central nervous system

3.5 Ectopic expression of activated form of *Drosophila* Toll or Toll-1 receptor and Toll-6 receptor in the adult *Drosophila* CNS

To test if expression of *Drosophila* Toll receptors has any effects on the architecture of the neurons in the adult *Drosophila* central brain or the optic lobe region, we ectopically expressed Toll (Toll-1) and Toll-6 receptors, as their activated form i.e. $Toll^{10b}$ and $Toll6^{CY}$, in the *Drosophila* system using the targeted GAL4/UAS expression system. In here, the activated form of the Toll receptors are the active mature form of the receptors that is devoid of the signal peptide. The LPTC GAL4 progenies expressing UAS mCD8RFP were used as control.

Here, the Toll-7 receptor was not considered for experimentation because they are not found to be expressed in the region surrounding protocerebrum or optic lobe or central complex, which made it an unlikely candidate to have any activity on the LPTC neuron, the ellipsoid body or its associated R-neurons.

Analysis of the result showed that expression of activated form of Toll (Toll-1) receptor ($Toll\ 10^{b}$) did have an effect on the structure of the ellipsoid body. In the adult brain, (Fig 3.4 B) even though the two concentric ring structures of the ellipsoid body were distinguishable, but the hole in the inner concentric ring region, where the R-neuron's axon passes, seems to have increased in size when compared to control (Fig. 3.4 A). Also, the R-neurons associated with EB were found to have changes in their structures, with the axon being defasiculated and the soma cell being scattered (Fig. 3.4 B). In case of the LPTC neurons (Fig. 3.4 E), neither the soma cells or the dendrite in the optic lobe region or the axon projecting into the proto-cerebrum region were found to have any changes in their structure, when compared to the control.

In flies ectopically expressing activated Toll-6 receptors ($Toll\text{-}6^{CY}$), the effect was seen only in the R-neurons and the LPTC neuron, and not on the structure of EB. The EB seemed to be normally distinguishable in the central brain region as two concentric ring structures that were similar to the control. But however expression of $Toll6^{CY}$ seems to have resulted in the soma cells to be scattered in the R-neurons (Fig. 3.4 C). Another difference that was observed was in the structure of LPTC neuron (Fig. 3.4 F), where the dendrite arborisation in the optic

lobe region seemed to be different from the control. However apart from this, the axon projection or the soma cell cluster seems to be not disturbed. In Toll-6^{CY} and Toll 10^{b}, the assumption is just through visual analysis, therefore in the future further analysis needs to be carried out to clearly find out if there is any change in the dendrite arborisation.

From the above observations, it can be stated that expression of the activated form of Toll (toll-1) and Toll-6 receptors does seem to have an effect on the structure of the neurons and the EB in the central brain region. To understand the possible effect of activated Toll receptors on the VS and HS cell dendrites branching and their axons, further investigation should be carried out in the future using MARCM clones to understand the exact changes that occur due to the over-expression of receptors in the neurons.

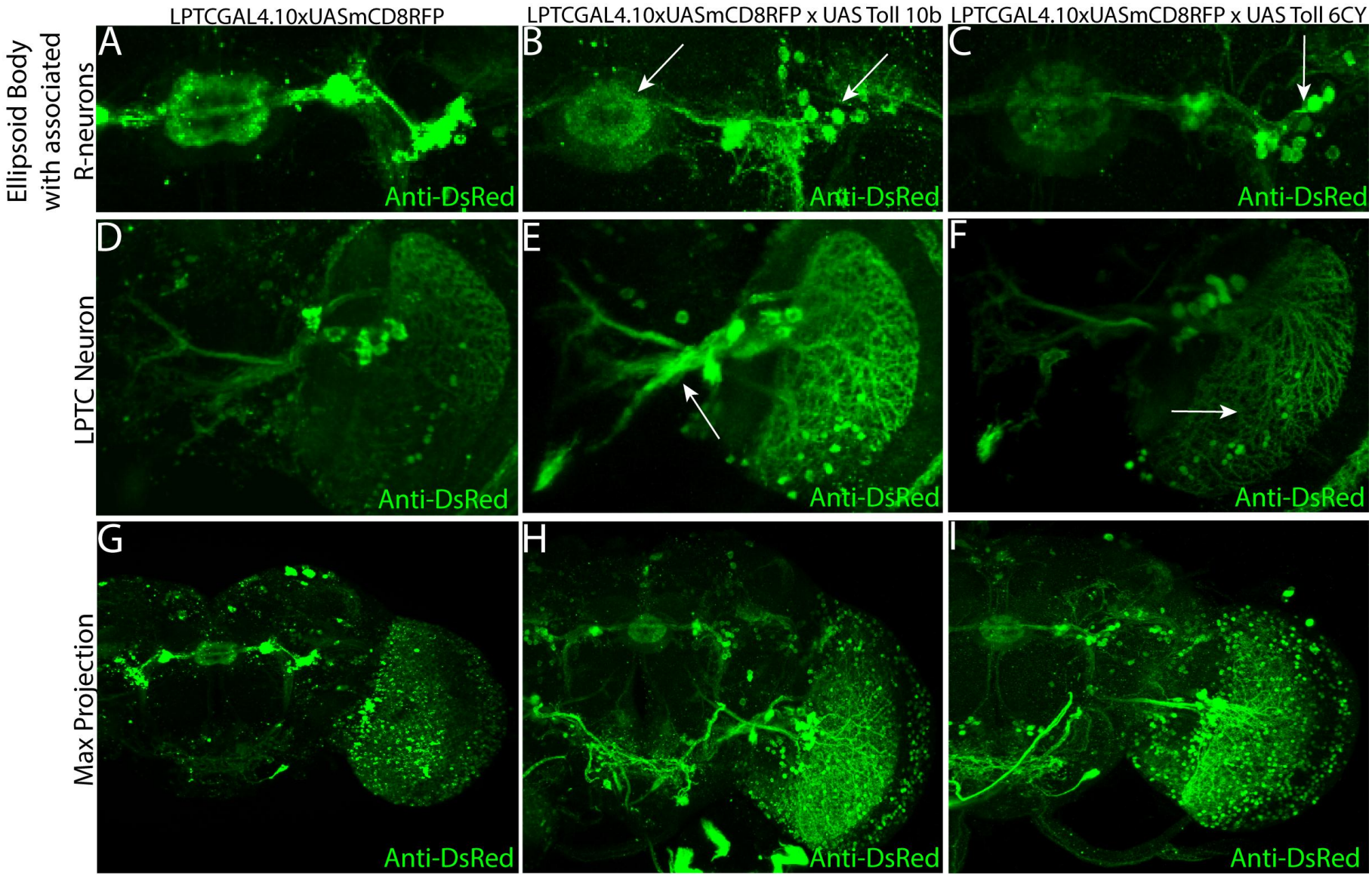

LPTCGAL4.10xUASmCD8RFP
LPTCGAL4.10xUASmCD8RFP x UAS Toll 10b
LPTCGAL4.10xUASmCD8RFP x UAS Toll 6CY
Ellipsoid Body with associated R-neurons
LPTC Neuron
Max Projection
A
B
C
D
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Anti-DsRed

Fig 3.4: Expression pattern of *Drosophila* Toll receptors with the driver line LPTC GAL4. (A) and (D) shows the structure of the ellipsoid body and LPTC neuron in control, with (G) representing the overall projection of the control. (B) and (C) shows the activated forms of Toll and Toll6 receptors expression in the LPTC GAL4 genotype, with the ellipsoid body showing an increase in the inner concentric ring hole of EB and scattering of R-neuron soma cell bodies in the flies expressing $Toll^{10b}$ and $Toll6^{CY}$, respectively(indicated by arrow). (E) and (F) shows the LPTC neuron structure, with the dendrite arborisation being different in appearance in $Toll6^{CY}$ expressing flies and VS axon being defasiculated in $Toll^{10b}$ expressing flies (indicated by arrow). (H) and (I) shows the overall projection of the adult *Drosophila* brain expressing the activated forms of Toll and Toll-6 receptors, respectively.

Driver	Responding line	Number of brains analyzed	Penetrance		Expressivity [Phenotypes]						
			Brains with Phenotype	Percentage	EB and associated R-neurons				LPTC Neuron		
					Axon defasiculation	EB Structure defect	Soma clustering disturbance	Reduced dendrite arborisation	Axon defasiculation	Soma clustering disturbance	LPTC dendrite arborisation
LPTC GAL4;;10x UAS mCD8RFP (Control)		5	0	0	0	0	0	0	0	0	0
LPTC GAL4;;10x UAS mCD8RFP	UAS Toll ^{10b}	5	3	60	3	0	4	Unknown	4	0	0
	UAS Toll6 ^{CY}	5	3	60	0	0	3	Unknown	0	0	2

Table 3.3: Penetrance and expressivity of UAS Toll^{10b} and UAS Toll6^{CY} with the driver line LPTC GAL4. In here, the number of flies taken into consideration was 5 (n=5). The control flies doesn't have any structural defects. Flies expressing Toll^{10b} has shown structural changes in the EB, R-neurons and in the axons of the LPTC neurons, and flies expressing Toll6^{CY} has shown structural changes in the R-neurons soma cluster and LPTC dendrite. As further analysis is required on the dendrites of the R-neurons they have been labelled unknown.

3.6 Identification of suitable GAL4 driver(s) for labelling interneuron dendrite branching in the mechanosensory domain of larvae ventral nerve cord

To identify a suitable circuitry neuron that has its interneuron dendrites projecting into the ventral neuropile region i.e. the mechanosensory domain in the VNC of the larvae CNS, we expressed HB9 GAL4 (*HB9* expressing ventral motorneurons and interneurons), D42 GAL4 (Toll 6 receptor expressing interneurons), RRK GAL4-EVE GAL4 (*Eve* expressing dorsal motorneurons), EL GAL4 (Eve-positive lateral interneuron) and CHA GAL4 (any neuron capable of acetylcholine secretion) with UAS DenMark, respectively, and stained the larvae brain with BP102 and DsRed. Staining with BP102 helped differentiate the mechanosensory domain in the highlighted neuropile region as a hole in the VNC, while DsRed helps in better visualisation of the dendrites. Expression of all the above GAL4 drivers with UAS myristoylated tomato or UAS mCD8GFP was used as control.

Observation of the pattern of dendrite projection via transverse view clearly showed difference between the circuit neuron projections, that wasn't observed through the horizontal view. Upon analysing the data, expression of myr::tomato and DenMark in D42 GAL4 (Fig. 3.5) clearly labelled the Toll-6 receptor expressing interneurons that were surrounding the neuropile region in the dorsal and ventral side of the VNC, but these neurons showed no projection of dendrites into the mechanosensory domain. In EL GAL4 (Fig. 3.6), the expression of either myr::tomato or DenMark showed only minimal presence of eve-lateral (EL) interneurons in the VNC. Interneuron dendrite projection was hard to be observed in DenMark expressing larvae because of the background disturbance from the staining, but however with mCD8GFP the dendrite projection was observed from the interneuron, but was weak in signal. As the GFP signal was weak, further analysis needs to be carried out to confirm the dendrite projection by amplifying the GFP signal using Anti-GFP staining. In here, the minimal number of EL interneurons appearance with mCD8GFP and DenMark expression may be due to the site of GAL4 insertion, as different inserts have different expression range.

In CHA GAL4 and HB9 GAL4 genotypes, even though in the ventral region the expressions of myr::tomato clearly labelled the cholinergic neurons (Fig. 3.7) and inter/motor neurons expressing HB9 protein (Fig. 3.8), respectively, but due to weak fluorescence the observation of dendrites branching into the mechanosensory domain was quite difficult. However, when DenMark was expressed (Fig. 3.7 and 3.8 E&F), the staining with DsRed clearly labelled the

extension of the dendrites from the neurons present in the ventral side of the VNC into the mechanosensory domain.

In RRK GAL4-EVE GAL4 (Fig 3.9), expression of myr::tomato showed indication of presence of neurons (aCC and RP2) in the region surrounding the mechanosensory domain (Fig. 3.9C: Merge), but endogenous RFP expression wasn't able to clearly conclude the dendrite's projection because of low fluorescence intensity of RFP and background disturbance by BP102 staining. This was however overcome when expression of DenMark and staining with DsRed clearly labelled the dendrite (Fig. 3.9F transverse view). The transverse view showed the dendrite of the neurons to project into the mechanosensory domain region as a thicker bundle, and it was also observed that these dendrites were also able to interconnect the two mechanosensory domains (Fig. 3.9F transverse view), which was clearly seen as a thin line of connection.

On a whole, observation of the data with the dendrite specific marker, DenMark, clearly helped in better differentiation of the neurons and their dendrite projection in the larvae ventral nerve cord region. The results showed that of all the GAL4 lines tested CHA GAL4, HB9 GAL4 and RRK GAL4-EVE GAL4 showed potential projection of the dendrites into the mechanosensory domain. But upon comparing these GAL4 lines, RRK GAL4-Eve GAL4 genotype showed increased and better visibility of dendrite projections.

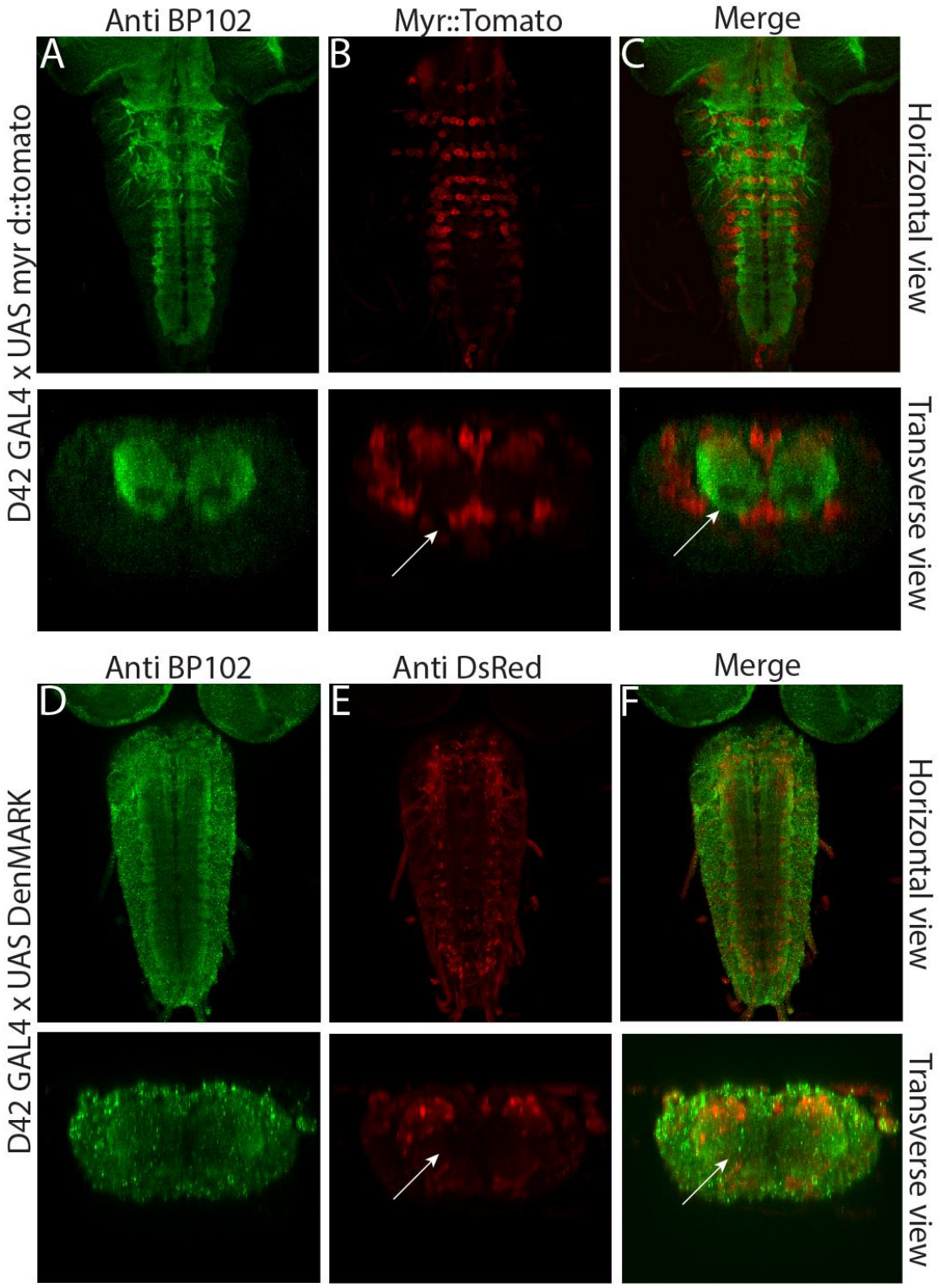


Figure 3.5: Expression pattern of the D42 GAL4 with UAS myr::tomato and UAS DenMark. (A) and (B) shows the horizontal and transverse view of staining with BP102 and myr::tomato expression. (C) shows the merged image with the absence of any dendrite projection from interneurons (indicated by arrow). (D) and (E) shows the transverse and horizontal view of staining for BP102 and DsRed for DenMark expression. (F) shows the merged image, which is a little disturbed from background staining, but still shows no dendrite projection.

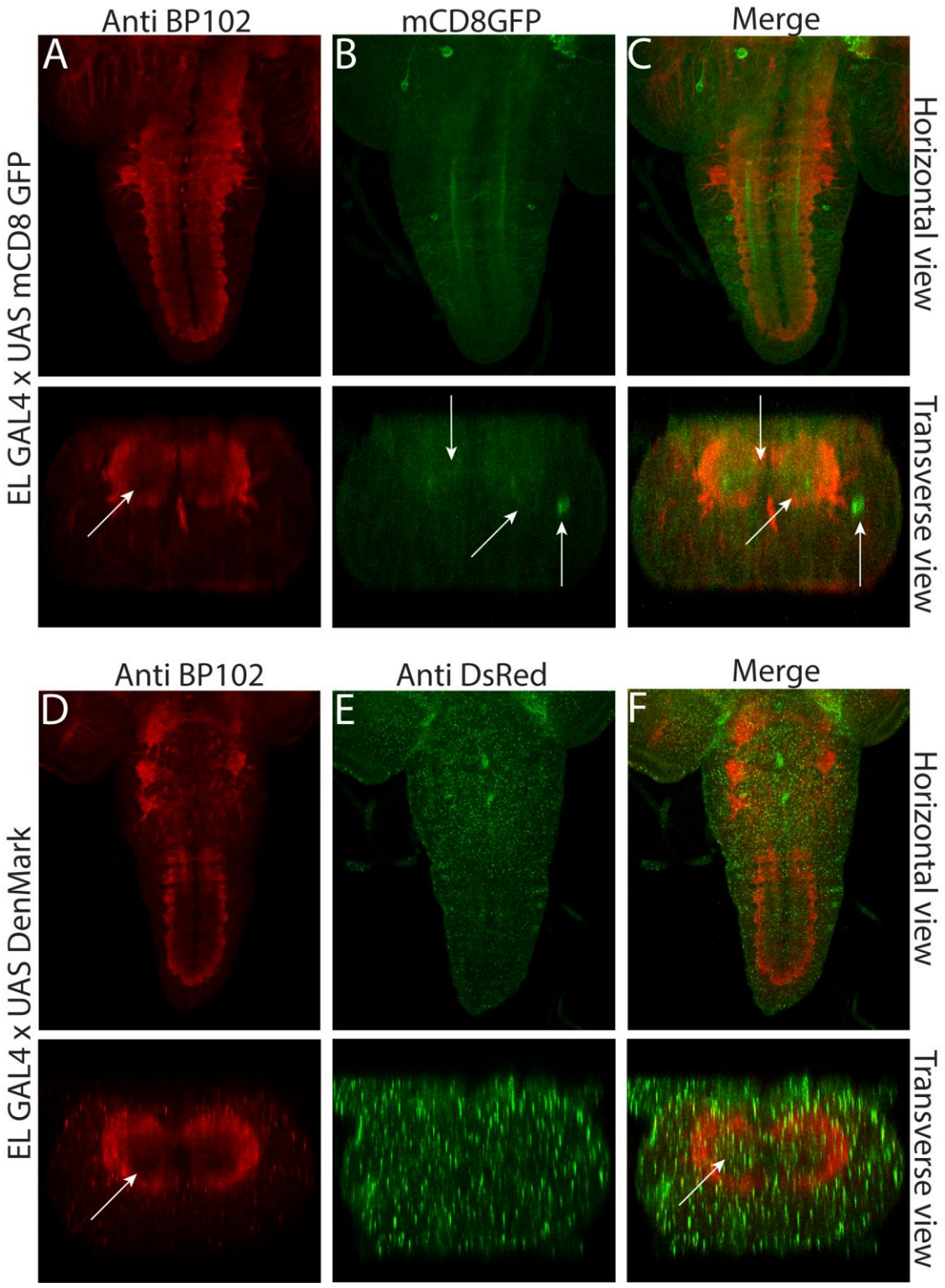


Figure 3.6: Expression pattern of the EL GAL4 with UAS mCD8GFP and UAS DenMark. (A) and (B) shows horizontal and transverse view of staining with BP102 and endogenous GFP for mCD8GFP expression (C) shows the merged image with the presence of eve-lateral neurons projecting their dendrites into the mechanosensory domain (indicated by arrow). (D) and (E) shows the horizontal and transverse view of staining for BP102 and DsRed for DenMark expression, but no dendrite projection is seen clearly into the mechanosensory domain due to background disturbance by staining.

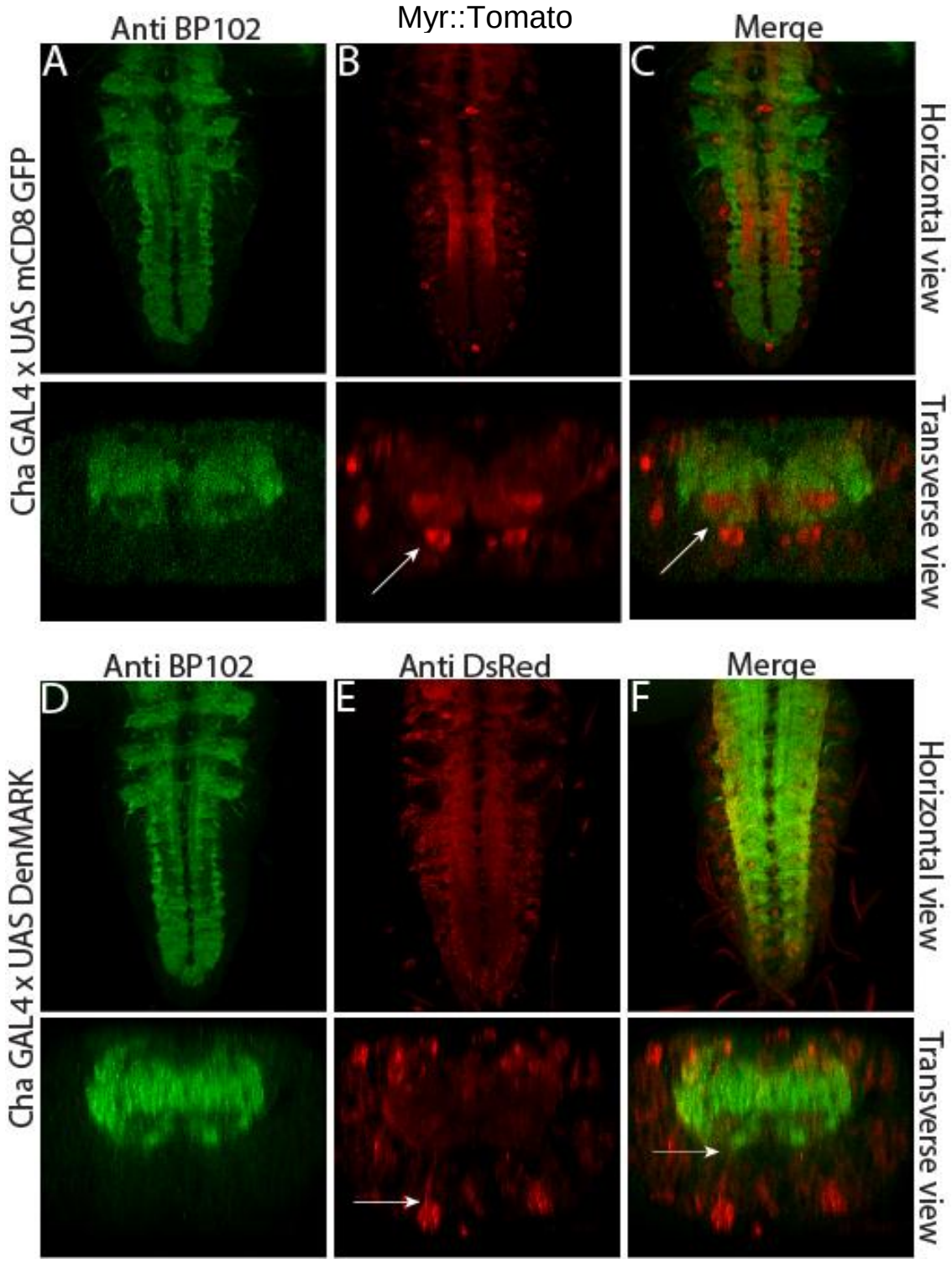


Figure 3.7: Expression pattern of the Cha GAL4 with UAS myr::tomato and UAS DenMark. (A) and (B) shows the horizontal and transverse view of staining with BP102 and myr::tomato expression, with the indication of presence of the cholinergic neurons (C) shows the merged image with the presence of cholinergic neurons and the blurred dendrite projection (indicated by arrow). (D) and (E) shows the horizontal and transverse view of staining for BP102 and DsRed for DenMark expression, with proper dendrite projection from the cholinergic neuron (F) shows the merged image, which shows the dendrite to enter into the mechanosensory domain (indicated by arrow).

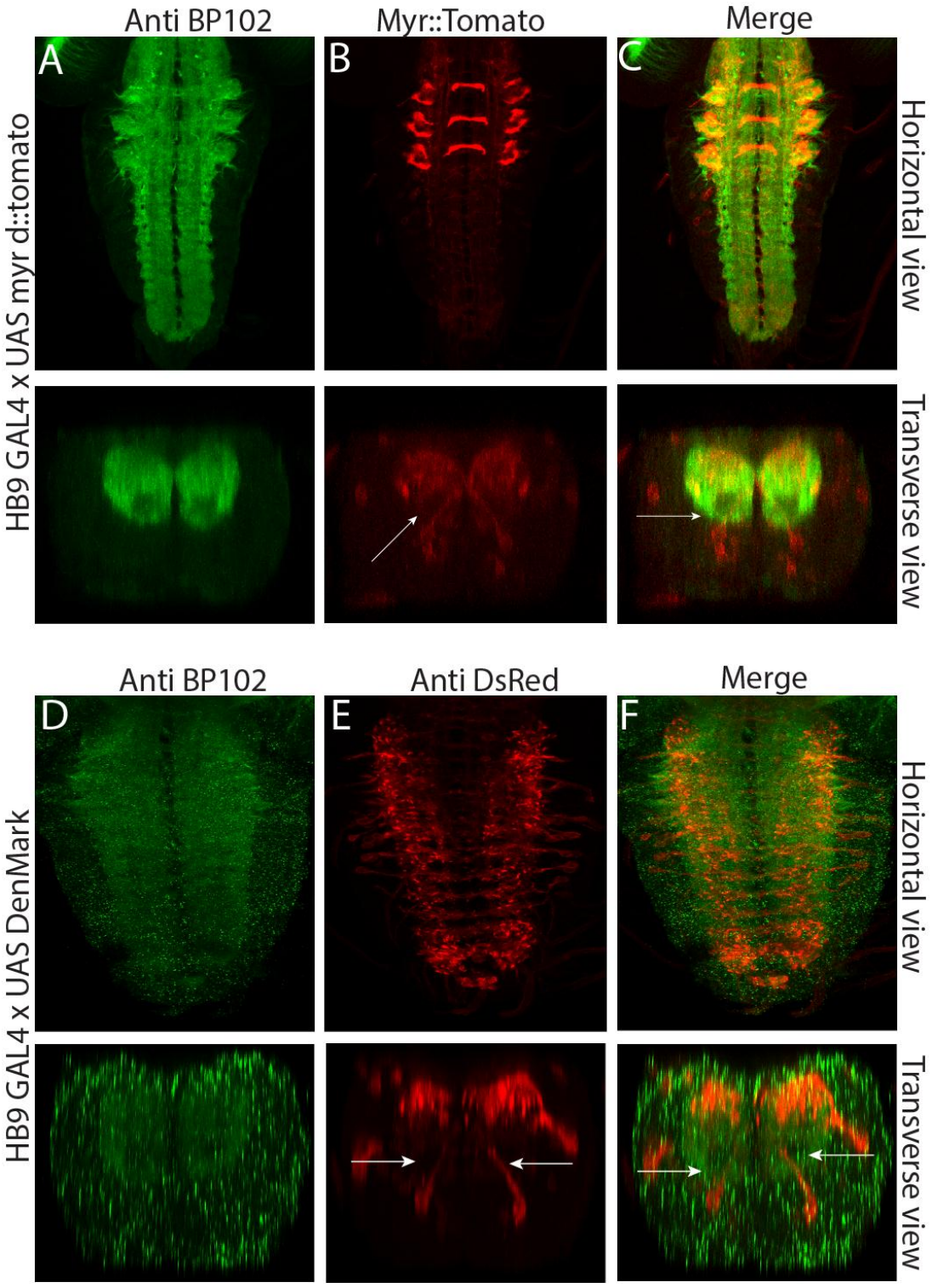


Figure 3.8: Expression pattern of the HB9 GAL4 with UAS myr::tomato and UAS DenMark. (A) and (B) shows the transverse and horizontal view of staining with BP102 and for myr::tomato expression, with the presence of neurons surrounding the mechanosensory domain (C) shows the merged image, but the neuronal dendrite projection is not clearly seen. (D) and (E) shows the transverse and horizontal view of staining for BP102 and DsRed for DenMark expression, with the DsRed staining (E) and the merged image (F) clearly showing the projection of dendrite into the mechanosensory domain.

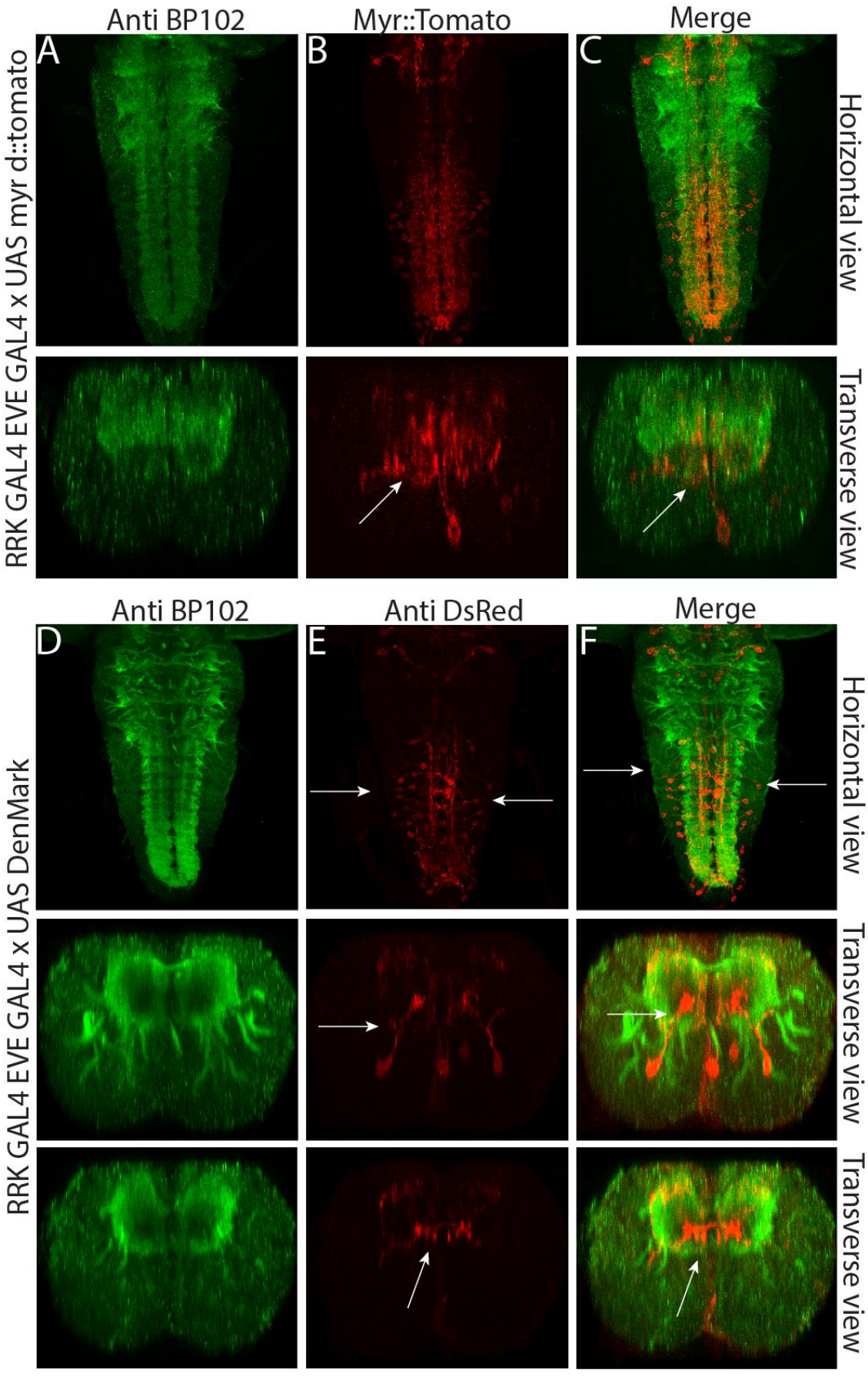

Anti BP102
Myr::Tomato
Merge
A
B
C
RRK GAL4 EVE GAL4 x UAS myr d::tomato
Horizontal view
Transverse view
Anti BP102
Anti DsRed
Merge
D
E
F
RRK GAL4 EVE GAL4 x UAS DenMark
Horizontal view
Transverse view
Transverse view

Figure 3.9: Expression pattern of the RRK GAL4-Eve GAL4 with UAS myr::tomato and UAS DenMark. (A) and (B) shows the horizontal and transverse view of staining with BP102 and endogenous RFP for myrRFP expression, with the presence of motor neuron surrounding the mechanosensory domain of the VNC. (C) shows the merged image, with the motor neuron dendrite projecting into the mechanosensory domain. (D) and (E) shows the horizontal and transverse view of staining for BP102 and DsRed for DenMark expression, with the DsRed staining and the merged image (F) clearly showing the thick bundle of dendrite projection from three different motor neurons into the mechanosensory domain. The interconnection between the two mechanosensory domain is also clearly visible (indicated by arrow) in the transverse section of the VNC.

3.7 Identification of dendrite arborisation of DA IV neurons in the larvae peripheral nervous system (PNS).

To identify the pattern of dendrite arborisation of DA IV in the peripheral nervous system (PNS), we expressed PPK GAL4 with UAS mCD8GFP and UAS DenMark. In here, the DA neurons were chosen for studying the dendrite arborisation pattern in the PNS because the dendrites are widely branched and they tend to not cross one another. But the reason for specifically looking into DA IV neurons is because the arborisation pattern of DA IV neurons is highly branched in the PNS, which makes it easy to observe and analyse the dendrite branching (Grueber *et.al*, 2003). The PPK GAL4 progenies expressing UAS mCD8GFP were used as control.

On observing the epidermis of the larvae expressing GFP (Fig 3.10A), the sensory neurons and their branching dendrites seemed to be evenly distributed throughout the larvae in a segmental manner. The expression of endogenous GFP in the larvae brain labelled the interconnection between the neuropile domains in the VNC (Fig. 3.10D). For the purpose of understanding the branching of dendrite in the PNS, the sensory neurons present in the 4^{th} abdominal segment was taken into consideration, because of wider branching and surface area. The endogenous mCD8GFP expression shows the dendrite to be arborized and the neurons to be in contact with each other in the epidermis (Fig. 3.10B), but the intricate branching isn't clearly seen with GFP. Magnification of a single neuron (Fig. 3.10C) present in the abdominal segment also didn't clearly show the dendrite branching with GFP.

However, expression of DenMark with PPK GAL4 and then staining the open-preps with DsRed seemed to have labelled the dendrite better than the expression of endogenous mCD8GFP. The immunostaining showed the dendrites to be diversely distributed, and the branching to be present throughout the epidermis with the dendrites interconnecting every neurons to another neighbouring neuron (Fig. 3.10 E and F), which on the other hand was difficult to be observed with endogenous GFP expression. Upon looking into the branching of a single neuron (Fig. 3.10 G), the dendrites also seemed to extend to nearby segment, but however further analysis is required to confirm the observation on whether the dendrite really extends to a nearby segment.

The data showed the DA IV neuron dendrite branching to be widely spread in the epidermis, which is in correlation with the DA IV neuron arborisation pattern showed by Grueber, *et.al*, 2003. This initial observation of the sensory neuron using PPK GAL4 has proven to be

effective and has helped in considering it as a suitable candidate for future studies that shall involve on observing the effect of neurotrophin and toll receptors activity on dendrite arborisation. This approach in the future can provide new insights on the importance of neurotrophins and Toll receptors in the peripheral nervous system.

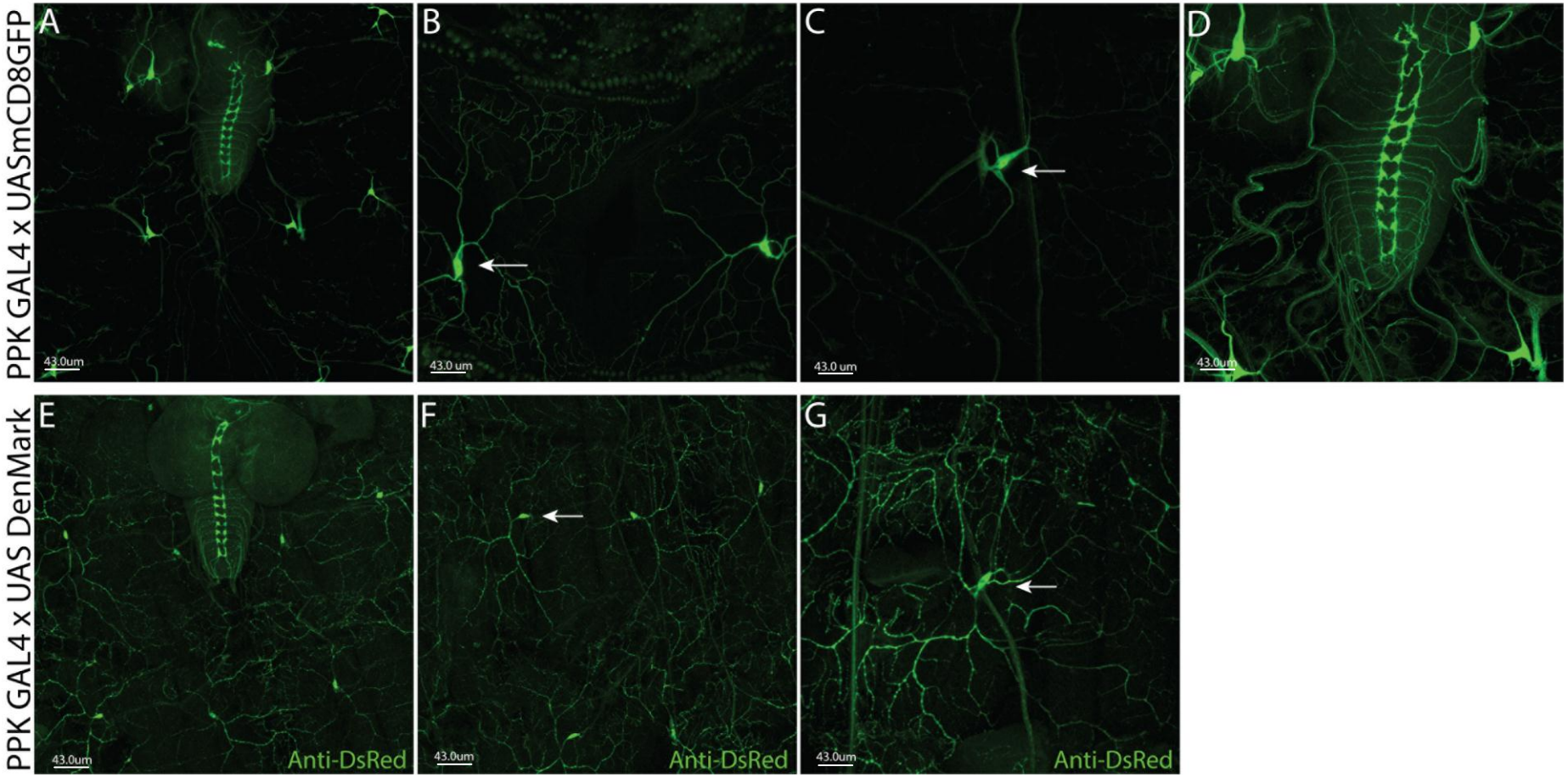


Figure 3.10: Expression pattern of the PPK GAL4 with UAS mCD8GFP and UAS DenMark. (A), (B) and (C) shows the neuron and dendrite arborisation in the epidermis of the larvae expressing mCD8GFP. (E), (F) and (G) shows the neuron and dendrite arborisation with DenMark expression and DsRed Staining. The dendrite arborisation seems to be more easily distinguishable with DenMark than with mCD8GFP. (D) shows the brain with neuropile as two interconnected lines. The axons from the neurons present in the epidermis are seen entering into the CNS via the nerve cord elongations and are found to reach the neuropile on either sides.

CHAPTER 4

DISCUSSION AND CONCLUSION

Being the core component of the brain, the neurons play an important role in the brain's proper function and response to any internal or external stimulus. Dendrite arborisation and axon projection has always been crucial for synapse transmission, with the expression of neurotrophin being crucial for neuron survival, dendrite restructure and branching and proper axon guidance (Snider, 2004). Even though neurotrophins were discovered in the vertebrates long time ago, it was not until the last decade that *Drosophila* neurotrophins were identified, which rendered the invertebrate model free from being considered hard-wired in their neural network. In this aspect, we wanted to study the effect *Drosophila* trophic factors have on the dendrites present in the central nervous system of the adult flies. To test this, we ectopically expressed the *Drosophila* neurotrophins and their respective Toll receptors in the developing nervous system and observed the effect they had on LPTC neuron, ellipsoid body and it's associated R-neurons, using the GAL4/UAS expression system. In addition, we also looked at the circuitry neuron dendrite branching pattern in the mechanosensory domain of the larvae VNC, using GAL4 lines HB9 GAL4, RRKGAL4-EVEGAL4, Cha GAL4, D42 GAL4 and EL GAL, and also visualized the dendrite arborisation of the DA IV neurons in the peripheral nervous system using PPK GAL4. This investigation is carried out to provide us with an answer in the future as to from which region i.e. is it from the mechanosensory domain or is it from the PNS that the Spz is secreted and to look into the effect of neurotrophin and Toll receptors in the dendrite arborisation in the *Drosophila* peripheral nervous system.

Drosophila Neurotrophins have an effect on the architecture of the adult brain

To test the effect of *Drosophila* neurotrophins in the adult central nervous system, we ectopically expressed DNT1, DNT2 and Spatzle using GAL4/UAS targeted expression system. In here, we used the driver line LPTC GAL4 for analysis, as the LPTC neuron has a massive dendrite in the lobula region that would help in better visualisation of any major changes that occurs to the dendrite structure. Apart from this, the LPTC GAL4 genotype we used also outlines the ellipsoid body structure with its associated R-neuron in the central brain region, which we felt could be of an advantage so that we could get an insight on the neurotrophins effect in the visual (LPTC neuron) and learning and memory (Ellipsoid body and associated R-neuron) system.

To analyse the effect on the dendrites, we expressed two different forms of the neurotrophins i.e. the full length (FL), which contains the pro-domain in between the signal peptide and

mature protein, and the Cysknot (CK), which is devoid of the pro-domain, in the *Drosophila* system. The idea to use two different forms of neurotrophin is because full length (FL) on its own needs to undergo enzymatic cleavage to form an active and functional dimer, which in the case of cysknot (CK) is not required, as they don't have the pro-domain and therefore can easily dimerize and become functional upon expression (Schweigreiter, 2006).

The analysis of the data clearly showed the full length form of DNT1 (DNT1FL) to have an effect in *the Drosophila* brain than DNT1 Cysknot (DNT1 CK). The over-expression of DNT1 FL seemed to have resulted in the R-neuron cell bodies to be dispersed, the axon to be less densely packed and have reduction of axonal entry into the EB inner concentric ring, but seemed to have caused no major changes to the EB or LPTC neuron. The effect of DNT1 FL on the R neuron might have been due to the unavailability of the activated form of DNT1 or presence of excessive inactive full length DNT1 at the early stages of neuron structuring and branching, which might have caused the neurons to have restricted contact between each other and result in scattering and limited axon projection from the R-neurons into the EB inner concentric ring. However as this is an assumption, further investigation needs to be carried out in the near future to see even if the structural defect in the R-neuron is due to loss of cells i.e. by staining the brain with anti-cleaved Caspase-3 antibody and then quantifying through the DeadEasy software (Zhu *et.al*, 2008), as pro-form of neurotrophin (Full length) have been associated in inducing apoptosis in neurons in the vertebrates by binding with $p75^{NTR}$-Sortilin receptor (Teng *et.al*, 2005).

In the case of ectopic expression of spatzle, the outcome of the result was quite contrary to what was observed with DNT1. In spatzle, it was found that the activated form of Spatzle (Act Spz) had a drastic effect on the structure of the EB and LPTC neuron. The expression of activated form of spatzle resulted in the EB's concentric ring structures to fuse together as a single ring and caused the axon projecting from the R-neuron towards the EB centre to be defasiculated. The same was also observed in the LPTC neurons, where the VS axon projecting from the soma cells were found to be less densely packed when compared to that of the control. These observation shows that over-expression of activated/functional spatzle at the time of development of the structure might have had a negative effect on the neuropile rings of the EB and the axons of the R-neuron and LPTC neurons, and this could have resulted in the structural disorganisation of the adult brain that are important for learning and

visual pattern memory. The effect of these disorganisations on the memory and learning centres of the adult *Drosophila* can be made only as an assumption and not as a conclusive remark without carrying out additional experimental analysis that looks into the locomotion or visual pattern memory of the adult flies.

All the above analysis on dendrite arborisation was performed by comparing the differences visually after processing the image through Image J, which can be considered only as a preliminary data. In these analyses that were carried out, at some point or the other changes in the dendrite volume or their pattern could have be overlooked. Thus taking this into account, to perfectly understand the effects of DNTs on the LPTC, EB and R-neuron structures, detailed analysis in the future should be done by (i) generating MARCM clones that can help visualise individual VS and HS neurons and their dendrite branches (Raghu *et.al*, 2009), and (ii) carryout analysis through 3D modelling using software like AMIRA (Scott, *et.al*, 2003) to provide not only a qualitative data, but to observe and understand even the minute changes that might have occurred in the neuron or axon or dendrite structure due to DNTs expression.

Inability of data generation in DNT2 FL, DNT2 CK and Spz FL is due to hindrance by PhiC31-based integration system

When DNT2 FL, DNT2 CK and Spz FL was expressed with the driver line LPTC GAL4 in the *Drosophila* for understanding their activity on the structure of adult brain, visualisation of the LPTC neuron or EB or the R neuron wasn't quite possible, even after scanning with laser scanning confocal microscopy. Upon having a careful look into the possible causes; it was found out that the effect was due to the PhiC31-based integration system present in the *Drosophila* genome. The PhiC31-based integration system developed by Konard Basler (Bischof *et.al.*, 2007) has a docking site [M{3xP3-RFP*attP*}] mapped into the 3rd chromosome in region 86F that helps in site specific insertion of transgene. As the inbuilt RFP present in the docking site is not removable, the flies that were generated expressed RFP strongly in their eyes and brain, and it was because of this reason that the progenies expressing the GAL4/UAS was not helpful in visualizing the neuronal structures.

However in the future, if analysis needs to be done for DNT2 FL, DNT2 CK and Spz FL, the above mentioned drawback can be rectified by either generating new genotypes for DNT2 and Spz by using *P*-element based insertion system, which is a random insertion, or by generating

a new double stock genotype for LPTC GAL4 that has UAS mCD8GFP on the 2nd,instead of UASmCD8RFP on the 3rd, which can be helpful in avoiding the hindrance produced by endogenous RFP by getting excited at a different wavelength (i.e. 488nm).

Drosophila Toll receptors seems to have the same effect like neurotrophins in structure formation

To test if ectopic expression of *Drosophila* Toll receptors has the same effect as their ligand (i.e. neurotrophins) on the structural organisation of the central brain, we ectopically expressed the activated form of Toll and Toll-6 receptors i.e. $Toll^{10b}$ and $Toll\text{-}6^{CY}$ in the adult *Drosophila*. In here, we didn't test Toll-7 receptor activity (binds DNT1), as it was found that Toll-7 was not to be expressed in the region of LPTC neuron or ellipsoid body, and is observed to be more likely to be expressed in the fan-shaped body layers.

Analysis of the data clearly showed that activated form of the receptors did have an effect on the structure of the brain. $Toll6^{CY}$ resulted in the reduction of dendrite arborisation of the LPTC neuron in the lobula region and loosening of one of the vertical axon in the LPTC neuron, and $Toll^{10b}$ resulted in the alteration of the structure of EB and its associcated R-neruon, by enlarging the hole in the middle of the concentric ring and loose packing of the R-neuron's axon projection.

Toll receptor is involved in the embryonic dorsoventral axis and axon targeting during early developmental processes (Gay and Gangloff, 2007) and Toll-6 that are membrane bound is found to be present in the interneurons and important for locomotion and motor axon targeting (McIlroy, *et.al*, 2013). Therefore, over-expression of these receptors in the developing nervous system might have resulted in the disruption of structural orientation, as report (Zhu, *et.al*, 2008) on mutational studies of DNTs have been shown to be rescued by the over-expression of Toll receptors i.e. causing an mechanism of interaction that shifts the balance of neurotrophic factors. In addition, activated *Drosophila* neurotrophin Toll receptors have also been shown to elevate the dorsal proteins like Dif and cactus, upon over-expression, and these proteins have been important for neuronal development process and axon targeting (Gay and Gangloff, 2007), so their untimely expression at a wrong place at a wrong time might have also aided in affecting the structural organisation of the neurons.

In addition, it might be possible that excessive receptor availability during the normal neural developmental process might result in adverse effect, as the *Drosophila* neurotrophins even though have been shown to have distinct function by binding only to their specific receptors under natural conditions, but under GAL4/UAS expression system they are more robust and function in a cell-autonomous manner (Chao, 2003), which is quite inclined towards creating imbalance between the neurotrophins in *Drosophila* system due to excessive presence of activate form of neurotrophin receptors.

Circuitry neurons do have its interneuron dendrite projections in the mechanosensory domain of the VNC

Mei Ann Lim of the Hidalgo lab has observed *Drosophila* neurotrophin Spatzle to be present in the mechanosensory domain in the larvae VNC (Unpublished work).And in this regard, to obtain further details on the activity of Spatzle in the mechanosensory domain, we wanted to identify a suitable GAL4 line that can label interneuron dendrite branching into the mechanosensory domain of the *Drosophila* CNS. To do this, we expressed Cha GAL4 (cholinergic neurons), EL GAL4 (Eve-lateral interneurons), HB9 GAL4 (ventrally projecting motoneurons and interneurons), RRK GAL4-Eve GAL4 (dorsal motorneurons) and D42 GAL4 (i.e. Toll-6 expressing interneurons) with the dendritic marker genotype, UAS DenMark and stained it with DsRed.

The reason as to why we used DenMark and DsRed staining was due to (i) it is a marker that is actually a hybrid protein produced by introducing mCherry fluorescent protein (target for DsRed) between the transmembrane and extra cellular domain of the mouse protein intercellular adhesion molecule (ICAM5), which perfectly recognizes *Drosophila* dendrite arborisation (Nicolai *et.al*, 2010), (ii) expression of the marker doesn't have any effect on the morphology of the neuron and (iii) in neurons it clearly differentiates axonal compartments from dendrites and is also found to be independent of the neuron subtype (Nicolai *et.al*, 2010).

Analysis carried out by comparing endogenous RFP expression (control) with DenMark clearly showed differences in dendrite visualisation. The transverse section view of the VNC expressing DenMark clearly showed EL GAL4, CHA GAL4, HB9 GAL4 and RRK GAL4-EVE GAL4 to have their interneuron dendrites projection in the mechanosensory domain, but

the most obvious choice of these four was RRK GAL4-EVE GAL4 because the dendrite branching from the neurons were observed frequent and the visualization of the dendrites were easy as it showed high fluorescence intensity.

Studies have shown that distinct transcription factors are specific for neuron's identity and that specific expression of processing genes shows the characteristic feature of the neurons in the brain, which in the case of *Drosophila* are present in the neuropile region of the VNC (Landgraf *et.al*, 1999; Odden *et.al*, 2002). Even though acetylcholine (neurotransmitter) using neurons (CHA GAL4) or *HB9* gene expressing ventral motor neurons or inter neuron subsets (HB9 GAL4) had their dendrite arborisation in the mechanosensory domain, but they were not frequently seen. But however, in RRK GAL4-EVE GAL4 too the projection of the dendrites was from the neurons that were present ventral than from lateral region, which has now raised the question as to what kind of neurons these may be i.e. interneurons expressing EVE. Therefore, even though RRK GAL4-EVE GAL4 shall be considered as a candidate, but further analysis on what type of neuron (inter or motor neuron expressing Eve) projects its dendrite into the mechanosensory region needs to be carried out in the future by staining the GAL4 reporter line with antibody against *Eve* or by specifically looking into antibodies that can differentiate interneurons expressing *Eve* from motoneurons expressing *eve.*.In addition to this, EL GAL4 (Eve-expressing interneurons) also requires further analysis to be carried out because only minimal number of EL interneurons were seen in the GAL/UAS expressing larvae and even if seen they were weak in signal. Even though it is known that different inserts have different expression range, but to carry out staining against Eve or other specific interneuron gene can help in better understanding of the result and providing a conclusive remark.

Pickpocket1 expressing DA IV neurons are perfect for analysing peripheral nervous system dendrite branching

In the larvae, sensory structures in the cuticle segments are important for their coordinated rhythmic locomotion. Signal transmission and response to external stimulus in the larvae depends strongly on the balanced connection between the central and peripheral controls through the dendrites that is arborized throughout the epidermis (Grueber, *et.al*, 2003). In the aspect, to observe and characterise the DA IV neurons in the PNS expressing *pickpocket1* (PPK1), we expressed PPK GAL4 genotype with UAS mCD8GFP and UAS DenMark. The

DenMark expressing genotype was then stained with DsRed, to improve the fluorescence signalling.

Being a member of degenerin/epithelial sodium channel (DEG/ENaC) family, the PPK1expressing neurons are functionally important for the larvae development transition and their reversal of behaviour (Boiko *et.al,* 2012) and it has also been reported (Ainsley *et.al*, 2003) that loss of function of PPK1 in the developing larvae has a drastic effect on the locomotion. It was for these reasons that we chose to look into the dendrite arborisation of the PPK1 expressing neurons. Analysis of the data showed the dendrite arborisation to be widely spread throughout the cuticle of the larvae with segmental neurons making contact with each other.

As expression of the marker DenMark doesn't affect the morphology or function of the neuron and distinguishes axon compartment from the dendritic branching in the mature neurons, I find the observations to be in correlation with the absolute dendrite pattern of the PPK expressing DA IV neurons. With this data clearly showing PPK GAL4 genotype as a suitable candidate, studies can be carried out in the future that can focus on understanding the importance of neurotrophins and their toll receptors on dendrite arborisation in the PNS through over-expression and knockout analysis, which can provide an understanding on how dendrite branching is important for locomotion and connectivity between the CNS and PNS for coordinated functioning.

CONCLUSION

Our analysis showed that the ectopic expression of different *Drosophila* Neurotrophins and Toll receptors at the time of nervous system development does have an effect on the structural architecture of the neurons in the adult central brain. The effect of disorientation seems to be acceptable as prominent research works have shown that excessive availability of a protein at any developmental stages have more often led to detrimental effect on the normal activity of the cells. Observation of the results showed that the activated form of the Toll receptors (Toll and Toll6) and Neurotrophins (Act-Spz) to be a more dynamic in inducing structural disorientation than their respective full length forms. But however, all these can't be considered as conclusive and what would be suggestive it to perform further analysis on these structural changes using MARCM clones and AMIRA, in the future, to provide strong evidence on the importance of DNTs and NTRs during dendrite branching and remodelling. In addition to this, our research identified RRK GAL4-EVE GAL4 as a suitable circuitry neuron candidate to observe the dendrite arborisation in the mechanosensory domain, and also helped visualizing the dendrite arborisation pattern in the DA IV neurons expressing Pickpocket1 (PPK GAL4 genotype), which I felt can help in conducting experiment in the future that can focus on studying the importance of Neurotrophins and Toll receptors on the sensory neuron dendrite branching in the larvae PNS.

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