Regulation of neuronal number and connectivity by neurotrophins via Toll receptors in the visual system of *Drosophila*

by

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The most rewarding part of my PhD journey was
when experiments failed, each failure
sparked a drive to dig deeper

Abstract

As the brain grows, neuronal number is coupled to the emergence of neural circuits ensuring correct connectivity conducive to normal behaviour. Across animals, neurons are produced in excess, neurotrophic growth factors are secreted in limiting amounts and only neurons that receive trophic support are maintained alive (Levi-Montalcini, 1987). In mammals, this is regulated by neurotrophin ligands function via tyrosine-kinase Trk and TNF-family p75 receptors. *Drosophila* neurotrophins (DNTs) belong to the Spätzle (Spz), paralogue group that function via Toll receptors instead. Intriguingly, the link between these major protein families could be evolutionarily conserved, but still remains unexplored in mammals. DNTs and Tolls regulate cell survival during axon guidance in the *Drosophila* embryo, and neuronal survival in the adult brain (Zhu et al., 2008; McIlory et al.,2013; Li et al., 2020). However, the optic lobe is the ideal context to further probe neurotrophism in *Drosophila*: vast number of neurons die naturally during pupal development and connectivity patterns in the visual system are well known.

The data show that DNTs and Tolls are expressed dynamically during pupal visual system development, in distinct spatial profiles. Interfering with the functions of DNT-2/spz-5 and DNT-3/spz-3 can either prevent or promote neuronal survival. Data suggest that this is mediated via Toll-2, -6 and -8 as the effects can be rescued with epistasis. Furthermore, the alterations in neuronal number correlate with altered dendrite and axonal patterns and connectivity. Moreover, DNTs can promiscuously bind multiple Toll receptors, confirming previous reports (Foldi et al., 2017), and implying that this molecular mechanism is unlikely to function in cell-cell contact recognition synaptic matching.

Altogether, the data support a function for DNTs and Tolls in the control of cell number and connectivity during visual system development that supports the evolutionary conservation of neurotrophism as a fundamental principle of nervous system development.

بسم الله الرحمن الرحيم

أود أن أقدم خالص شكري لدولة ا لإمارات العربية المتحدة وحكامها لمنحي فرصة العمر لاستكمال دراساتي خارج الدولة (البكالوريوس، الماجستير، والدكتوراة)

شكرا لكم أبي، أمي فاطمة، وأمي شيخة على صبركم ودعمكم المستمر لي

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Table of Contents

List of Figures

List of Tables

List of abbreviations

NGF:	Nerve growth factor
BDNF:	Brain derived neurotrophic factor
NT-3:	Neurotrophin 3
NT-4:	Neurotrophin 4
Trk:	Tropomyosin-Receptor-Kinase
DNT-1:	*Drosophila* Neurotophin 1
DNT-2:	*Drosophila* Neurotrophin 2
Spz:	Spätzle
CRISPR:	Clustered Regularly Interspaced Short Palindromic Repeats
NT:	Neurotrophins
TLR:	Toll-like receptor
NFkB:	nuclear factor kappa-light-chain-enhancer of activated B cells
SOS:	son of sevenless
MiMIC:	Minos mediated integration cassette
MARCM:	mosaic analysis with a repressible cell marker
GRASP:	GFP reconstitution across synaptic partners
MCFO:	Multi-color Flip-Out
DCP-1:	*Drosophila* caspase-1
VNC:	Ventral nerve cord
APF:	After puparium formation
Lawf1:	Lamina wide-field neuron
PR:	Photoreceptors
FRS2:	factor receptor substrate 2
GRB2:	growth factor receptor-bound protein 2

AD:	Alzheimer's Disease
FL:	Full length
GOF:	Gain of function
LOF:	Loss of function
PAMPs:	pathogen associated patterns
CNS:	Central nervous system
LPS:	Lipopolysaccharide
LRR:	Leucine rich repeat
Rh:	Rhodopsin
OPC:	outer proliferation center
LMC:	lamina monopolar cells
Lai:	lamina intrinsic cell
IPC:	inner proliferation center
M1:	medulla layer 1
LPTCs:	The Lobula Plate Tangential Cells
GFP:	Green flourcent protein
YFP:	Yellow flourecent protein
gRNA:	guide RNA
L3W:	3rd instar larva

Chapter 1

INTRODUCTION

The nervous system undergoes an initial overproduction of neurons during development. The regulation of proper neuronal number, as well as the impact on glia and their progenitors, is crucial for regular connectivity and function (de la Rosa and de Pablo, 2000; Vicario-Abejón et al., 2002). However, the exact processes underlying these events are still not fully understood.

The discovery of neurotrophism in mammals has shed light on the developmental process in the nervous system. They are important factors that regulate neural survival, development, function and plasticity (Levi-Montalcini, 1987). Also, the discoveries of these NTF's lead to the broad interest in the use of these factors in investigating neurodegenerative diseases (Blesch, 2006).

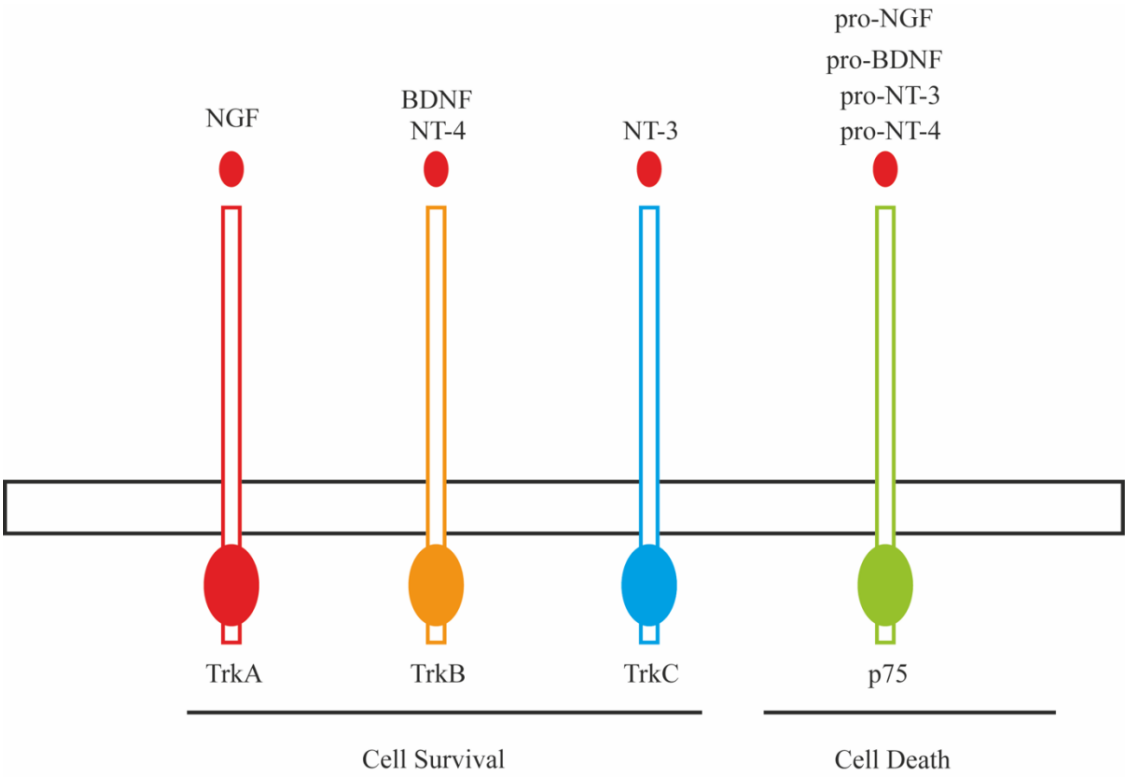
Neurotrophins such as Nerve Growth Factor (*NGF*) and Brain-derived Neurotrophic Factor (*BDNF*) regulate neuronal survival via specific receptors and signaling pathways (Huang and Reichardt, 2001). They achieve this by binding to the Tropomyosin-Receptor-Kinase (Trk) family, including TrkA, TrkB, TrkC, and p75, with each receptor binding to specific neurotrophins. For example, TrkA binds to NGF, TrkB binds BDNF and Neurotrophin 4 (NT-4), and TrkC binds Neurotrophin 3 (NT-3) (Huang and Reichardt, 2001) **(Figure 1.1)**. There is evidence that neurotrophism occurs in insects such as fruit flies. The discovery of neurotrophins in fruit flies suggested a common evolutionary origin in both invertebrates and vertebrates (Zhu et al., 2008). *Drosophila* Neurotrophin 1 (*DNT-1*), *Drosophila* Neurotrophin 2 (*DNT-2*) were identified as neurotrophin homologues in *Drosophila*, with sequence and

functional conservation, involved in nervous system development in the insect nervous (Zhu et al., 2008). When DNT1 was used as a query in structure-based searches using FUGUE, it identified human neurotrophins with over 99% certainty as probable homologs (Zhu et al., 2008). This high level of structural similarity suggests a conserved evolutionary relationship between *Drosophila* and mammalian neurotrophins. It's important to note that while the structural similarity is high, the sequence similarity may be lower, as neurotrophins have diverged considerably outside the Cystine-knot domain. The researchers focused on the Cystine-knot domain for phylogenetic analysis due to this divergence (Zhu et al., 2008). The study demonstrates that despite potential differences in sequence, the *Drosophila* neurotrophins maintain functional similarity to their mammalian counterparts, regulating neuronal survival and targeting (Zhu et al., 2008). This functional conservation, along with the structural similarity, supports the notion of a common origin for neurotrophins in bilateral organisms.

They are paralogues spätzle (spz), and like spz, *Drosophila* neurotrophins bind to the *Drosophila* Toll receptors (Foldi et al., 2017; McIlroy et al., 2013). It has been documented that *DNT-1* binds to *Toll-7*, and *DNT-2* binds to *Toll-6* (McIlroy et al., 2013). Additionally, it has been reported that these DNTs can be promiscuous and bind to different Tolls (McIlroy et al., 2013). The interactions between DNTs and their Toll receptors, such as *Toll-6* and *Toll-7*, are required for larval locomotion and motor-axon targeting, as well as maintaining neuronal survival in the embryo (McIlroy et al., 2013), to promote cell death in the pupa (Foldi et al., 2017), and to regulate synaptogenesis in the larval neuromuscular junction (Ulian-Benitez et al., 2017). *Toll-2* is required to maintain cell survival in the adult brain (Li et al., 2020).

Little is known about neurotrophic factors in the *Drosophila* visual system. However, the

Figure 1.1 Mammalian neurotrophins and their receptors



The mammalian neurotrophins and their receptors p75 and Trk family receptors. Mature forms of neurotrophins bind to Trk family receptors to promote cell survival (Red, orange, and blue). Pro-NTs bind to p75 receptor to promote cell death (Green).

expression and function of multiple Tolls that bind these trophic factors have been documented and shown to regulate neuronal number and survival in the adult and pupal visual system (Li et al., 2020). My thesis aims to test whether neurotrophic factors operate during the development of the *Drosophila* visual system and whether DNTs and Tolls are responsible for controlling cell survival during connectivity.

1.1 The neurotrophic theory

Rita Levi-Montalcini proposed the neurotrophic theory, which suggests that during nervous system development, neurons are initially produced in excess (Levi-Montalcini, 1987). At this stage, target tissues would produce limited amounts of trophic factors. Neurons would then compete for these trophic factors, which are taken up by the dendrites. Only the neurons that receive these factors will survive, while those that do not receive them will die and be eliminated by apoptosis. The trophic factors are then transported back to the cell body to activate survival signaling pathways. Additionally, neurotrophins can regulate the differentiation and plasticity of neurons (Huang and Reichardt, 2001).

Four neurotrophic factors have been discovered, with *NGF* being the first trophic factor, followed by *BDNF*, *NT-3*, and *NT-4* (Huang and Reichardt, 2001a). The discovery of these tropic factors significantly influenced the field of developmental biology. The experiments that led to the discovery of *NGF* have shown the essential role of cellular interactions during development (Guthrie, 2007).

When neurotrophins were discovered, *NGF* was found to promote the survival of sensory spinal and sympathetic neurons in culture (Huang and Reichardt, 2001). Studies using anti-*NGF* injections to inhibit the activity of *NGF* have shown that *NGF* is necessary for maintaining the survival of sympathetic neurons both in *vivo* and in *vitro*. Additionally, other

studies have revealed that neurotrophins have various sources. Before peripheral nerve injury, macrophages infiltrate the damaged nerves as part of an inflammatory response and release cytokines that induce the synthesis of *NGF* in Schwann cells and fibroblasts in the injured nerve. Moreover, *NGF* can also be synthesized in mast cells and released during mast cell activation (Huang and Reichardt, 2001).

Apart from promoting cell survival, each neurotrophin has been shown to promote neurite growth in *vitro*. *NGF*, for example, has been demonstrated to regulate the size of sympathetic neuron growth cones (Blesch, 2006), which implies that the presence of *NGF* is essential for axons to grow and find their targets. In a previous experiment in primate models, neurons treated with *NGF* had axons that invaded only the location where *NGF* was present, while neurons with no neurotrophins did not exhibit similar growth. When the *NGF* was removed from that location, neurons retracted their axons and ceased growing, demonstrating that neurotrophins can also promote growth and guide neurons to their targets besides promoting cell survival (Huang and Reichardt, 2001).

In summary, researchers paid close attention to *NGF* and other neurotrophins because they recognized the importance of cell-cell interactions between neurons and target cells for the survival of most neuronal populations during development (Huang and Reichardt, 2001). This observation also suggested a mechanism by which vertebrates might use apoptosis to eliminate mis-projecting neurons, thereby allowing the generation of complex neural systems.

1.2 Why must neurons die?

During nervous system development, there is an overproduction of neurons that are not needed (Huang and Reichardt, 2001a). To have the correct number of neurons for normal nervous system function, extra neurons must be eliminated. Neuronal death is critical

component of nervous system development, ensuring that the nervous system is properly wired and functions correctly by removing extra or incorrect connections (Hollville et al., 2019).

Neurons die during development for many reasons, which are crucial for the proper formation and function of the nervous system. First, the excessive production of neurons must be controlled. During nervous system development, neurons are produced in excess. These neurons are later eliminated through apoptosis ensuring the correct number of neurons required for normal function (Davies, 2003). Second, during synapse formation, cell death is required to eliminate the incorrect connections as synapses that form during development. Programmed cell death helps refine neural circuits ensuring only necessary connections remain (Oppenheim, 1991). Third, neuronal death can occur at different developmental stages. This occurs due to the requirement needed for each stage at different time points. For example, in *Drosophila*, neuronal cell death in the VNC is required for the proper structure formation, while in the optic lobes, cell death occurs in two different phases each with its own purpose (Togane et al., 2012). The first phase occurs from the start of metamorphosis to 48 hours after puparium formation (APF), with a peak at 24 hours APF. During this phase, dying cells are detected in all four cortices: the lamina, medulla, lobula plate cortices, and the region of T2/T3/C neurons. This phase coincides with a sharp rise in ecdysone levels, suggesting ecdysone may control optic lobe cell death (Togane et al., 2012). The second phase extends from 48 hours APF to eclosion, with a small peak of cell death at 84 hours APF. In this phase, a smaller number of cells continue to die, with dying cells concentrated in the dorsal and ventral medulla cortex. This phase appears unrelated to changes in ecdysone levels, suggesting different underlying mechanisms from the first phase (Togane et al., 2012).

1.3 Evidence in support and against neurotrophism in the Drosophila optic lobe

The controversy regarding the regulation of synaptic connectivity and cell survival has always been a spotlight in developmental biology. Researchers have always argued whether these precise connections are regulated during development as the result of neurotrophism or through guidance with specific molecular cues. Multiple studies have supported the neurotrophic theory by Rita Montalcini and the chemoaffinity hypothesis by Roger Sperry. The neurotrophic theory argues that competition for survival factors maintains the survival of neurons, which establishes the correct connectivity (Levi-Montalcini, 1987). However, the chemoaffinity hypothesis argues that these connections are established based on the molecular cues expressed on the pre and postsynaptic targets without relying on the survival of these neurons (Sperry, 1963). Although these two hypotheses are not exclusive, both mechanisms could operate together during nervous system development.

1.3.1 Massive cell elimination at 24 hr APF in the optic lobes driven by ecdysone

In the *Drosophila* optic lobes, cell death occurs at two different phases each for its own specific requirement (Hara et al., 2013).

The first phase occurs at the start of embryonic development up until 48 hr APF, peaking at 24 hr APF. During this phase, a massive number of cells die in the optic lobes, specifically in the lamina and medulla at 24 hr APF, eliminating excess of cells that aren't needed for the adult visual system. This phase of elimination corresponds to the time when the hormone ecdysone levels are high, suggesting that ecdysone controls cell death occurring in the optic lobe at this initial phase (Togane et al., 2012). Ecdysone controls cell death in the *Drosophila* optic lobe through multiple mechanisms. During early metamorphosis, cell death occurs independently of ecdysone. However, after 24 hours post-puparium formation, ecdysone-

dependent cell death becomes prevalent, requiring the EcR-B1 isoform of the ecdysone receptor (Hara et al., 2013). Ecdysone signaling activates cell death by regulating the expression of key genes involved in apoptosis. For instance, the promoters of reaper, a cell death-inducing factor, and Dronc, an initiator caspase, contain ecdysone response elements. The hormone's effects are mediated through the EcR/USP heterodimeric nuclear receptor, which binds to these response elements to control target gene expression. Importantly, the timing of ecdysone-dependent cell death does not directly correlate with EcR-B1 expression, suggesting additional regulatory mechanisms (Hara et al., 2013; Togane et al., 2012).

The second phase occurs from the end of 48 hr APF to eclosion. During this phase, a small number of dying cells can be seen, with a slight peak at 84 hr APF. Most cell death in this phase occurs in the dorsal and ventral medulla cortex. Unlike the first phase, cell death in the second phase does not seem to have a relationship with ecdysone levels. This suggests that the mechanism in which the second phase is regulated by is a different one from the mechanism in the first phase (Hara et al., 2013; Togane et al., 2012).

Overall, the first phase has been shown to have a massive cell death at 24 hr APF linked to the levels of ecdysone. However, in the second phase, less cell death is observed with no relation to changes in ecdysone levels. This also suggests that apoptosis is being controlled by changes in ecdysone levels to induce metamorphosis and is not linked or regulated by neurotrophism in the developing visual system (Togane et al., 2012).

1.3.2 Neuronal survival depends on factors released by glia

Glial cells play crucial and multifaceted roles in the development and function of the *Drosophila* visual system, making it an excellent model for studying neuron-glia interactions (Chotard and Salecker, 2007). The fly visual system contains a diverse array of glial subtypes

that closely resemble vertebrate oligodendrocytes and astrocytes in their morphological complexity. These glial cells extensively ensheath neuronal cell bodies, axon bundles, and neuropil compartments, providing structural and functional support throughout the visual system (Chotard and Salecker, 2007).

During larval development, retinal basal glia (RBGs) are essential for photoreceptor axon pathfinding. As photoreceptor axons extend from the eye disc towards the optic lobe, RBGs provide critical directional information (Hummel et al., 2002; Rangarajan et al., 1999). If RBG migration is disrupted, such as by overexpressing a dominant-negative form of Ras, photoreceptor axons stall and fail to enter the optic stalk (Hummel et al., 2002; Rangarajan et al., 1999). Conversely, premature entry of RBGs into the eye disc can cause aberrant axon projections, highlighting the importance of precisely timed glial migration (Hummel et al., 2002; Rangarajan et al., 1999).

Glial cells also play a crucial role in modulating neural proliferation and survival throughout visual system development. In early larval stages, surface glial cells near the outer proliferation center (OPC) express the secreted glycoprotein Anachronism, which prevents premature neuroblast proliferation (Ebens et al., 1993). This temporal regulation of neurogenesis is critical for proper optic lobe development.

The importance of glia in neuronal survival becomes evident in later stages of development and in the adult visual system (Dearborn and Kunes, 2004; Ebens et al., 1993). In flies with the viable hypomorphic allele repo1, lamina glial cells fail to terminally differentiate and undergo apoptosis from late pupal development onwards (Xiong and Montell, 1995). This glial cell death coincides with apoptosis of lamina neurons, demonstrating the interdependence of neuronal and glial survival (Xiong and Montell, 1995). Similarly, in optic lobes lacking photoreceptor innervation, medulla neurons in areas devoid of glial cell

coverage show increased apoptosis, further emphasizing the neuroprotective role of glia (Dearborn and Kunes, 2004).

In the adult visual system, glial cells continue to play critical structural and functional roles. They extensively enwrap lamina cartridges, reflecting the organized layers and columns in the medulla (Chotard and Salecker, 2007). Within each lamina cartridge, glial processes surround individual R1-R6 photoreceptor terminals, potentially modulating synaptic function (Chotard and Salecker, 2007). In the medulla, glial cells form characteristic deep invaginations called capitate projections into R7 and R8 axon terminals, likely derived from medulla neuropil glial cells (Chotard and Salecker, 2007).

In a previous study highlighting the importance of glial-neuronal interactions in the *Drosophila* Visual system, they have shown that glia in the lamina are required to prevent neuronal cell death. This study investigated reversed polarity (repo) mutants and their consequence on cell death in neurons (Xiong and Montell, 1995). *repo* encodes a glial-specific homeodomain protein required for terminal differentiation of glia in the *Drosophila* visual system. When lamina glia are mutant for *repo*, they found that the visual system went under cell death. Since *repo* is expressed in glial cells around the lamina, this suggests that lamina glial cells supply one or more factors required for neuronal survival. This evidence suggests that glial cells are required to prevent neuronal cell death in the lamina neurons (Xiong and Montell, 1995). Additionally, the study has provided evidence that *repo* expression is required for glial cells' survival in the lamina. In the *repo1* mutant, the lamina glial cells initiate the differentiation pathway but fail to differentiate terminally, causing it to undergo cell death in the adult visual system. Also, they show that the lamina glial cells in *repo1* mutant show glia feature as they develop in the third larval stage. However, these glial cells do not reach terminal differentiation as shown when stained with a glial marker anti-

ORTHODENTICLE. This failure in differentiation is likely due to the absence of *repo* expression (Xiong and Montell, 1995).

The *repo1* mutant also shows morphological features indicating glial cells in the lamina undergoing apoptosis, as shown by TUNEL staining, which reveals DNA fragmentation when cells are undergoing cell death. This study proves that neuronal survival depends on signals from glial cells (Xiong and Montell, 1995).

These findings show that glial cells are required for preventing neuronal cell death, providing evidence of the supporting role of glial cells in communicating with neurons to regulate cell survival. This also shows that cell death is the default pathway in the *Drosophila* visual system, and survival is controlled genetically. The evidence suggests that cell death could be the default pathway in *Drosophila* visual system development for several compelling reasons. Extensive degeneration occurs in repo mutant neurons and retina, despite the absence of repo expression in these cells, indicating cell death may be the default state unless actively prevented (Xiong and Montell, 1995). The cell death observed in the repo mutant lamina appears to be apoptotic, exhibiting DNA fragmentation and morphological features characteristic of PCD. Additionally, mutations in other *Drosophila* genes like eyes absent and sine oculus lead to ectopic cell deaths in the visual system, further supporting the idea that cell survival requires active genetic control (Xiong and Montell, 1995). This aligns with proposals in vertebrate nervous system development suggesting that most cells may be programmed to die unless they receive survival signals from other cells. Collectively, these findings indicate that both programmed cell death and cell survival are under genetic control during *Drosophila* visual system development.

The diverse and essential functions of glial cells in the *Drosophila* visual system highlight

their importance in nervous system development and function. From axon guidance and neuroblast proliferation to synaptic modulation and neuroprotection, glia are involved at every stage of visual circuit formation and maintenance (Chotard and Salecker, 2007; Dearborn and Kunes, 2004; Ebens et al., 1993; Hummel et al., 2002; Rangarajan et al., 1999). The striking parallels between *Drosophila* and vertebrate glial cells, both in morphology and function, make the fly visual system an invaluable model for understanding glial biology across species (Chotard and Salecker, 2007). As research continues, it is likely that even more roles for glia in visual system development and function will be uncovered, further emphasizing their critical importance in nervous system biology (Chotard and Salecker, 2007).

1.3.3 The chemoaffinity and synaptic matching hypothesis

In the nervous system, the mechanism by which synaptic partners recognize each other and establish connectivity to form functional circuits has always been an interesting debate in neurobiology. The chemoaffinty hypothesis states that presynaptic and postsynaptic partners express specific molecular tags or cell surface molecules that enable them to precisely recognize their targets and form a connection (Meyer, 1998; Sperry, 1963).

In a previous study, it was shown that the interactions between cell surface molecules, such as DIP/DPR interactions, can regulate connectivity and cell survival, providing evidence to support the chemoaffinty hypothesis (Morey, 2017).

The study focuses on DIP-α and Dpr6/10 heterophilic and DIP-α homophilic interactions regulating multiple processes such as circuit assembly, layer-specific targeting, cell survival, and synapse number and distribution in the *Drosophila* visual system.

They begin by showing the expression pattern of DIP/DPRs in the developing visual system, specifically in the medulla neuropile. They were shown to be expressed in medulla Dm4 and

Dm12 neurons as well as the lamina L3 neurons. Moreover, they wanted to investigate the role of DIP/DPRs in the regulation of circuit assembly in the medulla M3 layer. In the DIP-α null mutant, Dm4 mutant neurons did not show any targeting defects compared to the wild type (Xu et al., 2018). However, the branching of Dm4 mutant neurons did not cover as many columns as the wild type. On the other hand, Dm12 neurons showed a targeting phenotype extending an additional branch to the M8 layer (Xu et al., 2018).

Additionally, they explore the role of DIP/DPRs in controlling cell numbers in the context of medulla Dm4 neurons. The heterophilic interactions between DIP-α and Dpr6,10 promote cell survival by disrupting the hid-activated cell death pathway (Xu et al., 2018). However, in a different experiment regarding Dm12 neurons, the expression of p35 in Dm12 mutant neurons did not rescue the targeting defects. These findings suggest that the wiring of these neurons are independent of cell survival (Xu et al., 2018).

In summary, the study provides evidence that specific molecular interactions between DIP and DPRs play an important role in forming the precise connections in the *Drosophila* visual system, which is independent of cell survival, suggesting that the control of cell survival is not required for connectivity as argued in the neurotrophic theory (Morey, 2017; Xu et al., 2018).

1.3.4 Neuronal survival is controlled during connectivity

The control of neuronal survival during connectivity is a vital step in the developing nervous system. This regulation allows the precises formation of functional circuits. Cell signals produced by cells can influence the axons through interactions with their receptors located on

the growth cones (McAllister et al., 1995). This can involve the matching of axonal numbers and target neurons through the actions of trophic factors and topographic map formation through graded expression of molecular cues (Markus et al., 2002).

A recent study has demonstrated how cell survival control is regulated in the *Drosophila* visual system. They show that Jelly belly (Jeb) produced by R1-R6 axons lnteracts with its receptor, anaplastic lymphoma kinase (Alk), to control the survival of Lamina L3 neurons which then triggers these L3 neurons to produce Netrin that will regulate the connectivity of other neurons within the same circuit (Pecot et al., 2014).

They begin by investigating the role of the receptor *Alk* and its consequence on L3 neurons (Pecot et al., 2014). When knocking down *Alk* with RNAi, L3 neurons were not detected. Also, L3 neurons were not detected in a homozygous *Alk* null mutant. This suggests that *Alk* is required for the survival of L3 neurons. Moreover, they investigated the *Alk* ligand *Jeb*. They knocked down *Jeb* protein levels in R cells with RNAi. No L3 neurons were seen in the lamina innervated by *Jeb* deficient R cells. Also, L3 neurons were missing from a wild-type animal innervated By *Jeb* mutant R cells. These findings suggest that *Jeb* interacts with *Alk* to regulate the survival of L3 neurons (Pecot et al., 2014).

Additionally, they showed that the interaction between *Jeb*/*Alk* influences the targeting of R8 photoreceptors in the medulla indirectly. This was done through the manipulation of Netrin (Pecot et al., 2014). It has been reported that netrin is required for R8 targeting in the M3 medulla layer (Timofeev et al., 2012). Since L3 neurons express netrin, the loss of L3 neurons reduced the levels of netrin in the medulla, influencing the targeting of R8 PRs. Also, since *Jeb*/*Alk* interactions in R cells influence L3 survival, this would mean that blocking cell death with p35 would restore the levels of netrin produced by L3 neurons, which has been

shown in this study (Pecot et al., 2014).

A recent study have also shown that the survival of neurons is linked to connectivity. For example, the loss of *Toll-2* in the lamina with MARCM clones impaired the connectivity and survival of lamina neurons during development (Li et al., 2020).

Another study has demonstrated that the survival of R cells in adults depends on the connections they establish with the optic ganglia (Campos et al., 1992). They also investigate the *disco* gene in the context of connectivity. The *disco* gene plays a crucial role in the development and maintenance of photoreceptor neurons in the compound eye of *Drosophila* melanogaster (Campos et al., 1992). Mutations in *disco* typically result in a failure of photoreceptor axons to connect with their target cells in the optic lobes during larval development, leading to an "unconnected" phenotype where the optic lobes are severely reduced (Campos et al., 1992). While photoreceptors can initially develop normally without these connections, they progressively degenerate after the fly emerges as an adult if connections are not established (Campos et al., 1992). This demonstrates that photoreceptor survival depends on retrograde trophic support from the optic ganglia (Campos et al., 1992). The *disco* gene is important because it reveals a critical trophic interaction between the retina and brain that is required for long-term maintenance of photoreceptors, similar to trophic interactions seen in vertebrate nervous systems. Understanding this process could provide insights into mechanisms of neuronal survival and degeneration relevant to human retinal diseases (Campos et al., 1992).

1.4 Molecular mechanisms underlying neurotrophism in mammals: the neurotrophins

1.4.1 p75 neurotrophin receptor-mediated signalling pathways

The p75NTR receptor is a transmembrane protein that belongs to the tumour necrosis factor family. It binds with the pro-form of the NTs such as *NGF*, *BDNF*, *NT3* and *NT4*. The pro-form has one more additional pro-domain that is present on the N- terminus and this domain is removed through hydrolysis to yield the biologically active form of neurotrophin (Huang and Reichardt, 2001). Pro-neurotrophins are known to have a range of biological activities that are different, and in some cases even oppose, the activities of the mature forms. The best example can be seen in the role of neurotrophins: whereas mature neurotrophins are capable of sustaining cells and promoting their differentiation in the case of cells, pro-neurotrophins within certain types of cells, can activate cell death via apoptosis in specific cells after binding to certain receptors (Huang and Reichardt, 2001).The p75 receptor can activate two different pathways: pro-apoptotic as well as pro-survival pathways. The extracellular domain contains four Cystine-rich domains followed by a death domain in the intracellular domain. Due to the lack of tyrosine kinase domain, this receptor activates the signalling through different adapter proteins which will lead to different outcomes (Roux et al., 2002).

The pro-apoptotic pathways get initiated in the case of loss of Trk receptors or NTs which will lead to the activation of the c-Jun N-terminal kinase (JNK) pathway that will promote cell death through apoptosis. This can occur when the three adapters tumour necrosis factor receptor-associated factor 6 (TRAF6), neurotrophin receptor-interacting factor (NRIF), and neurotrophin receptor-interacting MAGE homologue (NRAGE) are being activated following the activation of the p75 receptor (Reichardt, 2006).

The p75 receptor can also activate a pro-survival pathway through different adaptors. When the nervous system encounters damage or injury, p75 binds to the Rho family GTPase, which

promotes neuronal regeneration, repair, and axonal growth (Roux, 2002).

It also can activate Ras, a small G-protein that will activate the MAP kinase ERK to promote cell survival (Blochl et al., 2004) p75 can promote cell survival via NFkB (Carter et al., 1996). **(Figure 1.2)**.

Overall, p75 receptor can promote both cell survival and cell death through these different pathways mentioned. It can also regulate cell proliferation, differentiation and synaptic plasticity (Roux, 2002).

1.4.2 Trk receptor-mediated signaling pathways

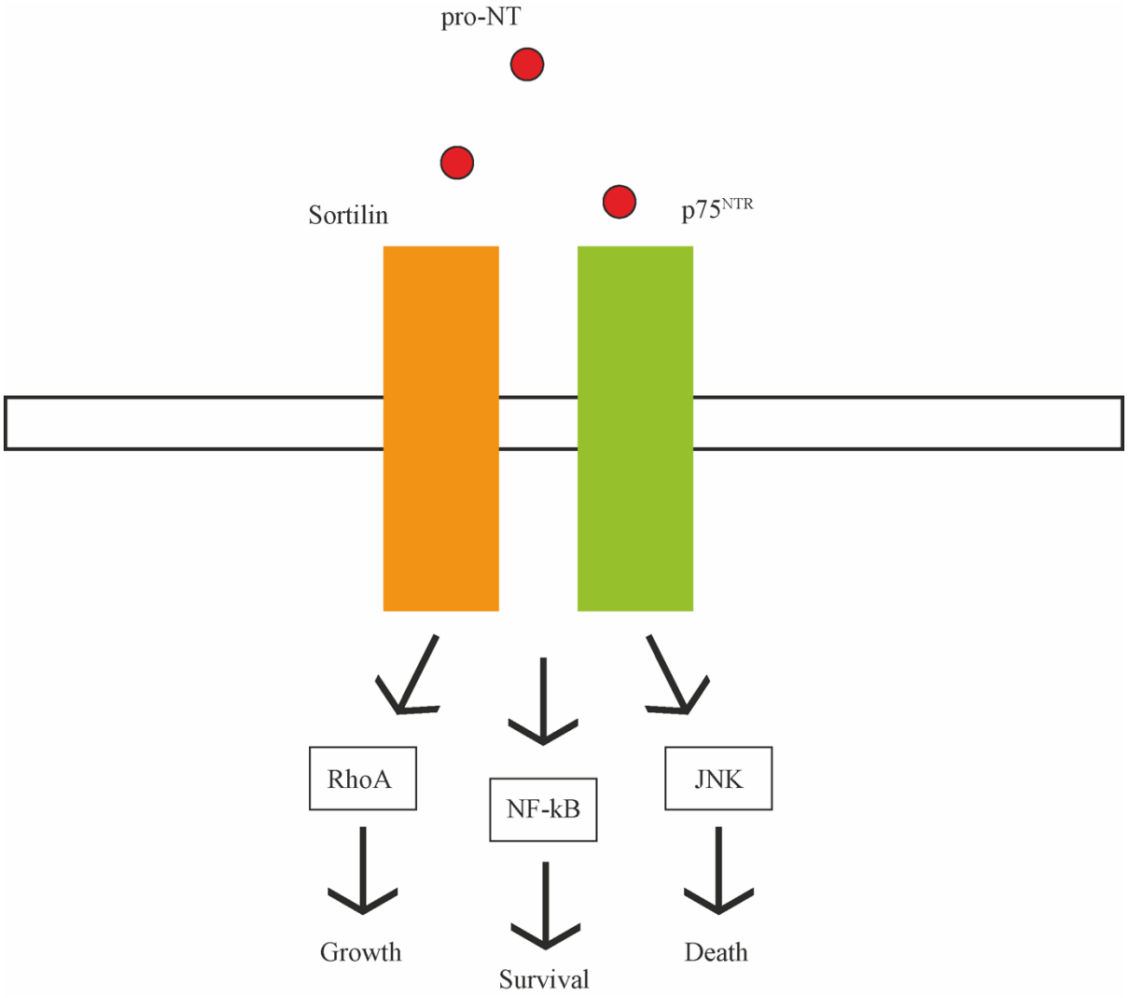
The neurotrophin Trk receptor pathway plays an important role in the development, function, and survival of neurons. These receptors are a family of three receptor tyrosine kinase: *TrkA*, *TrkB*, and *TrkC*. These receptors can be activated by one or more of four NTs; *NGF*, *BDNF*, *NT3* and *NT4* (Huang et al., 2003).

The extracellular domain of these receptors is composed of three components. There are three LRRs flanked by Cystine-rich clusters on the N- and C-terminals. Downstream, there are two immunoglobulin C2 domains. Passing the plasma membrane into the intracellular domain, there is a tyrosine-kinase domain with two tyrosine residues that play a role in phosphorylating downstream proteins (Reichardt, 2006).

The binding of NTs to Trk receptors results in the activation of PLCγ through the Y816 residue that will promote the release of Ca2+. This pathway plays an important role in regulating synaptic plasticity (Gartner et al., 2006).

Another pathway that gets activated by the Trk receptors is through the phosphorylation of

Figure 1.2 p75 mediated signalling pathway



The p75 receptor signalling pathways. Pro-NTs binds the receptor to activate different downstream signalling pathway. When JNK signalling pathway is activated, cell death is promoted. However, when NF-kB pathway is activated, cell survival is triggered. Finally, when the nervous system is going through injury, the RhoA pathway is activated to promote neuronal regeneration, repair, and growth.

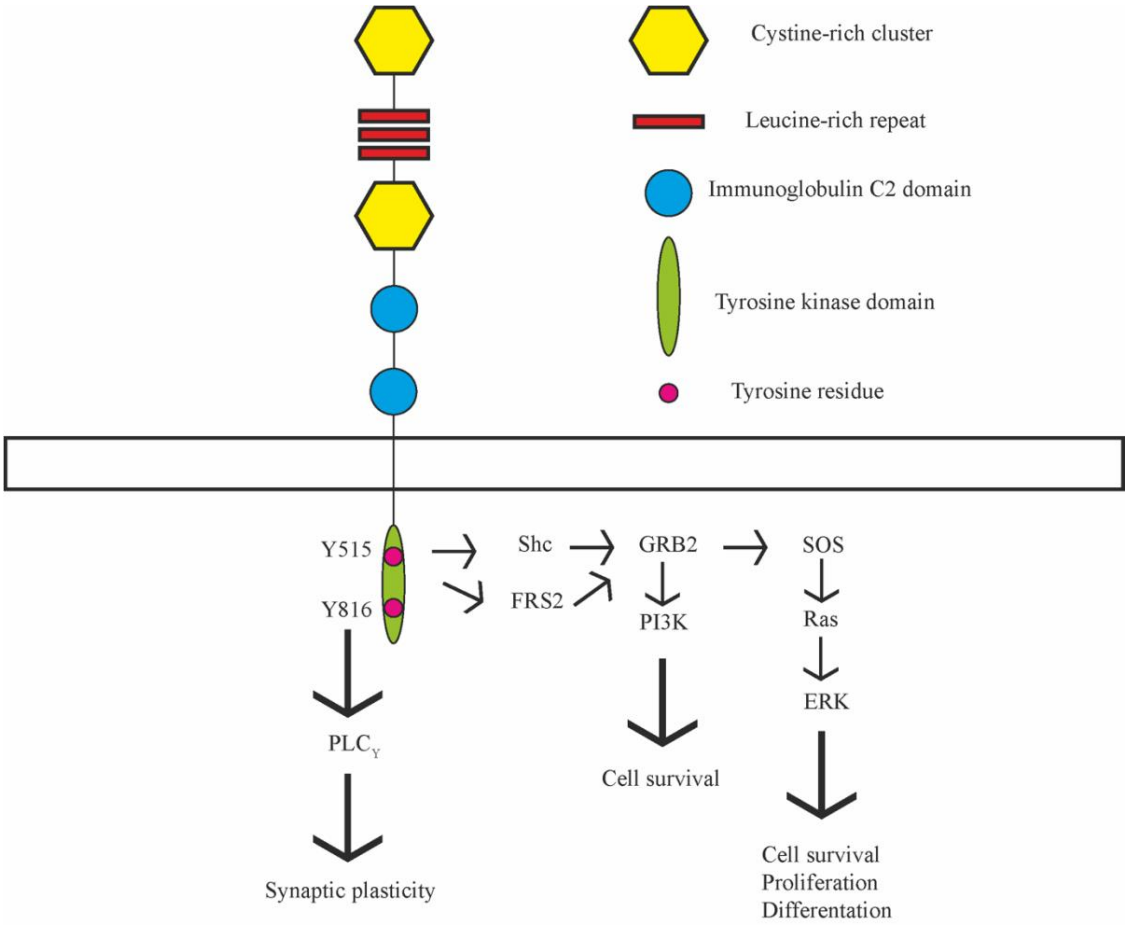
tyrosine residue at position 515 (Y515) which will bind to two different adapters, Shc and the fibroblast growth factor receptor substrate 2 (FRS2) (Uren and Turnley, 2014). When Shc adapter is activated, it leads to the activation of growth factor receptor-bound protein 2 (GRB2) and son of sevenless (SOS). When Sch is trying to bind to GRB2, there is also competition from the FRS2 adaptor to bind to GRB2. The downstream signaling pathway of GRB2 can lead to the activation of phosphatidylinositol 3-kinase (PI3K) which will lead to the regulation of cell survival (Huang and Reichardt, 2003). The other route that this pathway can take is through the GRB2 and moving to the SOS that will activate the Ras-ERK pathway in which will activate the promotion of cell survival, proliferation and differentiation (Huang, 2003) **(Figure 1.3)**.

1.4.3 Mammalian neurotrophins during cell death and cell survival

Neurotrophins play a major role in regulating cell survival and cell death during development (Guthrie, 2007). They regulate these events by interacting with specific receptors, such as the Trk family and p75 receptors (J. Allen et al., 2011). The regulation of cell death and cell survival in the nervous system is crucial for several reasons. First, during development, the nervous system produces more neurons than needed. Cell survival and cell death regulation ensure that only neurons that receive trophic factors and form proper connections survive, while apoptosis will eliminate the rest (Levi-Montalcini, 1987). Also, NTs can prevent apoptosis by biding to Trk receptors activating pathways that promote cell survival and growth (Vicario-Abejón et al., 2002).

Second, the dysregulation of cell death pathways can cause neurodegenerative disease (J. Allen et al., 2011). For example, during the process of ageing one characteristic is the decline in the number of cells and tissue function (Blesch, 2006). NGF is well known to be involved

Figure 1.3 Trk receptor mediated signalling pathway



The Trk receptor family that function via three different pathways to cause different outcomes. The different colours represent each component of the ectodomain. The PLC_y pathway induces synaptic plasticity, PI3k pathway promotes cell survival and the Ras-ERK pathway promotes cell survival, proliferation, and differentiation.

in promoting cell survival in the nervous system (Davies, 2003). One of the most famous neurodegenerative disease that is present in older people is Alzheimer's Disease (AD), which causes cell death in neurons (Blesch, 2006). Memory loss is a prominent symptom in Alzheimer's patients and is related to neuronal loss in the hippocampus (J. Allen et al., 2011). Multiple studies have shown that there is a link between *NGF* and AD where i.c.v infusions of *NGF* can prevent the lesion-induced degeneration of cholinergic neurons in the medial septum (Blesch, 2006).

1.4.4 Mammalian neurotrophins in neuronal connectivity, dendritic complexity and synaptic function

Mammalian neurotrophins are vital for neuronal connectivity, dendritic complexity, and synaptic function, which is important for the overall function of neuronal networks (McAllister et al., 1995).

During connectivity, the neurotrophins play a major role in axon guidance while establishing correctly wired and functional neural networks. Any failure in maintaining the appropriate synaptic connections can lead to alteration in the neural circuits leading to a breakdown in the entire neural system (Poo, 2001). It has been documented that neurotrophins can guide axons to their correct targets during development. in *vivo*, demonstrations have shown that developing axonal projections extend their axonal endings to the appropriate region where they are meant to establish a connection. They are able to navigate accurately to these regions by detecting molecular guidance cues presented by target cells (Tuttle and O'Leary, 1998).

Early studies regarding *NGF* have shown that microinjections of *NGF* into the rat brain have stimulated a massive outgrowth of sympathetic fibres into brain tissue following the source of *NGF*. Moreover, this outgrowth was shown to be linked to *NGF* as *NGF* levels declined.

Additionally, the axonal endings retracted upon the withdrawal of *NGF* (Turney et al., 2016). These findings lead to the idea that during development, *NGF* is secreted from target tissues to attract developing axons and guide them to their correct target. The idea of which *NGF* acts as a guidance molecule was then supported by a study in tissue cultures showing a chick DRG neurites changed their direction and grew depending on the NGF concentration gradient, where growth cones have shifted toward *NGF* sources (Tuttle and O'Leary, 1998).

Dendrite growth and branching are crucial for synaptic input. Neurotrophins have been associated with the regulation of dendritic complexity in the nervous system, and any change in the number of dendrites or morphology can lead to neurodevelopmental and neurodegenerative diseases (Blesch, 2006). It has been documented that BDNF promotes the maturation of dendritic spines and has been shown to influence the width of these dendrites in the hippocampus (Chapleau et al., 2009; Tylerand Pozzo-Miller, 2003; Verpelli et al., 2010).

It has been shown that *BDNF* and *Wnt* signalling can regulate dendritic spine formation. In this study, they showed that inhibiting *Wnt* signalling disrupts the development of dendritic spines in cortical neurons reduces arbour size and affects overall dendritic complexity. The research also shows the interaction between *BDNF* and a specific *Wnt2*. When altering *BDNF*, the expression of *Wnt2* is regulated, and *Wnt2* overexpression has been shown to promote cortical dendrite growth and spine formation. This study suggests that neurotrophins such as *BDNF* are essential for nervous system development (Hiester et al., 2013).

Neurotrophins play an important role in regulating synaptic function, plasticity, and morphology. They are well known for their involvement in regulating the differentiations, maintenance, and survival of neurons, but they can also influence the strength and structures of synapses. There are two ways neurotrophins can regulate synapse number: by promoting

synaptogenesis or by stabilizing existing synapses (Vicario-Abejón et al., 2002).

A previous study has documented the role of neurotrophins in regulating synapse number. The study involved *Trkb* and *Trkc* knockout mice, where they showed a significant reduction in synapse number in the hippocampus as well as a reduction in axonal arborization during development. Their findings suggest that neurotrophins have a significant role in synapse formation (Martínez et al., 1998).

In another study in *Xenopus* visual system, they showed that *BDNF* could increase the number of synapses. Also, *BDNF* was shown to enchase the optic axon arborization, but the increase in synapse number surpassed the increase in axonal branches, suggesting a different role of *BDNF* in synapse formation (Alsina et al., 2001).

Overall, neurotrophins have been shown to be involved not only in the differentiation and survival of neurons but also in regulating dendritic complexity, synapse function, and plasticity, indicating more complex roles of neurotrophins in nervous system development (Huang and Reichardt, 2001).

1.5 The *Drosophila* neurotrophin system

The discovery of neurotrophins initially took place in the field of mammalian research. In the 1950s, Levi Montalcini and Hamburger made progress by identifying *NGF* as the first discovered trophic factors. Their groundbreaking work showcased *NGF*'s role in supporting the survival and development of neurons marking a milestone in neurobiology. During that period, the existence of tropic factors in *Drosophila* was still undiscovered (Guthrie, 2007).

1.5.1 Molecular mechanism of DNTs and Tolls

DNT-1 was discovered by searching for vertebrate neurotrophin homologues in flies (Zhu et al., 2008). This led to the discovery of *DNT-1*, *DNT-2* and *spz* as close neurotrophin homologues. It was found that as well as being homologues of vertebrate neurotrophins, they were also paralogues of the well-known gene *spz* in *Drosophila* (Zhu et al., 2008). There are six *spz* paralogues in flies – *spz-1*, *spz2*/*DNT-1*, *spz-3*, *spz4*, *spz5*/*DNT-2* and *spz-6*. *Spz-6* is only very distantly related (Parker et al., 2001; Zhu et al., 2008). Spz had also been previously shown to share biochemical properties with *NGF* (De Lotto and De Lotto 1998). Spz was later crystallised and shown to share the characteristic cystine-know domain structure with *NGF* (Hoffmann et al., 2008). Structural modelling of *spz-1*, *DNT-1* and *DNT-2* demonstrated close evolutionary conservation with mammalian *BDNF* and *NGF* (Foldi et al., 2017). Our lab demonstrated functional conservation as well as sequence and structural conservation. Firstly, in their mature form, *DNT-1* and *DNT-2* can promote neuronal survival in embryos, larvae and adult CNS (Foldi et al., 2017; Li et al., 2020; Zhu et al., 2008). Second, this is linked to connectivity and synaptogenesis, as alterations in DNTs impair connectivity in embryos and synapse formation in larvae (Sutcliffe et al., 2013; Ulian-Benitez et al., 2017; Zhu et al., 2008). Spz is the well-known Toll ligand. *DNT-1* and *DNT-2* are the ligands of *Toll-7* and *Toll-6*, respectively (McIlroy et al., 2013). Importantly, DNTs are also ligands for receptors of the Trk family, the Kekkons (*Kek*) (Ulian-Benitez et al., 2017). Keks are closely related to the Trks, but lack the tyrosine kinase domain (Mandai et al., 2009). At least *Kek-6* and *Toll-6* form a receptor complex for *DNT-2* that regulates synapse formation and axonal branching (Ulian-Benitez et al., 2017).

Like with mammalian neurotrophins, DNTs and their receptors can have multiple functions, and they can regulate the balance between cell survival and cell death through different mechanisms (Foldi et al., 2017).

First, The DNT cleavage pattern. Each DNT is being processed differently which leads to different cellular outcomes (Foldi et al., 2017). Pro-*DNT1* can work via the JNK signalling pathway to activate apoptosis, while mature *DNT1* and *DNT2* activate survival via NF-κB and ERK signalling pathways (Foldi et al., 2017). This molecular mechanism is evolutionary conserved, as mammalian pro-NTs promote cell death, while furin-cleaved mature NTs promote cell survival (Lu et al., 2005). NF-κB, JNK, and ERK signaling pathways are shared with the mammalian NTs, downstream of p75NTR (NF-κB and JNK) and Trks (ERK), to regulate cell survival and cell death (Roux and Barker, 2002; Lu et al., 2005; Minichiello, 2009). This means that although the receptors may differ between *Drosophila* and mammals, the downstream signalling outcomes are evolutionarily conserved.

Second, the specificity of Toll receptors and to which DNT they bind to is crucial. Different Toll receptors could promote cell survival, while other Tolls could promote cell death (Foldi et al., 2017). It has been shown that *Toll-6* and *Toll-7* could maintain neuronal survival. However, *Toll-1* has been shown to have proapoptotic effects (Tauszig et al.,2000; Yagi et al., 2010; McIlroy et al., 2013; Meyer et al., 2014; Paré et al., 2014). Also, different Tolls can have distinct functions in immunity and development. So far, only *Toll-1* has been shown to have clear functions in innate immunity, activating the expression of anti-microbial peptides (Valanne et al., 2011).

Third, the downstream adaptors. The availability and distribution of downstream adaptors of Toll receptors determine the outcome, whether it's cell survival or cell death. *Toll-6* and *Toll-7* activate cell survival through MyD88- NF-κB signalling pathway. Also, *Toll-6* can activate cell death through Wek-Sarm-JNK signalling pathway. The cellular outcome downstream of DNTs and Tolls are time dependent. Promoting cell survival or cell death will depend on

which proteases are expressed nearby, which ligand receives it, and what form that ligand is in (Foldi et al., 2017) **(Figure 1.4)**.

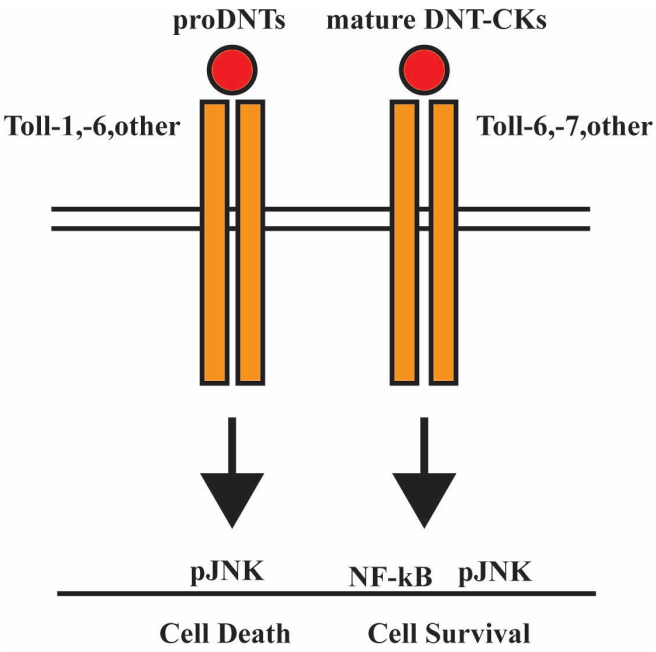
The balance between cell survival and cell death in the *Drosophila* nervous system is being regulated by the interaction between the different cleaved patterns of DNTs, the particular Toll receptors each DNT/Spz interacts with, and the downstream adaptors that will lead to either cell survival or cell death (Foldi et al., 2017). These mechanisms are context and time dependent (Foldi et al., 2017).

1.5.2 Functional Conservation of DNTs in *Drosophila*

The *Drosophila* neurotrophins show functional conservation with mammalian neurotrophins (Zhu et al., 2008). Having similarities in the structure, their role in neuronal survival, and the signalling pathways they activate to cause these outcomes (Zhu et al., 2008).
The structural similarities of DNTs, including *spz, DNT1*, and *DNT2* with mammalian neurotrophins include the pro-domain and the conserved CK domain of a 13-15 kD that forms a disulfide-linked dimer (Zhu et al., 2008). Despite the structural similarities, DNTs have unique features in their pro-domains and are processed differently (Foldi et al., 2017). Spz was identified as *NGF* biochemically and structurally (De Lotto and de Lotto 1998; Hoffmann et al., 2008). Spz in *Drosophila* binding to *Toll-1* shares structural conserved features to the binding of *NGF* to p75NTR in mammals (Lewis et al., 2013). *DNT1* and *DNT2* regulates neuronal survival and are required for connectivity and synaptogenesis, similar to the functions of *BDNF* and *NGF* in mammals (Zhu et al., 2008; Sutcliffe et al., 2013; Foldi et al., 2017; Ulian-Benitez et al., 2017).

The regulation of cell survival and cell death is evolutionary conserved between DNTs and

Figure 1.4 Molecular mechanism of *Drosophila* NTs with Toll receptors



The *Drosophila* neurotrophins binding to different Toll receptors. Pro-DNTs binds to Toll-1, Toll-6 to promote cell death via the JNK pathway. Moreover, the mature DNTs binds Toll-6 and Toll-7 to promote cell survival via the ERK-NF-kB pathway (Illustration adapted from Foldi et al., 2017).

mammalian neurotrophins (Zhu et al., 2008; McIlroy et al., 2013; Foldi et al., 2017; Li et al., 2020). DNTs and Toll receptors in *Drosophila* regulate cell number plasticity by promoting both cell survival and cell death in the nervous system through different mechanisms (Anthoney et al., 2018; Foldi et al., 2017). This is functionally similar to regulating cell survival and cell death in neurons by mammalian neurotrophins, which can promote cell survival via Trk and p75NTR receptors and cell death via p75NTR and Sortilin (Roux, 2002). Interestingly, the signaling pathways activated by DNTs is similar downstream signaling pathways to mammalian Neurotrophins, including NF-κB, JNK, and ERK pathways. These pathways are involved in regulating neuronal survival and death, suggesting a conserved mechanism of action (Foldi et al., 2017; McIlroy et al., 2013).

Overall, DNTs show functional and structural conservation with mammalian neurotrophins. They also share a role in promoting cell survival and cell death as well as downstream signaling pathways. However, the specifics of their processing, interactions, and the context in which they act may differ.

1.5.3 Function of Tolls in embryonic development

Drosophila Toll receptors play an important role in development (Valanne et al., 2011). They can influence embryonic patterning as well as tissue differentiation. During embryogenesis, Toll receptors are involved in establishing the dorsoventral polarity of the embryo. They do that by activating the downstream signaling pathways through the binding with the ligand *spz*. This activation results in the nuclear localization of the NF-κB factor dorsal, which controls gene expression along the dorsoventral axis (Stathopoulos and Levine, 2002).

Additionally, these receptors also play an important role in germ cell migration (Fauvarque and Williams, 2011). It is involved in the specification of pole cells, the precursors of the

germline, their migration to the embryonic gonads (Keith and Gay, 1990, Eldon et al., 1994). Toll receptors can have many roles in development that are crucial for the proper formation and patterning of the embryo by integrating cues and activating pathways that aid in the development of the *Drosophila* embryo.

1.5.4 Function of Tolls in innate immunity

Drosophila Toll receptors were initially identified for their involvement in controlling dorsoventral patterning during development (Anthoney et al., 2018). Subsequently, research showed that these receptors are responsible for innate immunity by recognizing pathogen-associated patterns (PAMPs) in response to fungal and Gram-positive bacterial infections (Lemaitre et al.,1996).

A study conducted by Lemaitre and colleagues demonstrated the significance of Toll receptors in responses to infection (Lemaitre et al., 1996). A mutation in the *Toll* gene rendered the flies more vulnerable to the fungus leading to a mortality rate compared to the control group. This research highlighted the role of Toll receptors as regulators of immunity in *Drosophila* (Lemaitre et al.,1996).

In another investigation by Tanji et al. the focus was on understanding how *Toll 9* influences the expression of peptides (AMPs) when fruit flies are infected with bacteria. The absence of *Toll 9* resulted in reduced production of AMPs making the flies more susceptible to infections. Conversely, overexpression of *Toll 9* led to an increase, in AMP gene expression enhancing the fly's ability to combat infections effectively (Narbonne-Reveau et al., 2011). According to their research, it was found that flies with *Toll 9* mutations exhibited no decrease, in their response (Narbonne Reveau et al., 2011).

1.5.5 Function of Tolls in the Nervous System

Toll receptors also have functions outside innate immunity and early embryonic development (Anthoney et al., 2018). In the nervous system, an original study investigated the involvement of Toll receptors in muscle development during embryogenesis. They showed that when flies were Toll mutants, they experienced a loss in motoneurons in the CNS that affected the neuronal circuits innervating the muscle (Halfon et al., 1995).

Our lab showed that *Toll-6* and *Toll-7* function as neurotrophin receptors in the CNS, binding *DNT-2* and *DNT-1*, respectively, to regulate neuronal survival and connectivity (McIlroy et al., 2013). When altering *Toll-6* and *Toll-7*, the motor axons experienced mistargeting phenotypes (Mcllroy et al., 2013). Also, it has been shown that *Toll-6* has a function in regulating cell survival in the CNS of pupa as well as promoting cell death via different pathways (Foldi et al., 2017).

Toll-6 and *Toll-7* play crucial roles in mediating the wiring of neurons between dendrites and axons in the adult brain during the pupal stage (Ward et al., 2015). *Toll-7* functions cell-autonomously in olfactory receptor neuron (ORN) axons, regulating their targeting to specific glomeruli in the antennal lobe, particularly a cluster of anterolateral glomeruli (Ward et al., 2015). Similarly, *Toll-6* functions cell-autonomously in projection neuron (PN) dendrites, guiding their targeting to a similar cluster of anterolateral glomeruli (Ward et al., 2015). Both receptors participate in synaptic partner matching between ORN axons and PN dendrites in the VA1d glomerulus, with *Toll-6* functioning in PNs and *Toll-7* in ORNs (Ward et al., 2015). This partner matching may be part of a larger strategy of target selection for both PN dendrites and ORN axons (Ward et al., 2015). Misexpression experiments demonstrated that *Toll-6* and *Toll-7* act instructively to direct PN dendrites and ORN axons, respectively, to

target the *Toll-6*/*Toll-7* enriched region of the antennal lobe (Ward et al., 2015). Interestingly, the wiring functions of *Toll-6* and *Toll-7* do not require their cytoplasmic domains or canonical signaling pathways, suggesting they signal through novel mechanisms involving unknown ligands and co-receptors (Ward et al., 2015). These findings highlight the essential instructive roles of *Toll-6* and *Toll-7* in guiding the targeting and matching of specific ORN axons and PN dendrites during olfactory circuit assembly. However, no direct evidence was found of their involvement in synaptic matching or connectivity between *Toll-6* and *Toll-7* expressing neurons (Ward et al., 2015). *Toll-6* was also shown to be involved in regulating structural synaptic plasticity in neurons at the larval NMJ (Ulian-Benitez et al., 2017).

In another study, it has been documented the involvement of *Toll-2* in regulating neuronal number and plasticity in the adult brain (Li et al., 2020). Loss of *Toll-2* caused the number of neurons to decrease as well as the size of the brain. Also, the flies showed a defect in locomotion when altering *Toll-2* (Li et al., 2020).

Toll-2 also promotes adult neurogenesis via Wek (Li et al., 2020). That wek can function either on its own or together with Yki to promote adult progenitor cell proliferation and neurogenesis. Moreover, Wek antagonizes MyD88 in this function, and Toll signalling via MyD88 keeps adult progenitor cells quiescent, whereas Wek induces their proliferation (Li et al., 2020).

Overall, this shows that Toll receptors can have versatile functions besides being only involved in the development of immunity. These studies confirm the importance of Toll receptors in regulating the nervous system's development and function.

1.6 TLRs in the mammalian nervous system

The Toll gene was first discovered in *Drosophila* (Lemaitre et al., 1996). They introduced a mutation into the gene that resulted in defects in dorsal-ventral patterning in *Drosophila* (Lemaitre et al.,1996). Additionally, Toll mutation in *Drosophila* also enhanced susceptibility to function infections, providing evidence that Toll is involved in innate immunity response in *Drosophila*.

In 1997, the search for mammalian TLRs began, and the first Toll homolog in human monocytes were discovered and named TLR4 (Medzhitov et al., 1997). Studies have shown that TLR4 is the receptor for LPS, a major component of the outer cell wall of Gram-negative bacteria.

The structure of the mammalian TLRs is similar to that of *Drosophila* in the TIR domain (Gay and Gangloff, 2008). They consist of an ectodomain made up of LRRs and Cystine-rich clusters and an intracellular tail with a TIR domain. The mammalian ectodomain TLRs contains only one cystine cluster flanking the C-termina end of the LRR domain in comparison to *Drosophila* in which they have two or more cystine clusters flanking both C- and N-terminal (Luo and Zheng, 2000, Gay and Gangloff, 2008).

Mammalian TLRs are well known for their role in innate immunity. They recognize Pathogen-associated molecular patterns (PAMPs) located on pathogens which then will trigger the activation of innate immunity (Kielian, 2009). These PAMPs are recognized by receptors referred to as pattern recognition receptors (PRRs), such as TLRs. In addition to being involved in innate immunity, TLRs could also initiate adaptive immunity (Hanke and Kielian, 2011). Currently, there are a total of 13 TLRs in mice and 11 in humans. To date, TLR2 and TLR4 are well known for their role in recognizing PAMPs. Moreover, TLR1 and TLR6 form heterodimers with TLR2 to identify triacylated from diacylated lipoproteins.

TLR5 is known for sensing the flagella of moving bacteria, while TLR11 recognizes pathogenic bacteria associated with urinary tract infections. Additionally, TLR3, TLR7/8, and TLR9 recognize intracellular pathogen-derived nucleic acid motifs that lead to viral infections (Kielian, 2009).

In addition to their well-known role in immunity, TLRs are also expressed in the mammalian nervous system and have significant roles in neuroinflammation, neurodevelopment, and neurodegenerative diseases (Chen et al., 2019).

Mammalian TLRs are expressed and function in different cell types in the CNS. Microglia, are known for their role in innate immunity in the CNS. They have the capability of recognizing infectious agents through different PRRs, including TLRs, Nod-like receptors (NLRRs), and scavenger receptors. They express all known members of the TLRs known to date (Chen et al., 2019).

Moreover, Astrocytes can also express TLRs but in a limited manner compared to microglia. They are the major glial cell type in the CNS that can play a role in responding to inflammation. Multiple studies have shown that TLR2 expression in astrocytes is crucial for recognizing both intact *S. aureus* and PGN (Phulwani et al., 2009). Additionally, another study has shown that when astrocytes are exposed to proinflammatory stimuli, that exposure leads to an increase in the expression of TLR2 (Hanke and Kielian, 2011).
Studies have demonstrated that neurons in the CNS express intracellular TLRs such as TLR3, TLR7/8, and TLR9, suggesting a role of these TLRs in the physiology and pathology of the nervous system (Cameron et al., 2007). Also, some neurons have been shown to express TLR4, which interacts with CD14 at the cell surface. However, the function of TLR4 in neurons remain a mystery (Gorina et al., 2011; Hanke and Kielian, 2011).

Another study involving TLR2, investigated the role of this TLR in microglia activation and neuroinflammation in Parkinson's disease. The study showed that a protein called alpha-Synuclein related to the disease activated the glial cell via TLR2. The activation of this pathway then led to the production of pro-inflammatory and neurotoxic factors by the glial cells (Doorn et al., 2014).

However, the intrinsic activators of mammalian TLRs within the central nervous system (CNS) remain relatively understudied. Recent investigations in cell culture settings have unveiled that mammalian neurotrophins (NTs), including *BDNF* and *NGF*, possess the capability to interact with TLRs and initiate their downstream signalling pathways. For instance, both *BDNF* and *NGF* have been shown to elicit the activation of NFκB in cells expressing TLR-4 (Foldi et al., 2017; Fukuda et al., 2020). This observation implies that mammalian neurotrophins might stimulate neuronal functions of TLRs, thereby modulating structural brain plasticity.

1.7 The *Drosophila* visual system as a context to investigate neurotrophism

The *Drosophila* visual system provides an incredibly attractive model for investigating the formation of neuronal circuits and, at a highly intricate level, their connectivity with individual neurons. Santiago Ramon Y Cajal's ground-breaking work in 1915 provided the basis for this by highlighting similarities between the neural circuits involved in vision of flies and those of other vertebrates. Such knowledge formed the starting point for further research in this area, including important investigations such as that of Fischbach and Dittrich in1989 which explained how neural networks are assembled within fly eyes (Fischbach and Dittrich 1989).

The *Drosophila* visual system comprises four major neuropils: lamina, medulla, lobule and lobule plate. Each of these neuropile has well-defined roles in processing visual information hence by studying them researchers can get insights into molecular and cellular mechanisms that underlie the development of neural circuitry and connectivity in a cell-type-specific manner (Chiang et al. 2011). This level is crucial to understanding the complexities of neural networks and their role in visual processing and behaviour in flies. Researchers may exploit *Drosophila*'s genetic resources highly manipulate or monitor neurons, enabling them to clarify what any specific neuron does or plays.

1.7.1 Structure of the *Drosophila* visual system

The retina:

The *Drosophila* compound eye is a highly organized structure that represents a system that can be used to study the molecular and cellular mechanisms of pattern formation (Wolff and Ready, 1993). The retina contains 750-800 simple eyes called ommatidia, each ommatidium containing eight photoreceptor neurons or R cells (R1-R8). The R1-R6 photoreceptors are located in the outer regions of the ommatidium, while the R7 and R8 PRs occupy the inner regions. Photoreceptors of the retina project their axons to the optic lobes in the brain. These axons form retinotopic projections in two neuropils, the lamina, and medulla (Clandinin and Zipursky, 2002). R cells can be classified into three types: R1-R6 photoreceptors that express the broad-spectrum opsin Rh1, which plays a role in motion detection. R7 photoreceptors express the UV-sensitive opsins Rh3 and Rh4 (Courgeon and Desplan, 2019), and R8s express blue (Rh5) or green (Rh6) opsins. The third type is found at the dorsal margin of the retina: they are called Dorsal Rim Area (DRA) R7 and R8 that express Rh3, which are important for detecting the vector of polarized light (Courgeon and Desplan, 2019). The three types of ommatidia are known to be dependent on the opsin expression in the R7 and R8

photoreceptors (Morante, Desplan & Celik, 2007). Ommatidia are classified into yellow (70%), which contain R7s and R8s expressing Rh4 and Rh6, and pale (30%), where R7s express Rh3 and R8s express Rh5. The arrangement of photoreceptors in the fly with different rhodopsins is similar to the human retina, suggesting *Drosophila* has color vision (Yan Zhu, 2013). The retinal R cells take up visual information into the fly's optic lobe, known as the visual processing center. The optic lobe contains four major types of neuropils: the lamina, medulla, lobula, and the lobula plate. The last two neuropils are known to form the lobula complex that sends visual inputs to higher-order brain centres through different pathways (Nériec and Desplan, 2016).

In the *Drosophila* visual system, R1-R6 axons extend into the lamina, where they form synaptic units called lamina cartridges with lamina neurons L1-L5, amacrine cells, and centrifugal interneurons (Sasieta et al., 2017). Conversely, R7 and R8 axons extend to the medulla columns, passing through the lamina without forming synaptic connections. The medulla also receives axons from lamina neurons L1-L5 (Morante and Desplan, 2008). Electron Microscopy (EM) studies have elucidated the connectivity relationships between neurons within lamina cartridges and medulla columns. These studies have identified mini-circuits within these columnar modules that facilitate the processing of visual information in different ways, enabling features such as motion and color to be distinguished (Shinomiya et al., 2015; Takemura et al., 2011, 2013, 2017) **(Figure 1.5)**.

The lamina:

The lamina serves as the initial neuropil beneath the retina within the fly's visual system and is primarily responsible for motion detection. Comprising approximately 6000 cells, its development is intricately linked with the retina's (Tuthill et al. 2013). As photoreceptors in

Figure 1.5 Structure of the *Drosophila* visual system

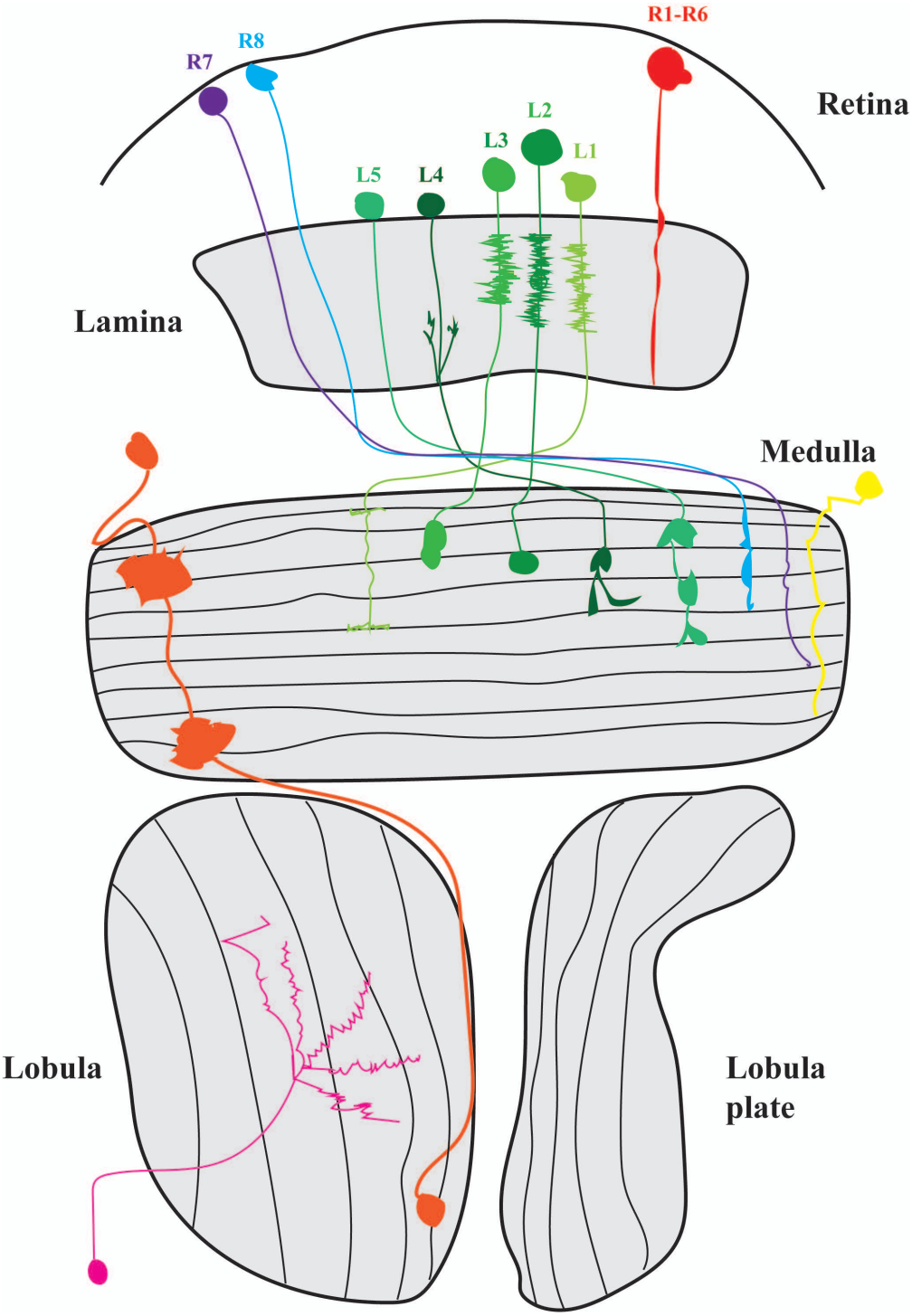


Figure 1.5 Structure of the *Drosophila* visual system

The *Drosophila* visual system is made up of four different neuropiles each with its own function (Lamina, medulla, lobula, and lobula plate). As light hits the retina, information passes through the photoreceptors R1-R8. R1-R6 PRs extend and reside in the lamina to form synaptic connections with lamina neurons. On the other hand, R7-R8 make their way into the medulla without stopping at the lamina where they aid in colour detection function in the medulla. The lamina is mostly responsible for motion detection that consist of 5 monopolar lamina neurons (L1-L5) that form connections in different medulla layers. The medulla is the largest neuropiles responsible for colour detection and processing. The lobula complex is made of two parts: the lobula and the lobula plate. These two last neuropiles act as the pilot for the fly in detecting and monitoring all motion related connections. And finally, the lobula is the link between the visual system and the central brain to send in information for final processing (Illustration adapted from Pecot et al., 2014).

the retina develop during the third larval instar, the posterior axons extend to the site of the future lamina, situated within the inner portion of the outer proliferation centre (OPC), a neuroepithelium from which the lamina and medulla derive. As additional photoreceptor axons arrive and signal for lamina differentiation, this region of the neuroepithelium transforms the lamina (Nériec and Desplan, 2016).

The differentiation of these cells into the lamina involves a signalling relay, where photoreceptors initially signal to glial cells using the EGF ligand spitz. Subsequently, glial cells express insulin-like peptides, inducing differentiation and forming five distinct lamina monopolar cells (LMCs). The lamina is organized into cartridges, with the induction of these cartridges commencing during the third larval stage (Chan et al., 2011). While R1-R6 receptors are positioned close to their targets at this stage, they do not establish direct connections. Instead, the growth cones of these six photoreceptors halt between two glial layers in the lamina in response to glial signalling. R1-R6 photoreceptors project their axons into each cartridge, whereas R7-R8 photoreceptors traverse the lamina without forming synaptic connections, targeting the medulla. Alongside the six photoreceptor axons, the lamina accommodates 12 other neuronal cell types (Nériec and Desplan, 2016).

Among these 12 neurons are five lamina output neurons, six putative feedback neurons, and one lamina intrinsic cell (Lai) (Fischbach and Dittrich, 1989). Notably, eight of these neurons exhibit a columnar organization. Columnar neurons, including L1-L5, project their axons to the medulla. T1, C2, and C3 are columnar neurons with cell bodies in the medulla, projecting their axons back to the lamina. Other lamina neurons, termed multi- columnar, are inferred to function as feedback neurons based on their anatomical characteristics. Wide-field feedback from the medulla to the lamina is mediated by two types of lamina neurons (Lawd1, Lawf2), with Lawf2 distinguished from Lawf1 by the specific layer they integrate within the medulla.

Lastly, the lamina tangential neuron (Lat) projects from the ipsilateral central brain to the distal surface of the lamina (Tuthill et al., 2013).

The medulla:

The medulla stands out as the largest neuropil among the four in the fly's visual system. Organized into columns corresponding to the 750-800 ommatidia of the retina, it comprises approximately 40,000 cells of various types, rendering it more complex than the other neuropils. Primarily tasked with processing motion and color information, the medulla receives direct input from R7 and R8 photoreceptors, as well as from lamina monopolar cells (LMCs) (Takemura et al., 2008).

Neurons within the medulla can be categorized into two subtypes: uni-columnar neurons, whose processes are confined to a single column and present in each column, and multi-columnar neurons, which branch across multiple columns and are not confined to a single column (Raghu and Borst, 2011).

The development of the medulla is closely intertwined with the formation of photoreceptors and the lamina, originating from a single neuroepithelium known as the outer proliferation centre (OPC) during the third larval instar and early pupal stages. This coordinated development underscores the intricate interplay between different components of the visual system during its maturation (Erclik et al., 2017).

The lobula complex: lobula and lobula plate

The lobule and lobula plate serve as the final two neuropils within the visual system. They receive input from the medulla to process visual information before transmitting it to the central brain. While relatively little is known about the lobule compared to the lobule plate, it

is believed that only the lobula plate contains retinotopic neurons (Perry et al., 2017).

Within the lobula plate, two primary classes of retinotopic neurons exist: T4 neurons, which receive input from the ON-motion pathway and detect bright moving edges, and T5 neurons, which receive input from the OFF-motion pathway and detect dark moving edges. These Neurons can further be subdivided into four subclasses, each projecting into one of the four layers of the lobule plate. The four T4 and T5 neuron subclasses must establish their own parallel retinotopic maps within the lobule plate to ensure that each of the approximately 750 columns contain one copy of each subtype (Ammer et al., 2015; Serbe et al., 2016).
The development of the lobula plate arises from the neuroepithelium known as the Inner Proliferation Center (IPC), located beneath the Outer Proliferation Center (OPC) in the larval brain. This developmental process underscores the complexity and precision involved in establishing the neural circuitry responsible for visual processing within the fly's brain (Sato et al., 2013).

1.7.2 Connections in the *Drosophila* visual system

Retina into the lamina:
R1-R6 photoreceptors of the retina establish connections with lamina neurons L1, L2, and L3, while L4 and L5 neurons do not receive direct connections from R cells. Instead, L2 forms synaptic connections with L4. Among these lamina neurons, L1 and L2 are consistently present at every R cell synapse, while L3 is found only at a subset of these synapses. In a cross-section of the lamina cartridge, L1 and L2 are situated within the inner side, surrounded by R1-R6 photoreceptors, while L3, L4, and L5 are positioned in the peripheral region of the cartridge (Zhu, 2013).

The organization of the lamina cartridge is heavily influenced by the expression of CadN,

with L1 and L2 neurons exhibiting high levels of CadN expression compared to other lamina neurons and R cells. Manipulating CadN expression, such as its removal from lamina neurons or overexpression in R cells, results in the displacement of L1 and L2 from the center of the lamina cartridge, indicating the importance of CadN adhesive interactions in neurite localization (Nern et al., 2008).

L1 and L2 neurons play crucial roles in motion vision, and the dendritic pairing of these neurons at each synapse is a complex process due to their extensive branching. Synaptic exclusion is the mechanism of this pairing, involving repulsion between processes of the same cell type (Nern et al., 2008). This process prevents postsynaptic pairing of elements from the same cell, such as L1-L1 or L2-L2 pairs. Synaptic exclusion is mediated by two transmembrane immunoglobulin proteins, Dscam1 and Dscam2, which are expressed in L1 and L2 neurons, facilitating self-avoidance (Nern et al., 2008). Alternative splicing of both Dscam1 and Dscam2 generates isoforms capable of homophilic binding, inducing repulsion. Over 38,000 Dscam1 isoforms resulting from alternative splicing are expressed in each neuron, but only neurons with identical isoforms can mediate homophilic binding, leading to repulsion. The elimination of Dscam1 from lamina neurons does not significantly disrupt synaptic exclusion. Dscam2 alternative splicing is regulated in a cell-type-specific manner, with two different isoforms, A and B, expressed in cells separately (Millard et al., 2007).

Lamina into the medulla:

Once visual stimuli are received from the retina into the PRs, the information must travel from the lamina through a set of neurons connecting them to the next neuropile, the medulla. The R1-R6 PRs project their axons into the lamina where they connect to lamina neurons to send information (Nériec and Desplan, 2016). In the lamina, there are five monopolar lamina

neurons (L1-L5) that extend their axonal projections into different medulla layers (Morante and Desplan, 2008). The first lamina neuron, L1, terminates into two different layers in the medulla, M1 and M5. L2 neurons sends out one axonal projection into the M2 layer. Similar to L2, L3 also send out a single axonal projection into the M3 layer. However, L4 and L5 neurons project two axonal terminals into the medulla. L4 residues in M2 and M4 layers while L5 terminates into M1 and M5 (Millard and Pecot, 2018).

Medulla into the lobula complex:

The medulla by far is the largest neuropile of all that consist of the majority of neuronal cell types of the *Drosophila* visual system. There are 10 layers (M1-M10). The medulla neuropile location serves a great purpose for acting as a bride between the lamina and lobula complex (Morante and Desplan, 2008). The lamina is known for its motion detection purposes as well as the loubla complex for Having the ON and OFF motion pathways of the fly (Tuthill et al., 2013). To be able to coordinate between these Two neuropiles, the medulla neurons receive input from the lamina where medulla neurons Will send projections into the lobula complex. For example, Tm3, Mi4, and Mi4 will have Their cell bodies in the medulla cortex will send projections through the medulla and Will finally terminate at the final 10th layer in the medulla, where the double plate T4 will Establish connections with them (Morante and Desplan, 2008). Also, T5 neurons from the lobual plate will send out projections into the lobula where some of medulla neurons are terminating in that neuropile such as Tm9, Tm2, Tm4, and Tm1 (Morante and Desplan, 2008; Nériec and Desplan, 2016; Millard and Pecot, 2018).

Lobula complex into the central brain:

The last step of visual process is when all the information has reached the lobula complex and now ready to be sent to a higher processing area in the central brain. The Lobula Plate

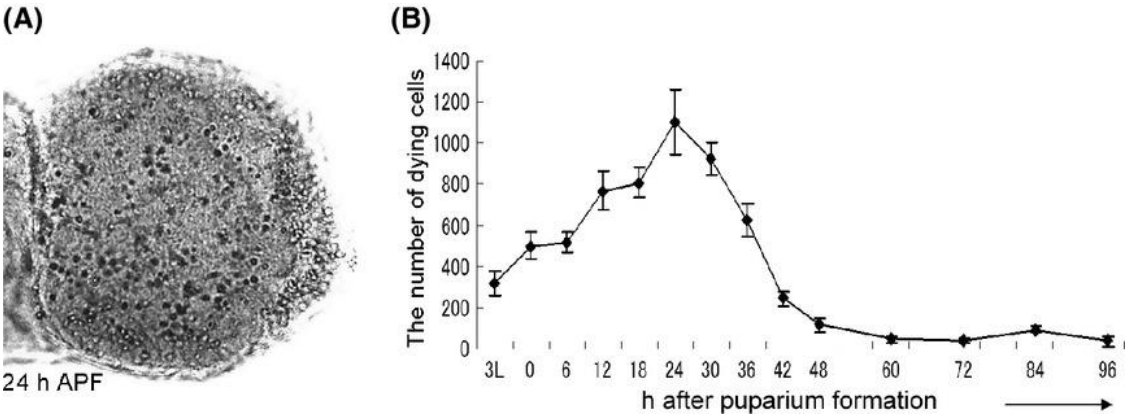
Tangential Cells (LPTCs) are projection neurons that has a role in motion computing located near the lobula complex (Nériec and Desplan, 2016). They receive direct input from T4 and T5 neurons and sends out the information to the ventro-lateral neuropils of the central brain for final Processing (Maisak et al., 2013).

1.7.3 The visual system as a context in this research

To better understand the roles of *Drosophila* neurotrophins and their receptors in the regulation of neuronal survival, cell number, and connectivity. I will be using specific developmental stages to answer these questions. For example, it has been documented that at 24 hr APF, a massive wave of cell death occurs to eliminate the excess of neurons (Hara et al., 2013). This stage will be used to answer the question of whether the DNTs and Tolls regulate cell survival at 24 hr APF. Also, it has been shown that cell death declines at around 48 hr APF (Togane et al., 2012). The 48 hr APF stage is an ideal stage to test wether cell number is affected after the elimination of cell at the earlier stage 24 hr APF **(Figure 1.6)**. Finally, it has been reported that at 72 hr APF the *Drosophila* visual system goes through the final stages of synaptic connectivity to establish the final connectivity patterns to get it ready for the adult visual system (Morante and Desplan, 2008). This stage will be used to answer the question of whether the interactions between DNTs and Toll influences the connectivity patterns during this developmental stage.

In my research, I will investigate two lamina neurons to answer the connectivity question: Lamina L1 neurons and lamina wide field neurons (Lawf1). The lamina L1 neurons have cell bodies in the lamina cortex with massive dendritic extensions in the lamina, and their axon passes through the lamina and resides in two medulla layers, M1 and M5 layer (Tuthill et al., 2013). The L1 axonal terminals in the medulla have been studied in the past, where Dscam2

Figure 1.6 Cell death in the developing optic lobe



A graph showing the peak of cell death in the developing optic lobe. A great context to investigate the regulation of cell survival and cell death at that stage. Also, as indicated in the graph, cell death starts to decline at around 48 hr APF which makes it a great context to investigate the regulation of cell number after the massive wave of cell death (Image taken from Togane et al., 2012).

has been shown to act as a repellent to regulate the targeting of these axonal projections in the medulla (Millard et al., 2007). The Lawf1 neuron is a lamina feedback neuron that has its cell body in the medulla and it sends out projections in both directions in the medulla and the lamina. Although there is some insight into the function of these neurons in motion detection (Chen et al.,2016). No evidence shows their involvement in regulating cell survival or connectivity **(Figure 1.7)**. The *Drosophila* visual system makes a great tool to investigate various questions. It is an ideal model for my research to investigate cell survival and connectivity due to the events mentioned above that occur at specific developmental stages.

1.8 Aims and Objectives

My thesis aimed to investigate the neurotrophic hypothesis by examining how neurotrophins regulate cell survival, alongside exploring how this regulation impacts the establishment of connectivity in the *Drosophila* visual system.. This is important, as the visual system offers a unique context, whereby many neurons are known to die and many to be maintained alive, at the time when neural circuits are established. Finding evidence for neurotrophism and a role for the *Drosophila* neurotrophin system in this context would provide compelling evidence for a universal evolutionary conservation of neurotrophism as shared mechanism of brain development across species. To test this hypothesis, I have asked whether the *Drosophila* neurotrophin system is involved in the regulation of cell number and whether this is linked to the establishment of connectivity patterns during the development of the visual system.

The experimental objectives of my thesis were:

1. To characterize the spatial and temporal distribution of DNT and Toll receptor gene expression in the visual system during development **(Figure 1.8)**. I looked at: L3W optic lobe, 24 hr APF, 48 hr APF, 72 hr APF.

2. To test the function of *DNT-2* and *spz3* in regulating cell survival and cell number in the optic lobe. This was done using DCP-1 to visualize and quantify dying cells when altering *DNT-2*, *spz3*, *Toll-2*, *Toll-8* and the nuclear marker Histone-YFP to test whether alterations in apoptosis or cell survival also modified cell number. The reason behind selecting *DNT-2* and *spz3* is due to the evidence we already know regarding what kind of receptors they work by. *DNT-2* binds *Toll-6* and *Toll-7* (Foldi et al., 2017). Also, it has been reported that *spz3* might interact genetically with *Toll-8* (Ballard et al., 2014).

3. To investigate whether altering the levels of *DNT-2* and *spz3* and their receptors could influence connectivity patterns in the optic lobe. This was done by: Looking at how altering *DNT-2*/*Toll-2* and *spz3*/*Toll-8* affected the connectivity of L1 at the medulla, and Lawf1 neurons in the lamina and measuring dendrite branching in the lamina.

Figure 1.7 The visual system as a context in this research

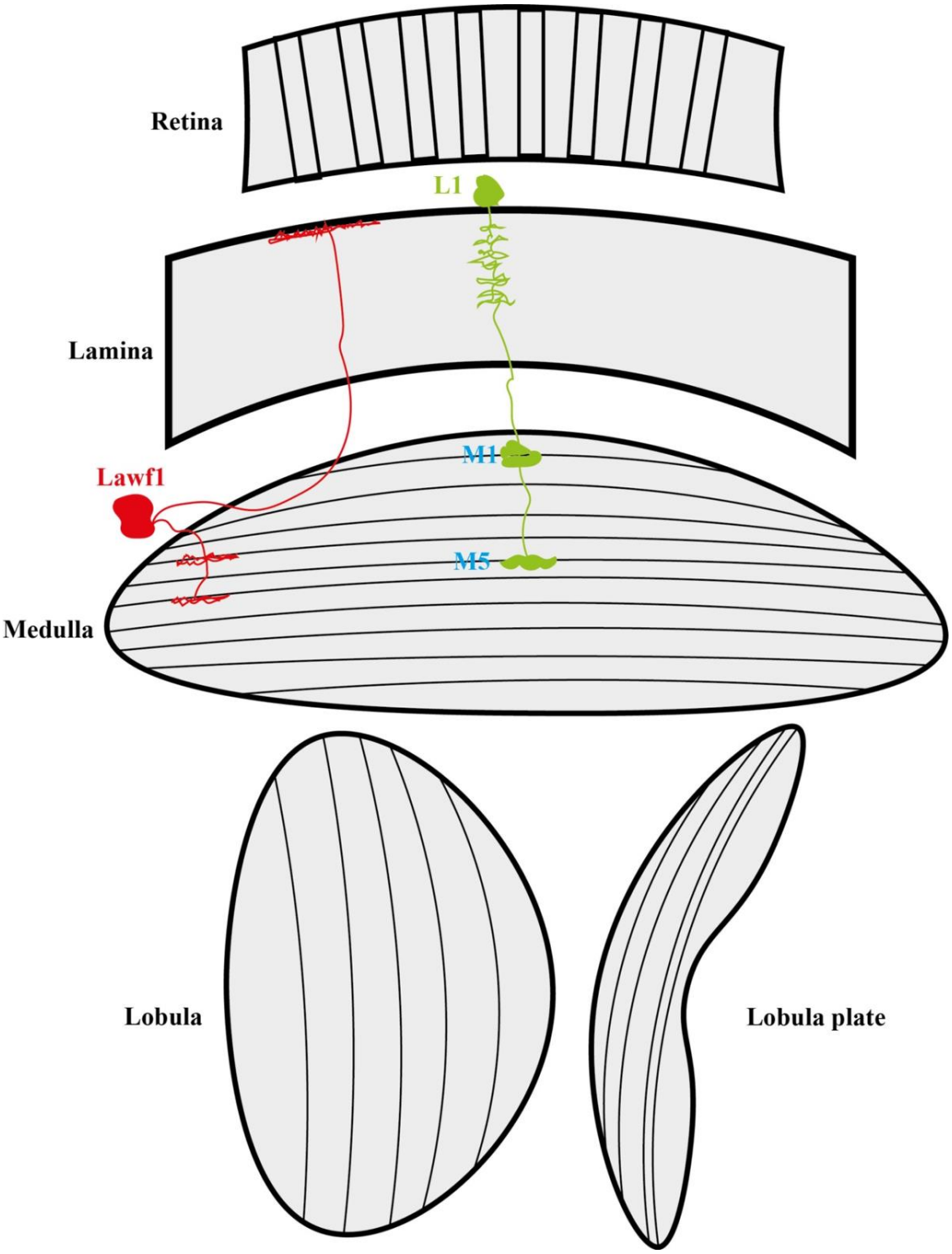


Figure 1.7 The visual system as a context in this research

The *Drosophila* visual system used as a model to investigate the regulation of connectivity patterns when manipulation spz3/Toll-8 and DNT-2/Toll-2 in L1 and Lawf1 neurons. In green is the L1 neurons with a cell body in the lamina cortex and massive dendrites within the neuropile. It sends out two projections into the medulla layers M1 and M5. The focus on this lamina neurons will be when altering DNT-2 and Toll-2 to study the dendrites size and the targeting in the medulla. Moreover, in red, the Lawf1 neurons is a lamina feedback neuron with a cell body in the medulla cortex. It sends out projections in both directions, in the medulla and the lamina. I will be focusing on the axonal terminals in the lamina when altering spz3 and Toll-8 together to investigate any connectivity phenotypes.

Figure 1.8 The *Drosophila* developing visual system

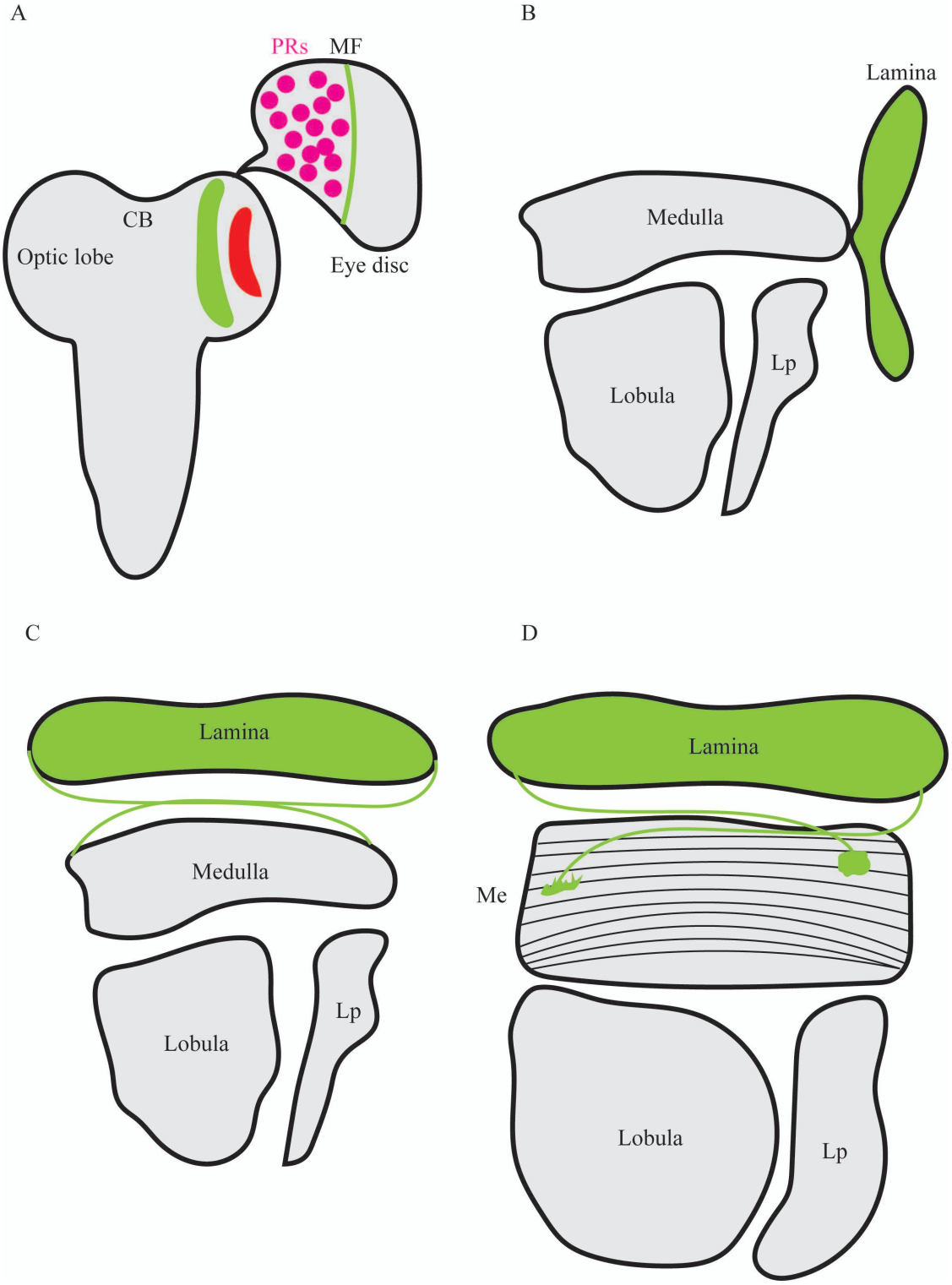


Figure 1.8 The *Drosophila* developing visual system

A schematic diagram of the developing visual system. (A) the third instar larval optic lobe where PRs are being born starting from R8 preceptor. (B) pupal optic lobe at 24 hr APF where the majority of cell death occurs. (C) pupa optic lobe at 48 hr APF where cell death starts to decline. (D) pupal optic lobe at 72 hr APF where connections in the optic lobes are being finalized in the their final targets for the adult optic lobes.

Chapter 2

Materials and Methods

2.1 Genetics

2.1.1 Fly maintenance

All flies were kept in a standard agar medium in bottles or vials. All flies were bred at 25°C with 12 hours Light/Dark cycle. Flies for virgin collection were kept at 25°C during the day and transferred to 18°C at the end of the day. All the stocks were kept at 18°C with 12 hours Light/Dark cycle and transferred once every 5 weeks. The list of stocks generated and used in this project are listed in **(Table 2.1)**.

2.1.2 Genetic protocols

Genetic techniques were used to combine the 2nd, 3rd, and X chromosomes and create recombinants on both 2nd and 3rd chromosomes **(Figure 2.1-2.4)**. All experiments were carried out during pupa, and it was important to pay attention to the markers that would help identify the genotype during this stage.

2.1.3 Temperature manipulation

In the Multi-color Flip-out (MCFO) experiments, the pupa was placed in a water bath at 37°C for 2-5 minutes for the change in temperature to activate the flip recombinase in the construct, to provide the random selection of cells.

2.2 Molecular cloning

2.2.1 Generation of UAS-*spz3*-FL and UAS-*spz4*-FL with Gateway Cloning

To investigate the gain of function of *spz3* and *spz4*, pUAS GW attB vector was used **(Table 2.3)**. these two constructs were generated using the standard protocol of Gateway® Technology with Clonase® II (Thermo Fischer Scientific) **(Figure 2.6)**.

2.2.2 Generation of pTL2-YPET-*spz4*gRNA-5'HA-3'HA

This method was used to generate a construct containing the *spz4* gene fused with a yellow flourcent protein (YFP) following the protocol **(Figure 2.5)**.

2.2.3 Genomic DNA extraction

Genomic DNA from adult flies was extracted using the PureLink® Genomic DNA Mini Kit. A total of 20 Oregon R wild type adult flies were anesthetized on ice in an Eppendorf tube and then homogenized with 180µl of PureLink® Genomic Digestion Buffer using tips. Following this, 20µl of Proteinase K was added to the homogenate and thoroughly mixed. The homogenate was then incubated at 55˚C for 3-4 hours until complete lysis, after which the genomic DNA was isolated according to the provided instructions and finally dissolved in 30µl TE buffer (10 mM Tris-HCl,1mM disodium EDTA, pH8.0, Invitrogen).

2.2.4 Polymerase Chain Reaction (PCR)

This method was performed by following the "Thermo Scientific Phusion High-Fidelity DNA Polymerase" protocol to amplify DNA fragments. GoTaq® Flexi G2 DNA polymerase (Promega) was used for diagnostic tests to check if the amplification settings in the cycle are correct. The reaction mix details of each system and the thermal cycle is provided **(Table 2.4-2.7)** and details of the primers can be obtained from **(Table 2.2)**.

2.2.5 Gel electrophoresis and gel purification

All gels were made using 0.8% agarose or 1% with 1X TAE buffer. 0.5 µL Serva DNA dye (SERVA) and ran at 70V for 30 minutes.

For the gel extraction protocol, 1% of agarose was made using 1X TAE buffer, 2 µL Serva DNA dye and run at 120V for 1 hour. After one hour, the gel was placed under UV light in GeneBox (SYNGENE) to be visualized. The wanted bands were cut and placed into Eppendorf tubes for purification. QIAquic gel extraction kit (QIAGEN) was used to dissolve the purified DNA into EB buffer. The full protocol was followed from the kit.

2.2.6 Spectrophotometer (Nanodrop)

This method was used to check for the concentration of the purified DNA and give an idea of how pure the genomic DNA is. Before taking the sample for testing, the tube was vortexed and centrifuged to make the solution homogenous (equal concentration of DNA in the tube). ND-1000 software was used to measure the concentration of the DNA. 1µL of the elution buffer from the Genomic DNA mini-kit was used to blank the reader. 1µL of DNA sample was placed on the reader, and the DNA concentration was measured to be 28.1 ng/µL. After measuring the DNA concentration, the tube was placed into the -20 °C freezer for later use.

2.2.7 PCR purification

The protocol for PCR purification was followed by the guide provided with QIAquick PCR Purification kit. 5 volumes of Buffer PB 1 were added to 1 volume of the PCR reaction. The PCR reaction was placed into a QIAquick column and centrifuged for 1 minute. The column was centrifuged again to remove any ethanol.

The column was placed into a new 1.5ml tube to elute the DNA. 30µL of elution buffer was added to the centre of the column. The column was left to stand for 1 minute at room temperature then centrifuged at max-speed for 1 minute.

2.2.8 Restriction enzyme digest and ligation

Restriction enzymes in this project were used to digest vectors and plasmids for the purpose of ligation reactions as well as to run diagnostics digest to confirm the results. A list of all the enzymes used in this project are provided **(Table 2.8)**.

2.2.9 Cell transformation

In this method, 30µL of competent *E. coli* cells (NEB-5α, C2987, New England Biolabs) were thawed on ice. 5µL of DNA was added to the cells and incubated on ice for 30 minutes. After 30 minutes, the cells were heat shocked for 30 seconds at 42 °C. The cells were placed on ice for 5 minutes. Lastly, 950µL S.O.C medium (NEB) was added to the mixture. Cells were placed into the 37 °C incubator shaking at 250 rpm for 1h. After 1 hour, 50µL of cells were plated out on agar plates with 17 carbenicillin and grown O/N at 37°C.

2.2.10 Plasmid amplification by Mini-prep and Midi-prep

In this method, one colony of the O/N grown cells was selected to be amplified. The protocol was followed by Plasmid Miniprep Kit (QIAGEN). After the plasmid extraction, the plasmid was digested and run on a gel to confirm the ligation of homology arms. After the confirmation of ligation, the miniprep cultures were used to be amplified on a larger scale by midiprep using the Plasmid Midi Kit (QIAGEN). The provided protocol was followed to complete the amplification.

2.2.11 Sequencing and PCR checking

When required, plasmids or PCR products underwent sequencing. To prepare a sequencing mixture of 10µl, 300ng of plasmid, 3.2pmol of primer (refer to Table 2.2), and the necessary volume of ddH2O were combined. The samples were then forwarded to the DNA sequencing services at the School of Biosciences, University of Birmingham.

2.2.12 Designing the sequence of gRNA oligos

To generate the oligos required, the gRNA for the Spz4 gene was designed using the online tool CRISPR Optimal Target Finder A 20nt sequence followed by a 3nt PAM sequence was positioned upstream of the Spz4 ATG to ensure maximal specificity. Following the selection of oligos online, SeqBuilder was employed to append 3bp "gtc" to the 5' end of sense oligos and 4bp "aaac" to the 5' end of antisense oligos.

2.2.13 gRNA oligos treatment with Polynucleotide kinase (PNK)

As the oligos were synthesized without a phosphatase group on either end, they underwent initial treatment with polynucleotide kinase (Promega). For each reaction, 8µl of 100µM oligo stock solution was combined with 1µl of PNK and 1µl of T4 ligase buffer. The mixture was then subjected to incubation at 37˚C for 30 minutes, followed by inactivation of the kinase at 65˚C for 20 minutes. Subsequently, both 10µl PNK-treated oligos were merged with 10µl of 10x annealing buffer (comprising 100mM Tris-HCl, pH 7.5, 1M NaCl, 10mM EDTA) and 70µl H2O to facilitate the formation of double-stranded gRNA. This mixture underwent incubation at 95˚C in a heating block for 5 minutes and was then allowed to gradually cool down to room temperature in the block, enabling the gradual annealing of the

oligos as the temperature decreased.

2.4 Confocal microscopy and imaging

2.4.1 Confocal microscopy

All the expression map samples were scanned by Zeiss (LSM 710). The resolution of all scans were either 512x512 or 1024x1024 pixels using the 20x lens. The speed was set at 7 for all samples, and the interval of each step was 1µm.

For the cell survival experiments with DCP-1, All samples were scanned at the Zeiss (LSM 900 with Airyscan 2) with a resolution of 512x512 using the 25x oil lens with 0.9x zoom. The speed was set to 7 and the step to 1µm.

The same microscope was used to scan the HistoneYFP samples. The resolution was set to 1024x1024 using the 25x oil lens with 0.8x zoom. The speed was set to 7 and the step to 1µm.

For the connectivity question, the same microscope was used with some changes. The resolution was set to 1024x1024 no zoom for the medulla and 2x zoom for the dendrites. The speed was set to 7 with 2x averaging for the medulla and 8x for the dendrites. The step was set to 1µm.

2.4.2 ImageJ

This software was used to visualize sample, and analysis for the expression map samples, MCFO, TransTango, GRASP, and some connectivity phenotypes. To analyse the connectivity phenotypes, 2-3 slides were set to max projection to focus on 2-3 L1 neurons and assess if they cross to the nearby columns. This software was also used

to count the number of HistoneYFP cell with the Deadeasy optic lobe plug in.

2.4.3 Imaris

Imaris is a 3D software that was used to measure the volume of DCP-1 signal intensity, Lawf1 axonal terminals and generate ROIs for the HistoneYFP data to then be analysed in ImageJ. For the DCP-1 experiments, an ROI was generated around the desired region in the optic lobe, saved as an ROI for reanalysis, and finally the volume was measured. For the Lawf1 axonal terminals, an ROI of the axonal terminals in the lamina only were selected the volume was measured. For the cell number with HistoneYFP, the ROI for the lamina was generated in imarsi software, saved as sperate TIFF files, then imported to ImageJ and analysed with the Deadeasy optic lobe plug in. This was one of the advantages of imaris in generating the ROI. It was time consuming to generate the lamina ROI in ImageJ where sections had to be saved one by one. This was avoided by using imaris where the whole ROI was generated at once without the need of sectioning.

2.4.4 Amira 3D

Amira was used to measure the volume of the L1 neurons dendrites. This method was selected due to its accuracy in recognising the dendrites. It also offers complete control of the area that needs to be analysed and the area that needs to be left out. Samples of L1 neurons were imported into the software, in the segmentation section, the magic wand was used to select the dendritic branching while adjusting the threshold to cover the desired area. After the selection, volume retendering was applied to measure the volume of the selected area. Finally, a statistical plug-in was applied to export the volume number of each L1 dendrite analysed.

2.4.5 Cell number quantification using DeadEasy

Histone YFP cells were quantified using an ImageJ plug-in called Deadeasy OpticLobe, developed by a former lab member, Dr. Manuel Forero. This software could identify cells within 3D image stacks and automatically counting them. Due to some difficulties generating the ROIs in ImageJ, they were generated using imaris and brought back into imagej for quantification.

2.5 Dissection and immunostaining of larval and pupal brains

All dissections were performed in ice-cold 1xPBS, and the dissected brains were immediately transferred to ice-cold 4% formaldehyde (FA) diluted in PEM solution following a 20-minute dissection period. Brains then were fixed for a period depending on the experiment (DCP-1 20 minutes) (All other experiments 40 minutes). Brains underwent a series of five 15-minute washes in 750µl of 0.3% PBT (0.5% Triton X-100 dissolved in 1xPBS). For visualization of endogenous fluorescent signals, brains were mounted with Vectashield (Vector Labs) immediately after washing. For immunostaining, brains were blocked in 750µl of 10% NGS (normal goat serum, #S1000, Vector Labs) dissolved in 0.5% PBT for 1 to 2 hours at room temperature. Following blocking, the NGS solution was replaced with 100µl of primary antibody and incubated over one night at 4°C. Subsequently, brains underwent five 15-minute washes in 750µl of 0.5% PBT, followed by overnight incubation in 100µl of secondary antibody at 4°C. Finally, stained brains were subjected to five 15-minute washes in 750µl of 0.5% PBT, one wash in 750µl of 1x PBS, each lasting 15 minutes, before being mounted with Vectashield (Vector Labs).

2.6 Data analysis

Excel (Microsoft) was utilized for data collection, and GraphPad Prism® was employed for data analysis. Conversely, data derived from cell number and volume intensity constituted

numerical continuous data. Normal distribution of data was assessed using One-Way ANOVA, with multiple comparisons conducted using Dunnett's test. In cases where data did not exhibit a normal distribution, the Kruskal-Wallis test was employed, followed by Dunn's test for multiple comparisons. For sample groups consisting of two sets of data, an unpaired t-test or Mann-Whitney U test was conducted, depending on whether the data were normally distributed or not, respectively. The connectivity phenotype data were analysed using Excel graph generator with the percentages.

Figure 2.1 Genetic protocol for combining 2nd and 3rd chromosomes

G0 ♀ w; IF/CYO; MKRS/TM6B X w; a/CyO ♂

G0 ♀ w; IF/CYO; MKRS/TM6B X w; ; ; b/TM6B ♂

F1 ♀ w; +/CyO; b/MKRS X w; a/IF; +/TM6B ♂

F2 **Stable Stock: w; a/CyO;b/TM6B**

This protocol was used to combine the alleles on 2nd and 3rd chromosomes.

Figure 2.2 Genetic protocol for generating a recombinant of the 2nd chromosome

G0 ♀ w; a/a or a/CyO X w; b/Sm6aTM6B ♂

F1 ♀ w; a/b X w; IF/CyO ♂

F2 ♀ w; IF/CyO X w; a,b/IF ♂

"F2 single mating [5 females to 1 male] 50 crosses"

F3 **Stable stock: w; a,b/CyO**

In this recombinant, a HistoneYFP stock was combined to a mutant line. To check for the HistoneYFP, it was crossed to a GAL4 line that worked well to spot the flourcent signal at the microscope to confirm the presence of HistonrYFP. To check for the mutant, genomic DNA was extracted from the flies followed by a PCR and was sent to be sequenced.

Figure 2.3 Genetic protocol for generating a recombinant on the 3rd chromosome

G0 ♀ w; a/TM6B X w; b/TM6B ♂

F1 ♀ w; a/b X w; MKRS/TM6BlacZ ♂

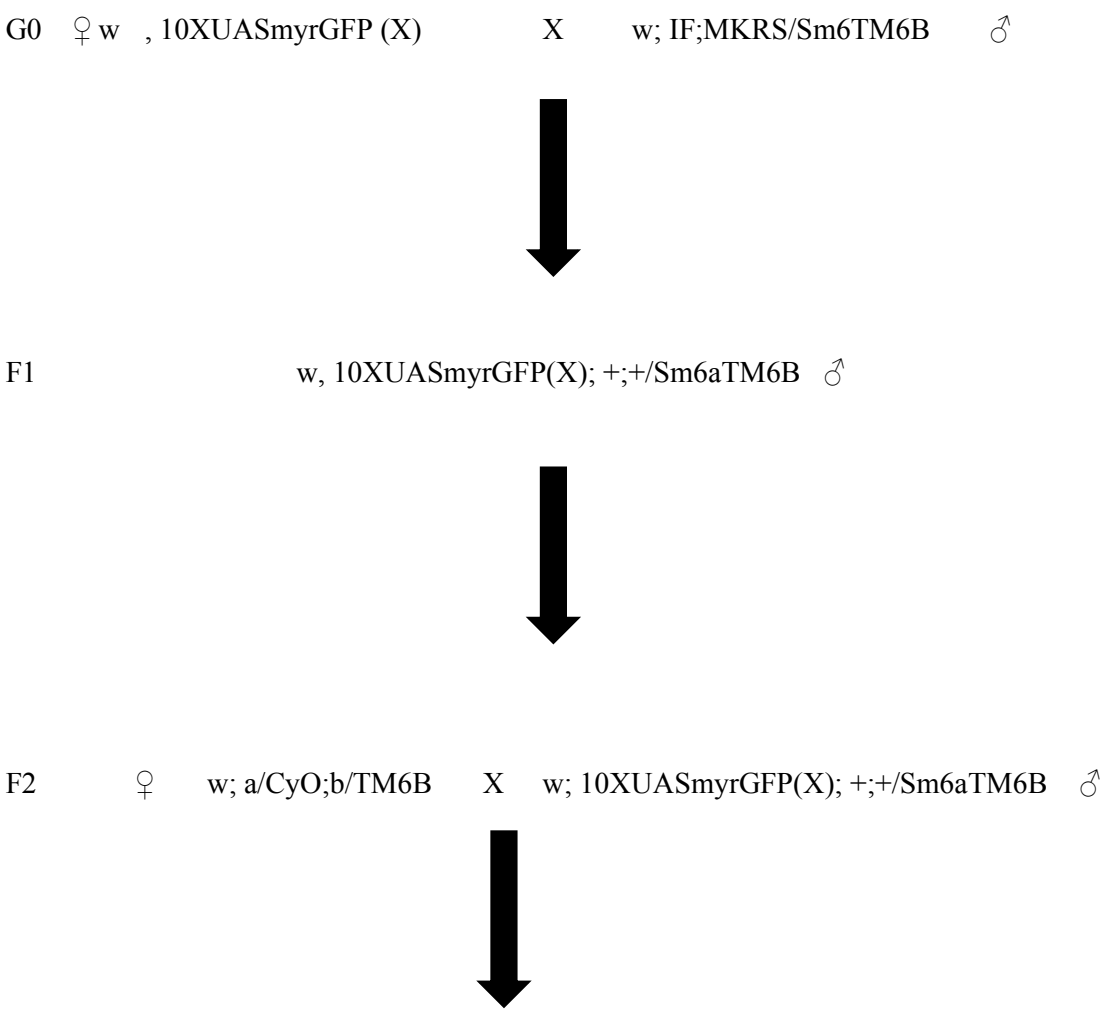
F2 ♀ w; MKRS/TM6BlacZ X w; a,b/MKRS ♂

"F2 single mating [5 females to 1 male] 50 crosses"

F3 **Stable stock: w; a,b/TM6B**

To check if the recombinant has worked, the GAL4 was checked by crossing it to 10XUASmyrGFP to check for the GFP signal. The other GAL4 was checked by following the eye colour

Figure 2.4 Genetic protocol for combining 10XUASmyrGFP on the X chromosome



The combined 10XUASmyrGFP with a stock would then be used to cross to a split-GAL4 lines.

Table 2.1 Stocks List

Name	Full genotype	Source
Oregon	Oregon	Hidalgo Lab
nsybGAL4	w; nsyb GAL4 attP2	BDSC#39171
10XUASmyrGFP 2nd	P{10XUAS-IVS-myr::GFP}attP40	BDSC# 32198
10XUASmyrGFP 3nd	P{10XUAS-IVS-myr::GFP}attP2	BDSC# 32197
10XUASmyrGFP X	P{10XUAS-IVS-myr::GFP}su(Hw)attP8	BDSC# 32196
UASHistoneYFP	UAS histone YFP(2)	Hidalgo lab
MCFO	w hsFLP:PEST ;; HA-V5-FLAG-OLLAS MCFO	BSC #64086
TransTango	QUAS mTd tomato, UAS myrGFP; TransTango attp40	BSC #77124
GRASP	w; P{UAS nsyb spGFP 1-10}2 P{lexAOP CD4 spGFP 11}2	BSC #64314
UAS mCD8 GFP	w; UAS mCD8 GFP	Hidalgo lab
Toll-1	w;+/(CYO);Toll-1 Gal4 [CRISPR]/ TM6B	Deepanshu
Toll-2pTV	y)w; pTV-Toll-2 1M-b/CyO	Guiyi Li
Toll-5		Guiyi Li
Toll-6	yw; Toll-6 MI02127 Gal4/TM3 Line 1-6M	Hidalgo lab
Toll-7	Toll-7 Gal4 MI13963/SM6a	Hidalgo lab
Toll-8	PGawB MD806/TM6B	BDSC #36548
Spz1	w; spz-1 [tgF04306D] / SM6aTM6B 234113-3-M1-M	Hidalgo lab
DNT-1	w; +; DNT-1 GAL4 [cripsr] 1-M2-M / SM6aTM6B	Hidalgo lab
DNT-2	w: + / CyO; DNT-2 [CRISPR] GAL4 26831-F1-Ma / MKRS	Hidalgo lab
Spz3	Spz3-IT-GAL4	Hidalgo lab

Name	Full genotype	Source
Spz4	yw; spz-4 MiMIC{MI15678} / SM6a	Hidalgo lab
$Spz3^{27}$	w; spz-3 [27PHo] / CyO	Hidalgo lab
$Spz3^{46}$	w; spz-3 [46] / CyO	Hidalgo lab
$DNT\text{-}2^{18}$	DNT-2 [18]	Jill Wentzell
$DNT\text{-}2^{37}$	DNT-2[37] / TM6B	Jill Wentzell
UASspz3FL	w; UAS spz-3 Full length	Naser Alshamsi
UASspz3CK	w; UAS spz-3 Cysknot 22A	Bangfu
UASToll-8RNAi;UASspz3FL	W;UASToll-8RNAi/CyO;UASspz3FL/Tm6B	Naser Alshamsi
UASDNT-2FL	w; UAS DNT-2 FL 22A	Bangfu
UASDNT-2CK	UAS DNT-2 CK6A	Suzana
UASToll-2RNAi	W; UASToll-2RNAi/CyO	VDRC:36305
UASToll-2RNAi;UASDNT-2FL	UASToll-2RNAi;UASDNT-2FL/Sm6aTM6B	Naser Alshamsi
UAShisYFP;UASDNT-2FL	w; UAS histone YFP; UAS DNT-2 FL	Jun
UAShisYFP;UASDNT-2CK	w; UAS histone YFP; UAS DNT-2 CK-6A	Alicia
Toll-2GAL4;$DNT\text{-}2^{18}$	Toll-2GAL4;$DNT\text{-}2^{18}$/Sm6aTM6B	Naser Alshamsi
UAShisYFP;$DNT\text{-}2^{37}$	UAShisYFP;DNT-2[37]/Sm6aTm6B	Naser Alshamsi
Spz3GAL4;Toll-8LexA	Spz3GAL4/CyO;Toll-8LexA/Tm6B	Naser Alshamsi
Spz3GAL4;Toll-6LexA	Spz3GAL4/CyO;Toll-6LexA/Tm6B	Naser Alshamsi
Spz3GAL4;Toll-2LexA	Spz3GAL4/CyO;Toll-2LexA/Tm6B	Naser Alshamsi
w; IF/CyO		Guiyi Li

Name	Full genotype	Source
UASmyrGFP;UASspz3FL	UASmyrGFP/CyO;UASspz3FL/Tm6B	Naser Alshamsi
Lawf1-Split-GAL4	W; 52H01-p65ADZp attp40; 17c11-ZpGdbd attp2	Tuthill Reiser Lab
UASToll-8RNAi	UASToll-8RNAi /+	VDRC: 27098
L1-Split-GAL4	W; 84A08-p65ADZp attp40; 66A01-ZpGdbd attp2	Tuthill Reiser Lab
UASmcd8GFP;UASDNT-2FL	UASmcd8GFP/CyO;UASDNT-2FL/Tm6B	Jun
w; IF/CyO lacZ;MKRS/TM6B		Hidalgo lab
+; IF/CyO;MKRS/TM6B		Guiyi Li

Table 2.2 Primers List

Primer No.	ORIGINAL NAME	GENE/ PL ASMID	PRIMER SEQUENCE(5'-->3')	DESCRIPTION
1	Spz4 gRNA senseoligo	Spz4	tcgGTTCACAGGAGGGAATCTAA	for CRISPR Golic+ spz-4YPET at C-terminus
2	Spz4 gRNA nonsenseoligo	Spz4	aacTTAGATTCCCTCCTGTGAAC	for CRISPR Golic+ spz-4YPET at C-terminus
3	Spz4 5'HA F	Spz4	ataacagggtaatgccggcaGGCTTCGTAATCTAAGCGGCCTAATA	for CRISPR Golic+ spz-4YPET at C-terminus
4	Spz4 5'HA R	Spz4	acatgccgcctgctccgccaGTCCTCCAAGAAATCGAACTCATCAC	for CRISPR Golic+ spz-4YPET at C-terminus
5	Spz4 3'HA F	Spz4	aacggtcctaaggtagcgagTCCCATTAGATTCCCTCCTGTGAACAA	for CRISPR Golic+ spz-4YPET at C-terminus
6	Spz4 3'HA R	Spz4	ccttcgaaagggtttaaacgAACTAGATTTCCTTCTCTGGCGACATTC	for CRISPR Golic+ spz-4YPET at C-terminus
7	attB1 F primer	Spz4	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGGTGCGCACCCAAATGGA	To PCR spz4 flanked by attB sites for Gateway cloning
8	attB2 R primer	Spz4	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTCCTCCAAGAAATCGAACT	To PCR spz4 flanked by attB sites for Gateway cloning
9	attB1 F primer	Spz3	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGCTAGCATATTTCGCACGCCC	To PCR spz3 flanked by attB sites for Gateway cloning
10	attB2 R primer	Spz3	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGGATTACATCTACAGACAC	To PCR spz3 flanked by attB sites for Gateway cloning
11	M13_Forward		GTAAAACGACGGCCAGT	M13 sequencing primer, forward. Can be used to sequence HA inserts in CRISPR cloning constructs into pGEM-T2AGAL4
12	M13_Rev		GGAAACAGCTATGACCA	M13 sequencing primer, Reverse. Can be used to sequence HA inserts in CRISPR cloning constructs into pGEM-T2AGAL4
13	UAS-Spz3-FLGW (F) New	Spz3	GAGGGTTCGTACTCCCGTTA	To sequence the missing parts in the middle of Spz3
14	UAS-Spz3-FLGW (R) new	Spz3	CGGCTTCGACGGTCTCCCCG	To sequence the missing parts in the middle of Spz3
15	UAS-Spz4-FLGW (F) New	Spz4	AGTTCCAAATGAGGTTACCG	To sequence the missing parts in the middle of Spz4-FL-entry clone in pDonr221
16	UAS-Spz4-FLGW (R) New	Spz4	CTCCCTTAGCAGTCCAGACT	To sequence the missing parts in the middle of Spz4-FL-entry clone in pDonr221

Table 2.3 List of plasmids and vectors

Constructs	Antibiotic-resistance gene	Inserts	Comments
pTL2-YPET	Ampicillin	YPET cDNA from pL452-C-YPET	Contains C-terminal YPET Tag and linker
pUAS GW attB	Ampicillin	Empty	Destination vector of gateway cloning, it carries UAS promotor and attB site
UAS-Spz-3-FL-GW-attB	Ampicillin	1.8 Kb full lentgh spz3	Expression clone containing spz3 FL
UAS-Spz-4-FL-GW-attB	Ampicillin	1.7 Kb full length spz4	Expression clone containing spz4 FL
Spz-4-FL-pOT2 (clone MIP13656	Chloramphenicol	2.9 kb of full length Spz4	Clone containing the full length spz4 gene
pDONR221	Kanamycin		Gateway donor vector
Spz-3-cDNA-full length. RE22741	CMP	Spz3 full length cDNA in pOT2	Clone containing the full length spz3 gene
Spz-3-FL-pDonr221	Kanamycin	1.8 Kb full lentgh spz3	Entry clone containing spz3 FL

Figure 2.5 pTL2-YPET-spz4 cloning strategy

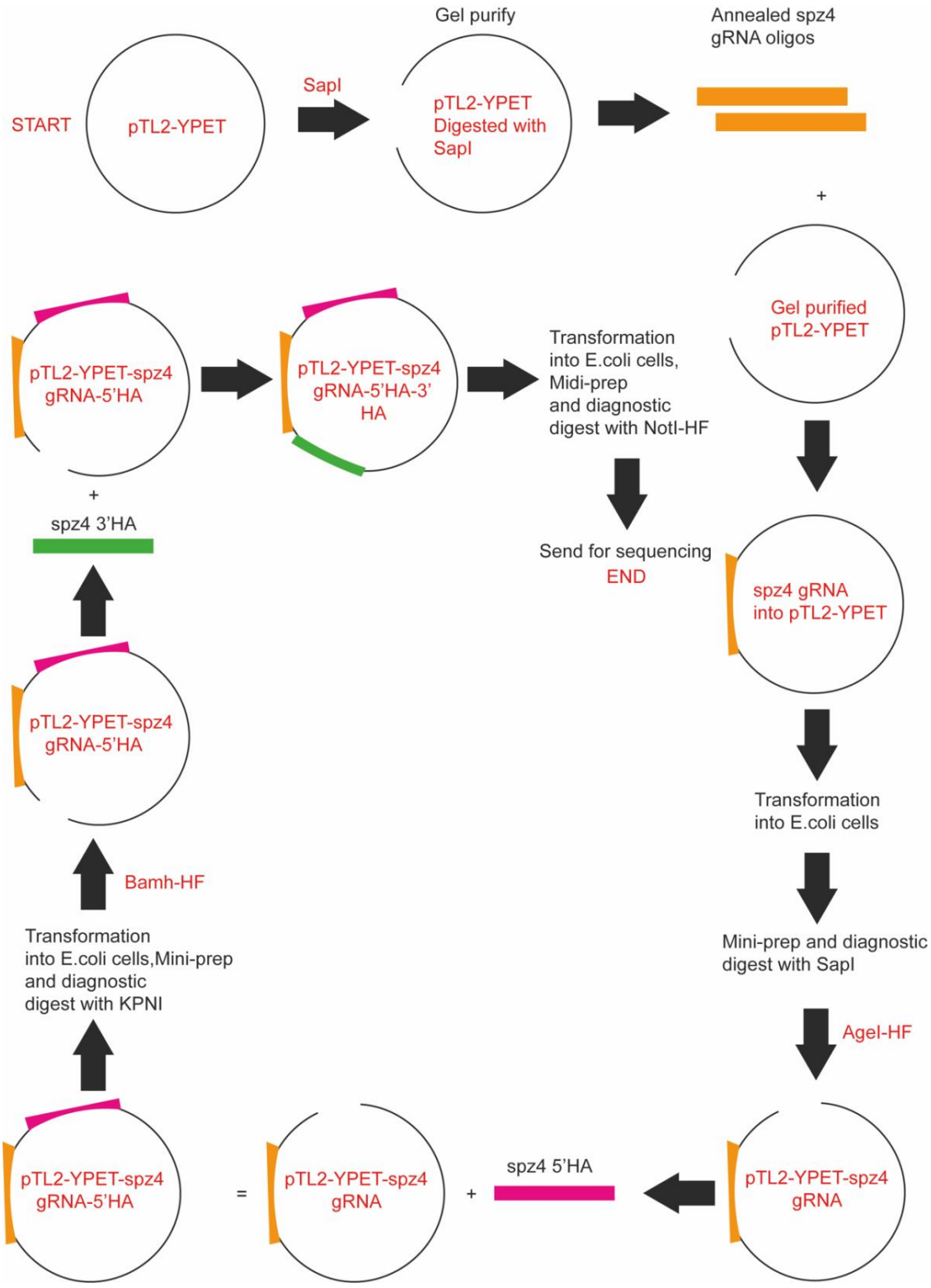


Figure 2.6 Gateway cloning strategy

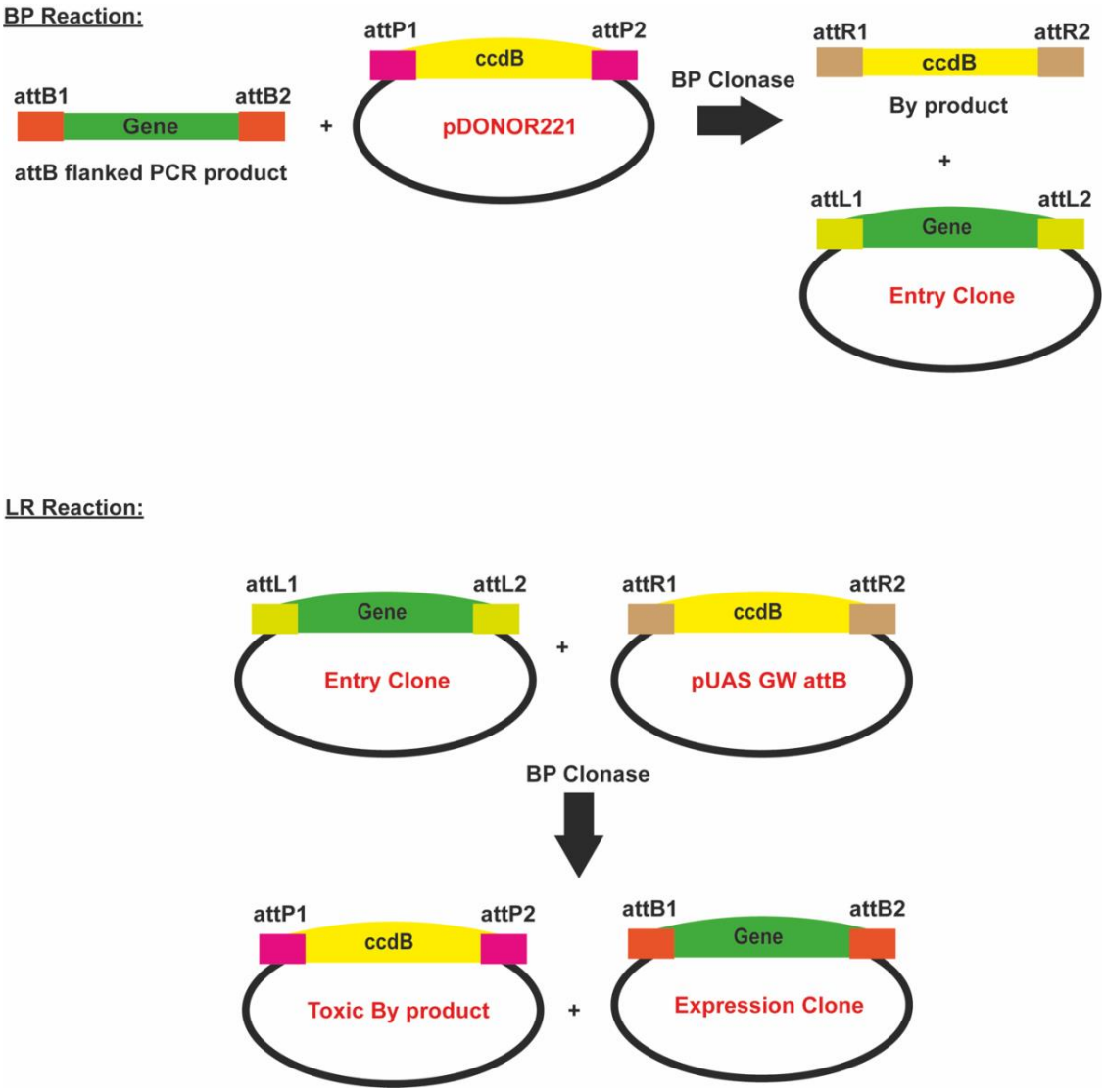


Table 2.4 Components of Expand High Fidelity DNA polymerase PCR System

Component	Volume
10xBuffer with MgCl2	5 µl
10mM dNTP	1 µl
2µM Forward primer	7.5 µl
2µM Reverse primer	7.5 µl
Template DNA	0.1-250ng
Enzyme Mix	0.75 µl
dd H2O	X µl
TOTAL	50 µl

Table 2.5 Components of GoTaq® Flexi G2 DNA polymerase PCR system

Component	Volume
5x Green Buffer	5 µl
25mM MgCl2	1.5 µl
2mM dNTP	2.5
2µm Forward primer	5 µl
2µm Reverse primer	5 µl
Template DNA	<250ng
Enzyme	0.25 µl
dd H2O	X µl
TOTAL	25 µl

Table 2.6 PCR program when using Expand High Fidelity DNA polymerase

Temperature	Time	Cycles
94°C	2min	1
94°C	15s	
45°C-65°C	30s	10
68°C	4min	
94°C	15s	
45°C-65°C	30s	25
68°C	45s-4min + 5s for each successive cycle	
68°C	7min	1
4°C	Unlimited	1

Table 2.7 PCR program when using GoTaq® Flexi G2 DNA polymerase

Temperature	Time	Cycles
95°C	2min (4min for colony PCR)	1
95°C	30s	
45°C-65°C	30s	25
72°C	1min/kb	
72°C	5min	1
4°C	Unlimited	1

Table 2.8 Restriction enzymes used in the experiments

Enzyme	Buffer	Company
AgeI	CutSmart	New England BioLabs
NotI-HF	CutSmart	New England BioLabs
BamH-HF	CutSmart	New England BioLabs
KpnI-HF	CutSmart	New England BioLabs
SapI	CutSmart	New England BioLabs

Table 2.9 List of antibodies

Antibody	Donor	Working dilution
Primary		
Anti-GFP	Rabbit	1:250
Anti-DsRED	Rabbit	1:100
Anti-Ncadh	Rat	1:250
Anti-24B10	Mouse	1:250
Anti-DCP-1	Rabbit	1:250
Anti-Flag	Rabbit	1:100
Anti-V5	Mouse	1:100
Anti-HA	Chicken	1:100
Secondary		
Anti-rabbit 488	Donkey	1:250
Anti-rabbit 546	Goat	1:250
Anti-mouse 647	Goat	1:250
Anti-rat 647	Goat	1:250
Anti-Chicken	Goat	1:250

Chapter 3

Drosophila Neurotrophins and Toll receptors are expressed in the developing optic lobe

3.1 Introduction

The aim of this chapter is to investigate and visualize the expression patterns of *Drosophila* NTs and their *Toll* receptors in the developing optic lobe across different stages (L3, 24, 48, and 72 hr) after puparium formation (APF). Understanding the expression dynamics of the neurotorphins and their *Toll* receptors during these stages is crucial for testing their roles in regulating cell survival and connectivity throughout development. The visual system serves as an excellent model for exploring how cell survival influences connectivity patterns in the optic lobe, an area where our understanding remains incomplete, particularly regarding the interactions between *Toll* receptors and spätzle (*spz*) in *Drosophila* visual system development.

To test the expression pattern of the *Drosophila* NTs and Toll receptors, the Gal4 lines in **(Figures 3.2 and 3.4) (Genotypes are provided in table 2.1)** were crossed to a membrane-bound reporter 10xUASmyrGFP, featuring 10 copies of GFP for enhanced signal and subsequently stained with anti-GFP antibody **(Table 2.9)**.

Another way of testing this was to employ Multi-color Flip-out (MCFO) technique utilized to label and track individual cells. This method employs the Flp-FRT system, wherein the Flp recombinase induces recombination between FRT sites, randomly activating a specific reporter. Three distinct reporters V5, Flag, and HA are stained for, considering their random nature. Subsequently, the channel displaying a signal is identified and scanned (Nern et al.2015).

Another aim of this chapter was to generate a construct through molecular cloning to visualize *spz4* expression in the optic lobes during development. This was due to the limited tools we have in the lab of *spz4* to be tested. The aim was to fuse the *spz4* endogenous gene with Yellow Fluorescent Protein (YFP) which offers a promising avenue for precise visualization of *spz4*, providing insights into its expression patterns that often elude detection with Gal4 lines. To achieve this, I used CRISPR-enhanced homologues recombination using Golic^{+} and cloned the *spz4* gRNA and the two homology arms into the pTL2-YPET vector **(Figure 2.5)**.

3.2 Results

3.2.1 Generation of pTL2-*spz4*-YPET

While the GAL4/UAS system serves as a potent genetic tool in *Drosophila* research, it is not without its limitations. One such limitation lies in the landing site of the GAL4 in the desired gene, where the Gal4 line may exhibit unexpected expression in certain tissues or display variability from one Gal4 line to another.
Additionally, issues with expression levels may arise, influenced by the strength and specificity of the promoter associated with each line (Casas-Tintó et al., 2017).

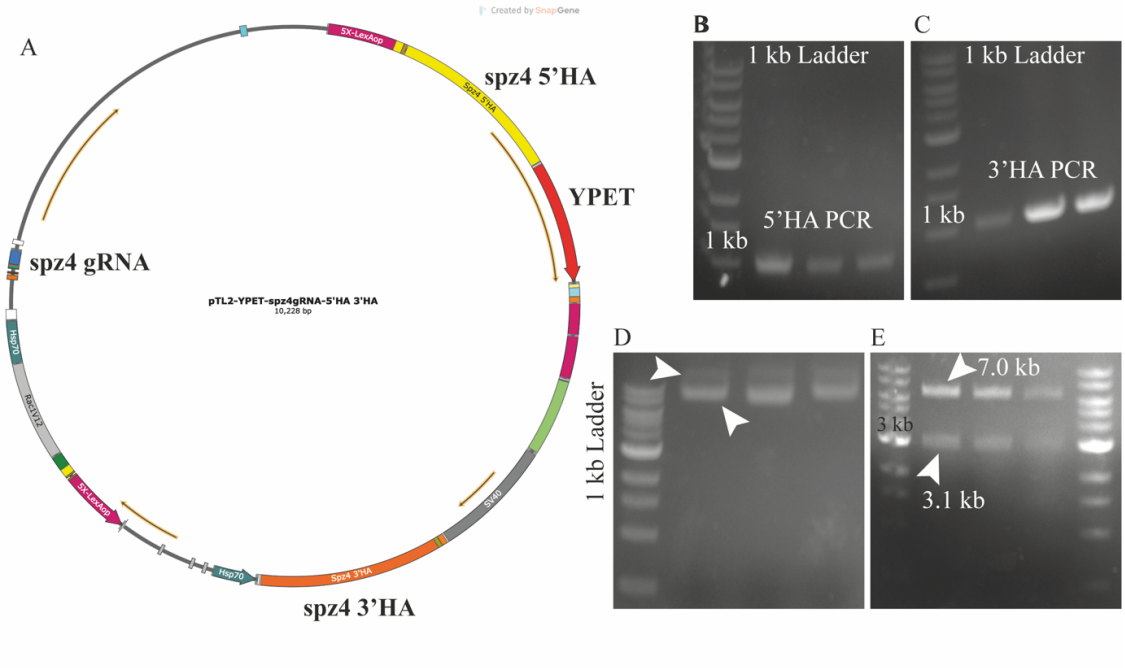
The cloning process commenced with the virtual design of the *spz4* gRNA flanked by SapI sites. Subsequently, both oligos were annealed using thermal cycle, and the empty plasmid was prepared for ligation by digesting it with the SapI enzyme, targeting the site where the gRNA scaffold is located **(Figure 3.1A)**. Following cloning of the *spz4* gRNA into the plasmid, 9 colonies were selected for mini-prep, and diagnostic digestion with SapI was conducted. The successful ligation of the gRNA into the plasmid was confirmed by observing two bands on the gel, indicative

of coiled and supercoiled plasmids. Colony 1 was chosen to undergo midi-prep of the plasmid containing the spz-4 gRNA **(Figure 3.1D)**. It is recommended to carry out a sequencing reaction to confirm the ligation of the gRNA. However, in this case, the sequencing reaction was not done to save time and money. This is because we have had multiple gRNA ligations in the past done by myself and other lab members and the method we used showed successful gRNA ligations when sequenced many times.

After the gRNA ligation, the design of the homology arms was undertaken by referencing genomic data on Flybase (Flybase.org) to obtain approximately 1kb fragments for each arm. Both arms were around 1kb in size. The 5' homology arm (5'HA) was derived from 1kb upstream of the *spz4* 2nd exon, with the stop codon removed at the end of the arm during primer design. Similarly, the 3' homology arm (3'HA) was obtained from 1kb downstream of the 3rd exon. Genomic DNA from wild-type flies was isolated for PCR to generate the homology arms using specific forward and reverse primers **(Table 2.2 3-6)**. The expected 1kb bands were observed on the gel, as depicted in **(Figure 3.1 B and C)**.

Proceeding to the next step, the plasmid containing *spz4* gRNA was digested with AgeI to prepare for the ligation of the 5'HA. Subsequently, an annealing reaction was set up to incorporate the 5'HA arm into the digested plasmid. Advancing to the subsequent phase, a midi-prep of the plasmid containing spz4 gRNA and 5'HA was digested with BamHI to prepare for the ligation of the 3'HA. The ligation reaction between the plasmid and the 3'HA arm product was initiated, followed by plating the reaction and setting up mini-prep cultures for diagnostic digestion. An enzyme was selected to provide insight into the successful ligation of both arms, and the mini preps

Figure 3.1 Molecular cloning of *spz4* into pTL2-YPET



F

```
TAGCTGTGGCCAGCAACTTGCGTGGAGTGTAAGTATATGACCCATTTGCTTATCAAATTA
************************************************************

tataatacaatattgatctctctgcagAAATGCCTGTCCCGTGAAGGATGAAGTGGTGGC
TATAATACAATATTGATCTCTCTGCAGAAATGCCTGTCCCGTGAAGGATGAAGTGGTGGC
************************************************************

TCCCTTTTGGGCAAACAATACCCGCGGCGAGGTCTTAGCCCTCCTCAATCTCTATCCCTT
TCCCTTTTGGGCAAACAATACCCGCGGCGAGGTCTTAGCCCTCCTCAATCTCTATCCCTT
************************************************************
                                  START OF spz4 5'HA
CGAGCAGTACGTCCACTGGGAGAAGTGCACCCACGAGTTCAAGCAAATGTTCTGCCGCGA
CGAGCAGTACGTCCACTGGGAGAAGTGCACCCACGAGTTCAAGCAAATGTTCTGCCGCGA
************************************************************

TGGATGCAGATGTGAACAGCAGTACCGCCTCCACAGATTGTTGGCCTACGATCCGCATAA
TGGATGCAGATGTGAACAGCAGTACCGCCTCCACAGATTGTTGGCCTACGATCCGCATAA
************************************************************

CGAGTGCCGCGGGATATTCTCCGACTGGTTCCGATTCCCGTCCAGCTGCATATGCAAGTG
CGAGTGCCGCGGGATATTCTCCGACTGGTTCCGATTCCCGTCCAGCTGCATATGCAAGTG
************************************************************

CTACAATATACCAATGGAGTTCCGCGCAACATCACGTAGTCCACGGTCCGACAGTCGCTT
CTACAATATACCAATGGAGTTCCGCGCAACATCACGTAGTCCACGGTCCGACAGTCGCTT
************************************************************

CGATGCTCCAAAATCCGATCCCATCGAGGCTGCCGAGGCGGAGGTCCAACGGGCCATTTA
TGATGCACCAAAATCCGATCCCATCGAGGCTGCCGAGGCGGAGGTCCAACGGGCCATTTA
 ***** ******************************************************

CGAACATGCCACGGAAGAGTGGTATCGCCCACGTGATGAGTTCGATTTCTTGGAGGACGC
CGAACATGCCACGGAAGAGTGGTATCGCCCACGTGATGAGTTCGATTTCTTGGAGGACGC
************************************************************

TGGCGGAGCAGGCGGC----------------------------------------------
TGGCGGAGCAGGCGGCATGTCTAAAGGTGAAGAATTATTCACTGGTGTTGTCCCCAATTT
****************

------------------------------------        1036
TGGTTGAATTAGATKGTKATKKTWAAAGKKKMMCMA        1082
```

G

```
----------------------------------------------------------------
CAWWMMSGGCTCCYGGATCGAATCCTTTAATTAAATAACCTTCGTATAATGTATGCTATA
                    START OF spz4 3'HA
------------------------------------TCCCATTAGATTCCCTCCTGTGAA
CGAAGTTATTAACTATAMCGGTCCTAAGGTAGCGAGTCCCATTAGATTCCCTCCTGTGAA
                                    ************************

CAATTGTACAGTAGTATTAGGTTTTATAGAATTAAGCTATAATTTCCAATTAGTCCTTAC
CAATTGTACAGTAGTATTAGGTTTTATAGAATTAAGCTATAATTTCCAATTAGTCCTTAC
************************************************************

AATACACATTTAAGAAACTTCAACCGATCAAGTTAATAACTTTCGATCATTCTTCGATCA
AATACACATTTAAGAAACTTCAACCGATCAAGTTAATAACTTTCGATCATTCTTCGATCA
************************************************************

TATTTCACACAGACCTTCAATCTCACTTTAAACCTATAAGTAATCAATAATTATAAATTA
TATTTCACACAGACCTTCAATCTCACTTTAAACCTATAAGTAATCAATAATTATAAATTA
************************************************************

AATCATAAAAGCGGCGTTATGTTAATGTAAATATAGTCCAACATAATGCCCGATTTACGG
AATCATAAAAGCGGCGTTATGTTAATGTAAATATAGTCCAACATAATGCCCGATTTACGG
************************************************************

AAAAACTGCAGAGCGAATGTACCTTGAAATACTTACGAACGCATTGCGGCCATTGAGCTG
AAAAACTGCAGAGCGAATGTACCTTGAAATACTTACGAACGCATTGCGGCCATTGAGCTG
************************************************************

CGTACGATGGTATTTACTAGAGTTATGTATTTATTTATTCAAATGATAGCTATAAGGATA
CGTACGATGGTATTTACTAGAGTTATGTATTTATTTATTCAAATGATAGCTATAAGGATA
************************************************************

ACCATAAACGTGGCCATAACATAAACCCCAAATCTTTTGATGGACCCCTAAACGAAAGCA
```

Figure 3.1 Molecular cloning of *spz4* into pTL2-YPET

(A). pTL2-spz4-YPET construct map with all the elements cloned into it.

(B). spz4 5'HA PCR product with 1kb size.

(C). spz4 3'HA PCR product with 1kb size.

(D). pTL2-spz4gRNA-YPET digest with SapI to confirm the ligation of spz4 gRNA into the vector. The two white arrows are indicating the coiled and supercoiled forms of the DNA confirming the successful ligation of the gRNA due to the loss of the SapI sites.

(E). pTL2-spz4gRNA-5'HA-3'HA-YPET construct diagnostic digest with NotI-HF to test the success of the ligation of spz4 gRNA and both homology arms that gives two bands with a size of 7kb and 3.1 kb.

(F). Sequencing alignment of spz4 5'HA against the reference sequence. The highlighted green area shows the start of the coding region of the spz4 gene. The two orange highlights indicate the mismatches in the construct which does not affect the coding of spz4 since these mismatches are silent mutations.

(G). Sequencing alignment of spz4 3'HA against the reference showing no mutations in the homology arm when compared to the reference.

were digested with NotI, yielding two bands on the gel with sizes of 3.1kb and 7.0kb **(Figure 3.1 E)**. Upon confirmation of the diagnostic digest results, a colony was selected for midi-prep to be sent for sequencing to ensure the absence of mutations in the arms. Following confirmation with sequence reactions **(Figure 3.1 F and G)**, the constructs were prepared for injection into fly embryos by BestGene.

3.2.2 *Drosophila* Neurotrophins are expressed in the optic lobe

The different ligands of *Drosophila* showed dynamic expression pattern in the optic lobes at different stages. In spz1 Gal4, there is an expression in the central brain and the VNC (Data not shown). At 48hr APF there is an expression in the lobula complex with the cell bodies located there and extending the axon through the medulla then reaching the lamina with its axonal terminals **(Figure 3.2A-A')**. In DNT-1 Gal4, the expression appeared at 48hr APF in the medulla, lobula complex and the central brain **(Figure 3.2B-B')**. However, no expression was detected at earlier stages or at 72 hr APF. In DNT-2 Gal4, the expression was localized to the lamina, medulla and central brain at 24 hr APF **(Figure 3.2C-C')**. Also, there was an expression detected at 72 hr APF in retinal cells that looked like glia more than photoreceptors (Data not shown in this section but was shown in the MCFO data). In spz3-IT-Gal4, the expression profile was localized in the retina specifically in R1-R6 PRs that extend their axonal terminals into the lamina as their destination (Pecot et al., 2014). The expression was detected in the larval stage up until 72 hr APF (Only 24 hr APF stage was shown) **(Figure 3.2D-D')**. Lastly, at 72hr APF, spz4^{MIMICGal4} showed an expression profile in the medulla, lobula complex and central brain **(Figure 3.2E-E')**. Moreover, there was an expression in the larval optic lobes and the VNC as well as 48 hr APF in the medulla (Data not shown). However, there was no expression detected at 24 hr APF.

To gain more insight into which cell types these neurotrophins are expressed in the optic lobes, I tested the expression using MCFO technique at 72hr APF, this is because the connections in the developing optic lobes are finalised at this stage for the adult visual system (Nériec and Desplan, 2016). This will help us understand the consequence of manipulating the neurotrophins on connectivity at this specific developmental stage. In DNT-2 Gal4 there was an expression in retinal cells that appear to be pigment cells or glia more than photoreceptors. This is because the morphology and layer termination of PRs are well-known and documented and by comparing my data to the literature, the cell morphology does not look like PRs (Nériec and Desplan, 2016) **(Figure 3.3A)**. In spz3-IT-Gal4, a single R1-R6 PR was isolated with this technique showing the cell body in the retina and extending its axon into the lamina where it terminates **(Figure 3.3B)**. The morphology and layer termination of R1-R6 is documented and I was able to compare my data to that in the literature (Pecot et al., 2014).

3.2.3 *Drosophila* Toll receptors are expressed in the optic lobe throughout development

I tested the expression of *Toll* receptors across four different stages in the optic lobe: L3W, 24hr, 48hr, and 72hr APF.

In Toll-1 Gal4, the expression profile was visible across all stages and in all four different neuropiles. In Toll-2^{PTV} Gal4, the expression was localized to the lamina and medulla in L3W. At 24h APF, the expression appears to be in some PRs, lamina, medulla and the lobula complex. At 48hr APF, the expression in the PRs has disappeared and only localized in the lamina, medulla and lobula complex. Lastly, at 72hr APF, the expression profile is like the previous stage with a slight decline in expression levels in the lobula complex.

In Toll-5 Gal4, the expression profile is like that in Toll-2^{PTV} Gal4 across all stages with only one difference, in Toll-5 Gal4, the expression levels in the lobula complex appears to increase and much higher compared to Toll-2^{PTV} Gal4.

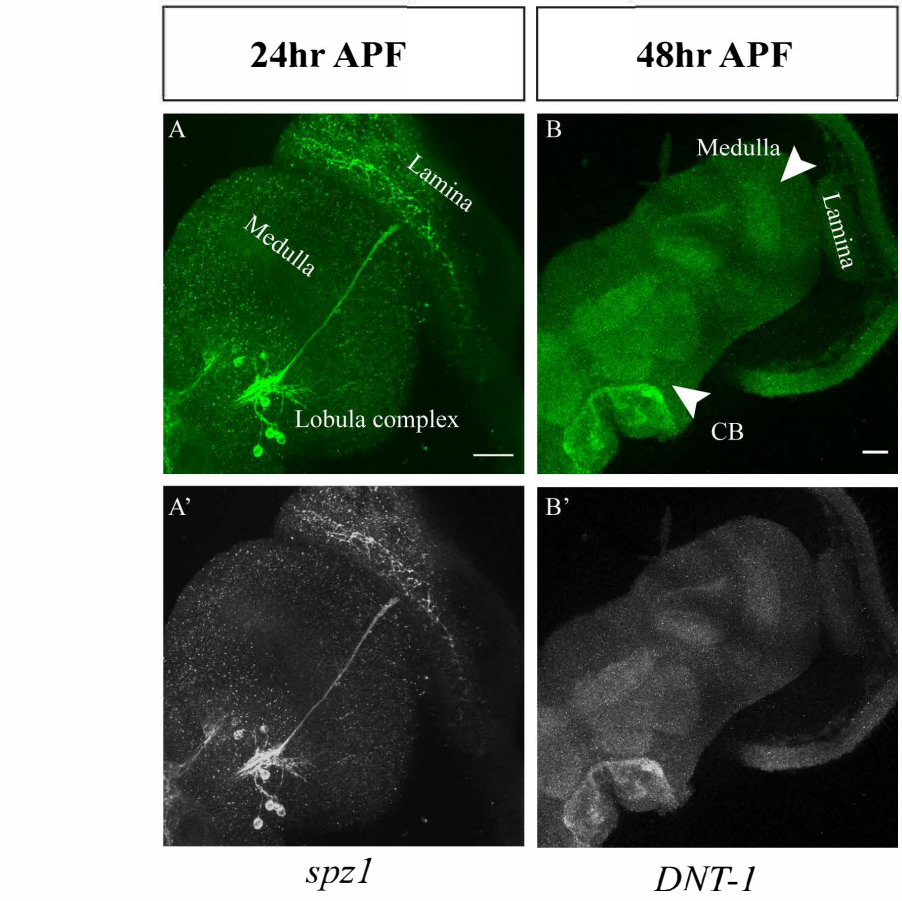

24hr APF
48hr APF
A
Lamina
Medulla
Lobula complex
B
Medulla
Lamina
CB
A'
B'
spz1
DNT-1
24hr APF
24hr APF
72hr APF
C
Medulla
Lamina
D
Retina
Lamina
E
Lobula complex
Medulla
C'
D'
E'
DNT-2
spz3
spz4

Figure 3.2 *Drosophila* Neurotrophins expression in the optic lobe

Expression profile of the *Drosophila* NTs in the developing pupal optic lobe. All genotypes were crossed to >10xUASmyrGFP and stained with anti-GFP (Rb) and were fixed in formaldehyde. The images in green are the 488 channel showing GFP staining. The black and white is images are similar channels but were made black and white to enhance the visualization of the expression pattern. Number of repeats >5.

Genotype:

(A). spz-1 GAL4>10XUASmyrGFP

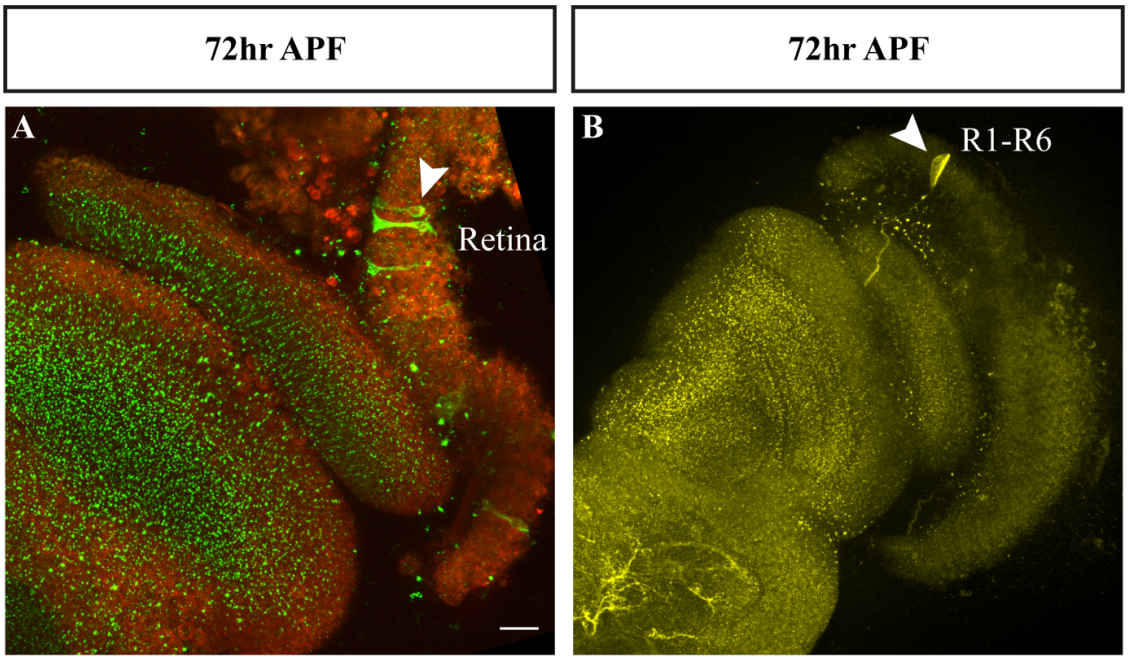
(B). DNT-1 GAL4>10XUAsmyrGFP

(C). DNT-2 GAL4>10XUAsmyrGFP

(D). spz3-IT-GAL4>10XUAsmyrGFP

(E). spz-4 ^{MIMICGAL4}>10XUAsmyrGFP

Figure 3.3 ***Drosophila*** **Neurotrophins expression with MCFO**



MCFO of the *Drosophila* ligands at 72 hr APF. All genotypes were crossed to w hsFLP:PEST ;; HA-V5-FLAG-OLLAS MCFO. The clones were generated by placing 24 hr APF pupa into a water bath 37 degrees for no more than 5 minutes. Pupa was then placed back into the 25 degrees incubator until the age of 72 hr APF. Brains were dissected and fixed in formaldehyde and stained with anti-HA, anti-V5, and anti-FLAG. The signal will be randomly expressed based on the Flipase activity and only the channels that have expression or clones are presented. A. anti-V5 and anti-FLAG are shown only. In B, only anti-V5 is shown. Number of repeats >5.

(A). DNT-2 GAL4>w hsFLP:PEST ;; HA-V5-FLAG-OLLAS

(B). spz3-IT-GAL4>w hsFLP:PEST ;; HA-V5-FLAG-OLLAS

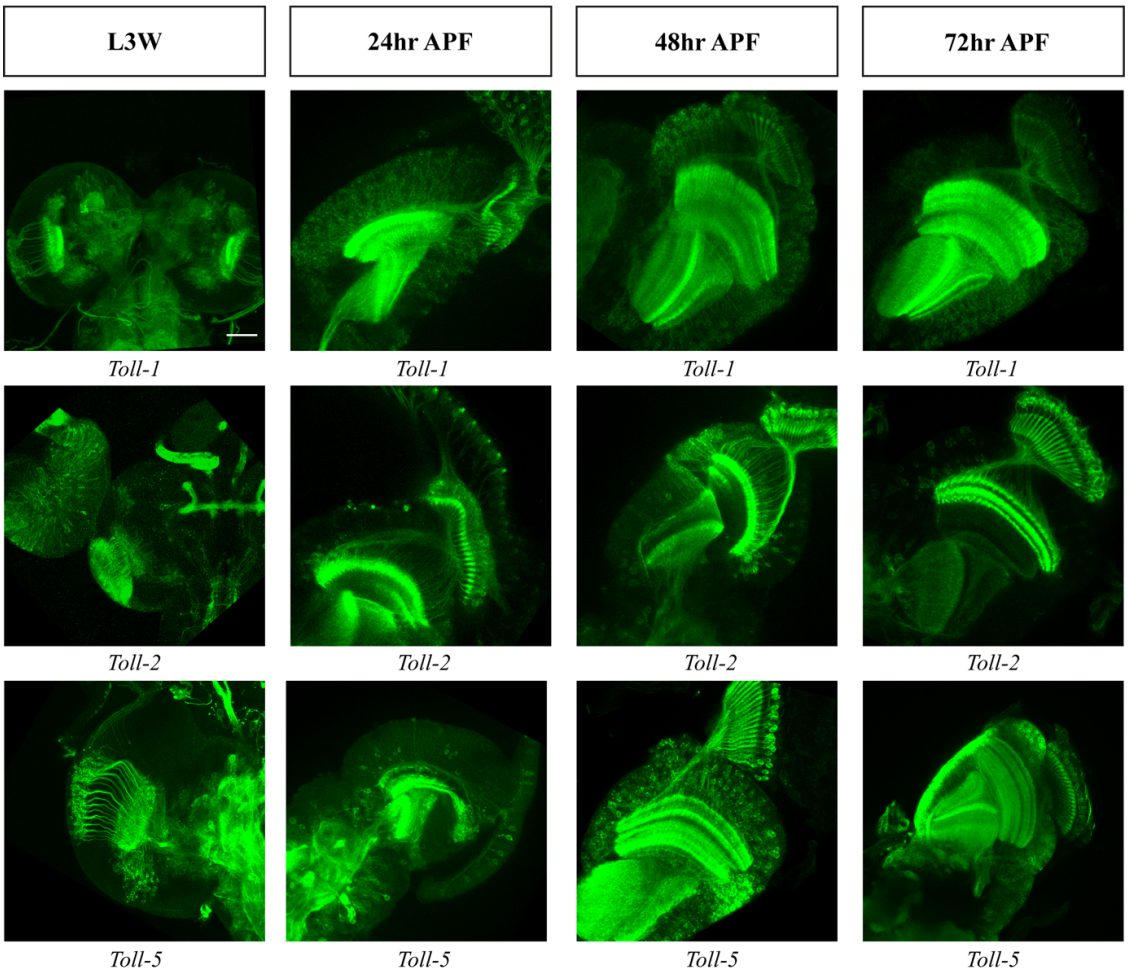
In Toll-6^{MIMICGFP} Gal4, there was no expression in the eye disc only in the central brain at L3W. At 24hr APF, the expression is localized to the lamina, medulla and the lobula complex. At 48hr APF, the expression declines in the lamina, medulla and lobula complex. In 72hr APF, the expression began to be more expressed in the lamina while the levels of the medulla and lobula complex remained the same as in the previous stage.

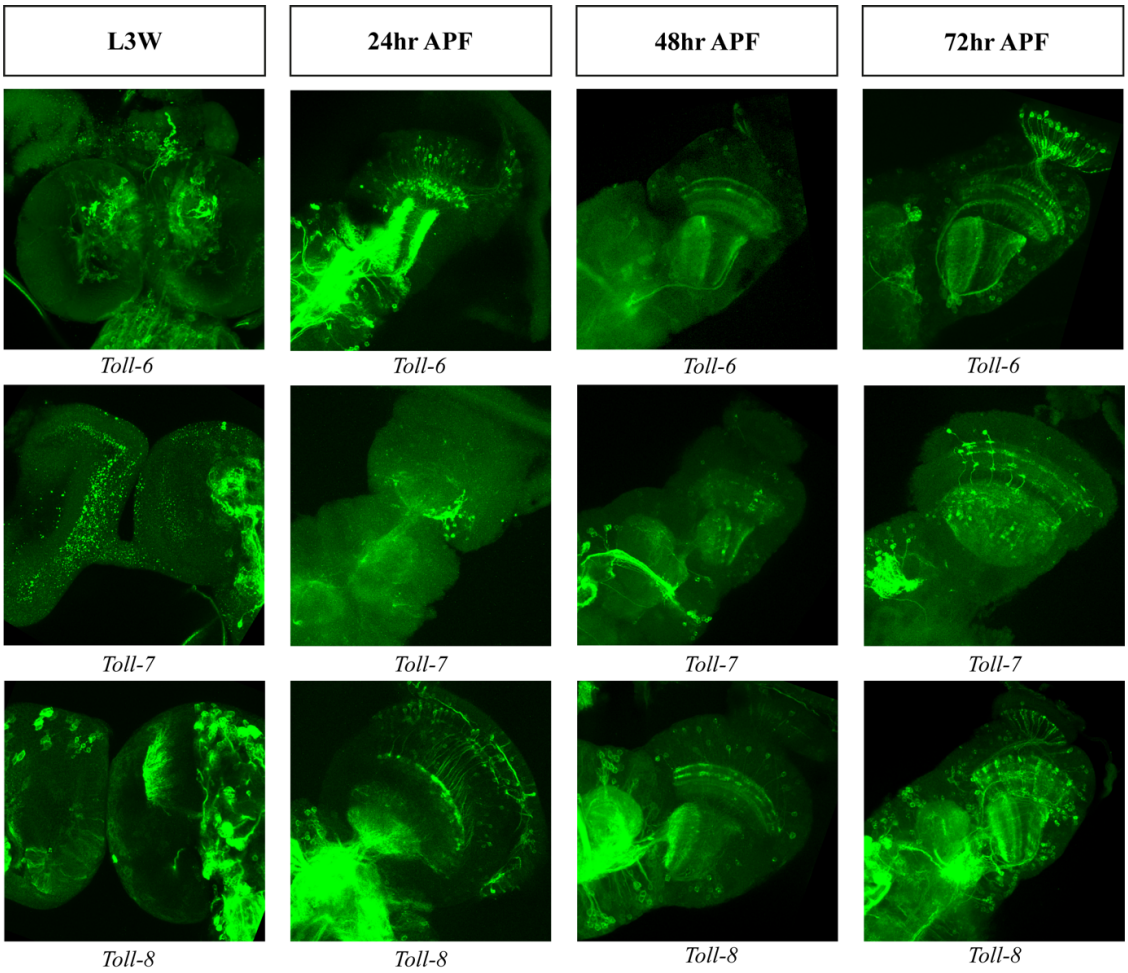
In Toll-7 Gal4, there was no expression in the eye disc at L3W but only in the central brain. At 24hr APF, there is a slight expression in the lobula complex. At 48hr APF, the expression slightly increases in the medulla and lobula complex. At 72hr APF, the expression increases in the medulla and lobula complex.

In Toll-8 Gal4, there is an expression in the medulla and some cells in the eye disc that are possibly glia at l3W. At 24hr APF, the expression increases in the lamina, medulla and lobula complex. At 48hr APF, the expression in the lamina declines but the same levels remain in the medulla and the lobula complex. At 72hr APF, the expression increases again in the lamina and in the other 3 neuropiles. The expression profile of these Tolls is shown in **(Figure 3.4)**.

like the ligands, I tested the expression pattern of Toll receptors at 72hr APF using MCFO to identify which cell type they are expressed in. The level of saturation can be controlled by adjusting the expression of the Flp recombinase, which triggers the excision of transcription-terminating cassettes (Nern et al., 2015). With short Flp induction periods, few cells are labeled (<10%), while longer induction times result in higher labeling densities (>50%) with a full spectrum of colour combinations (Nern et al., 2015). The saturation of clonal analysis is crucial for understanding the developmental origins and relationships between different cell types (Nern et al., 2015). For example, MCFO has been used to study the origins of C2 and C3 columnar medulla neurons in the fly optic lobes, revealing that these related cell types have

Figure 3.4 ***Drosophila*** **Toll receptors expression in the optic lobe**





Expression profile of the *Drosophila* Toll receptors in the developing optic lobe at l3W, 24, 48, and 72hr APF. All genotypes were crossed to >10xUASmyrGFP ,stained with anti-GFP, and brains were fixed in formaldehyde. Number of repeats 3.

Genotype:

. Toll-1^{CR} GAL4 >10XUASmyrGFP

. Toll-2^{PTV} GAL4 >10XUAsmyrGFP

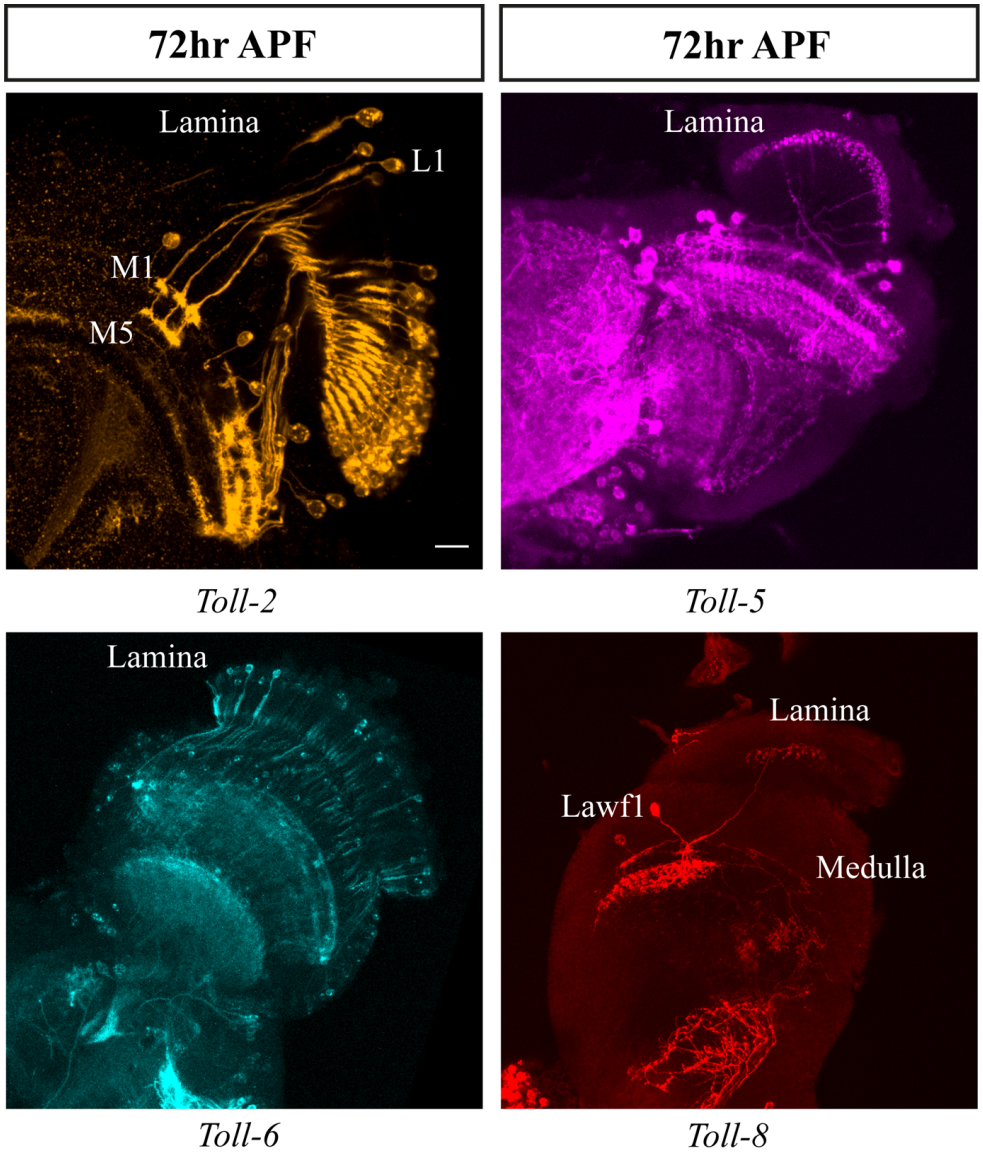
. Toll-5^{CR} GAL4 >10XUASmyrGFP

. Toll-$6^{MIO2127}$ GAL4 >10XUASmyrGFP

. Toll-7^{M13963} GAL4 >10XUAsmyrGFP

. Toll-8^{MD806} GAL4 >10XUASmyrGFP

Figure 3.5 ***Drosophila Toll*** **receptors expression with MCFO**



MCFO of the *Drosophila* Tolls at 72hr APF. All genotypes were crossed to w hsFLP:PEST ;; HA-V5-FLAG-OLLAS MCFO. Only anti-V5 was shown for all images. the different colors were selected for personal preferences to distinguish between the different Tolls Number of repeats 2.

Genotype:

.Toll-2^{PTV} GAL4 > hsFLP:PEST ;; HA-V5-FLAG-OLLAS

.Toll-5^{CR} GAL4 > hsFLP:PEST ;; HA-V5-FLAG-OLLAS

.Toll-$6^{MIO2127}$ GAL4 > hsFLP:PEST ;; HA-V5-FLAG-OLLAS

.Toll-8^{MD806} GAL4 > hsFLP:PEST ;; HA-V5-FLAG-OLLAS

distinct developmental origins (Nern et al., 2015).

In Toll-2^{PTV} Gal4, the expression was observed in one of the lamina neurons L1 which has its cell body in the lamina and then extends to the medulla to terminate into two different medulla layers: M1 and M2. In Toll-5 Gal4, the expression levels were very high in the central brain as well as the optic lobe. It was challenging to isolate single neurons. However, Toll-5 Gal4 is shown to be expressed in the medulla and lamina feedback neurons as well as the lobual complex. On the other hand, in Toll-6^{MIMICGFP} Gal4 there was an isolation of single neurons in the lamina that terminates into one layer in the medulla. In Toll-8 Gal4, I was able to isolate a single neuron referred to as Lawf1 that has its cell body in the medulla, and then sends its axons in both directions in the medulla as well as the lamina **(Figure 3.5).**

3.3 Discussion

In this chapter, I have generated a construct with molecular cloning using CRISPR-enhanced homologues recombination using Golic^{+} and cloned the *spz4* gRNA into pTL2-YPET.

Also, I have presented the expression patterns of *Drosophila* NTs with their *Toll* receptors across different developmental stages. This chapter aimed to explore how dynamically these ligands and their receptors are expressed in the optic lobes at different stages and times during development. The findings of this study make a significant contribution to our understanding of the temporal expression profiles of *Drosophila* NTs and their Toll receptors during development, with special emphasis on the optic lobes. It appears that these ligand-receptor systems are used in a critical window of time during the construction of the brain. The stage-wise change in expression suggests that such NTs may have different functions,

with some being more critical during the early stages of neuronal patterning and others during the late stages of synapse construction and optic lobe maturation.

Neurotrophins are particularly interesting as they are co-expressed with Toll receptors that are known to be involved in immune responses (Phulwani et al., 2009). In this context, it is possible that Toll receptors are more than just constituents of the immune mechanisms but also participate in the activities in the nervous system such as axon guidance or the survival of neurons where supportive evidence has been offered in the past (Foldi et al., 2017; Li et al., 2020). Given the mentioned important role of optic lobes in visual information processing, one can calmly assume that the neurotrophins as well as the Toll receptor are interfaced and highly coordinated to enhance normal development in structural and functional parameters. Such pathways, when disturbed, can affect structural formation and the circuitry of the neural systems.

In the future, functional studies such as loss- or gain-of-function studies should clarify how NT-Toll receptors interact during optic lobe development. Finally, related comparisons with similar patterns of expression in other brain areas or other species may elucidate the evolutionary origin of these signaling pathways.

In any academic work, it is important to evaluate one's work. In this section, I have generated the *spz4*-YPET construct that was successful in the cloning steps. However, after injecting the construct into the flies, it was challenging to obtain the desired flies through genetics but in the end, I was able to obtain 3 stocks that did not show the YFP after extracting genomic DNA and setting up a PCR reaction to identify if the flies carry that protein. This opened several options to try and diagnose the problem. One possibility is that the construct may have integrated into some inappropriate genomic region or that the integration region does

allow expression and transgenic silencing occurred, so transcription did not take place. Or it could be that the tissues or stage of development in which expression was to be directed did not

contain or had a weak expression of the appropriate promoter. Also, although PCR confirmed the presence of the construct, it could be that YFP expression was too low to allow detection of fluorescence or instability of the fusion protein complex. These specifically include further delimitation mapping of the integration site, using even more sensitive detection methods, trying other promoters or tags, etc. This approach of fusing genes to the YFP has been used in the past by previous members of the lab. They also faced some challenges obtaining these stocks where in some situations all flies would die during the genetics protocols and in other where they obtained the desired stock and there was no signal of that gene when stained with antibodies.

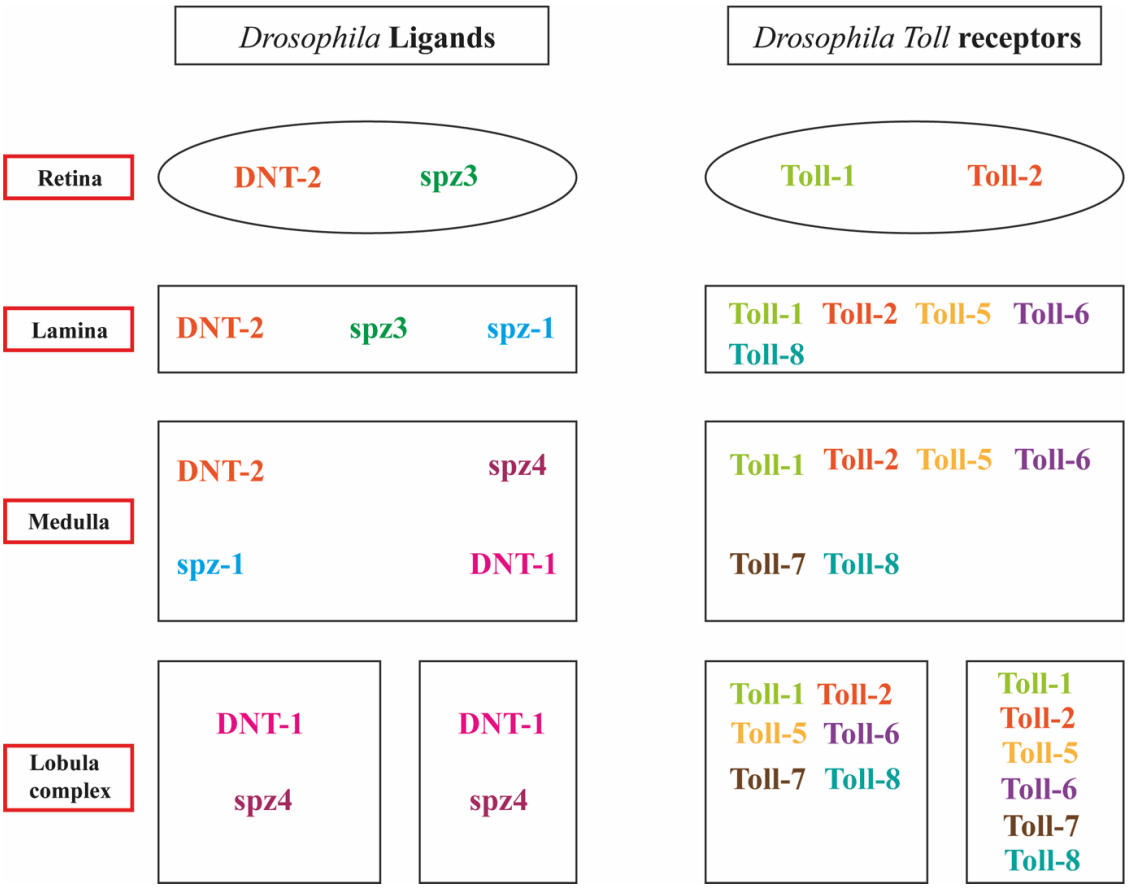
To resolve the issue regarding the demonstration of spz4 expression, a spz4 MIMIC-GFP line was obtained and dissected to study the localization of GFP-tagged spz4 molecules in the appropriate tissues. Expression was reported in the medulla, lobula complex and trachea after staining with GFP suggesting the presence of spz4 activity for the first time. However, to assure that the GFP signal was specific for endogenous spz4 expression, additional work was done. Anti-GFP immunohistochemistry would provide a much stronger signal to verify that fluorescence was indeed caused by spz4-GFP. Furthermore, in situ hybridization or qPCR would confirm that the observed signal corresponds to the appropriate mRNA transcripts, ensuring that protein expression is under the expectation of transcription **(Figure 3.2E-E')**.

Another challenge in this chapter was the expression profile of the ligands. The majority of the Gal4 lines of the ligands tested in **Figure 3.2** were not tested in the past in the optic lobes except for DNT-1 Gal4 where it was shown to be expressed in the lamina at L3W and central brain with in situ hybridization to *DNT-1* transcripts (Zhu et al., 2008). *Spz-1* and *DNT-2* transcripts were also shown in the CNS midline but were not shown where they are expressed in the optic lobe (Zhu et al., 2008). When testing these ligands in the optic lobes, I had to

consider the limitation of the UAS/Gal4 system. As these lines are promoter-based and do not represent an accurate expression of the genes. Further investigation is required to confirm the precise expression pattern of these ligands in the optic lobes.

In conclusion, The *Drosophila* NTs with their receptors are very dynamic and can be expressed in different neuropiles of the optic lobes at different stages during development **(Figure 3.6)**. After the exploration phase of this chapter, the research will continue answering the cell survival and connectivity with *DNT-2/Toll-6* and *spz3/Toll-8*. The reason for that, *DNT-2* and *Toll-6* binding has been documented in previous research of the lab (Mcllroy et al. 2013). Moreover, *spz3* and *Toll-8* genetic interactions have been reported (Ballard et al., 2014). Another reason for conducting the research with these pairs is that their expression profile timing is critical for investigating the link between cell survival and connectivity. Both ligands (*DNT-2/spz3*) as well as their receptors (*Toll-6/Toll-8*) are expressed at 24hr APF which is the ideal stage to investigate the regulation of cell survival due to the fact there is a massive wave of cell death occurring at 24hr APF (Togane et al., 2012). Also, these genes are also expressed at 72hr APF, a stage where connections in the optic lobe are finalised for the adult visual system. This is an ideal stage to investigate the regulation of connectivity when manipulating these genes (Nériec and Desplan, 2016).

Figure 3.6 Schematic diagram of *Drosophila Tolls* with their ligands across the optic lobe



Schematic diagram of the expression profile of both *Drosophila* ligands and Toll receptors across the visual system in four different neuropiles.

Chapter 4

Drosophila spz/DNTs Regulate cell survival and cell number in the optic lobes

4.1 Introduction

This chapter aims to test whether *Drosophila* NTs with Toll receptors regulate cell survival and cell number in the developing pupal optic lobes. It has been documented that there is a massive wave of cell death occurring at 24hr APF followed by a decline in cell death at 48hr APF (Kimura & Truman 1990).

It has been shown that *Toll-8* interacts genetically with *spz3* (Ballard et al., 2014). Also, it has been documented that *Toll-6* is the receptor for *DNT-2* (Mcllory et al., 2013). To gain more insight into the regulation of cell survival at 24hr APF, loss of function, gain of function and epistasis experiments were performed involving *spz3/Toll-8* and *DNT-2/Toll-6*. The reason for selecting *Toll-8* with *spz3* and *Toll-6* with *DNT-2* mark significant functional connections that have already been identified during genetic and biochemical investigations. For example, Ballard et al. found that there is a genetic interaction between *Toll-8* and *spz3* and this means that these two molecules are likely to function in the same biological pathway (Ballard et al., 2014). Such interactions could take place in neural development or immune signaling mechanisms because mutations in genes of interactant affect similar processes suggesting that they act together. In the same manner, Mcllory et al (2013) observed that the *DNT-2* molecule, which is crucial for the survival and growth of neurons in *Drosophila*, binds to *Toll-6* as its specific receptor (McIlroy et al., 2013). This observation raises the point about the multiplicity of the functions of Toll receptors. These findings also demonstrate that *Toll-6* is important for neurotrophic signaling because it mediates the action of *DNT-2*.

To test and visualize cell death, a developed antibody to cleaved DCP-1 (Cell Signaling Technology) provides a way to visualize caspase activation in dying cells (Sarkissian et al., 2014). This will help in understanding how cell death is being regulated when manipulating the ligands or Tolls at 24hr APF. The DCP-1 volume intensity will then be measure by the 3D software imaris of the desired ROI.

Toll-8 Gal4 will be used as the main Gal4 driver to investigate cell survival when manipulating *spz3* and Toll-6^{MIMICGFP} as the main driver when altering *DNT-2*.

As cell death declines at around 48hr APF (Kimura & Truman 1990), I wanted to investigate the regulation of cell number at this stage. This will be achieved by employing UAS*Histone*YFP as nuclear marker to count the number of cells by using imaris to generate the ROI and the Deadeasy to count cell number.

Another aim of this chapter is to design and generate constructs via gateway cloning for *spz3* FL (Full length) and *spz4* FL (Full length). These tools would be used to test the overexpression of these genes on cell death and cell number as well as epistasis experiments.

4.2 Results

4.2.1 Generating spz3 FL with GateWay cloning

The cloning of *spz3* started by designing the construct using SnapGene software to get an idea on the final map of the construct **(Figure 4.1A)**. using the standard protocol of Gateway® Technology with Clonase® II (Thermo Fischer Scientific) **(Figure 2.6)**, Firstly a PCR reaction using primers flanked with attB sites **(Table 2.2 9 and 10)** was used to amplify the full-length form of *spz3* gene (1.8kb) **(Figure 4.1B)** from the cDNA clone

RE22741 containing the full length *spz3* coding region **(Table 2.3)**. Secondly, a BP reaction was set up by mixing the amplified PCR product with a Gateway donor vector pDONR221 to generate the entry clone. After the transformation into the competent cells, a diagnostic digest was set up with EcoRV that expected to produce two bands with the sizes of 3.6 kb and 791bp **(Figure 4.1C)**. In the next step, an LR reaction was done by mixing the entry clone with a destination vector pUAS.GW.attB (Table 2.3). to produce the final expression clone carrying the UAS*spz3*FL. The expression clone was then subject to a diagnostic digest with NheI enzyme that expected to produce two bands with the sizes of 8.6 kb and 2.0 kb **(Figure 4.1D)**. Finally, the expression clone was sent to be sequenced using primers listed in (Table 2.2 11 and 12). The sequencing alignment shows the reaction set up with the forward primer showing the start of the coding region of *spz3* FL in green followed by an orange indication highlighting a mismatch in the sequence that is silent mutation which does not change the amino acids of *spz3* FL **(Figure 2.1E)**. In the reverse primer reaction, the sequence of *spz3* FL matches the reference sequence **(Figure 2.1F)**. The construct was then sent to be injected to be used for overexpression experiments as well as epistasis experiments when combined with RNAi lines.

4.2.2 Generating *spz4* FL with GateWay cloning

The cloning of *spz4* FL followed similar steps using the standard protocol of Gateway® Technology with Clonase® II (Thermo Fischer Scientific) **(Figure 2.6)**. The virtual construct was designed using SnapGene **(Figure 4.2A)**. A PCR reaction was set up to amplify the 1.7 kb coding region of *spz4* FL **(Figure 4.2B)**. The BP reaction was set up by mixing the PCR product with pDONOR to generate the entry clone. The entry clone was then sent to be sequenced with the M13 forward and reverse primers (Table 2.2 11 and 12). The sequencing reaction shows the start of *spz4* FL coding region highlighted in green with

Figure 4.1 Gateway cloning of pUAS-GW-spz3FL

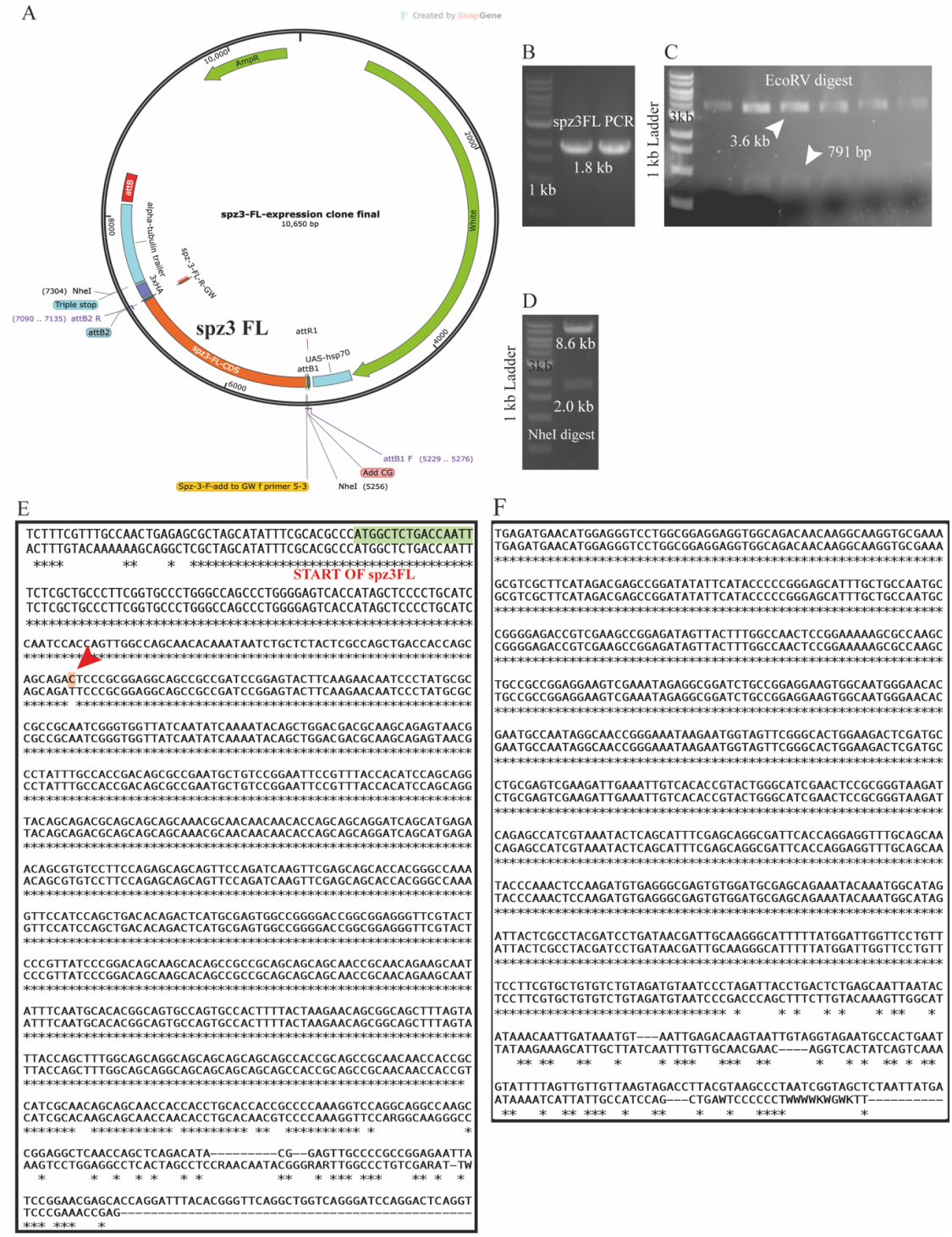


Figure 4.1 Gateway cloning of pUAS-GW-spz3FL

(A). pUAS-GW-spz3FL construct map

(B). spz3 FL PCR product of a 1.8kb size

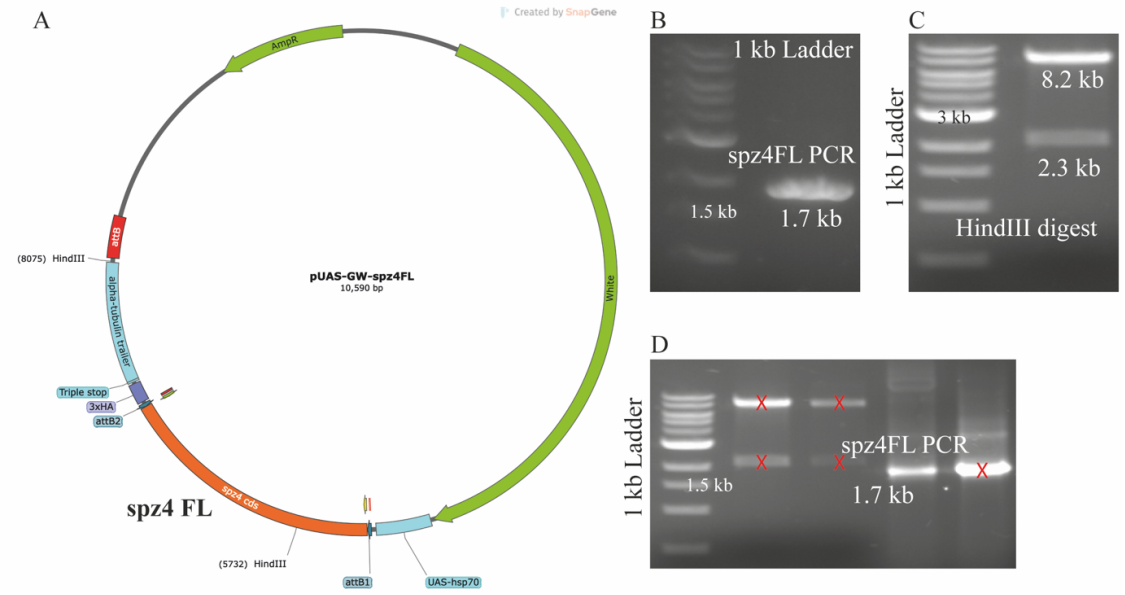
(C). spz3 FL in pDONOR BP reaction digested with EcoRV with two bands 3.6kb and 791bp.

(D). pUAS-GW-spz3FL expression clone digested with NheI with two bands 8.6kb and 2.0kb.

(E). Sequencing alignment of spz3 FL in pDONOR with forward primer to the reference sequence of spz3 from flybase.

(F). Sequencing alignment of spz3 FL in pDONOR with reverse primer to the reference sequence of spz3 from flybase.

Figure 4.2 Gateway cloning of pUAS-GW-spz4FL



E

```
------------------------------------------------------------
CSSKKSCCMAWWAAWKGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTG

                                   START OF spz4FL
------------------------------------------------ATGGTGCGCA
ATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCTCAATGGTGCGCA
                                                 **********

CCCAAATGGATAGGCGATCGCCGATCACTAGACTAATAACGATCGAGGTTATCGGGTATT
CCCAAATGGATAGGCGATCGCCGATCACTAGACTAATAACGATCGAGGTTATCGGGTATT
************************************************************

TGCTGATCCTAATGACGATTGCTCTACCACTTTCTTCCGCCGGATTTGGCTTCGACTCGG
TGCTGATCCTAATGACGATTGCTCTACCACTTTCTTCCGCCGGATTTGGCTTCGACTCGG
************************************************************

CCAACTCGTGCAGTCCCAATGGGAAGATATCGCGACGTGGTAGGGCCCAGATCCTGGCTG
CCAACTCGTGCAGTCCCAATGGGAAGATATCGCGACGTGGTAGGGCCCAGATCCTGGCTG
************************************************************

CGATTCCCTGCGACCTGGGTCAGCAGGCGTTCTGCCACCTGCCAGGATCCGCTTATCCAT
CGATTCCCTGCGACCTGGGTCAGCAGGCGTTCTGCCACCTGCCAGGATCCGCTTATCCAT
************************************************************

GGCACGCTGTACGACGATTCGTGCACGAGAACCAGGGTCTGATGAAGCGCATGTACGGCG
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************************************************************

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************************************************************

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F

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************************************************************

AGATGTGAACAGCAGTACCGCCTCCACAGATTGTTGGCCTACGATCCGCATAACGAGTGC
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************************************************************

CGCGGGATATTCTCCGACTGGTTCCGATTCCCGTCCAGCTGCATATGCAAGTGCTACAAT
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************************************************************

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************************************************************

CCAAAATCCGATCCCATCGAGGCTGCCGAGGCGGAGGTCCAACGGGCCATTTACGAACAT
CCAAAATCCGATCCCATCGAGGCTGCCGAGGCGGAGGTCCAACGGGCCATTTACGAACAT
************************************************************

GCCACGGAAGAGTGGTATCGCCCACGTGATGAGTTCGATTTCTTGGAGGAC---------
GCCACGGAAGAGTGGTATCGCCCACGTGATGAGTTCGATTTCTTGGAGGACGACCCAGCT
**************************************************

------------------------------------------------------------
TTCTTGTACAAAGTTGGCATTATAAGAAAGCATTGCTTATCAATTTGKTGCAACGAACAG


------------------------------------------------------------
GTCACTATCAGTCAAAATAAAATCATTATTGCCATCCAGCTGAWCCCYWAWWRGKG
```

Figure 4.2 Gateway cloning of pUAS-GW-spz4FL

(A). pUAS-GW-spz4FL construct map

(B). spz4 FL PCR product of a 1.7kb size

(C). pUAS-GW-spz4FL expression clone digested with HindIII with two bands 8.2kb and 2.3kb.

(D). A PCR reaction to amplify spz4FL CDS from pUAS-GW-spz4FL to confirm the presence of the gene in the clone.

(E). Sequencing alignment of spz4 FL in pDONOR with forward primer to the reference sequence of spz4 from flybase.

(F). Sequencing alignment of spz4 FL in pDONOR with reverse primer to the reference sequence of spz4 from flybase.

no mismatches or deletions compared to the reference sequence **(Figure 4.2E)**. The sequencing reaction with the reverse primer shows no mismatches or deletions compared to the reference sequence **(Figure 4.2F)**. After sequencing the entry clone, the LR reaction was set up by combining the entry clone with the destination vector pUAS.GW.attB (Table 2.3). The LR reaction was then digested with HindIII to check for the success of obtaining the expression clone. The expected bands were the sizes of 8.2 kb and 2.3 kb **(Figure 4.2C)**. To confirm the presence of *spz4* FL CDS in the clone, a PCR reaction was set up using the final expression clone to amplify *spz4* FL CDS with a size of 1.7 kb **(Figure 4.2D)**. The construct was then sent to be injected to generate flies. Due to the limitation of time, I supervised a masters student Myles Maddick who did an incredible work in a very short amount of time. He used the flies to investigate whether *spz4* is involved in regulating cell survival in the lamina and medulla of the pupal optic lobes at 24hr APF. Despite the great work done by Myles, there was no conclusion reached from the data he gathered whether *spz4* regulates cell survival or not.

4.2.3 *spz3* loss of function increased cell death in the lamina and medulla at 24hr APF

To test cell death in the loss of function of *spz3*, mutant lines were generated in the Hidalgo lab by Samaher Alahmad using the p-element hop-out approach. A trans-heterozygous combination of two alleles were used $spz3^{46}/spz3^{27}$ to eliminate the genetic background. This is because when a p-element is mobilized, they can jump into other locations on the genome that will induce mutations. The two stable stocks generated from 2 alleles will carry the same genetic background and the alleles were hopped out from spz3 [EY06670] locus.

In the lamina, the *spz3* null mutant showed a significant increase in DCP-1 signal intensity compared to the wild type. The ROI was set around the lamina region in the

optic lobe indicated with a white arrow **(Figure 4.3A-B)**.

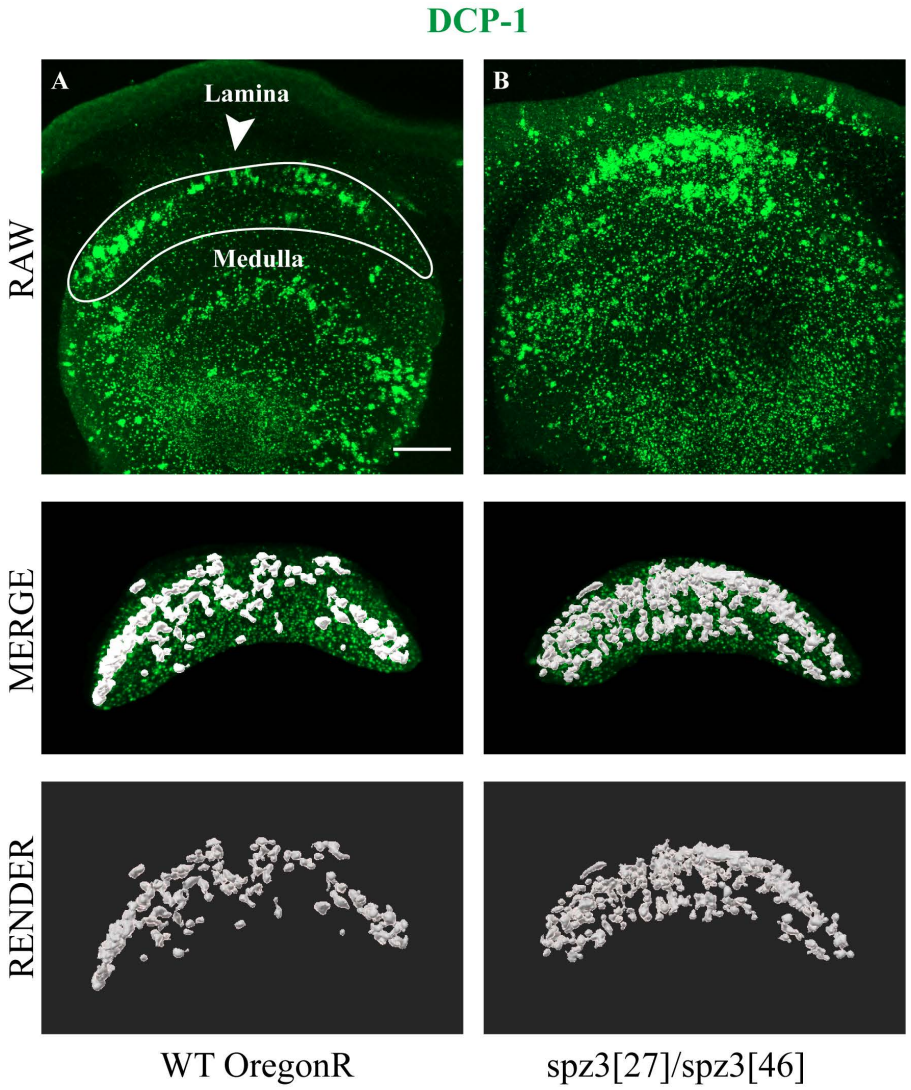
In the medulla, the *spz3* null mutant showed a significant increase in DCP-1 signal intensity compared to wild type. The ROI was set around the region of the medulla indicated with a white arrow **(Figure 4.4A-B)**.

4.2.4 *spz3* gain of function reduced cell death in the lamina and medulla at 24hr APF

To investigate cell death in the gain of function of *spz3*, both forms of *spz3* (full length and mature form CK) UAS-spz3-CK was made by Samaher which contains the signal peptide of DNT-2 plus the cystine knot of spz3. This signal peptide should enable the secretio. However, we have found that in S2 cells DNT-2 made in the same way is not secreted so we can assume that spz3-CK will not be secreted. *Toll-8*Gal4 (PGawB MD806/TM6B) was used as the main driver to observe the effects on cells that express *Toll-8*. It is known that *spz3* FL is secreted from the muscle (Coutinho-Budd et al., 2017). However, it is not known whether *spz3* CK is secreted or remains cell autonomous. For that reason, both forms were used to gain an insight into how *spz3* behaves. It has been shown that *DNT-2* FL gets secreted while *DNT-2* CK is not secreted which makes it likely that *spz3* CK will do the same (Foldi et al., 2017). In the lamina, there was no significant change in UAS*spz3*CK Dcp-1 signal intensity compared to wild type. However, when overexpressing the secreted UAS*spz3*FL, there is a significant reduction in DCP-1 volume intensity compared to wild type **(Figure 4.5A-C)**.

In the medulla, when overexpressing both UAS*spz3*FL and UAS*spz3*CK, the data shows a significant reduction in DCP-1 volume intensity when compared to wild type **(Figure 4.6A- C)**. More evidence is required to show that these two forms of spz3 FL and CK are functional.

Figure 4.3 spz3 mutant increases cell death in the lamina



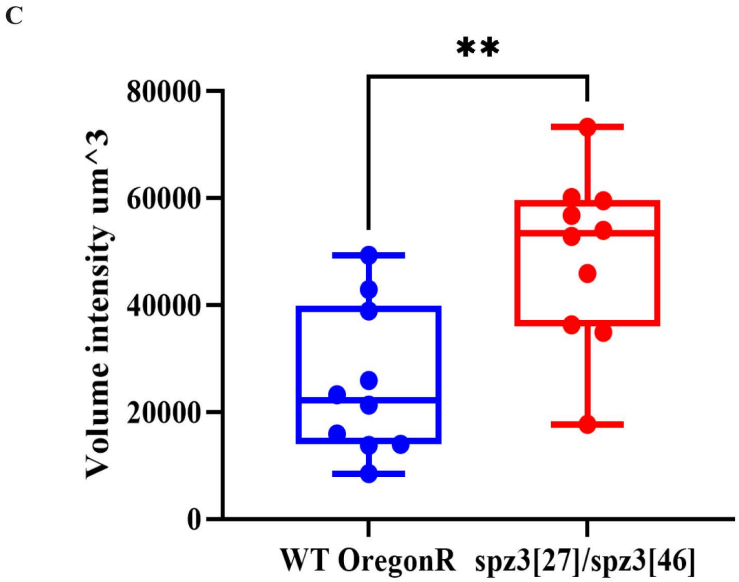


Figure 4.3 spz3 mutant increases cell death in the lamina

(A). anti-DCP-1 staining in wild type control

(B). anti-DCP-1 staining in spz3 null mutant

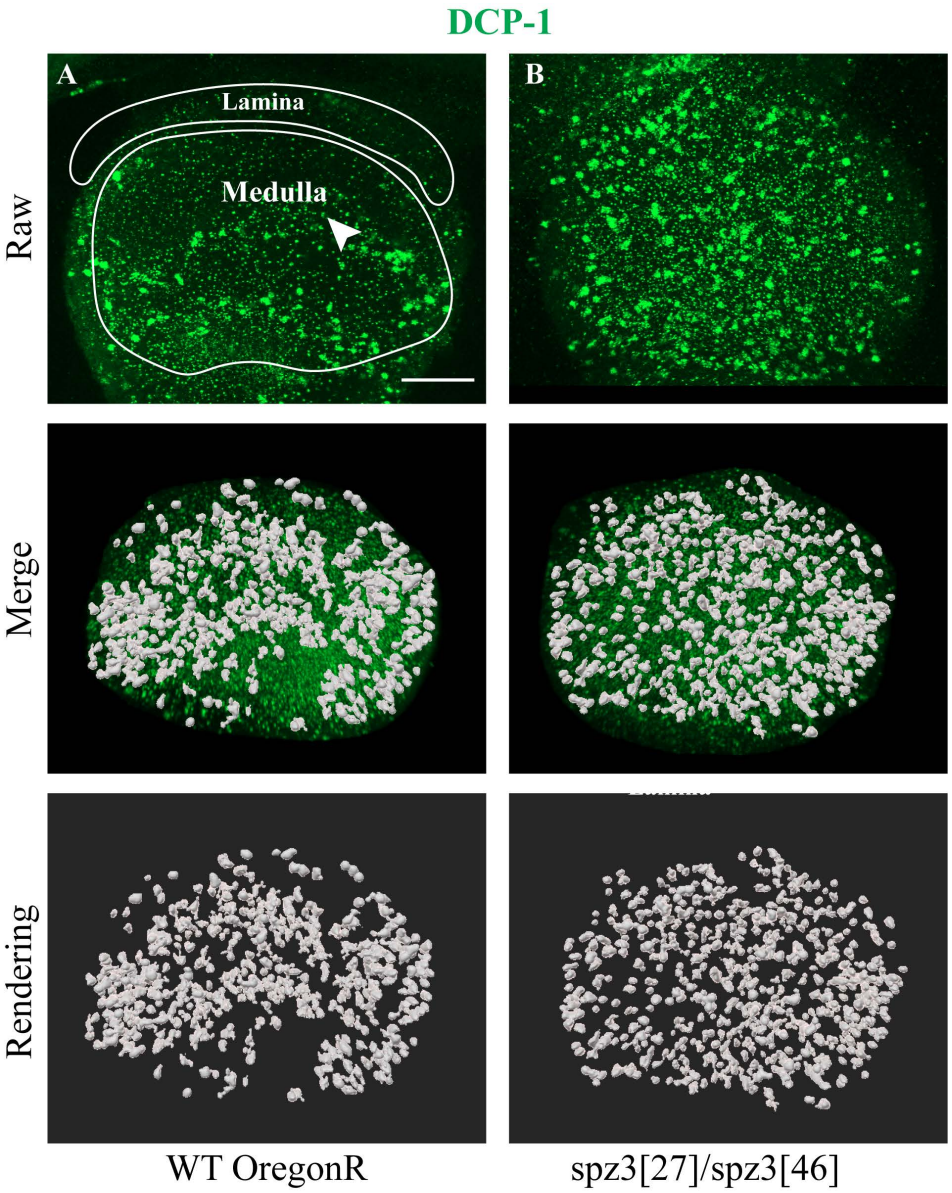
(C). Statistical analysis of DCP-1 volume intensity in the lamina. There is a significant increase in DCP-1 volume in the spz3 null mutant compared to the wild type control (Two-tailed Unpaired t test, $P \leq 0.01$ n= 10 (Control), n= 10 (spz3 mutant). Images A and B are single plains of a max projection done in imageJ. The merge and render images are the full stacks analyzed in Imaris software. Number of repeats = 2.

Genotypes:

Control: WT OregonR

LOF: spz3[27]/spz3[46]

Figure 4.4 spz3 mutant increases cell death in the medulla



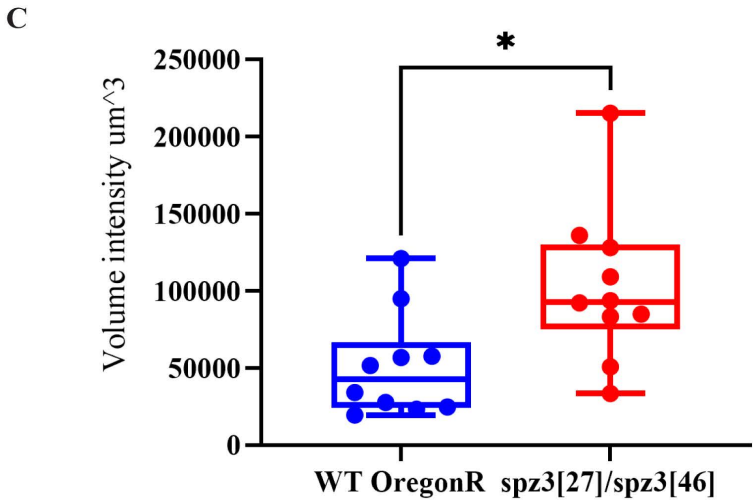


Figure 4.4 spz3 mutant increases cell death in the medulla

(A). anti-DCP-1 staining in wild type control

(B). anti-DCP-1 staining in spz3 null mutant

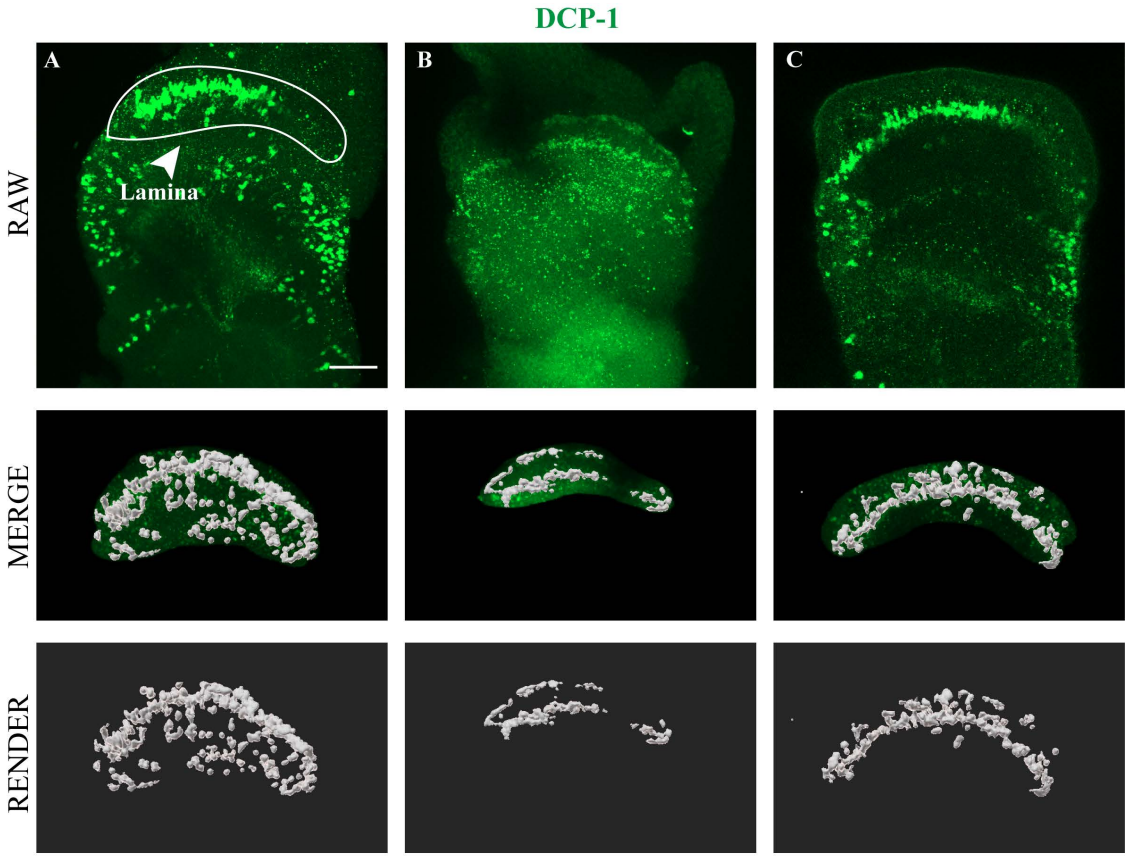
(C). Statistical analysis of DCP-1 volume intensity in the medulla. There is a significant increase in DCP-1 volume in the spz3 null mutant compared to the wild type control (Two-tailed Unpaired t test, $P \leq 0.05$ n= 10 (Control), n= 10 (spz3 mutant). Images in A and B are plain stacks done with max projection in imageJ. The merge and render images are full stacks done in Imaris software. In image A, the region of the medulla is selected with a white arrow to distinguish between the lamina and medulla where the analysis took place. Number of repeats = 2.

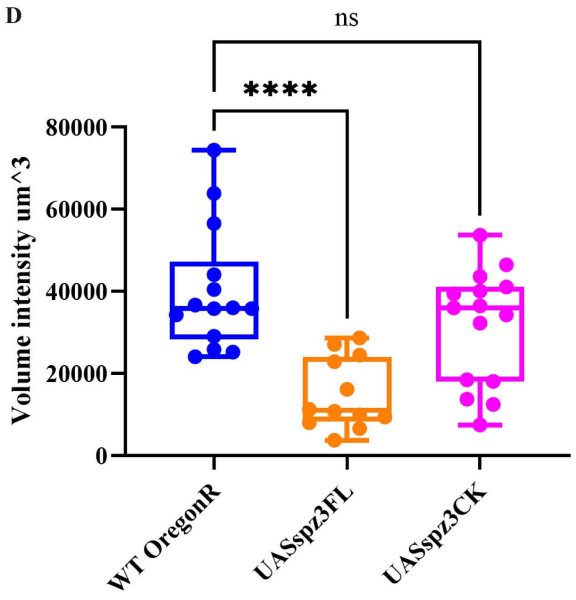
Genotypes:

Control: WT OregonR

LOF: spz3[27]/spz3[46]

Figure 4.5 spz3 overexpression reduces cell death in the lamina





Toll-8GAL4>

Figure 4.5 spz3 overexpression reduces cell death in the lamina

(A). anti-DCP-1 in wild type control

(B). anti-DCP-1 in UASspz3FL

(C). anti-DCP-1 in UASspz3CK

(D). Statistical analysis of DCP-1 volume intensity in the lamina. There is a significant decrease in DCP-1 volume in UASspz3FL compared to wild type control. However, there is no significant reduction in DCP-1 volume in UASspz3CK compared to wild type (Kurskal-Wallis test, Dunn's multiple comparison), $P \leq 0.001$, n= 14 (Control), n= 12 (UASspz3FL), n= 15 (UASspz3CK). Images in A-C are single plains. The images in merge and render are full stacks done in Imaris software. Number of repeats = 2.

Genotypes:

A. Control: Toll-8GAL4>WT OregonR

B. GOF: Toll-8GAL4>UASspz3FL

C. GOF: Toll-8GAL4>UASspz3CK

Figure 4.6 spz3 overexpression reduces cell death in the medulla

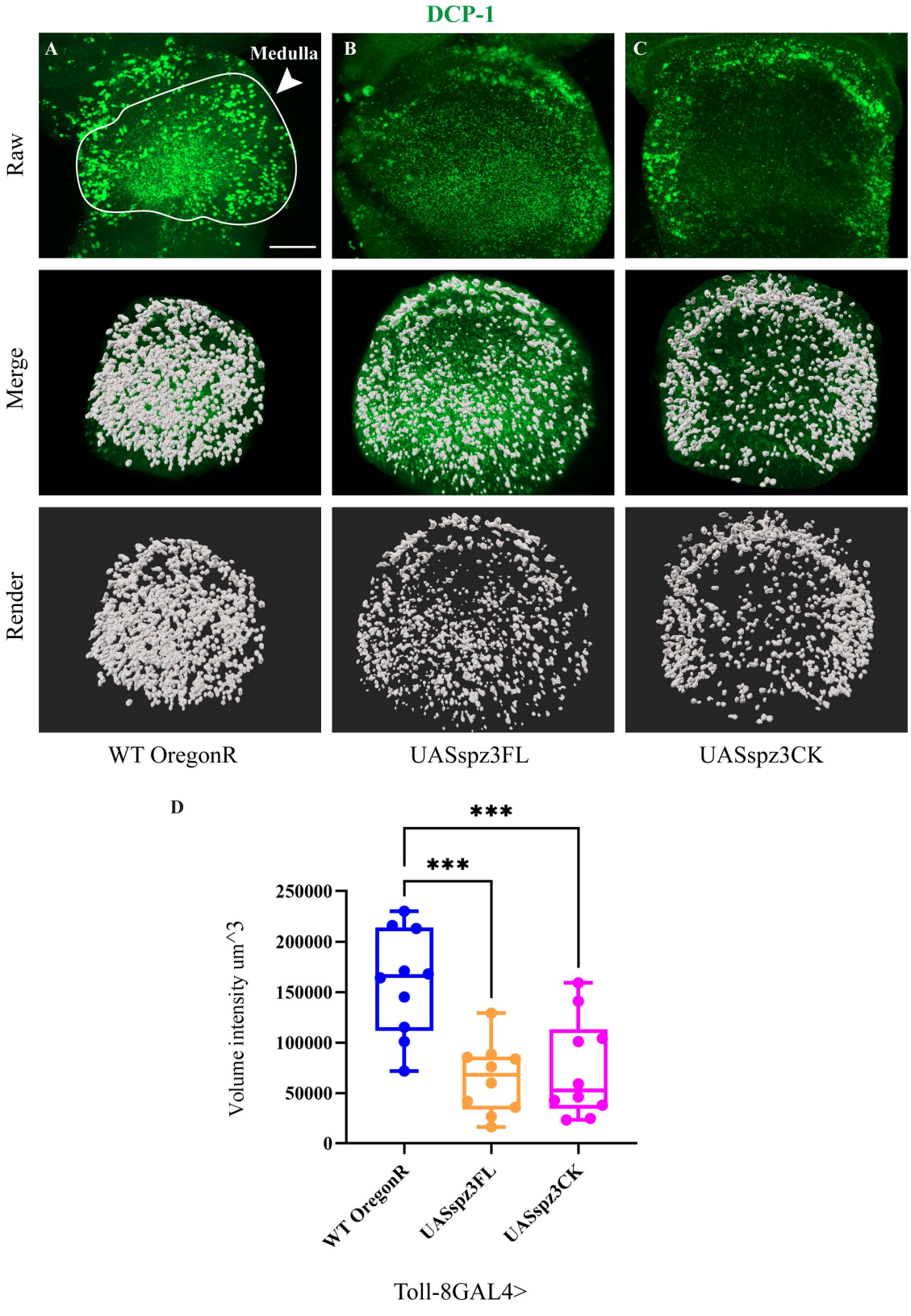


Figure 4.6 spz3 overexpression reduces cell death in the medulla

(A). anti-DCP-1 staining in wild type control

(B). anti-DCP-1 staining in UASspz3FL

(C). anti-DCP-1 staining in UASspz3CK

(D). Statistical analysis of DCP-1 volume intensity in the lamina. There is a significant decrease in DCP-1 volume in both UASspz3FL and UASspz3CK compared to wild type control (One-way ANOVA, Dunnett's multiple comparison), $P \leq 0.001$, n= 10 (Control), n= 10 (UASspz3FL), n= 10 (UASspz3CK). Images in A-C are single plains made in imageJ. The merge and render images are full stacks made in Imaris software. Number of repeats = 2.

Genotypes:

A. Control: Toll-8GAL4>WT OregonR

B. GOF: Toll-8GAL4>UASspz3FL

C. GOF: Toll-8GAL4>UASspz3CK

A typical test for that is a rescue experiment. Meaning that we have to overexpress spz3 FL or CK in a spz3 mutant background. Since we are assuming that spz3 CK is not secreted, we cannot express it in spz3 cells and the alternative to that is to express it in Toll-8-expressing cells. Genetically, this would be a spz3 homozygous mutant crossed to Toll-8 Gal4 while overexpressing UASspz3CK.

4.2.5 *spz3* works via different Tolls to regulate cell survival in the lamina and medulla

It has been shown that *spz3* interacts genetically with *Toll-8* (Ballard et al.,2014). To test whether the regulation of cell survival in the lamina and medulla is due to the interactions between *spz3* and *Toll-8* only, an epistasis experiment was done. To achieve this, knocked down *Toll-8* with *RNAi* (UAS*Toll-8RNAi*) and overexpressed UAS*spz3*FL. To be able to compare the difference in whether UAS*spz3*FL does rescue cell death, the UAS*Toll-8 RNAi* alone experiment was included.

In the lamina, knocking down UAS*Toll-8RNAi* showed a significant increase in DCP-1 volume intensity compared to the control group as well as the epistasis. However, in the epistasis, DCP-1 signal intensity was not significantly higher than the control group but showed a significant reduction in DCP-1 signal compared to UAS*Toll-8*RNAi **(Figure 4.7A-C)**. In the medulla, the epistasis UAS*Toll-8RNAi*;UAS*spz3*FL showed a significant reduction in DCP-1 volume intensity compared to the wild type but was not significant compared to the overexpression of UAS*spz3*FL alone **(Figure 4.8A-C)**.

4.2.6 Expression of Toll-6 in multiple cell types prevents automatic cell counting in the lamina at 48hr APF

To gain more insight on how altering *DNT-2* in the lamina affects *Toll-6* cell number, I crossed *Toll-6*GAL (*Toll-6*^{MI02127}) to UAS*Histone*YFP alone as a wild type control. Also, the

Figure 4.7 spz3 works via Toll-8 and other Tolls in the lamina

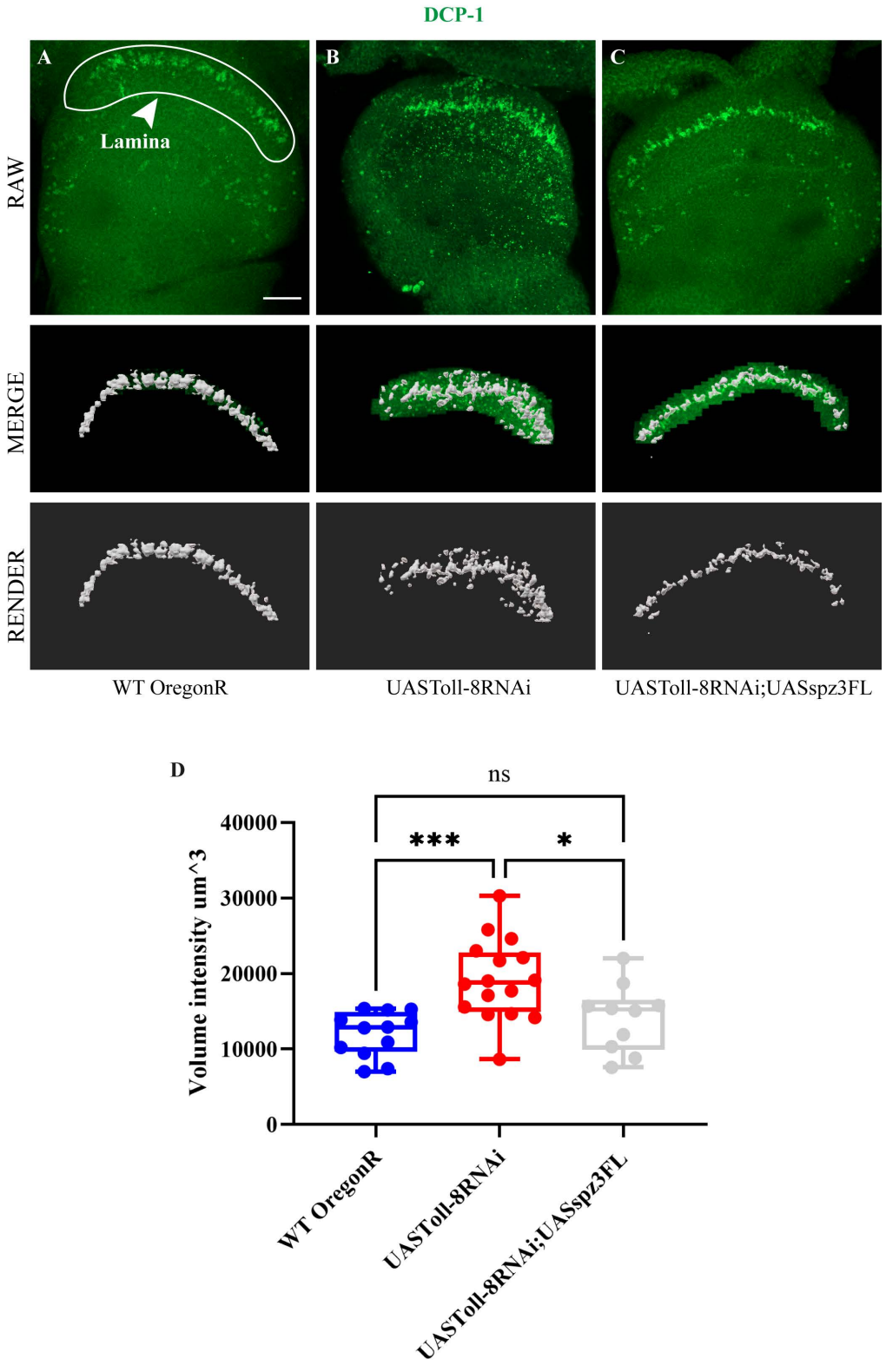


Figure 4.7 spz3 works via Toll-8 and other Tolls in the lamina

(A). anti-DCP-1 staining in wild type control

(B). anti-DCP-1 staining in UASToll-8RNAi (KD)

(C). anti-DCP-1 staining in UASToll-8RNAi;UASspz3FL (Epistasis)

(D). Statistical analysis of DCP-1 volume intensity in the lamina. There is a significant increase in DCP-1 volume when knocking down UASToll-8RNAi compared to wild type. However, there is no significant change in the epistasis UASToll-8RNAi;UASspz3FL compared to wild type but there is a significant decrease in DCP-1 volume compared to UASToll-8RNAi alone. (One-way ANOVA, Tukey's multiple comparison), $P \leq 0.001$, n= 12 (Control), n= 16 (UASToll-8RNAi), n= 10 (UASToll-8RNAi;UASspz3FL). Images in A-C are single plains made in imageJ with max projection. Images in merge and render are full stacks made in Imaris software. Number of repeats = 2.

Genotypes:

A. Control: Toll-8GAL4>WT OregonR

B. GOF: Toll-8GAL4>UASToll-8RNAi (KD)

C. Epistasis: Toll-8GAL4>UASToll-8RNAi;UASspz3FL (Epistasis)

Figure 4.8 spz3 works via Toll-8 and other Tolls in the medulla

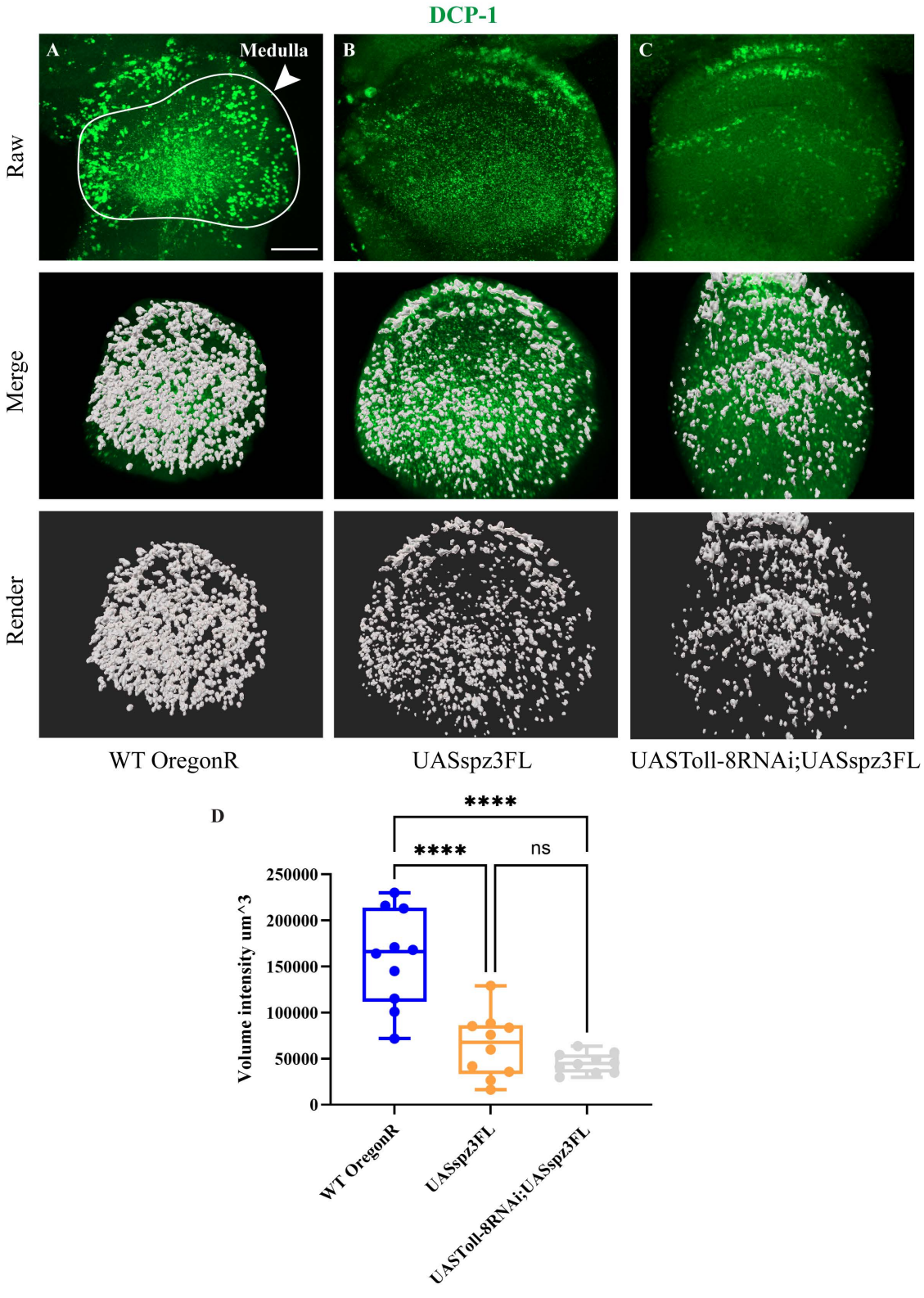


Figure 4.8 spz3 works via Toll-8 and other Tolls in the medulla

(A). anti-DCP-1 staining in wild type control

(B). anti-DCP-1 staining in UASspz3FL

(C). anti-DCP-1 staining in UASToll-8RNAi;UASspz3FL (Epistasis)

(D). Statistical analysis of DCP-1 volume intensity in the medulla. There is a significant decrease in DCP-1 volume in UASspz3FL (GOF) and UASToll-8RNAi;UASspz3FL (Epistasis) compared to wild type. However, there is no significant change between the GOF and epistasis in DCP-1 volume. (One-way ANOVA, Dunnett's multiple comparison), $P \leq 0.0001$, n= 10 (Control), n= 10 (GOF), n= 10 (Epistasis). Images in A-C are single plains made with imageJ. The images in merge and render are full stacks made in Imaris software. Number of repeats = 2.

Genotypes:

A. Control: Toll-8GAL4>WT OregonR

B. GOF: Toll-8GAL4>UASspz3FL (GOF)

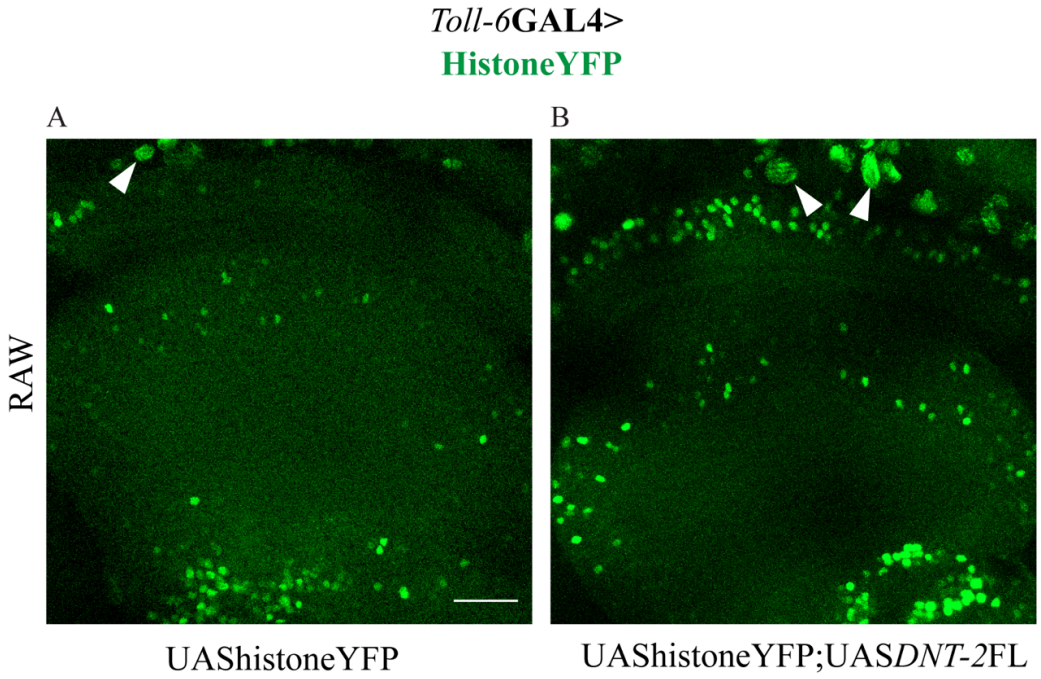
C. Epistais: Toll-8GAL4>UASToll-8RNAi;UASspz3FL (Epistasis)

combination of UAS*Histone*YFP;UAS *DNT-2* FL for the overexpression. In both genotypes, *Toll-6* was expressed in multiple cell types in the lamina that look like hemocytes more than *Histone*YFP cells **(Figure 4.9A-B)**. It has been shown that Toll-6 with DNT-2 is expressed in glia and is required for the phagocytosis of apoptotic cells (McLaughlin et al., 2019). Loss of Toll-6 causes an excess of cell derbies due to the limited interactions with DNT-2 (McLaughlin et al., 2019). From this study, the Toll-6 Gal4 expressing cells I observed could be hemocytes due to their role in phagocytosis. These results lead to the consideration of other Tolls to investigate cell number. While *Toll-7* can bind *DNT-2* (McIlroy et al., 2013) it is not present in the lamina to investigate cell number (see chapter 3). While testing other Tolls, *Toll-2* showed a unique organization of cells in the lamina that lead to the consideration of *Toll-2* in investigating cell number knowing that *Toll-2* is expressed in the lamina at 48hr APF (see Chapter 3).

4.2.7 *DNT-2* interacts genetically with *Toll-2* to regulate cell number in the lamina at 48hr APF

To test whether *DNT-2* regulates *Toll-2* cell number in the lamina, I tested the overexpression of UAS*DNT-2*FL and UAS*DNT-2*CK. Also, I tested *Toll-2* cell number in *DNT-2* mutant alleles ($DNT\text{-}2^{18}/DNT\text{-}2^{37}$) as well as the knockdown of *Toll-2* (UAS*Toll- 2RNAi*) and the epistasis (UAS*Toll-2RNAi*;UAS*DNT-2*FL). More evidence is provided in **(Figure 4.14)** suggesting a genetic interaction between *Toll-2* and *DNT-2* in the epistasis. In *Toll-2* knockdown with RNAi and the epistasis, the genotypes to test the regulation of cell number were not viable. All crosses showed $TM6B^{-}$ pupa **(Figure 4.11A)**. However, when overexpressing both forms of *DNT-2* (UAS*DNT-2*FL) and (UAS*DNT-2*CK), the data showed a significant increase in cell number compared to wild type. Moreover, in *DNT-2* null mutant, the data showed a significant reduction in

Figure 4.9 Expression of Toll-6 in multiple cell types prevents automatic cell counting in the lamina at 48hr APF



(A). HistoneYFP in wild type control

(B). HistoneYFP in UASDNT-2FL (GOF)

The Toll-6 GAL4 line is expressed in many cells that look like hemocytes. These cells would interfere with the Deadeasy plugin analysis of the smaller histoneYFP positive cells. The brains were dissected, fixed with formaldehyde and were not stained.

Genotype:

A. Toll-6GAL4>UAShistoneYFP

B. Toll-6GAL4>UAShistoneYFP;UASDNT-2FL

Figure 4.10 DNT-2 interacts genetically with Toll-2 to regulate cell number in the lamina

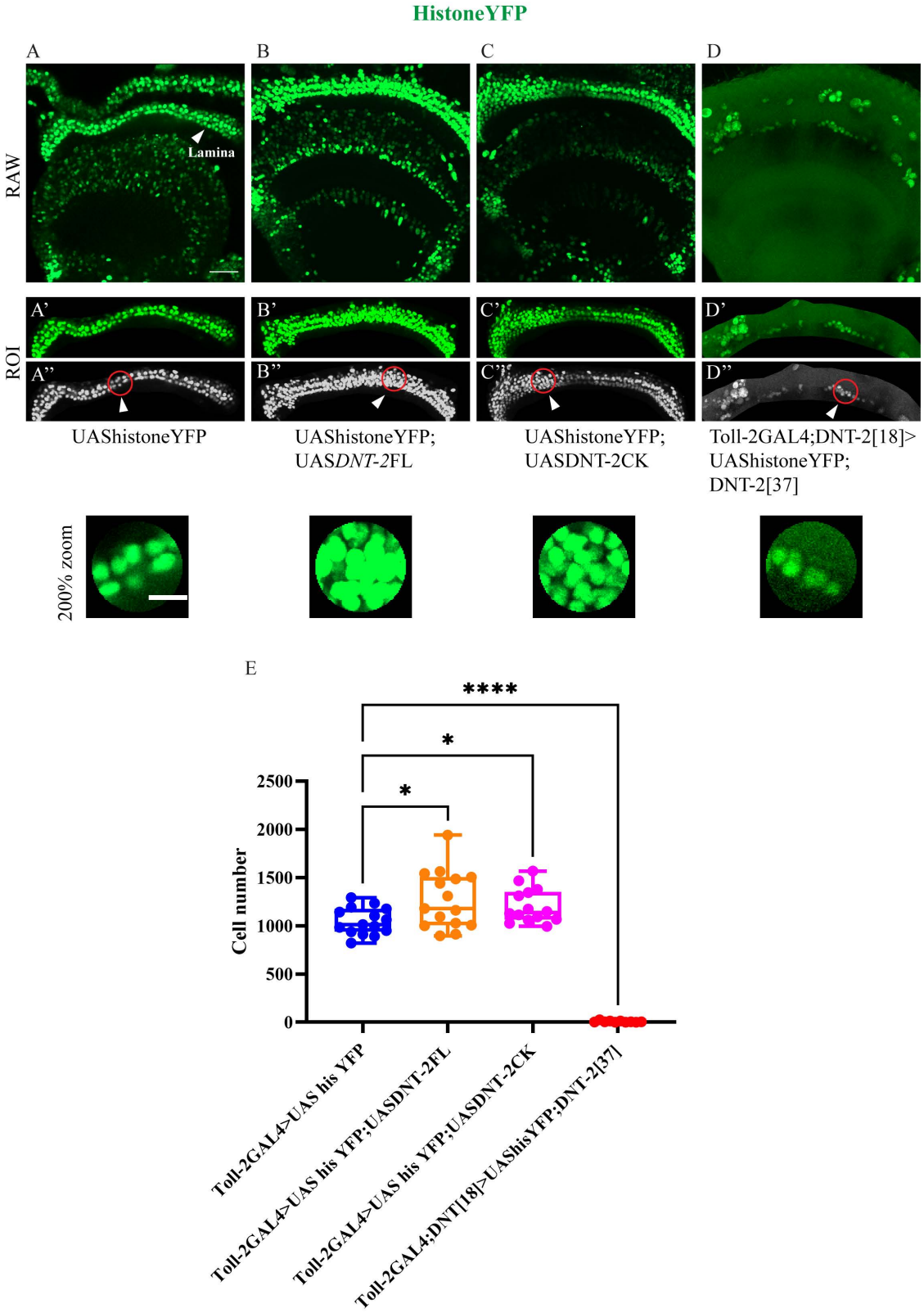


Figure 4.10 DNT-2 interacts genetically with Toll-2 to regulate cell number in the lamina

(A). HistoneYFP in wild type control

(B). HistoneYFP in UASDNT-2FL (GOF)

(C). HistoneYFP in UASDNT-2CK (GOF)

(D). HistoneYFP in DNT-2 null mutant (LOF)

(E). Statistical analysis of Toll-2^{+} HistoneYFP cells in the lamina at 48hr APF. There is a significant increase in cell number when overexpressing both UASDNT-2FL and UASDNT-2CK compared to wild type control. Also, there is a significant reduction in cell number in DNT-2 mutant alleles (DNT-2^{18}/DNT-2^{37}) compared to wild type control. (Brown-Forsythe and Welch ANOVA test), $P \leq 0.0001$, n= 15 (Control), n= 15 (UASDNT-2FL), n= 14
(UASDNT-2CK), n= 10 (DNT-2 mutant). Brains were dissected, fixed and not stained. Number of repeats = 2.

Genotypes:

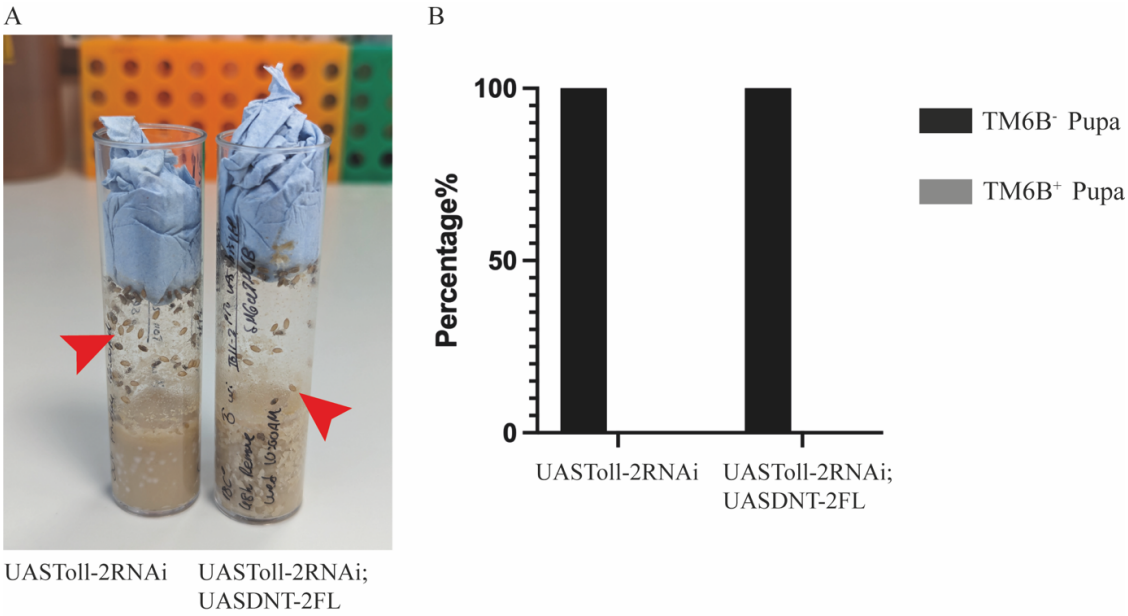
A. Control: Toll-2^{pTV}GAL4>UASHistoneYFP

B. GOF: Toll-2^{pTV}GAL4>UASHistoneYFP;UASDNT-2FL

C. GOF: Toll-2^{pTV}GAL4>UASHistoneYFP;UASDNT-2CK

D. LOF: Toll-2^{pTV}GAL4;DNT-2^{18}>UASHistoneYFP;DNT-2^{37}

Figure 4.11 TM6B^{+} pupa was not viable in the knockdown and epistasis experiment



(A). TM6B^{-} pupa in UASToll-2RNAi (Left) and UASToll-2RNAi;UASDNT-2FL (Right).

(B). Percentage of TM6B^{-} against TM6B^{+}. The graph shows both crosses did not have any TM6B^{+} pupa (The desired genotype for dissection).

cell number compared to wild type and the overexpression phenotypes **(Figure 4.10A-D'')**.

4.2.8 *DNT-2* loss of function increases cell death in the lamina at 24hr APF

To investigate whether loss of *DNT-2* affects cell survival in the lamina a null mutant of *DNT-2*^{18}/*DNT-2*^{37} were generated in the Hidalgo lab using the FRT-PBac mutagenesis by a previous member Jill Wentzell. The DNT-2 mutant alleles DNT-2[18] and DNT[37] were generated using site-directed mutagenesis to alter specific furin cleavage sites within the DNT2 gene (Foldi et al., 2017). This technique allowed for precise modifications to the DNA sequence, enabling the creation of mutant variants with altered protein processing capabilities (Foldi et al., 2017). The mutant constructs were then expressed in S2 cells to evaluate the effects of these mutations on protein cleavage and secretion (Foldi et al., 2017). Western blot analysis was performed on both cell lysates and secreted media from the transfected S2 cells, using anti-HA antibodies to detect the HA-tagged DNT2 proteins (Foldi et al., 2017). By comparing the protein bands of wild-type and mutant DNT2, they could assess whether the mutations resulted in changes to protein production, cleavage, or secretion (Foldi et al., 2017).

The data shows the loss of *DNT-2* in the lamina had a significant increase in DCP-1 volume intensity compared to wild type **(Figure 4.12A-B)**.

4.2.9 *DNT-2* gain of function decreases cell death in the lamina at 24hr APF

To test the effects of overexpressing *DNT-2* on cell death, both forms of *DNT-2* (UAS*DNT-2*FL and UAS*DNT-2*CK) were crossed to *nsyb*GAL4 to visualize the effects on all neurons instead of cells expressing only *Toll-6*. The reason for using *nsyb*GAL4 was that *Toll-*

Figure 4.12 DNT-2 null mutant increases cell death in the lamina

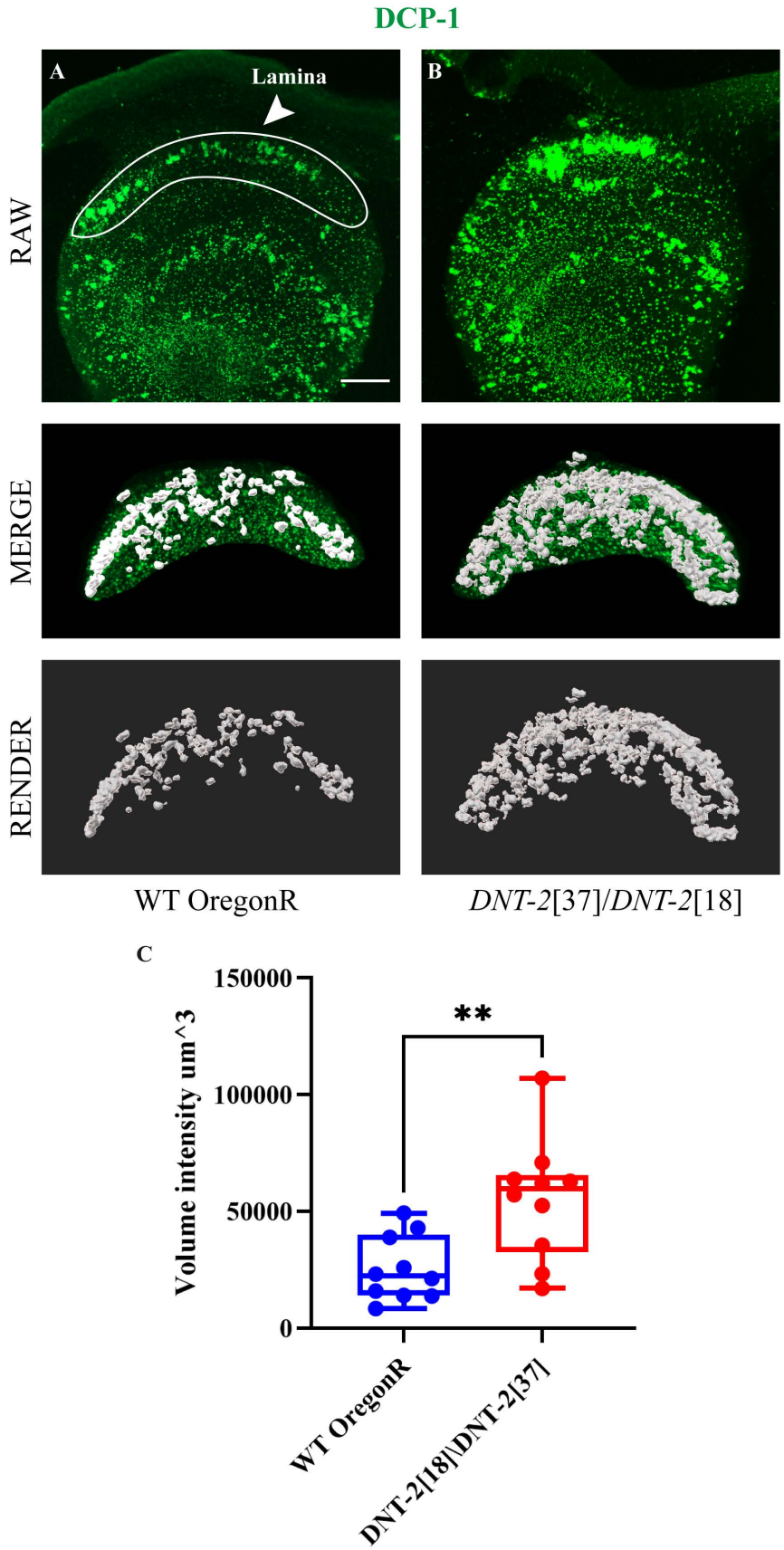


Figure 4.12 DNT-2 null mutant increases cell death in the lamina

(A). anti-DCP-1 staining in wild type control

(B). anti-DCP-1 staining in DNT-2 null mutant

(C). Statistical analysis of DCP-1 volume intensity in the lamina. There is a significant increase in DCP-1 volume in DNT-2 null mutant compared to the wild type control (Two-tailed Unpaired t test, $P \leq 0.01$ n= 10 (Control), n= 10 (DNT-2 mutant). Images in A and B are single plains made in imageJ with max projection. The merge and render images are full stacks made in Imaris software. Number of repeats = 2.

Genotypes:

A. Control: WT OregonR

B. LOF: DNT-2[18]/DNT-2[37]

*6*GAL4 was giving strange phenotypes in the lamina **(Figure 4.9A-B)**, to overcome that, I tested cell death using *nsyb*GAL as the main driver.

The data show that when overexpressing UASDNT-2FL, DCP-1 volume intensity is significantly reduced compared to wild type. Moreover, when overexpressing UAS*DNT-2*CK, DCP-1 volume intensity is significantly reduced compared to wild type **(Figure 4.13A-C)**.

4.2.10 *DNT-2* regulates cell survival in the lamina via *Toll-2*

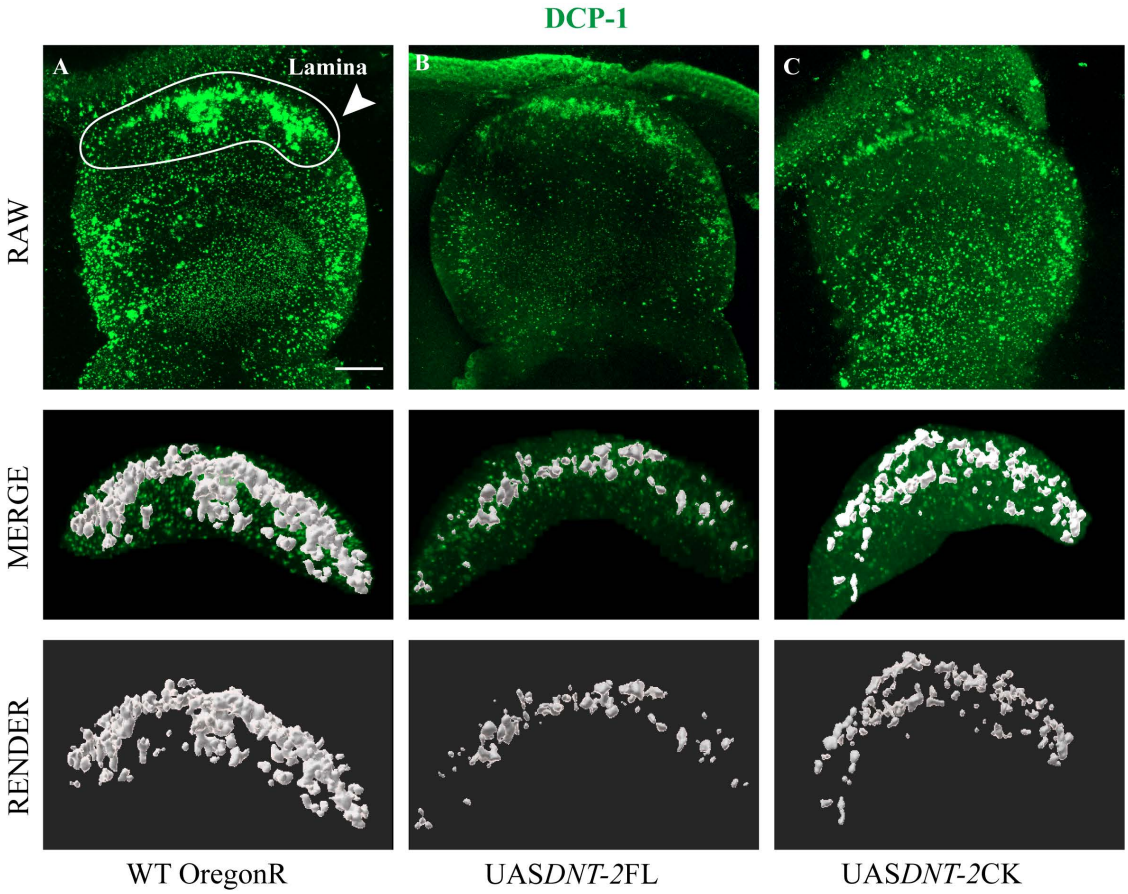
Toll-6 is the receptor for *DNT-2* (Mcllory et al., 2013). However, due to the challenges of observing phenotypes with *Toll-6*GAL4, using *Toll-2*GAL4 to investigate cell number at 48hr APF showed a genetic interaction between *DNT-2* and *Toll-2* **(Figure 4.10A-D'')**. This is possible since *DNT-2* can be promiscuous and binds both *Toll-6* and *Toll-7* (Mclorry et al., 2013). To further investigate whether *DNT-2* regulates cell survival in the lamina via *Toll-2*, an epistasis experiment was done by knocking down *Toll-2* with *RNAi* (w; UAS*Toll-2RNAi*) while overexpressing UAS*DNT-2* FL. Additionally, UAS*Toll-2RNAi* alone was tested to be able to compare if knocking down *Toll-2* with *RNAi* causes cell death in the lamina.

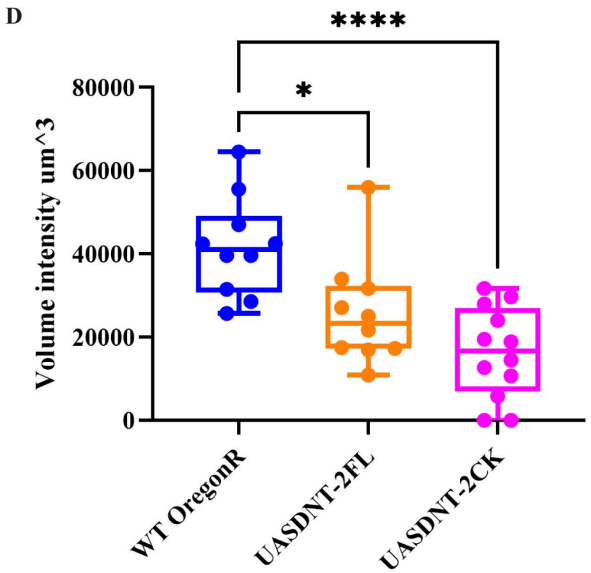
The data shows that upon knocking down *Toll-2* (UAS*Toll-2RNAi*), there is a significant increase in DCP-1 volume intensity compared to wild type. However, it did not show any significant changes compared to the epistasis. Finally, the epistasis data showed a significant increase in DCP-1 volume intensity compared to wild type these data suggests that *DNT-2* can interact genetically with *Toll-2* to regulate cell survival **(Figure 4.14A-C).**

4.3 Discussion

In this chapter, I showed the successful construction of spz3FL and spz4FL, which was

Figure 4.13 DNT-2 overexpression reduced cell death in the lamina





NsybGAL4>

Figure 4.13 DNT-2 overexpression reduced cell death in the lamina

(A). anti-DCP-1 staining in wild type control

(B). anti-DCP-1 staining in UASDNT-2FL (GOF)

(C). anti-DCP-1 staining in UASDNT-2CK (GOF)

(D). Statistical analysis of DCP-1 volume intensity in the lamina. There is a significant decrease in DCP-1 volume in UASDNT-2 and UASDNT-2CK compared to wild type (one-way ANOVA, Dunnett's multiple comparisons test, $P \leq 0.001$ n= 10 (control), n= 10 (UASDNT-2FL), n= 12 (UASDNT-2CK). Images in A-C are single plains made in imageJ with max projections. The merge and render images are full stacks made in Imaris software. Number of repeats = 2.

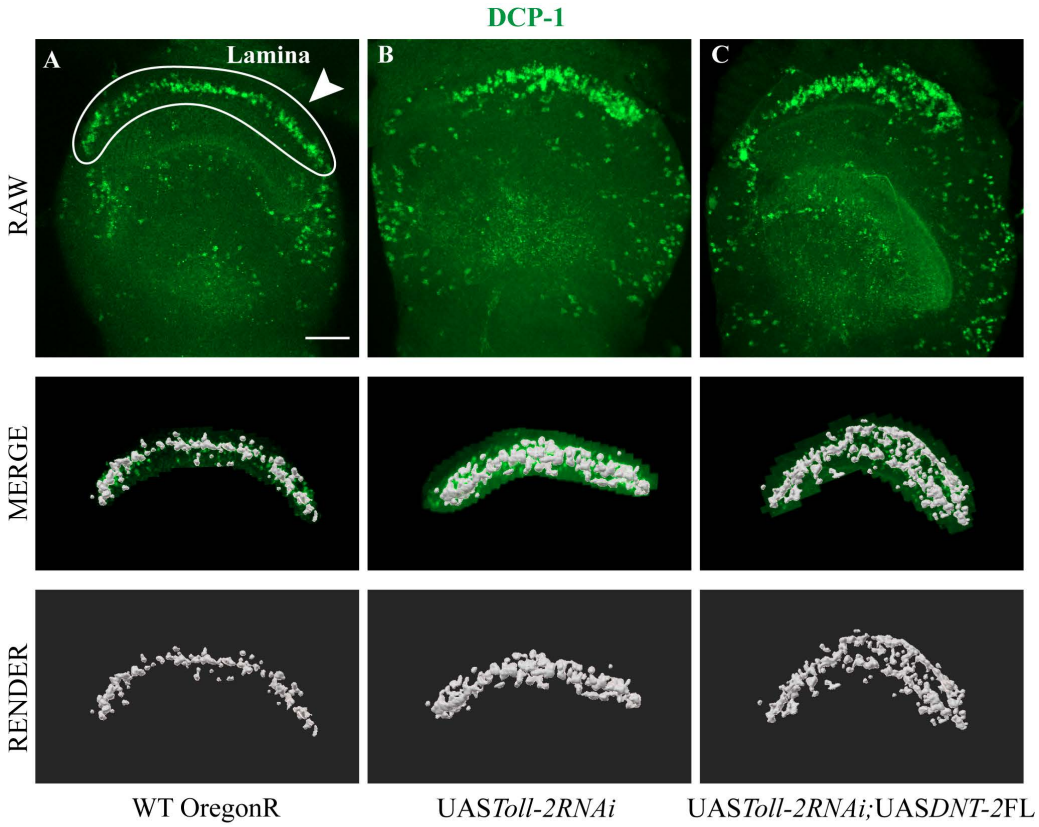
Genotypes:

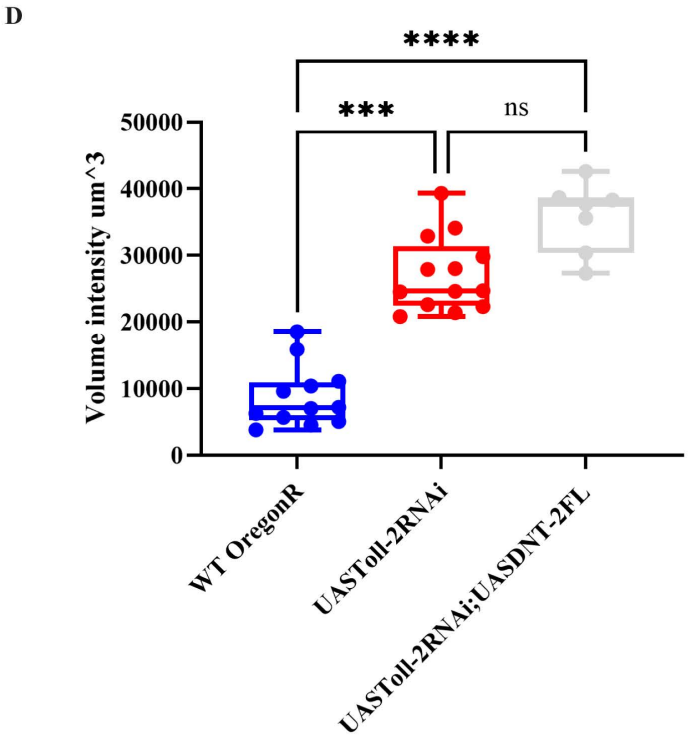
A. Control: nsybGAL4>WT OregonR

B. GOF: nsybGAL4>UASDNT-2FL

C. GOF: nsybGAL4>UASDNT-2CK

Figure 4.14 DNT-2 interacts with Toll-2 to regulate cell survival in the lamina





NsybGAL4>

Figure 4.14 DNT-2 interacts with Toll-2 to regulate cell survival in the lamina

(A). anti-DCP-1 staining in wild type control

(B). anti-DCP-1 staining in UASToll-2RNAi (LOF)

(C). anti-DCP-1 staining in UASToll-2RNAi;UASDNT-2FL (Epistasis)

(D). Statistical analysis of DCP-1 volume intensity in the lamina. There is a significant increase in DCP-1 volume in UASToll-2RNAi knockdown. Also, the epistasis UASToll-2RNAi;UASDNT-2FL shows an increase in DCP-1 volume intensity compared to wild type (Kurskal-Wallis test, Dunn's multiple comparisons test $P \leq 0.0001$ n= 12 (control), n= 13 (LOF), n= 7 (Epistasis). Images in A-C are single plains made in imageJ with max projection. the merge and render images are full stacks made in imaris software. Number of repeats = 2.

Genotypes:

A. Control: nsybGAL4>WT OregonR

B. LOF: nsybGAL4>UASToll-2RNAi

C. Epistasis: nsybGAL4>UASToll-2RNAi;UASDNT-2FL

accomplished through gateway cloning, allowing me to explore their functions in neural development. Importantly, through my experiments, I was able to establish that spz3 has a role in controlling cell survival at the 24 APF timepoint in both the lamina and medulla, which is an important stage. This finding suggests that spz3 is involved in controlling the number of neurons that survive during the final periods of optic lobe development so that developmental circuits can be formed with the correct number of neurons. Also, It was interesting to observe that DNT-2 not only modulates cell survival but also modulates cell number in the lamina. This regulation arises through genetic interactions with Toll-2 and so there is a specific receptor-ligand relationship that mediates control of cell fate during development. As such, these findings highlight the individual but complementary functions of spz3 and DNT-2 patterns in the formation of the optic lobes. However, it raises the question of how these effects occur, including the significant signaling pathways involved.

In this chapter, there were some challenges when conducting the experiments. For example, the regulation of cell number involving *spz3* and *Toll-8* was not included in this chapter. After experimenting, the *Toll-8 Histone*YPF positive cells were condensed into very small clusters that made it impossible to quantify by the Deadeasy software. Also, I have tried using Imaris to measure the volume intensity, but the program was missing a huge number of cells in all genotypes. Because of that, I did not reach a conclusion for this experiment and decided to leave it out. This question could be investigated by a different method. For example, the use tubulin GAL80^{ts} to control the expression of *Toll-8*GAL4, this would allow more control on how many cells are being expressed which may help make the analysis of counting cell number easier.

When testing the regulation of cell survival at 24hr APF in the lamina, in *spz3* GOF, only *spz3*FL showed a significant decrease in DCP-1 compared to wild type but not *spz3*CK. This might be due to the expression pattern of *Toll-8*. In Chapter 3, I have shown that *Toll-8* is highly expressed in the lobula complex and central brain. There is also moderate expression levels in the medulla and very little in the lamina. When overexpressing *spz3* FL and CK, this overexpression is present in cells that express *Toll-8* GAL4. Since *spz3*FL is secreted (Coutinho-Budd et al., 2017), that means cells in the lamina are receiving *spz3* molecules from the medulla and possibly the lobual complex to cause the decline in cell death. In the case of *spz3*CK, it is not known if it acts cell autonomously or gets secreted. It has been shown that *DNT-2*CK does not get secreted (Foldi et al., 2017), opening the possibility that *spz3*CK may do the same as well. If *spz3*CK remains in the cell, this would explain the non-significance in cell death reduction since there are only a few *Toll-8* neurons in the lamina and they are not receiving any *spz3*Ck from nearby neuropiles such as the medulla and lobula complex.

To support this argument, when looking at cell death at 24hr APF in the medulla, both *spz3*FL and *spz3*CK were able to significantly reduce cell death. Since *Toll-8* expression is moderate in the medulla and very high in the lobula complex, *spz3*CK levels were enough to reduce cell death in the medulla.

It has been documented that *Toll-8* interacts genetically with *spz3* (Ballard et al., 2014). However, the epistasis data argues that *spz3* can work via *Toll-8* as well as other possible Tolls. In the lamina, The DCP-1 volume intensity in the epistasis was significantly lower than *Toll-8 RNAi*, this suggests that *spz3* was able to bind a different Tolls to reduce cell death without the need of *Toll-8* to achieve that. When comparing the epistasis to the wild type, there are no significant changes in DCP-1 which suggests that *spz3* can work via *Toll-8*.

This argument is also shown in the medulla data. Moreover, As I reflect on the result from my experiments, the knockdown of UASToll-8RNAi led to enhancement of DCP-1 signal intensity in lamina which was stronger as compared to control. However, in the case of epistasis experiments, where the additional UAS constructs were included, the DCP-1 signal was not significantly greater than control and it was lower than DCP-1 signal following a single UASToll-8RNAi knockdown. As it is, such a puzzling observation prompted a possible clarification based on the Gal4 dilution effect. As Gal4 is activated to express UAS constructs, if several UAS elements are employed, then less Gal4 becomes available for each individual target and therefore lowers the expression of each of the UAS-linked genes. In this instance, UASToll-8RNAi and other UAS constructs may have stretched Gal4 too thin in the epistasis experiment, resulting in a less efficient Toll-8 knockdown and therefore, a decline in the DCP-1 signal.

To address this situation, a few options seem plausible. The first would be to use a more effective Gal4 driver to prevent the spalling of all the UAS constructs due to a lack of sufficient activation. Or better still, UAS constructs containing less assertive elements from epistasis group, might allow more effective driving of UASToll-8RNAi by Gal4 and therefore be able to better understand the function of DCP-1.

To conclude, there is still no strong evidence confirming the binding between *spz3* and *Toll-8* as well as the binding between *DNT-2* and *Toll-2*. However, these data have demonstrated that *spz3* and *DNT-2* are required for and can promote cell survival during visual system development.

Chapter 5

The Role of *Drosophila spz/DNTs* in regulating connectivity in the optic lobes

5.1 Introduction

This chapter aims to test whether altering *spz3/Toll-8* and *DNT-2/Toll-2* regulates connectivity during optic lobe development at 72hr APF.

In Chapter 4, the data suggests that *spz3* interacts genetically with different Tolls and not *Toll-8* only. To test whether *spz3*-expressing neurons connect to other Toll-expressing neurons, I used *trans-tango* and GFP reconstitution across synaptic partners (GRASP) to answer that. Trans-Tango is a powerful method for anterograde transsynaptic tracing and manipulation of neural circuits (Talay et al., 2017). The principle behind trans-Tango is based on a synthetic signaling pathway that is introduced into all neurons in an animal, which converts receptor activation at the cell surface into reporter expression through site-specific proteolysis (Talay et al., 2017). Specific labelling is achieved by presenting a tethered ligand at the synapses of genetically defined neurons, thereby activating the pathway only in their postsynaptic partners (Talay et al., 2017). Unlike previous Tango-based systems that detected endogenous neurotransmitters, trans-Tango employs an exogenous ligand-receptor pair, allowing it to be applied to any neural circuit regardless of the neurotransmitter used (Talay et al., 2017). The ligand is tethered to a synaptic protein via an extracellular spacer, which directs it to the synapse and prevents diffusion away from the synapse (Talay et al., 2017). This design makes trans-Tango a flexible and general transsynaptic

labelling technique that can discover unknown synaptic partners without assumptions about the nature of the connections. This would allow me to visualize the input (presynaptic) neurons with one channel and the output (postsynaptic) neurons with another (Talay et al., 2017).

GRASP is a method developed to label membrane contacts and synapses between two cells in living animals (Feinberg et al., 2008). The principle behind GRASP involves expressing two complementary fragments of GFP on different cells, tethered to extracellular domains of transmembrane carrier proteins (Feinberg et al., 2008). When the two cells come into close proximity (within 100 nm), the GFP fragments can reconstitute and form a fluorescent signal, indicating a membrane contact or synapse (Feinberg et al., 2008). By using different carrier proteins, GRASP can be used to detect general membrane contacts or specifically label synapses (Feinberg et al., 2008). The method allows visualization of synaptic connections that are challenging to resolve with conventional light microscopy, especially in dense nerve bundles. GRASP has been demonstrated to accurately label known synaptic contacts in *C. elegans*, detect changes in mutants with altered synaptic specificity, and reveal new information about synaptic locations, as confirmed by electron microscopy (Feinberg et al., 2008). This technique offers the potential to greatly facilitate synaptic mapping in complex nervous systems, providing an anatomical framework for understanding neural circuitry. This method would allow me to test whether *spz3*-expressing neurons connect to different Toll-expressing neurons in the lamina.

In Chapter 3, I showed with MCFO that *Toll-8* is expressed in lamina wide-field (Lawf1) neurons. Also, I showed that *Toll-2* is expressed in lamina neurons (L1). Lawf1 neuron is a lamina feedback neuron that has its cell body in the medulla cortex and sends out axonal

terminals in the medulla as well as the lamina (Fischbach and Dittrich1989). L1 lamina neuron has its cell body in the lamina cortex with a unique dendrites shape and sends out its axonal terminals into two medulla layers (M1 and M5) (Fischbach and Dittrich1989).

To test whether *spz3/Toll-8* and *DNT-2/Toll-2* regulate connectivity during optic lobe development, I used the *Drosophila* Split Gal4 system to test whether altering both ligands and Tolls would affect the connectivity of Lawf1 and L1 neurons. The principle behind Split Gal4 is based on the modular nature of the Gal4 transcription factor, which consists of distinct DNA-binding (DBD) and transcription activation (AD) domains (Luan et al., 2020). These domains, when separated, are incapable of promoting gene expression independently (Luan et al., 2020). However, by fusing the DBD and AD to strong, heterodimerizing leucine zippers, researchers created a system where the two Gal4 domains could be independently targeted to different cells using distinct enhancers (Luan et al., 2020). This approach allows for precise targeting of gene expression, as only cells in which both enhancers are active will express both Gal4 components and reconstitute Gal4 activity (Luan et al., 2020). The Split Gal4 method has found its greatest application in targeting single cells or cell types in the *Drosophila* nervous system, becoming a powerful tool for mapping neural circuits in flies. This technique offers a significant advantage over traditional Gal4-UAS systems by providing more refined control over gene expression patterns, enabling researchers to achieve highly specific targeting of cell populations based on the intersection of two gene expression patterns

. While researching, I found Split Gal4 lines generated to specifically be expressed in 12 classes of lamina neurons in which Lawf1 and L1 lamina neurons are included (Tuthill et al., 2013). These two Split Gal4 lines would be used to investigate any connectivity phenotypes. For example, I will be looking at the axonal terminals projected back into the lamina in Lawf1 neurons, and the volume intensity of these axonal terminals will be

measured by Imaris software. This method will allow me to quantify the total amount of a fluorescently labelled molecule within a defined three-dimensional space. This process begins with the software identifying the 3D volume where the fluorescent signal is present, typically based on thresholding or segmentation of the image obtained through confocal microscopy. Once the region is defined, Imaris calculates the intensity of the signal in each tiny 3D pixel, or voxel, which reflects the brightness and, by extension, the concentration of the fluorescent marker. By summing the intensity values of all the voxels within this volume, the software provides a total volume intensity measurement. Moreover, in L1 neurons I will be focusing on the dendric branching in lamina and axonal terminals in the medulla. The dendritic branching volume intensity will be measured using Amira3D software due to its significant accuracy in highlighting the dendrites. However, the L1 axonal terminals in the medulla phenotypes will be assessed by using the Ab24B10 antibody to highlight the columns in the medulla and any noticeable misrouting phenotypes will be taken as a phenotype based on the analysis done in (Millard et al., 2007).

5.2 Results

5.2.1 *spz3* expressing neurons connect to different Tolls besides *Toll-8*

It has been reported that *spz3* interacts genetically with *Toll-8* (Ballard et al., 2014). However, data in Chapter 4 suggested that *spz3* can work via multiple Tolls and not only *Toll-8* to regulate cell survival in the lamina and medulla.

To test whether *spz3*-expressing neurons connect to other Toll-expressing neurons besides *Toll-8*, I used *trans-tango* and GRASP to answer that.

In *trans-tango*, the aim was to test if *spz3*-expressing neurons connect to the *Toll-8* Lawf1 expressing neurons and other possible lamina neurons. *spz3*-IT-GAL4 was crossed to *trans-*

tango line, dissected 72hr APF pupa and stained with GFP and DsRed. The GFP channel shows the *spz3* expressing neurons in R1-R6 PRs **(Figure 5.1B)**. In the DsRed channel, the output neurons are shown in cyan; these are lamina neurons that receive input from R1-R6 PRs which then will terminate in the medulla **(Figure 5.1C)**. The data shows output neurons in cyan of lamina neurons that express different Tolls **(see Chapter 3)**.

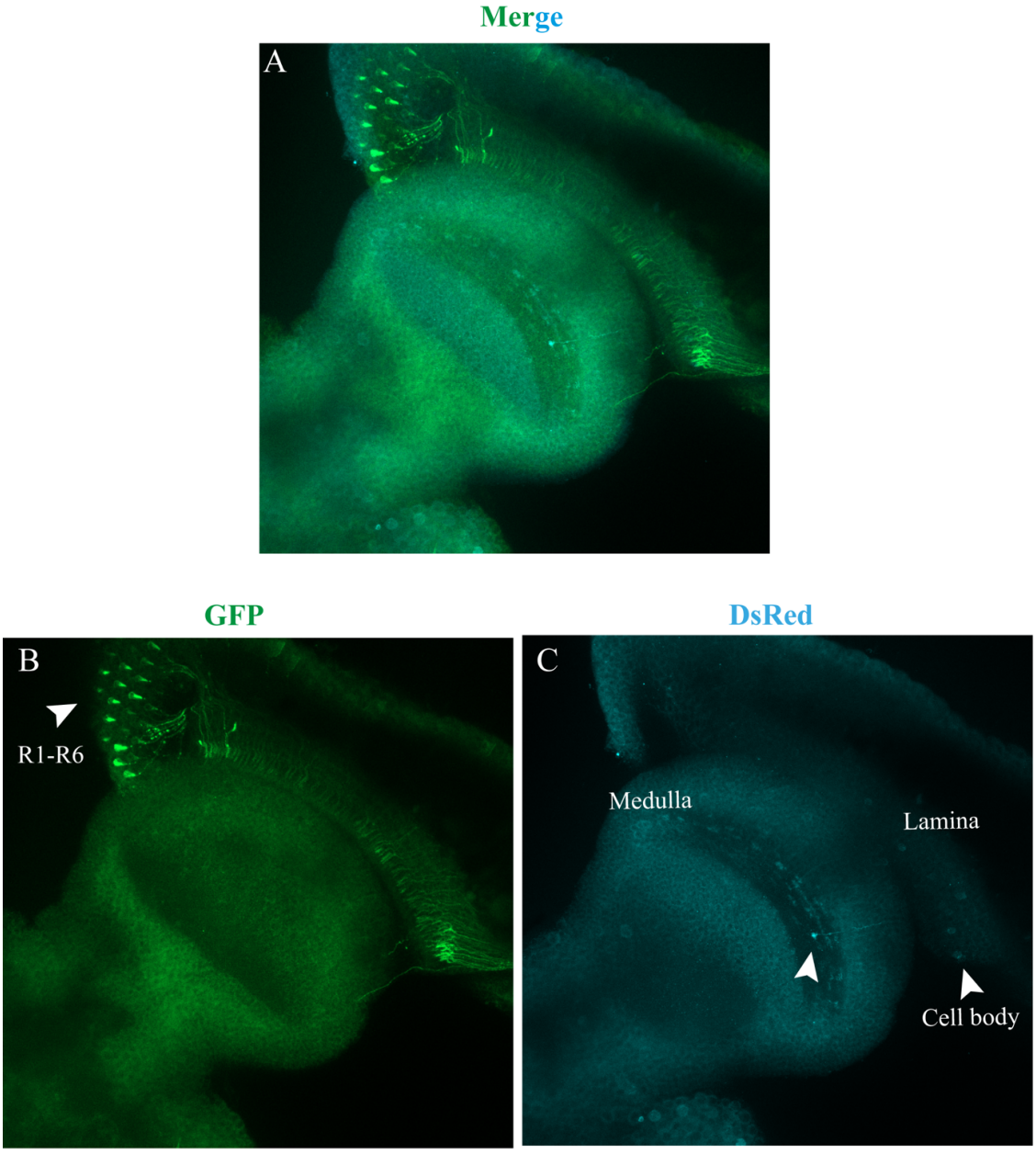
In the GRASP expirement, the aim was to test whether *spz3*-expressing neurons connect to specific Tolls known to be expressed in the lamina such as *Toll-8*, *Toll-6* and *Toll-2* **(see Chapter 3)**.

Gal4 lines combined with LexA lines were crossed to a GRASP line that contains the Gal4 region at the presynaptic site while the LexA region is at the postsynaptic site. Brains were dissected at 72hr APF and were not stained with GFP, only with Ncadh to label the optic lobe neuropiles. The wild-type control (GRASP>oregonR) does not show any signs of GRASP signal. However, the *spz3*-IT- GAL4 combined with different Tolls (*Toll-8*, *Toll-6* and *Toll-2*) showed GFP reconstitution signal at the lamina where all these Tolls are present at the termination site of *spz3* expressed in R1-R6 PRs. This means that the location where *spz3*-expressing neurons terminate in the lamina is met by the neurons that express the Tolls mentioned above suggesting a potential genetic interaction between these Tolls and the ligand *spz3* **(Figure 5.2A-D)**.

5.2.2 Overexpression of *spz3FL* increased neuronal number in the lamina

To ask whether overexpression of *spz3*FL could regulate the connectivity of *Toll-8* expressing neurons, I crossed *Toll*-8GAL4 to UASmyrGFP; UAS*spz3*FL. Brains were dissected at 72hr APF and stained with GFP and Ncadh. *Toll-8* is not highly expressed in the lamina (see Chapter 3) this means that the analysis of connectivity phenotypes (mistargeting

Figure 5.1 spz3-IT-GAL4 output neurons with TransTango



spz3-IT-GAL4>TransTango

Figure 5.1 spz3-IT-GAL4 output neurons with TransTango

(A). GFP and DsRed merged channels.

(B). 488 GFP channel of spz3 input expressing neurons in R1-R6 PRs.

(C). 546 DsRed channel of lamina output neurons that connects to R1-R6 PRs.

Genotype:

. spz3-IT^{1408}-GAL4>TransTango.

Figure 5.2 spz3-IT-GAL4 expressing neurons connect other Tolls alongside Toll-8

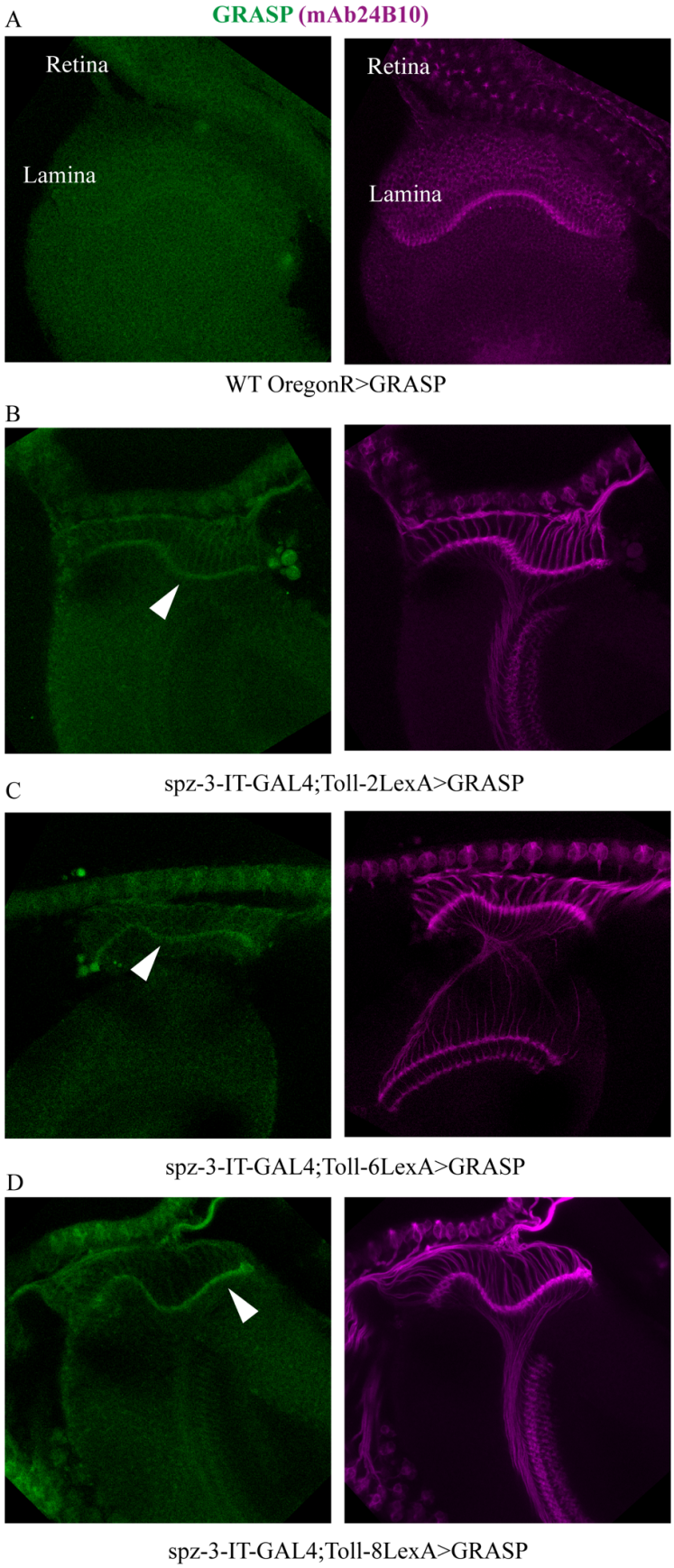


Figure 5.2 spz3-IT-GAL4 expressing neurons connect other Tolls alongside Toll-8 (A). GRASP of wild type control.

(B). GRASP of spz3 expressing neurons and Toll-2 expressing neurons in the lamina.

(C). GRASP of spz3 expressing neurons and Toll-6 expressing neurons in the lamina.

(D). GRASP of spz3 expressing neurons and Toll-8 expressing neurons in the lamina.

Genotypes:

. OregonR>GRASP

. spz3-IT^{1408}-GAL4;Toll-2LexA>GRASP.

. spz3-IT^{1408}-GAL4;Toll-6LexA>GRASP.

. spz3-IT^{1408}-GAL4;Toll-8LexA>GRASP.

or retraction of neurons) of Toll-8 Gal4 expressing neurons would be simpler since there are not that many neurons in the lamina. The data in this section shows an increase in neuronal number of the lamina neurons. However, whether these neurons are showing any sort of connectivity phenotype in the lamina would require better genetic tools such as MARCM clones or split Gal4 to identify these phenotypes. The data does not offer a conclusion as to whether overexpression of *spz3* affects the connectivity in the lamina. The data showed that there was an increase in the number of *Toll-8*-expressing neurons in the lamina compared to the wild-type control. In the overexpression samples, the majority exhibited an increase in neuronal number phenotype (n=9 out of 13), while the remaining samples showed a pattern like the control (n=4) **(Figure 5.3A-B)**.

5.2.3 There is no evidence suggesting that lamina wide-field 1(Lwaf1) connectivity is altered when manipulating *spz3* or *Toll-8*

To test whether manipulating *spz3* and *Toll-8* would affect the connectivity of Lawf1 neurons, I used a specific split-Gal4 line (R52H01AD;R17C11DBD) that has a promoter particularly for Lawf1 neurons to answer this question.

The overexpression of *spz3*FL volume intensity of Lawf1 axonal terminals was not significant compared to the wild type. Moreover, I tested the knockdown of *Toll-8* using *UASToll-8RNAi* and that did not show any significant change in volume intensity compared to the wild type. Lastly, I tested the epistasis by knocking down *Toll-8* and overexpressing *spz3*FL (*UASToll-8RNAi; UASspz3FL*). This also showed no significant change in volume intensity compared to the control **(Figure 5.4A-D)**.

5.2.4 Altering *DNT-2* and *Toll-2* affects the routing of L1 neurons in the medulla

To ask if altering *DNT-2* and *Toll-2* affects the routing of L1 neurons in the medulla, I

Figure 5.3 spz3 overexpression increased neuronal number in the lamina

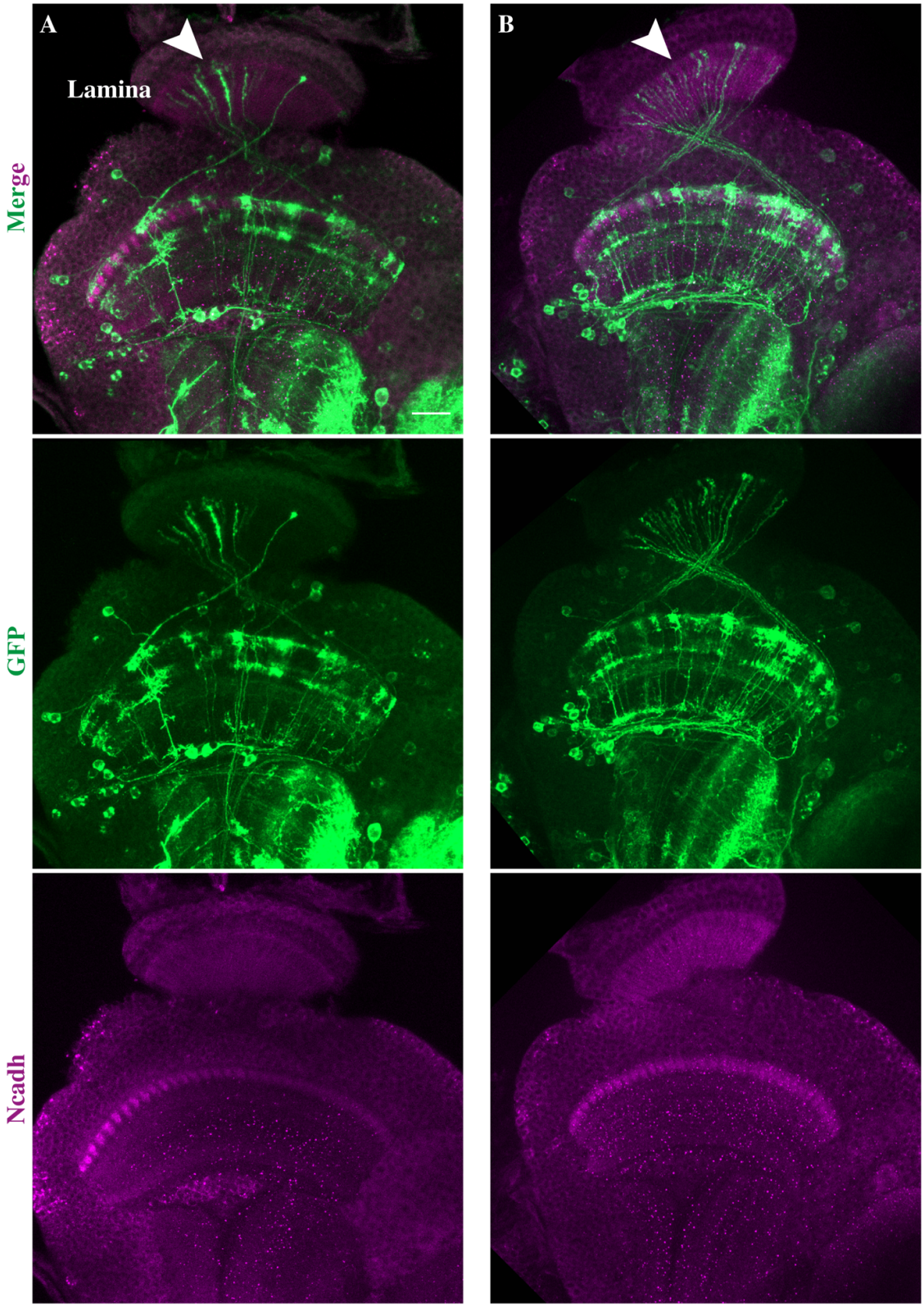


Figure 5.3 spz3 overexpression increased neuronal number in the lamina

(A). Toll-8 lamina feedback neurons in the lamina (Control).

(B). spz3FL overexpression in Toll-8 lamina feedback expressing neurons (GOF).

The data in this figure was not quantified with statistical analysis. The phenotypes in the lamina were observed and there is no evidence of any significance in the increase of neuronal number in the lamina. This was not further quantified due to the limitation of the tools used in this experiment. The brains were dissected, fixed, and stained with anti-GFP (Rb).

Genotypes:

A. Toll-8^{MD806}GAL4>10XUASmyrGFP.

B. Toll-8^{MD806}GAL4>10XUASmyrGFP;UASspz3FL.

used L1-split-GAL4 (R48A08AD;R66A01DBD) line to answer that. This was done at 72 hr APF.

First, I overexpressed *DNT-2*FL (*UASDNT-2FL*) in L1 neurons. When overexpressing *UASDNT-2FL*, the axonal terminal of L1 neuron in the M1 layer gets misrouted and crosses to a nearby column labeled with mAb24B10. In the wild type control, the L1 axonal terminal remains restricted to a single column in the medulla **(Figure 5.5A-B')**.

To test whether altering *Toll-2* would affect the routing of L1 neurons, I knocked down *Toll- 2* with *RNAi* (*UASToll-2RNAi*). Moreover, I tested the possibility of this misrouting phenotype is caused by the interaction between *DNT-2* and *Toll-2*, I tested an epistasis experiment by knocking down *Toll2* with *RNAi* while overexpressing *DNT-2*FL (*UASToll-2RNAi;UASDNT-2FL*). The knockdown of *Toll-2* with *RNAi* alone showed two different phenotypes. A mild phenotype and a severe phenotype. In the mild phenotype, the *UASToll- 2RNAi* knockdown, L1 neurons misrouted by crossing their axonal terminal in the M1 layer to the nearby column. Similarly, in the epistasis, L1 neuron axonal terminals in the M1 layer misrouted to a nearby column **(Figure 5.6A-C')**.

In the severe phenotype, the majority of L1 neurons die when knocking down *Toll-2* with *RNAi*. However, the L1 neurons that survive remain restricted to a single column similar to the wild-type control **(Figure 5.7A-B')**.

Findings from these experiments suggest that *Toll-2* plays a critical role in the proper routing of L1 neuron axonal terminals in the M1 layer. When *Toll-2* was knocked down using RNAi, the misrouting of these axons to nearby columns was observed, indicating that *Toll-2* is necessary for maintaining correct axon guidance. The fact that two phenotypes (mild and severe) were observed implies that the extent of misrouting might vary

Figure 5.4 There is no evidence whether spz3 and Toll-8 regulate the connectivity of Lawf1 neurons

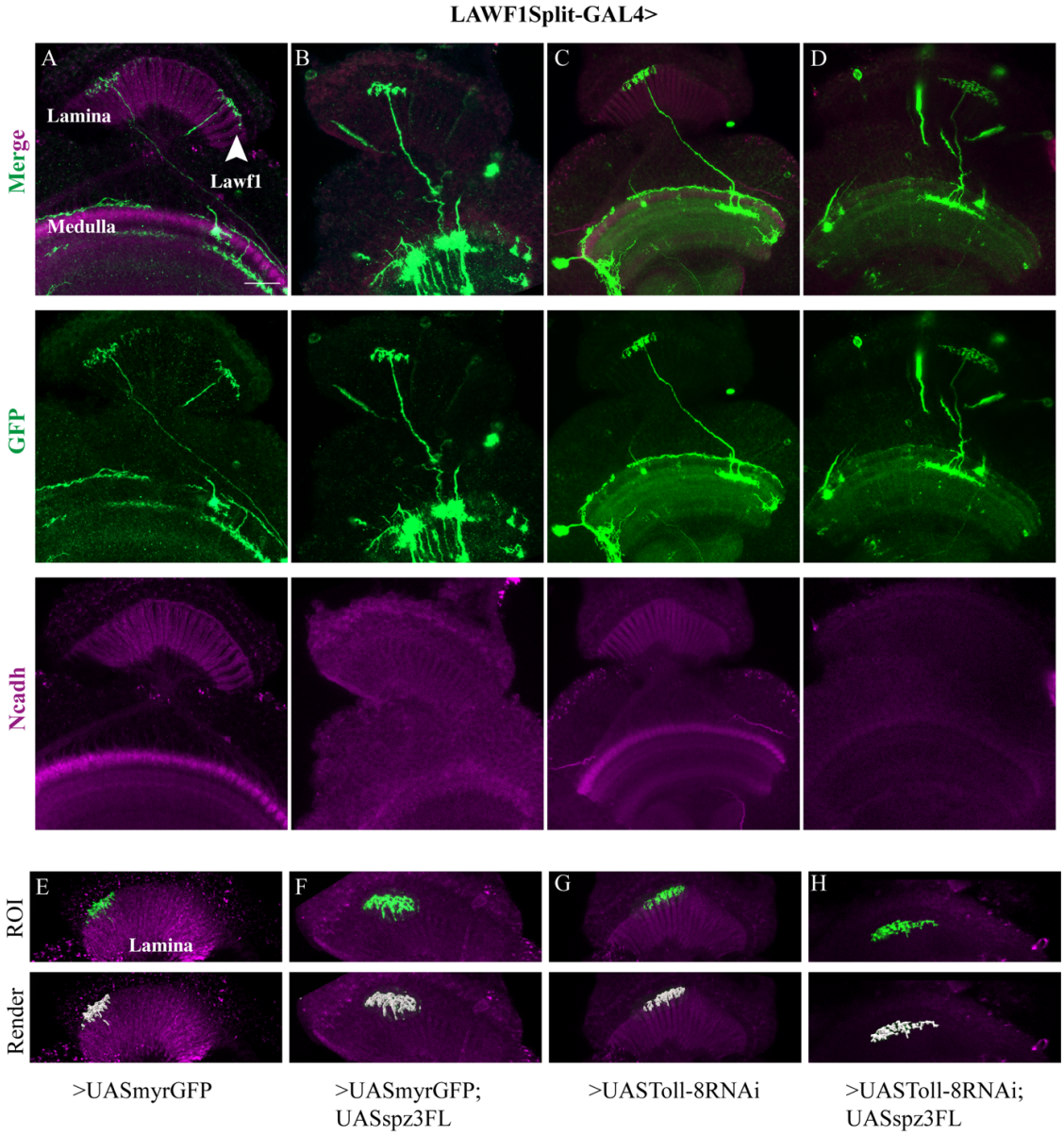


Figure 5.4 There is no evidence whether spz3 and Toll-8 regulate the connectivity of Lawf1 neurons

I

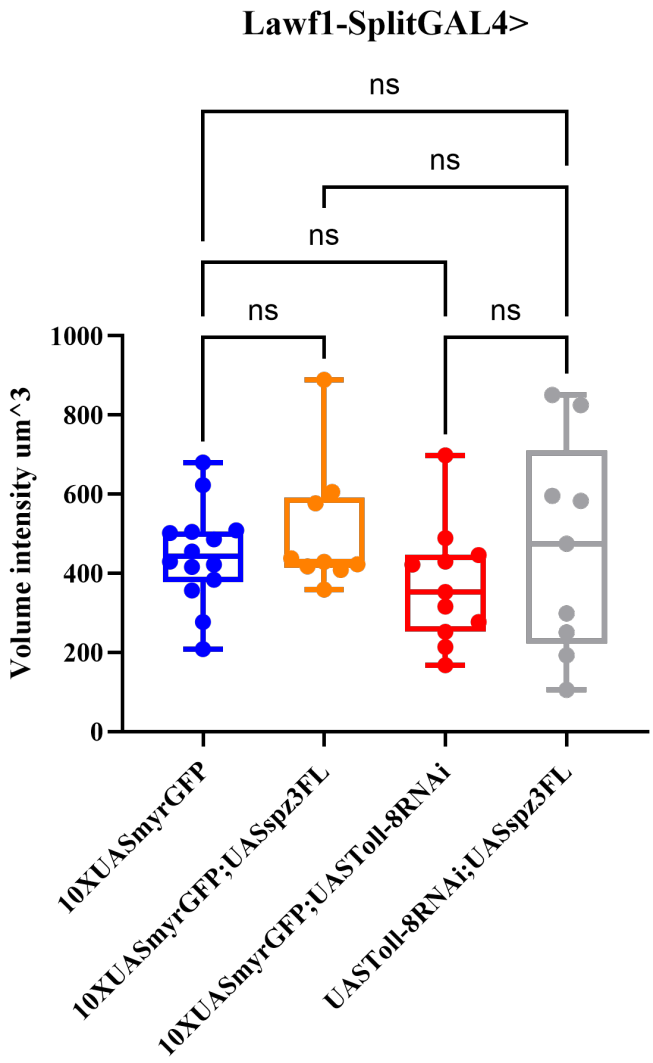


Figure 5.4 There is no evidence whether spz3 and Toll-8 regulate the connectivity of Lawf1 neurons

(A). Lwaf1 feedback neurons in wild type control.

(B). Lwaf1 feedback neurons in UASspz3FL overexpression.

(C). Lwaf1 feedback neurons in UASToll-8RNAi knockdown.

(D). Lwaf1 feedback neurons in UASToll-8RNAi;UASspz3FL epistasis.

(E-H). Cropped ROI of the axonal terminals of Lawf1 feedback neurons terminating in the lamina.

(I). Statistical analysis of Lawf1 axonal terminals volume intensity in the lamina. There is no significant change in the axonal terminals volume across all genotypes compared to wild type control as well as in between each genotype (Kruskal-Wallis test), $P > 0.05$, n= 14 (Control), n= 9 (UASspz3FL), n= 11 (UASToll-8RNAi), n= 9 (UASToll-8RNAi;UASspz3FL). The number of repeats = 3.

Genotypes:

. R52H01AD;R17C11DBD>10XUASmyrGFP.

. R52H01AD;R17C11DBD>10XUASmyrGFP;UASspz3FL.

. 10XUASmyrGFP(X);R52H01AD;R17C11DBD>UASToll-8RNAi.

. 10XUASmyrGFP(X);R52H01AD;R17C11DBD>UASToll-8RNAi;UASspz3FL.

depending on the degree of *Toll-2* knockdown or other underlying factors in the neural environment.

Furthermore, the epistasis experiment, in which *Toll-2* was knocked down while *DNT-2* was overexpressed, showed similar misrouting. This suggests that *DNT-2* overexpression could not rescue or compensate for the *Toll-2* knockdown, pointing to a functional relationship between *Toll-2* and *DNT-2* in guiding L1 axons. It implies that although *DNT-2* may interact with *Toll-2*, the correct functioning of *Toll-2* is crucial for the precise guidance of these neurons, and *DNT-2* alone cannot fully regulate this process. Therefore, this suggests that *Toll-2*'s role is not solely dependent on *DNT-2* but may involve other downstream signaling mechanisms or interacting molecules to ensure correct axonal pathfinding.

5.2.5 Altering DNT-*2* and *Toll-2* affects the dendritic branching of L1 neurons in the lamina

To ask whether altering *DNT-2* and *Toll-2* affects the dendric branching of L1 neurons in the lamina, I used the same L1-split-GAL4 (R48A08AD;R66A01DBD) line to answer this.

First, I overexpressed *DNT-2*FL (*UASDNT-2FL*) in L1 dendrites. The overexpression of *DNT-2* (*UASmcd8GFP; UASDNT-2FL*) showed a significant increase in the dendrites volume compared to the wild type. Also, the shape of the dendric branching is more complex in the overexpression compared to the wild type **(Figure 5.8A-B')**.

Second, I knocked down *Toll-2* with *RNAi* alone (*UASToll-2RNAi*) and *Toll-2 RNAi* while overexpressing *DNT-2* FL to test the epistasis (*UASToll-2RNAi; UASDNT-2FL*). The knockdown of *Toll-2 RNAi* alone showed two different phenotypes: a mild and severe phenotypes.

In the mild phenotype, *UASToll-2RNAi* volume of the L1 dendrites is significantly reduced compared to the wild type **(Figure 5.9A-B')**. In the severe phenotype, the volume of L1 dendrites were also significantly reduce compared to the wild type. Moreover, in the severe phenotype the dendric branching of the knockdown appears to be less complex when compared to the wild type **(Figure 5.10A-B')**.

In the epistasis (*UASToll-2RNAi; UASDNT-2FL*), the dendrites volume of L1 neurons is significantly reduced compared to the wild type. However, it was not significant compared to *UASToll-2RNAi* alone suggesting an interaction between *DNT-2* and *Toll-2* **(Figure 5.9C- C')**.

The data suggests that *DNT-2* and *Toll-2* have important, potentially interacting roles in regulating the volume and complexity of L1 dendritic branching. When I overexpressed *DNT-2FL* in L1 dendrites, it led to a significant increase in dendritic volume and branching complexity compared to the wild type, indicating that *DNT-2* promotes dendritic growth and complexity. On the other hand, the knockdown of *Toll-2* using RNAi caused a reduction in dendritic volume in both mild and severe phenotypes, with a noticeable loss of dendritic complexity in the severe cases, suggesting that *Toll-2* is required for maintaining proper dendritic architecture.

The epistasis experiment, where I knocked down *Toll-2* while overexpressing *DNT-2FL*, showed a reduction in dendritic volume similar to the *Toll-2* knockdown alone. The lack of a significant difference between *Toll-2 RNAi* alone and the epistasis condition suggests that *DNT-2* cannot fully compensate for the loss of *Toll-2*. This points to a potential interaction between *DNT-2* and *Toll-2*, where both factors are involved in regulating dendritic structure, but *Toll-2* might be the dominant player in maintaining proper dendritic volume and complexity.

Figure 5.5 DNT-2 overexpression misrouted L1 neurons in the medulla

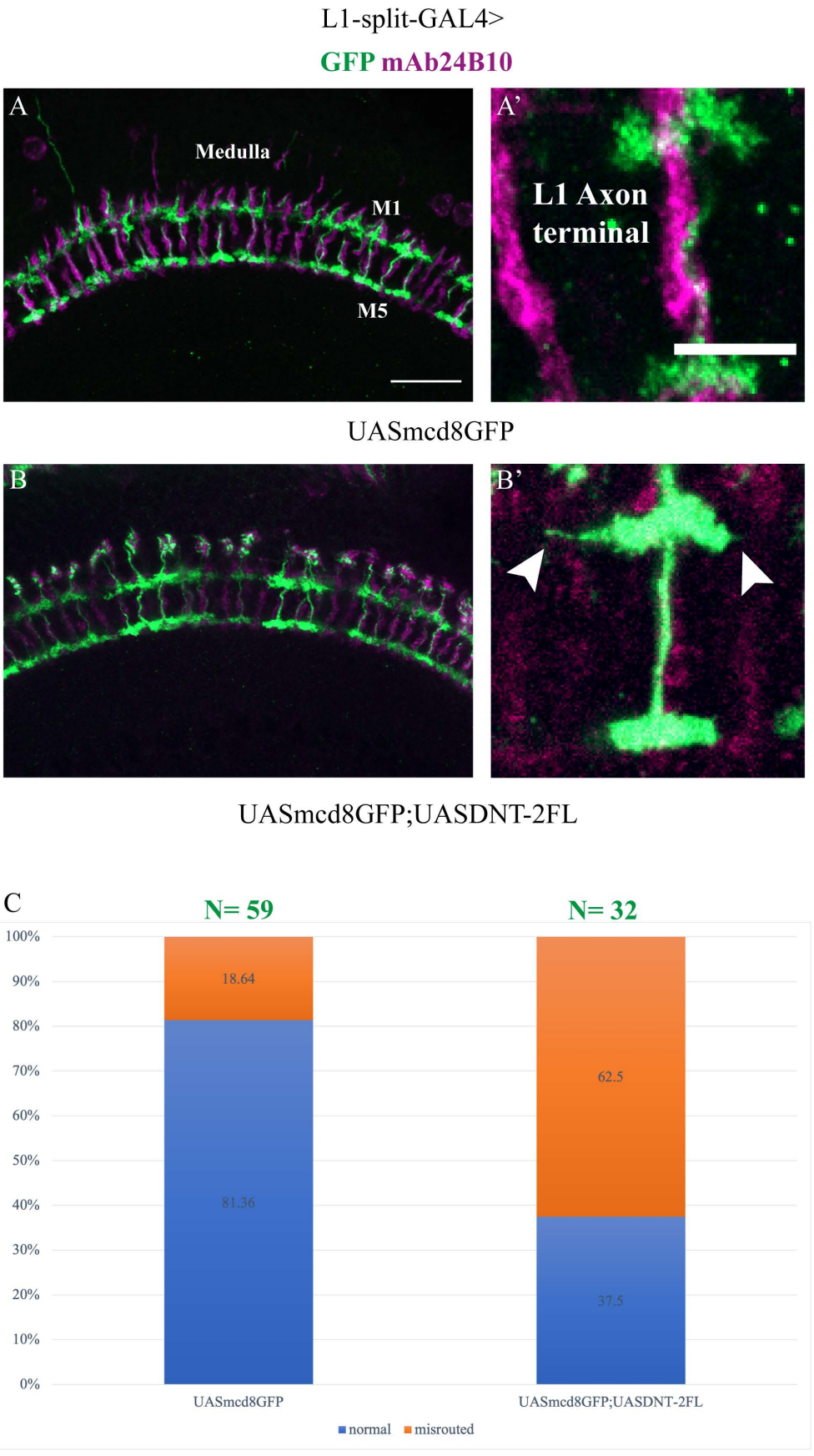


Figure 5.5 DNT-2 overexpression misrouted L1 neurons in the medulla

(A-A'). Lamina L1 neuron terminating in M1 and M5 medulla neurons, restricted to a single column in wild type control.

(B-B'). Lamina L1 neuron terminating in M1 and M5 medulla neurons, misrouted to a nearby column in the medulla in UASDNT-2FL overexpression.

(C). The graph shows the number of L1 neurons (Green), percentage of normal L1 neurons that are restricted to a single column (Blue) and the percentage of L1 neurons that are misrouted and crosses to a nearby column in the medulla (Orange). In DNT-2FL overexpression, there are more L1 neurons that are misrouted to nearby columns compared to wild type control. The number of brains scanned N=10 for each genotype. With each brain, 3-5 neurons were analysis. Fisher's exact test was used for this categorical data to identify the significance of L1 neurons bypassing the column in the medulla or no bypassing.

Genotypes:

A. R48A08AD;R66A01DBD>UASmcd8GFP.

B. R48A08AD;R66A01DBD>UASmcd8GFP;UASDNT-2FL.

Figure 5.6 DNT-2 and Toll-2 are required for L1 neurons routing in the medulla

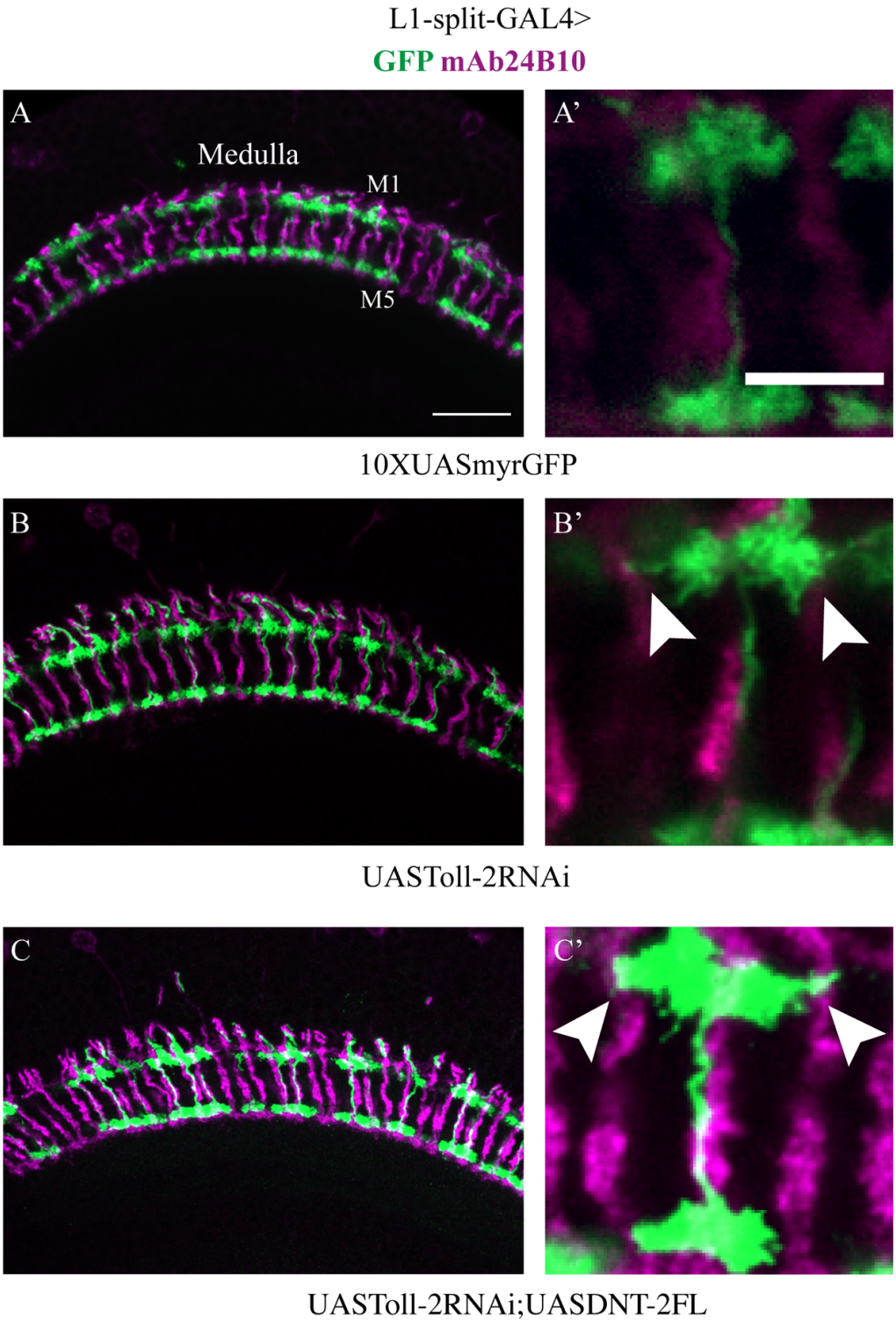


Figure 5.6 DNT-2 and Toll-2 are required for L1 neurons routing in the medulla

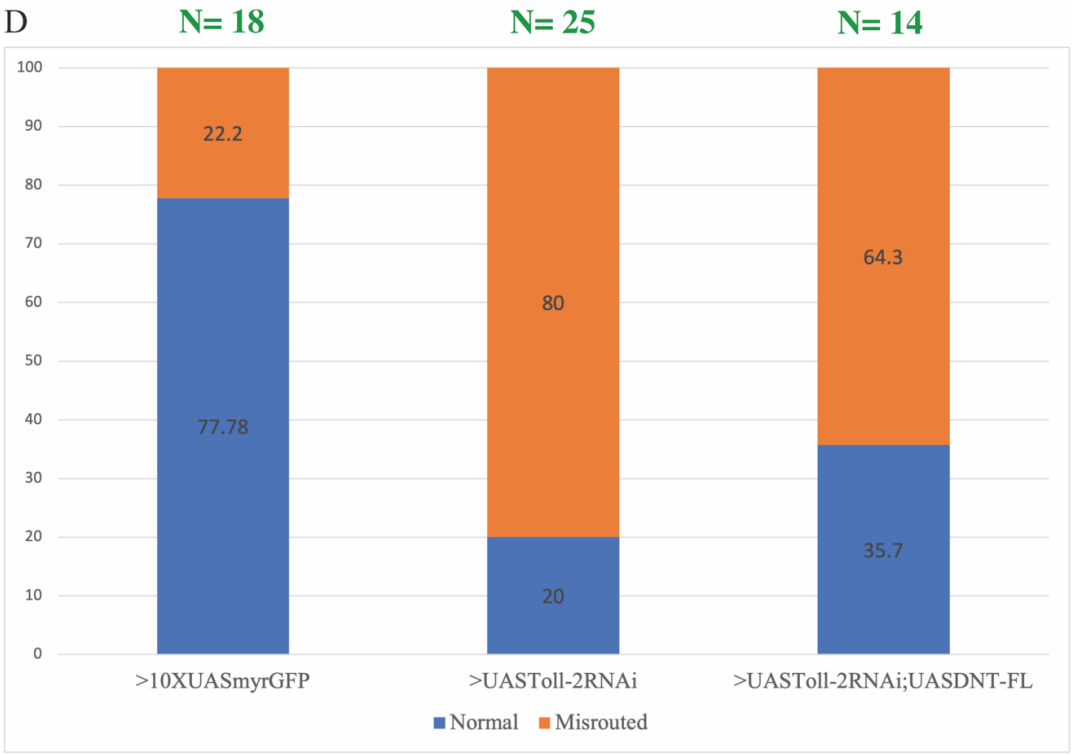


Figure 5.6 DNT-2 and Toll-2 are required for L1 neurons routing in the medulla

(A-A'). Lamina L1 neuron terminating in M1 and M5 medulla neurons, restricted to a single column in wild type control.

(B-B'). Lamina L1 neuron terminating in M1 and M5 medulla neurons, misrouted to a nearby column in the medulla in UASToll-2RNAi knockdown.

(C-C'). Lamina L1 neuron terminating in M1 and M5 medulla neurons, misrouted to a nearby column in the medulla in the epistasis showing similar phenotype to the UASToll-2RNAi alone.

(D). The graph shows the number of L1 neurons (Green), percentage of normal L1 neurons that are restricted to a single column (Blue) and the percentage of L1 neurons that are misrouted and crosses to a nearby column in the medulla (Orange). In both UASToll-2RNAi knockdown and epistasis (UASToll-2RNAi;UASDNT-2FL) more L1 neurons misrouted to a nearby column in the medulla compared to wild type control. The number of brains scanned N=10 for each genotype. With each brain, 3-5 neurons were analysis. Fisher's exact test was used for this categorical data to identify the significance of L1 neurons bypassing the column in the medulla or no bypassing.

Genotypes:

A. R48A08AD;R66A01DBD>10XUASmyrGFP.

B. 10XUASmyrGFP(X);R48A08AD;R66A01DBD> UASToll-2RNAi (KD).

C. R48A08AD;R66A01DBD>10XUASmyrGFP(X);UASToll-2RNAi;UASDNT-2FL (Epistasis).

Figure 5.7 L1 neurons die in UASToll-2RNAi severe phenotype

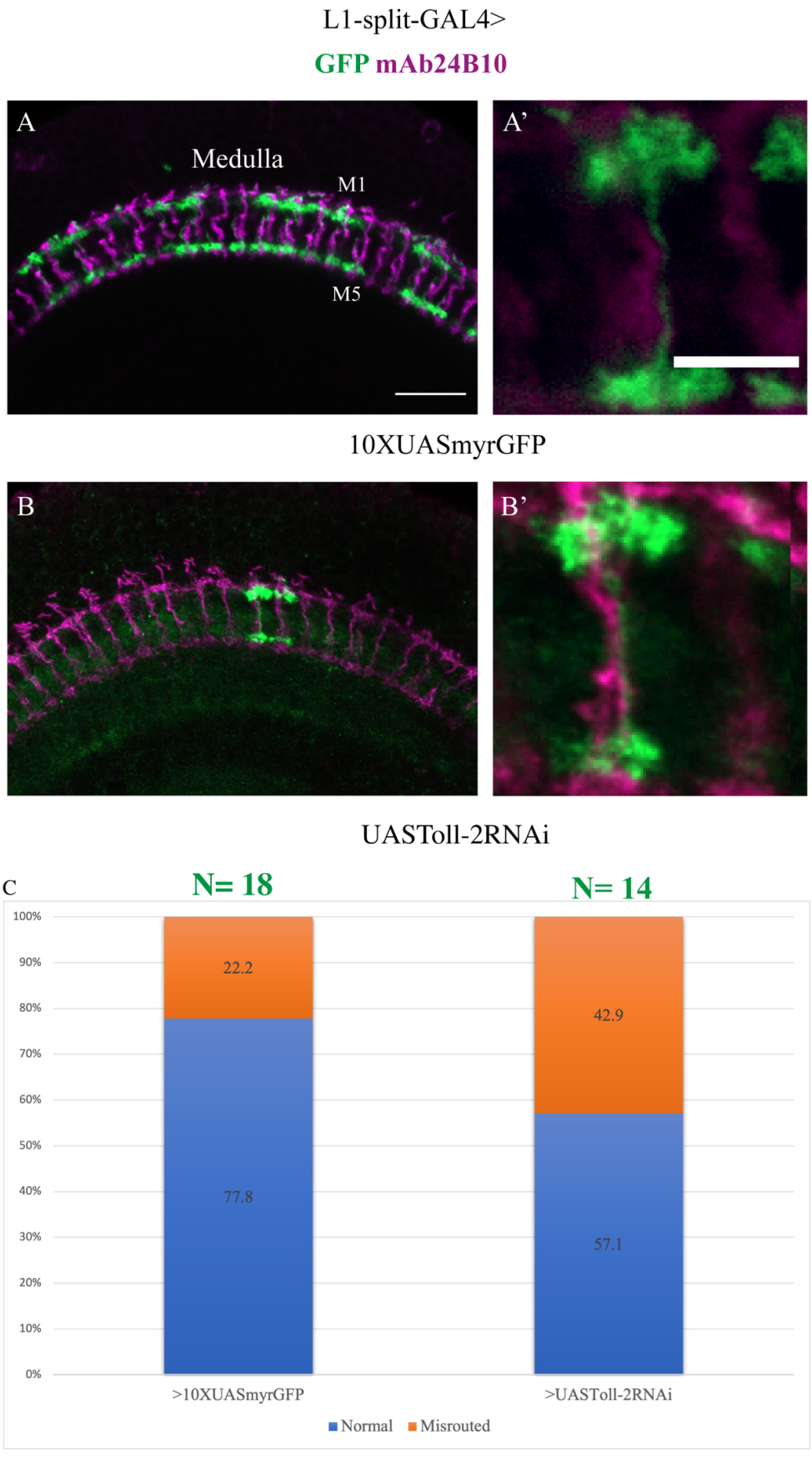


Figure 5.7 L1 neurons die in UASToll-2RNAi severe phenotype

(A-A'). Lamina L1 neuron terminating in M1 and M5 medulla neurons, restricted to a single column in wild type control.

(B-B'). Lamina L1 neuron terminating in M1 and M5 medulla neurons, in the UASToll-2RNAi severe phenotype, the L1 neurons that remain alive remain restricted to a single column in the medulla like that in wild type control.

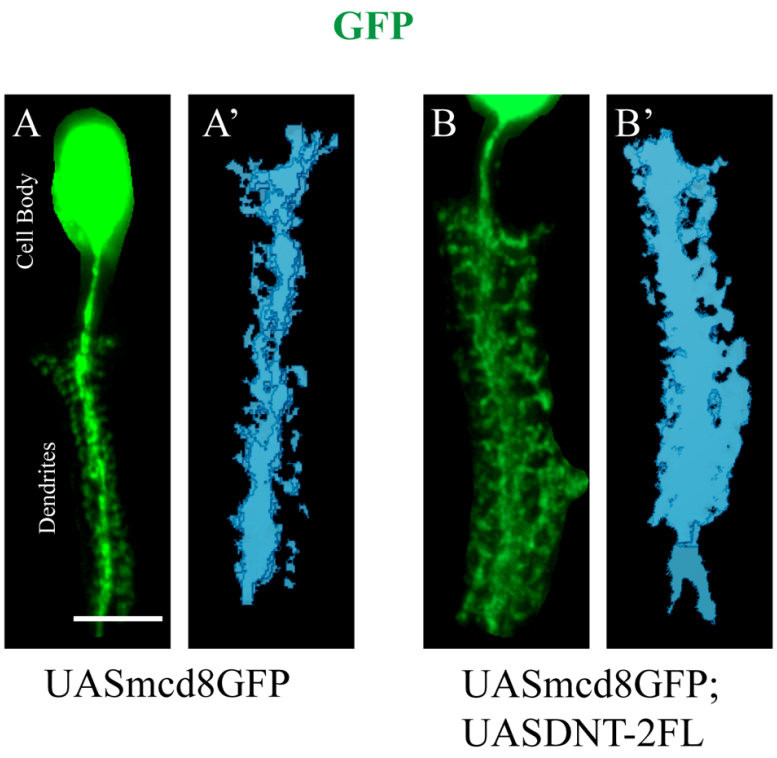
(C). The graph shows the number of L1 neurons (Green), percentage of normal L1 neurons that are restricted to a single column (Blue) and the percentage of L1 neurons that are misrouted and crosses to a nearby column in the medulla (Orange). In UASToll-2RNAi, more than 50% L1 neurons show similar phenotype to that in wild type control. The number of brains scanned N=10 for control and N=3 for the knockdown genotype. With each brain, 3-5 neurons were analysis. Fisher's exact test was used for this categorical data to identify the significance of L1 neurons bypassing the column in the medulla or no bypassing.

Genotypes:

A. R48A08AD;R66A01DBD>10XUASmyrGFP.

B. 10XUASmyrGFP(X);R48A08AD;R66A01DBD> UASToll-2RNAi (KD).

Figure 5.8 DNT-2 GOF increases L1 dendrite size



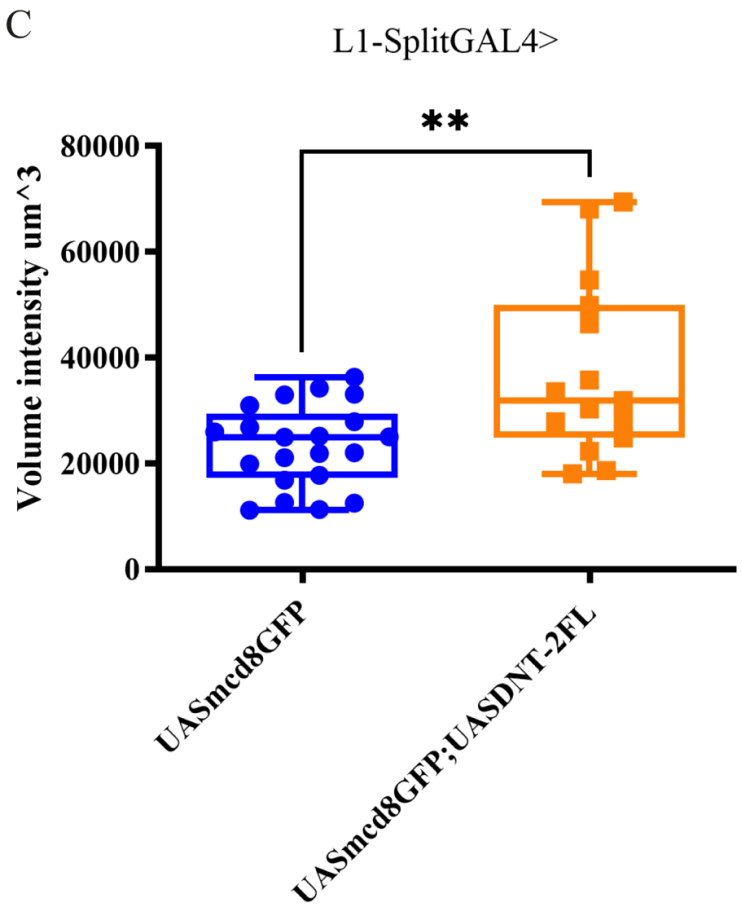


Figure 5.8 DNT-2 GOF increases L1 dendrite size

(A-A'). Lamina L1 dendrites in wild type.

(B-B'). Lamina L1 dendrites in UASDNT-2FL overexpression.

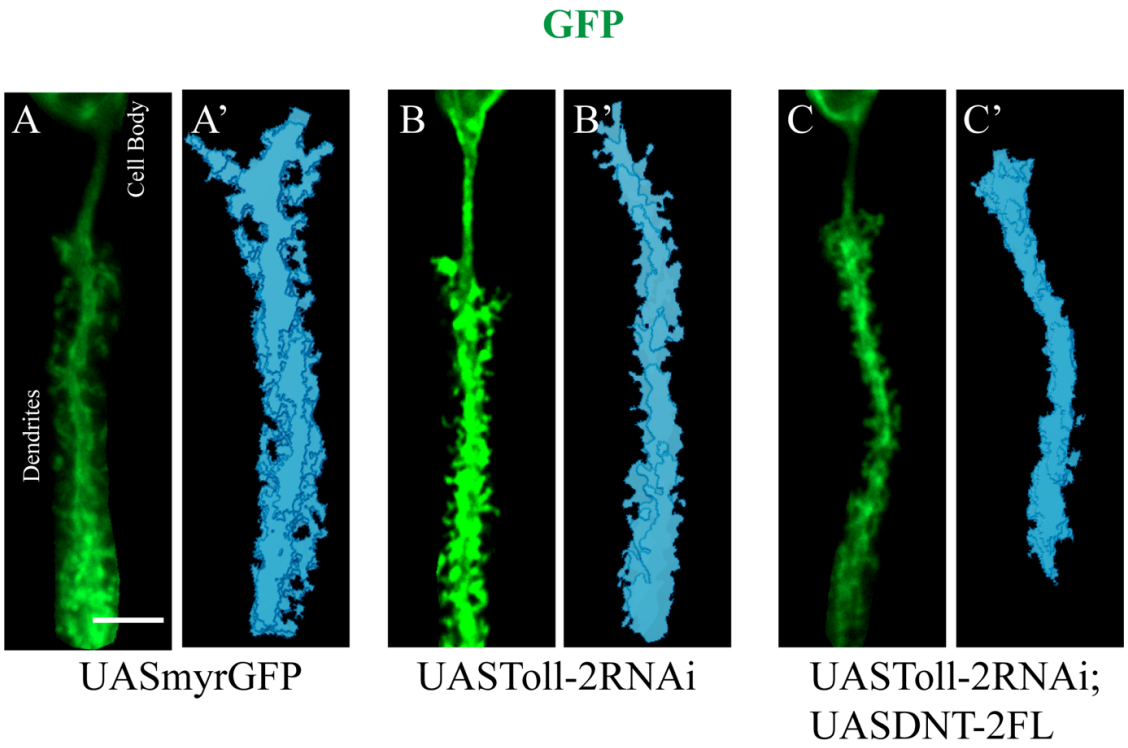
(C). Statistical analysis of L1 dendrites volume. There is a significant increase in dendrite volume in UASDNT-2FL overexpression compared to wild type control (Unpaired t test , two-tailed), $P \leq 0.01$, n= 21 (Control), n= 15 (UASDNT-2FL). The number of brains scanned for each genotype = 10 and for each brain 3 L1 dendrites were analaysed with Amira3D software. Number of repeats =2.

Genotypes:

A. R48A08AD;R66A01DBD>UASmcd8GFP.

B. R48A08AD;R66A01DBD>UASmcd8GFP;UASDNT-2FL.

Figure 5.9 DNT-2 and Toll-2 interact genetically to regulate L1 dendrite size



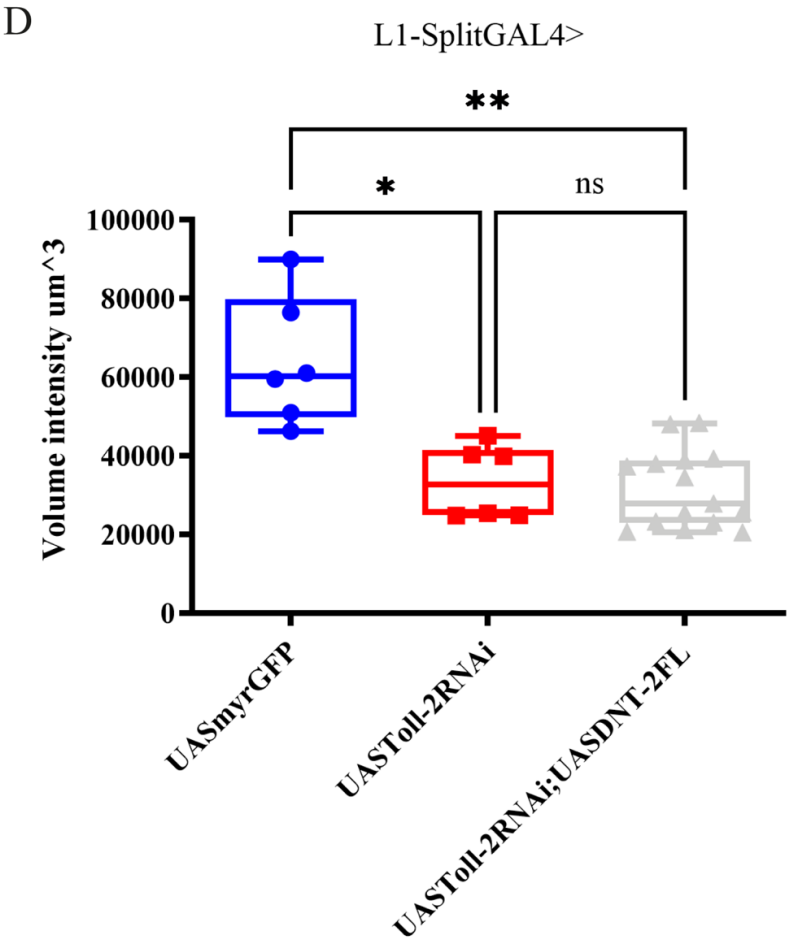


Figure 5.9 DNT-2 and Toll-2 interact genetically to regulate L1 dendrite size

(A-A'). Lamina L1 dendrites in wild type.

(B-B'). Lamina L1 dendrites in UASToll-2RNAi (KD) mild phneotype.

(C-C'). Lamina L1 dendrites in UASToll-2RNAi;UASDNT-2FL (Epistasis).

(D). Statistical analysis of L1 dendrites volume. There is a significant decrease in dendrite volume in both knockdown and epistasis compared to wild type control. However, there is no significant change in dendrite volume between the knockdown and epistasis (Kurskal-Wallis test), $P \leq 0.01$, n= 6 (Control), n= 6 (UASToll-2RNAi), n= 15 (UASToll-2RNAi;UASDNT-2FL). The number of brains scanned for each genotype = 10 and for each brain 3 L1 dendrites were analaysed with Amira3D software. Number of repeats =2.

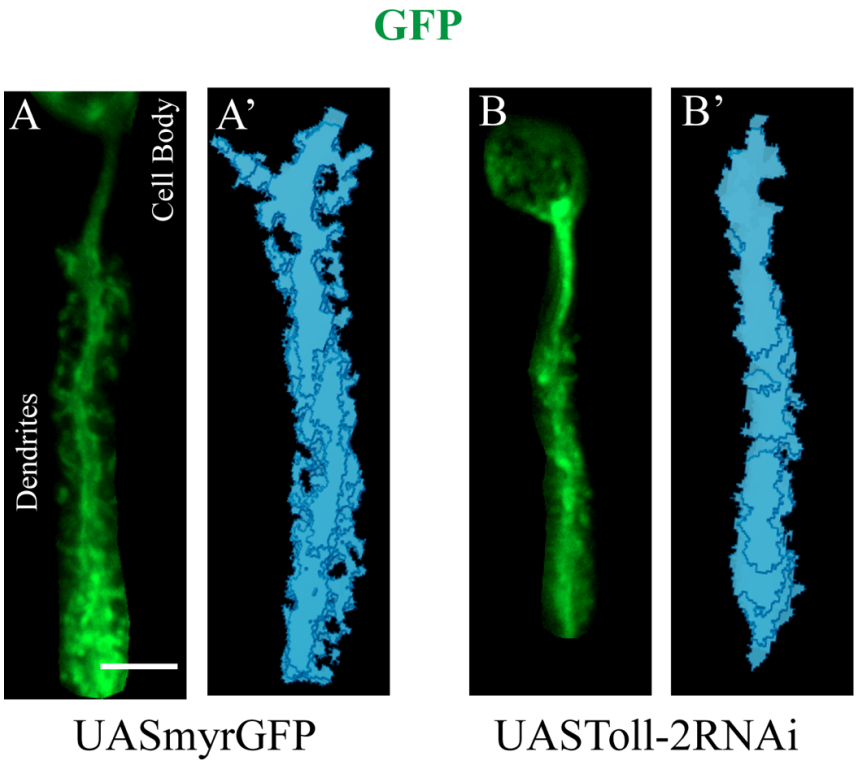
Genotypes:

A. R48A08AD;R66A01DBD>10XUASmyrGFP.

B. 10XUASmyrGFP(X);R48A08AD;R66A01DBD> UASToll-2RNAi (KD).

C. R48A08AD;R66A01DBD>10XUASmyrGFP(X);UASToll-2RNAi;UASDNT-2FL (Epistasis).

Figure 5.10 L1 dendrite size reduced in Toll-2 knockdown severe phenotype



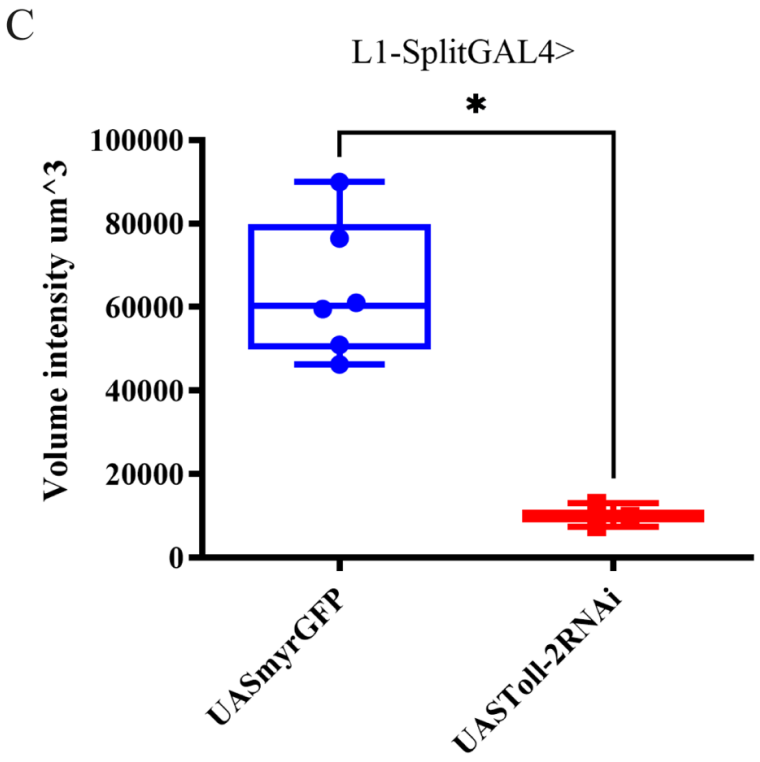


Figure 5.10 L1 dendrite size reduced in Toll-2 knockdown severe phenotype

(A-A'). Lamina L1 dendrites in wild type.

(B-B'). Lamina L1 dendrites in UASToll-2RNAi (KD) severe phenotype.

(C). Statistical analysis of L1 dendrites volume. There is a significant reduction in dendrite volume in the severe phenotype of UASToll-2RNA knockdown compared to wild type control (Mann-Whitney test, two-tailed), $P \leq 0.05$, n= 6 (Control), n= 3 (UASToll-2RNAi)The number of brains scanned for each genotype = 10 and for each brain 3 L1 dendrites were analaysed with Amira3D software. Number of repeats =2. .

Genotypes:

A. R48A08AD;R66A01DBD>10XUASmyrGFP

B. 10XUASmyrGFP(X);R48A08AD;R66A01DBD> UASToll-2RNAi (KD)

5.3 Discussion

In this chapter, I have shown that *spz3*-expressing neurons connect to different Toll-expressing neurons in the lamina with *trans-tango*. Moreover, data have shown that *spz3*-expressing neurons connect to multiple Toll-expressing neurons in the lamina such as *Toll-2* and *Toll-6* and not only *Toll-8* by using GRASP. Also, I have shown that overexpression of *spz3*FL increased neuronal numbers in the lamina. Additionally, data showed that *DNT-2* and *Toll-2* interact genetically to regulate L1 axonal terminal routing in the medulla as well as their dendrite size and shape in the lamina.

This section aimed to test whether Lawf1 neuron connectivity is being regulated by *spz3* or *Toll-8* during optic lobe development. The data have shown no significant changes in axonal terminal volume in the lamina across all genotypes. However, these data do not conclude the fact that these neurons are being affected.

One of the challenges in answering this question was with the stock itself (R52H01AD;R17C11DBD). The promoter in this stock was expressing lamina L3 neurons alongside Lawf1 neurons. When scanning the brains, L3 neurons would always be expressed across all genotypes with high abundance. However, Lawf1 neurons would sometimes be expressed and most of the time they won't be expressed. This variability could be attributed to several factors. For one, the differences in genetic backgrounds among the genotypes might be influencing the regulatory elements that control the split Gal4 system, leading to inconsistent activation of Lawf1 neurons. Additionally, the efficiency of the split Gal4 system itself may play a crucial role; if the fragments of the Gal4 transcription factor don't reassemble effectively in Lawf1 neurons, this could result in sporadic expression. Furthermore, there could be cell-type-specific factors at play perhaps

Lawf1 neurons require unique transcriptional co-factors or signaling cues that aren't consistently present across all conditions. Finally, environmental factors such as developmental timing might also contribute to this inconsistency, affecting the overall expression levels of Lawf1 neurons.

When Lawf1 neurons are being expressed, the volume intensity of their axonal terminals across genotypes is too variable. This meant that I had to collect a massive sample size to conclude. However, this was not an easy task due to the limitation of time and the rate at which Lawf1 neurons would be expressed in each dissected brain. Another challenge I came across during the analysis was that L3 neurons would overlap with Lawf1 neurons which made analyzing only Lawf1 axonal terminals more difficult. To avoid that, I only scanned brains that showed Lawf1 neurons distant from L3 neurons and that meant collecting a significant sample number is a limitation. These challenges could be solved in the future by using different approaches to answer the question. For example, the use of MARCM clones. Near the end of my PhD in the lab, I found a tool that uses a specific promoter to express Lawf1 neurons with MARCM clones. This method would allow me to visualize a single mutant Lawf1 neuron that will make analysis much simpler (Chen et al., 2016), but due to the limitation of time, I was not able to take that approach to tackle these challenges.

RNAi lines come with their limitations. The RNAi used in this experiment is UAS*Toll-2*RNAi**,** which was specifically designed to knock down *Toll-2* expression. The presence of two different phenotypes mild and severe could be attributed to variability in the efficiency of the RNAi knockdown, which can result from differences in Gal4 expression levels**,** tissue-specific variability, or stochastic gene silencing effects. These factors may cause the knockdown to be more effective in some cells than in others, leading to a range of

phenotypic outcomes.

To ensure the RNAi is working as intended, further verifications are required to test its effectiveness by confirming *Toll-2* knockdown through qPCR or Western blot to measure reduced *Toll-2* mRNA or protein levels, respectively. Additionally, consistent misrouting of L1 neurons in both the UAS*Toll-2*RNAi knockdown and the epistasis experiment suggest that the RNAi is effectively altering *Toll-2* levels, even if the phenotypic severity varies. The persistence of the misrouting phenotype across both conditions supports that *Toll-2* plays a key role in regulating L1 neuron axon guidance, regardless of the degree of knockdown efficiency. This also applies to the data showing the difference in the lamina dendrites of L1 neurons.

In conclusion, this chapter has shown that overexpression of *spz3*FL increased neuronal number in the lamina. However, more investigation is required to test whether the interaction between *spz3* and *Toll-8* regulate connectivity. Additionally, this chapter showed that the genetic interaction between *DNT-2* and *Toll-2* can regulate connectivity in the medulla and affect the dendrite size of L1 neurons.

Chapter 6

DISCUSSION

6.1 Summary of Findings

This project aimed to investigate whether *Drosophila* neurotrophins with Toll receptors regulate cell survival and connectivity in the developing optic lobe.

I showed that DNTs and Toll receptors are expressed in the visual system throughout the developing optic lobes. After drawing an expression map, my research focused on the interactions between spz3/Toll-8 and DNT-2/Toll-2 in regulating cell survival during connectivity in the developing visual system.

I first investigated the effects of altering spz3 and DNT-2 on cell survival and cell number. My data have shown that when knocking down these two ligands, apoptosis increased. Also, I have shown that overexpression of spz3 and DNT-2 reduced cell death. This suggests that spz3 and DNT-2 are required and can promote cell survival in the optic lobe at 24 hr APF. Additionally, I tested whether altering DNT-2 and Toll-2 could alter cell number at 48 hr APF. The data showed that when overexpressing DNT-2, the cell number of Toll-2 positive cells increased in the lamina. Also, when I knocked down DNT-2, There was a massive reduction in the number of Toll-2 positive cells in the lamina. These findings suggest that DNT-2 can regulate cell number and can interact genetically with Toll-2 as these DNTs can be promiscuous and bind different Tolls such as Toll-6 and Toll-7 (McIlroy et al., 2013). These findings add further evidence to those of Zhu et al 2008, McIlroy et al 2013, Foldi et al 2017, Li et al 2020 that neurotrophism is a fundamental mechanism of development. This means that if neurotrophism operates in the brains of arthropods and vertebrates, it is most likely a widespread mechanism across the animals, and a mechanism that was already present

in the common ancestor and used shared molecular mechanisms.

In addressing what controls developmental apoptosis at 24 hr APF and the relevance of apoptotic cells to neurotrophin-dependent neurons, it is important to consider both intrinsic and extrinsic factors that regulate cell death at this stage. Apoptosis at 24h APF is controlled by a balance between survival signals, such as neurotrophins like *Spz3* and *DNT-2*, and pro-apoptotic signals. My data demonstrate that knocking down *Spz3* and *DNT-2* results in a significant increase in cell death, whereas their overexpression reduces apoptosis, suggesting that these neurotrophins are crucial for promoting neuronal survival in the optic lobe at 24h APF. This likely occurs through interactions with Toll receptors, such as Toll-2 receptors, which modulate key downstream pathways like NF-κB and JNK.

In terms of how many of the apoptotic cells are neurons and their relevance to neurotrophins, it is important to consider that neurotrophins typically act on neurons by providing survival cues necessary to prevent apoptosis (Huang and Reichardt, 2001). My study suggests that a significant portion of the cells undergoing apoptosis at 24h APF are indeed neurons, particularly those that depend on neurotrophic signals like *Spz3* and *DNT-2* to survive. These neurons likely require neurotrophic support to withstand the developmental pruning process, which eliminates excess neurons while preserving those receiving appropriate survival signals. In the future, more evidence could be added to confirm that the dying cells are neurons by using anti-Elav staining, a neuronal marker and signal could be colocalised with the DCP-1 signal. These findings further emphasize the critical role of neurotrophins in preventing apoptosis during a key developmental window, reinforcing the notion that neurotrophic regulation of cell survival is an evolutionarily conserved mechanism.

Secondly, I then investigated the effects of altering spz3/Toll-8 and DNT-2/Toll-2 on the connectivity of Lawf1 and L1 neurons. When altering spz3/Toll-8, I could not find any

evidence to conclude that altering these interactions could affect the connectivity of Lawf1 neurons as further investigation is required to confirm that. However, when altering DNT-2/Toll-2, lamina L1 neurons exhibited connectivity defects. In the lamina, the dendrites of L1 neurons changed morphology and size when overexpressing DNT-2 and knocking down Toll-2. This shows that the L1 dendrites can be plastic and change their size and shape as required. Also, I have shown that when altering DNT-2/Toll-2, L1 axonal terminals in the medulla were misrouted and established connections in nearby columns where they are not supposed to be. These findings provide evidence that DNT-2 interacts genetically with Toll-2 regulates the connectivity of L1 neurons. To address how DNT-2/Toll-2 potentially regulates L1 neuron connectivity at the molecular level compared to known mechanisms controlling L1 neuron axon targeting, it is important to consider both neurotrophic signaling and established axon guidance cues. DNT-2, as a neurotrophin, likely interacts with Toll-2 to activate intracellular signaling pathways, such as NF-κB or JNK, which are known to regulate processes like neuronal survival, synaptic plasticity, and cytoskeletal dynamics. These pathways could influence structural changes in L1 dendrites and axons, such as branching and size alterations, that I observed in my experiments. The plasticity is seen in L1 dendrites suggest that DNT-2/Toll-2 signaling plays a critical role in modulating the growth and targeting of neurons, potentially influencing axonal routing in the medulla.

In contrast, well-established mechanisms for L1 axon targeting, such as Slit/Robo, Semaphorins, and Netrins, provide spatial cues for guiding axons to their appropriate destinations during development. While these guidance cues are responsible for the initial pathfinding of L1 neurons, it is likely that DNT-2/Toll-2 signaling functions at a later stage, refining or stabilizing these connections by supporting synaptic formation and preventing misrouting. My data, show that altering DNT-2/Toll-2 leads to misrouted axonal terminals and dendritic changes suggest that this signaling is crucial for the fine-tuning of

connectivity in L1 neurons after the initial axon guidance has occurred. This highlights the complementary role of neurotrophin signaling via Toll receptors in regulating both the structural plasticity and final connections of neurons in the developing visual system.

There have been many studies in the mammalian visual system showing evidence that neurotrophism operates in the visual system's development (Marshak et al., 2007; Sanchez et al., 2006). It has been shown that BDNF can increase synapse density in dendrites of developing tectal neurons in the developing visual system of Xenopus (Sanchez et al., 2006). They showed that increasing the levels of BDNF in the optic tectum significantly increased both axon arborization and synapse density per axon terminal with a few hours of treatment (Sanchez et al., 2006). In another study, it has been documented that the cell-autonomous TrkB receptor signalining in presynaptic ganglion cells mediates axonal arborization and synapse maturation during the retinotectal synaptic connectivity . They showed that disruption of TrkB signaling in individual RGCs influenced The branching and synaptic maturation of presynaptic axon arbors (Marshak et al., 2007). Their findings suggest that the presynaptic TrkB signaling in RGCs is a key determinant in the establishment of visual connectivity (Marshak et al., 2007). This is important because this was precisely the context in which the chemoaffinity hypothesis of Sperry was originally proposed and provides evidence that neurotrophism operates in visual system connectivity.

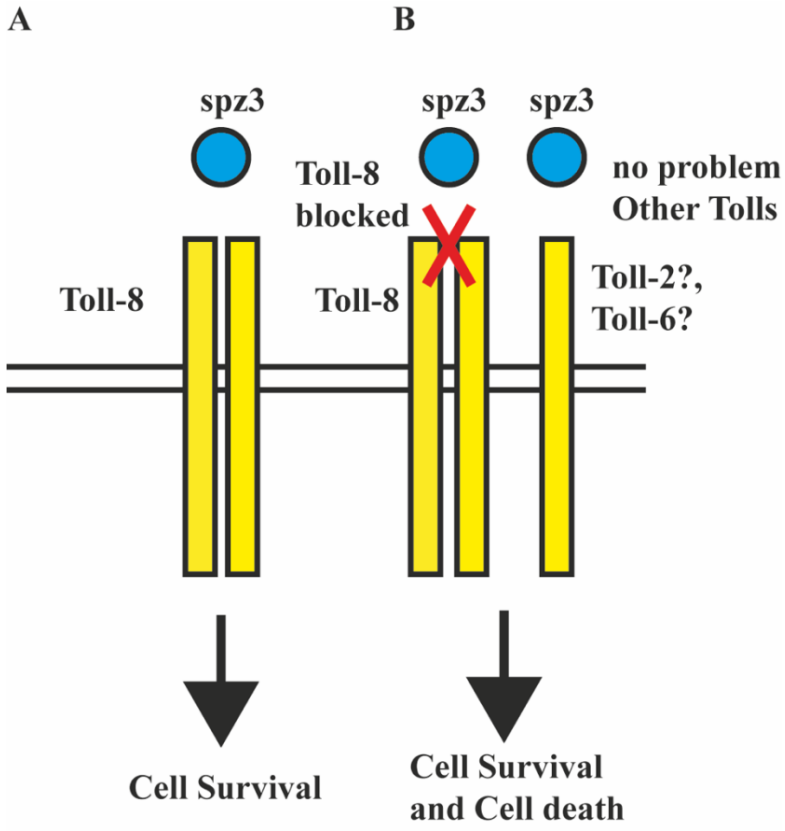
6.2 The regulation of cell survival and number in the developing optic lobe.

During nervous system development, there is an overproduction of neurons that are being produced in excess (Davies, 2003). Neurons that do not form connections are eliminated by apoptosis to regulate the correct number of neurons required for normal function. Understanding the mechanism and the vital role of cell death is important for understanding

the development of the nervous system. In the *Drosophila* optic lobe, many of these neurons are eliminated during development (Fischbach and Technau, 1984; Togane et al., 2012). The aim of this part of my PhD was to investigate whether the interactions between spz3/Toll-8 and DNT-2/Toll-2 regulate the survival of neurons in the developing optic lobe at 24 hr APF. Also, the interactions between DNT-2/Toll-2 in regulating cell number at 48 hr APF. While there are multiple studies supporting neurotrophism or the release of growth factors by glia in controlling cell survival in the visual system (Coutinho-Budd et al., 2017; Foldi et al., 2017; McIlroy et al., 2013).Others argue that these events do not require the existence of survival cues, such as neurotrophins and other growth factors that regulate cell survival during development (Agi et al., 2024; Campos et al., 1992; Malin et al., 2024). It has been documented that cell death in the developing optic lobe happens in a specific spatiotemporal pattern (Togane et al., 2012). This cell death can occur and go through two different phases. The first phase starts at the beginning of metamorphosis up until 48 hr APF. Most neurons die in this phase peaking at 24 hr APF and slowing down at around 48 hr APF. The second phase starts from 48 hr APF up until eclosion. In this phase, there is only a few cells that die (Togane et al., 2012). In a previous study, there was an investigation regarding cell death in the optic lobe and whether it was dependent on the insect hormone ecdysone. Ecdysone is an insect steroid hormone that has a role in larval development and metamorphosis (Hara et al., 2013). In this study, they document the roles of two ecdysone isoforms EcR -A and EcR-B1, in regulating cell survival in the developing optic lobe (Hara et al., 2013). They show that the number of dying cells at 24 hr APF depends on EcR-B1. Mutant animals for EcR-B1 showed less cell death at 24 hr APF compared to the wild type suggesting that cell death in the optic lobe at 24 hr APF required EcR-B1. Moreover, they test mutant animals for EcR-A, and they saw no change in the number of dying cells at 24 hr APF suggesting that EcR-A is not required at this stage to regulate cell death (Hara et al., 2013). Although the claim in this

study suggests the direct involvement of ecdysone in controlling cell death; they also argue that the ecdysone isoform EcR-B1, which is responsible for cell death at 24 hr APF, is not the decision maker for the optic lobe cell death. They saw no direct relationship between the expression of EcR-B1 and the emergence of cell death. Even though cell death at 24 hr APF required EcR-B1, its expression at this stage was weak in the lamina cortex. All their findings suggest that cell death decision is not related to the expression levels of EcR-B1, but they depend on the switch between ecdysone-dependent cell death and ecdysone-independent cell death (Hara et al., 2013).The data in Chapter 4 regarding the regulation of cell survival suggests that cell survival is promoted by *Drosophila* neurotrophins. The data shows that when knocking down spz3 and DNT-2 in the lamina at 24 hr APF, cell death increased compared to wild-type animals. Also, when overexpressing spz3 and DNT-2, the levels of dying cells were reduced significantly. These findings suggest that in the developing optic lobe neurotrophins are required to regulate cell survival at 24 hr APF. *Drosophila* neurotrophins work via Toll receptors and the data showed that DNT-2 interacts genetically with Toll-2 to promote cell survival and that spz3 works via Toll-8 and possibly other Tolls to promote cell survival in the optic lobe **(Figure 6.1)**. It has been documented that Toll-6, the known receptor for DNT-2 can promote cell survival via the MyD88-NF-kB pathway in the embryonic VNC and cell death via Wek-Sarm-JNK pathway in the pupal VNC (Foldi et al., 2017; McIlroy et al., 2013). These findings suggest the dual role of neurotrophins in regulating both cell survival and cell death via different pathways in different contexts during nervous system development. Also, this suggests that it is possible that Toll-2 goes through a similar pathway as Toll-2 to promote cell survival, as shown in my data (see Chapter 4). In fact, Toll-2 does work via the MyD88-NF-kB pathway to promote cell survival quiescence and knock-down of Toll-2 causes apoptosis in the pupal brain and loss of MyD88+ neurons (Li et al., 2020).

Figure 6.1 spz3 interacts genetically with Toll-8 and other Tolls



(A). Schematic diagram of how spz3 promotes cell survival via Toll-8

(B). Schematic diagram of how spz3 can promote cell survival by working with other Tolls in the case of Toll-8 KD with RNAi. The epistasis data (see chapter 4) suggests that spz3 can work via Toll-8 as well as other Tolls to regulate cell survival in the lamina and medulla.

When considering the tissue-specific roles of the Toll-2 and Toll-6 receptors their activities seem to be tissue and stage-specific. For example, during embryonic development, Toll-6 is important in the VNC, causing cell survival via the MyD88-NF-κB pathway, however in immature pupal VNC the receptor later causes cell death through a different pathway which is Wek-Sarm-JNK pathway (Foldi et al., 2017; McIlroy et al., 2013). This kind of mediation of life and death and its sign in the context of development rather speaks for the secured nature of the signaling of the neurotrophins. My data suggests that Toll-2 acts in the same manner, although in this case, my context is within the developing optic lobe. Such an event seems to be the case where 24 hr APF Toll-2 is suggested to activate so that upon the knockdown of this protein, the level of apoptosis increases. Taken together with the evidence of the interaction of DNT-2 with the receptor Toll-2 provides another factor for controlling neuronal survival in the optic lobe through these neurotrophins. It can be considered that Toll-2 may use the identical MyD88-NF-κB network as Toll-6 as it was shown that Toll-2 does work via that pathway (Li et al., 2020). These findings provide confidence that even though Toll-2 and Toll-6 had highly conserved mechanisms, their functions were used for other needs of the tissues in which they were expressed.

In the optic lobe, Toll-2 is critical in controlling the number and organization of neurons, while Toll-6 is more involved in neurogenesis and apoptosis in the VNC. This tissue-specificity is a reflection of the neurotrophin and Toll receptor interactions in the context of development and cellular environment.

Although there is no direct evidence suggesting that DNT-2 binds directly to Toll-2, previous studies have shown that DNT-2 can be a promiscuous ligand that can bind at least both Toll-6 and Toll-7, and also multiple Keks, at least Kek-6 and Kek-2 (McIlroy et al., 2013; Ulian-Benitez et al., 2017). While there is no structural evidence at present which can conclusively prove that DNT-2

binds to Toll-2, DNT-2's promiscuous nature indicates that its structure has the versatility required to interact with several different receptors, like Toll-6, Toll-7 and the Kek family (McIlroy et al., 2013; Ulian-Benitez et al., 2017). The members of Toll receptor family are known to have several LRR domains which are crucial for the recognition of ligands and therefore for the activation of the receptors. Like all neurotrophins DNT-2 may also have particular, but so far unidentified, sites that could recognize one or more of the LRR domains present in multiple Toll receptors. It is also possible that DNT-2 flexibility in binding various receptors is due to the fact that Keks have common structural features or motifs with Toll-2, Toll-6 and Toll-7 which have LRR domains. Furthermore, the described ability of DNT-2 to bind with several Keks supports the idea that this neurotrophin can adjust its binding with various families of receptors due to the structural compatibilities. Such promiscuity may provide DNT-2 an opportunity to cause context-dependent signals that can either be anti-apoptotic or cause an apoptosis state depending on the specific receptor.

Building on the findings of Hara et al 2013 on ecdysone's function in inducing cell death in the optic lobe by investigating *Drosophila* neurotrophins such as spz3 and DNT-2 and their interactions with Toll receptors to promote cell survival. Hara et al 2013 have shown that the steroid hormone ecdysone, especially the EcR-B1 isoform, provides regulation to apoptosis at 24 hr APF (Hara et al., 2013). In this regard, my data indicate a different pathway that is not regulated by Ecdysone but involves neurotrophin signalling through Toll receptors. In the course of that investigation, the requirement of EcR-B1 for cell death was also demonstrated, while it was predicted that EcR-B1 could never be the sole determining factor due to its weak level of expression in the lamina cortex (Hara et al., 2013). It implies that Ecdysone is not the only factor that regulates cell death during that time in development.

My research complements this by arguing that spz3 and DNT-2 act through Toll-8 and Toll-2, which are important factors for the survival of cells located in the optic lobe at 24 hr APF. Spz3 and DNT-2 knockdown resulted in increased cell death, while overexpression of these ligands resulted in the depletion of dying cells. This indicates, together with ecdysone-

dependent mechanisms, that neurotrophic factors are important in the maintenance of neurons in the developing nervous system. Most importantly, however, from the findings of my experiments, I was also able to demonstrate that DNT-2 interacts genetically with Toll-2 furthering the complexity of the interactions that regulate cell survival and apoptosis.
From the combined ecdysone and neurotrophins studies, both embryonic and developmental reduction of the cell population in the optic lobe is mentioned as the result of many regulatory mechanisms acting in harmony. Ecdysone signalling may be responsible for triggering and or controlling the timing of cell apoptosis, but neurotrophins acting through Toll receptors, in this case, may be important survival signals that maintain adequate amounts of neurons for this developmental stage. I do not only endorse the idea that there is a dual regulatory mechanism present which is ecdysone dependent and independent of neurotrophins, but I have also introduced Toll-2 and DNT-2 as important molecular tools in the optic lobe's development.

In a recent study, it has been reported that a certain type of glial cells referred to as xgO located proximal to the lamina, releases two ligands (Spi, Col4a1) which activates MAPK signalling pathway to control the correct number of L5 lamina neurons and eliminate unwanted ones (Prasad et al., 2022). This activation is vital for neuronal survival and differentiation. The study shows that LPCs that fail to activate MAPK signalling pathways do not differentiate and are eliminated by apoptosis (Prasad et al., 2022). This suggests that the survival of these cells is dependent on the growth factors released by Glial cells control the survival of the L5 lamina neuron. This mechanism in which cell number is regulated during development, supports the idea of the findings of my data regarding the requirement of growth factors as survival cues in regulating cell survival and cell number in the developing visual system (Prasad et al., 2022). In a different study, it has been suggested that spz3 functions autonomously in cortex glia to regulate cell survival. They have shown that the loss of spz3 included a loss of glial ensheathment of neuronal cell bodies that increased neuronal cell death (Coutinho-Budd et al., 2017). These data provide evidence that spz3 is involved in regulating cell survival, as shown in my results. However, there was no evidence in my results showing the influence of spz3 on glial cells.

This could be tested with anti-Repo, a marker that labels glial cells and the number of glia can be measured by the Deadeasy plugin.

In a previous study involving the survival of lamina neurons, it has been reported that Jeb produced by R1-R6 axons interacts with the receptor Alk to control the survival of lamina L3 neurons (Pecot et al., 2014). Jeb is a secreted protein produced by R cell growth cones in the *Drosophila* visual system, acting as a ligand for the Anaplastic Lymphoma Kinase (Alk) receptor, a receptor tyrosine kinase expressed in the optic lobe (Pecot et al., 2014). The Jeb/Alk signaling mechanism involves Jeb secretion from R1-R6 photoreceptor growth cones in the lamina, binding to Alk receptors on budding L3 dendrites, and activating Alk to promote L3 neuron survival during a critical developmental period (Pecot et al., 2014). This anterograde signaling mechanism is relevant to neurotrophins in several ways. Like neurotrophins, Jeb acts as a trophic factor regulating neuronal survival during development, but unlike classical neurotrophins which often act retrograde, Jeb functions anterogradely. While Jeb bears no significant homology to fly or vertebrate neurotrophins, its receptor Alk is distantly related to vertebrate neurotrophin receptors Trks (Pecot et al., 2014). Unlike *Drosophila* neurotrophins (DNT1, DNT2, and Spatzle) that act through Toll-like receptors, Jeb/Alk signaling involves a receptor tyrosine kinase (Pecot et al., 2014). This research demonstrates that anterograde trophic signaling can play a crucial role in neural circuit assembly, complementing our understanding of retrograde neurotrophin signaling and highlighting the diverse mechanisms of trophic signaling in neural development across species.

The interactions between Jeb and Alk are essential for the survival of L3 neurons during the developmental stages at around 20 to 40 hr APF, where the Most cell death occurs (Pecot et al., 2014; Togane et al., 2012). Upon the manipulation of Jeb/Alk L3 neurons face the fate of cell death by apoptosis. This was confirmed when further investigation was done, including the expression of p35 in the lamina, was able to rescue the survival of L3 neurons lacking Jeb/Alk, suggesting that this pathway directly influences the survival of these lamina neurons. These findings are another example on how cell survival requires the interactions between a ligand and a receptor at a specific time point

during development to regulate these survival events (Pecot et al., 2014). I have shown in my data that the interactions between DNT-2 and Toll-2 in the lamina are required for the survival of lamina L1 neurons. When knocking down Toll-2 receptor with RNAi, the majority of L1 neurons died in the severe phenotype **(Figure 5.7)**. The death of most neurons during the severe phenotype of L1 was due probably to the function of Toll-2 as a receptor important for cell survival following RNAi knockdown of the Toll-2 receptor. I have also shown in my study that DNT-2 a possible ligand for Toll-2 genetically interacts with Toll-2 to aid in the survival of the neurons. This interaction is lost whenever Toll-2 is knocked down such that the activation of cell survival pathways such as MyD88-NF-kB pathway have been reported to promote the survival of the cell cannot take place (Li et al., 2020). Without this pathway, the neurons are more susceptible to apoptosis, which explains the high degree of cell death in the severe phenotype. In this case, Toll-2 may be essential for preventing programmed cell death in L1 neurons, and its absence causes widespread apoptosis.

Since DNT-2 is expressed in the retina at 72 hr APF and in the lamina at 24 hr APF (see Chapter 3), and I have shown that DNT-2 interacts genetically with Toll-2 (see Chapter 4), this suggests that when knocking down Toll-2 with RNAi, the receptor is not capable of receiving the ligand DNT-2 from the retina or lamina to activate the downstream signalling pathways to promote the survival of L1 neurons **(Figure 6.3)**. These findings together with the findings of Pecot et al suggest a similar mechanism by which Jeb/Alk are regulating the survival of L3 neurons.

The use of different sets of ligands and receptors for various L neurons in the *Drosophila* visual system is crucial for achieving precise and selective control over the development and survival of specific neuronal subtypes (Pecot et al., 2014; Timofeev et al., 2012). This specificity enables fine-tuned regulation of different neuronal subtypes within the same circuit, allowing for the independent control of functionally diverse neurons involved in distinct aspects of visual processing, such as ON and OFF motion detection circuits and color processing pathways (Pecot et al., 2014; Takemura et al., 2013). The employment of distinct signaling systems also facilitates precise temporal and spatial control over neuronal development, as exemplified by the Jeb/Alk signaling in L3 neurons during a

specific developmental window and location (Pecot et al., 2014). Furthermore, the sequential action of different ligand-receptor pairs, like Jeb/Alk followed by Netrin/Frazzled, enables the coordination of circuit assembly across various regions of the visual system, ensuring that neurons in connected areas develop in a synchronized manner (Gao et al., 2008; Pecot et al., 2014; Takemura et al., 2013; Timofeev et al., 2012). This strategy likely represents an evolutionary adaptation to meet the specific developmental needs of distinct neuronal subtypes, providing greater flexibility and precision in circuit assembly (Pecot et al., 2014). By utilizing this approach, the *Drosophila* visual system can achieve the complex and precise wiring necessary for proper visual function, a mechanism that probably extends to other neural circuits and organisms, offering a general strategy for coordinating the development of diverse neuronal subtypes within intricate neural networks.

In a more recent study, Malin et al 2024 suggest that cell death plays a limited and minor role in the regulation of neuronal number in the visual system (Malin et al., 2024). The limited role of apoptosis in their argument suggests a different mechanism in which neuronal number is controlled, such as spatial patterning and morphogenetic signaling through the Dpp/BMP which provides a second layer of pattering that allows the control of cell proportions. They also argue that the initial regulation of cell number is established earlier and influenced by spatial patterning rather than regulated by trophic support (Malin et al., 2024). This however does not contradict the involvement of trophic support in regulating cell number in the optic lobe, but rather suggests that in the visual system, the role of trophic factors in cell death is less prominent than the spatial patterning, meaning that the control of cell survival via spz3 and DNT-2, as shown in my results, does not take full responsibility for guiding the full process of developing the nervous system. It should be noted that Malin et al 2024 state that their study allows for a relatively minor role of apoptosis in the control of cell number in the visual system, and then discuss the data to see if it supports or argues against their conclusions. In their scenario, they predict that morphogenic signals such as Dpp/BMP will structure the cells numbers more effectively. But my observations in which DNT-2 and spz3 are shown to interact genetically with Toll-2 and Toll-8 to enhance survival of cells in the optic lobe do not challenge but rather extend on the hypothesis. They argue, and rightly so,

that because more is known about the development of spatial patterning it is that which has a greater influence. However, my data would reflect that at later times during development neurotrophins like DNT-2 together with spz3 do provide some degree of trophic support. My results thus do not contradict their findings, but postulate a slightly different, yet involving, mechanism where trophic support through neurotrophins, such as DNT-2 and spz3, along with spatial patterning assist in the regulation of cell numbers, or survival of neurons in the optic lobe.

The major difference between my results and the ones provided by Malin et al 2024 is the range of developmental processes that were studied. My study investigated the 24 hr APF in the pupa, which marks a time of great intracellular remodeling and development of the optic lobe. By contrast, Malin et al 2024 looked at the L3W stage which is much earlier and characterized during development by active spatial patterning and cell division. This difference in the level of development could account for the differences in opinions concerning the role played by apoptosis.

Malin et al 2024 went further to suggest that, primitive signaling mechanisms such as the Dpp/BMP signals may be the only indicators at the L3W larval stage (Malin et al., 2024). However, the perspective shifts on the role of certain factors like DNT-2 and spz3 at the 24 hr APF pupal stage as greater emphasis is put on cell viability during the establishment of neuronal circuitry.

My results strongly suggest that the genetic interrelations between DNT-2/Toll-2 and spz3/Toll-8 are important for the promotion of cell survival at this later stage, which indicates that while the formation of spatial patterning might determine the initial conditions of the number of cells, concentration of the trophic factors becomes more critical during pupation in order to control the number of neurons relative to the volume of the brain that is changing dramatically.

This variation in the relative chronological order of the two events suggests that enhancement of spatial patterning and concentration of trophic support are required but do not occur in parallel as DNT-2 and spz3 in my studies were shown to be predominantly active in the later stages of neurodevelopmental processes while earlier stages saw spatial patterning as the driving force.

Overall, while apoptosis may be vital for fine-tuning neuronal numbers, it is not the primary mechanism for regulating neuronal number. Instead, there are different mechanisms that could be responsible for regulating cell survival and cell number during development. The findings in Malin et al. 2024, provide additional evidence of how complex biological systems are. The development of the complex nervous system does not depend on one single mechanism, in my opinion, but rather work collectively through different mechanisms, as redundancy is a key mechanism to provide robustness during nervous system development. In particular, the data showing that both DNT-2 and spz3 promote cell survival through genetic interactions with Toll-2 and Toll-8 respectively, suggests a form of redundancy. My research also shows the ability for redundancy in the regulation of neurotrophic receptors and the number of neurons due to the interaction of neurotrophins with their receptors. This is evident when considering that different Toll receptors Toll-2 and Toll-8 can mediate similar survival functions via their respective ligands, DNT-2 and spz3. Despite employing different receptors, these ligands perform similar functions in the end, which is enhancing survival of the developing optic lobe cells.

In addition, the promiscuity of DNT-2, which is reported to bind to other receptors (Toll-6 and T-7) and other Kek receptors, supports further the idea of redundancy in signaling pathways (Foldi et al., 2017; McIlroy et al., 2013; Ulian-Benitez et al., 2017). DNT-2's capability to engage with various receptors implies that if one receptor's pathway is altered, the receptor’s pathway would still develop through other routes.

These findings moreover tell us that redundant mechanisms, while their presence is not guaranteed, are indeed important and should be expected in order to achieve robustness during the development of the nervous system, especially at times when the neural circuits are being fine-tuned, as is the case at 24 hr APF. In this case, my data support the conjecture that several overlapping mechanisms, like neurotrophin signaling through distinct receptors, coordinate the survival of cells and the number of neurons in development preventing the failure of one of the pathways.

6.3 The regulation of connectivity in the developing optic lobe.

During nervous system development, the formation of neural circuits requires the axons to find their appropriate targets and form synaptic connections with them to end up with a functional nervous system (Hakeda-Suzuki and Suzuki, 2014). In developmental neurobiology it has been debated whether limiting amounts of trophic factors are necessary to guide neurons to their targets (Levi-Montalcini, 1987), or whether the molecular cues that are located on the pre and postsynaptic targets are sufficient to guide them (Gillespie, 2003; Sperry, 1963), or whether, most likely, both mechanisms operate in concert. Whilst it has been long thought that both mechanisms can co-exist in the vertebrate nervous system, it was thought that in the brains of arthropods and other smaller organisms, neurotorphism would not be involved (Venter et al., 2011; Jaaro et al., 2001). This was disproved with the discovery of the Drosophila neurotrophins (Zhu et al., 2008).

The aim of this section of my thesis was to investigate whether the interactions between Spz3/Toll-8 could regulate the connectivity of Lawf1 neurons and if DNT-2/Toll-2 interactions could also regulate the connectivity and dendrite size of L1 neurons. The findings were important because they demonstrate that neurotrophism operates during the development of the visual system in *Drosophila*, adding further evidence that neurotrophism is a fundamental mechanism of development across the animals.

In previous studies, it has been documented that there is a genetic interaction between spz3 and Toll-8. These interactions were shown to regulate synaptic growth in the larval neuromuscular junction (NMJ) (Ballard et al., 2014). Mutant animals for Toll-8 showed NMJ undergrowth, whereas overexpression of Toll-8 led to the overgrowth of the NMJ(Ballard et al., 2014). Moreover, the study have shown that spz3 interacts with Toll-8 to regulate and promote the growth of the NMJ (Ballard et al., 2014). In my research, the data shows that spz3 is expressed in R1-R6 PRs that extend their axons and terminate in the lamina (see Chapter 3). When overexpressing UASspz3FL in Toll-8 expressing neurons, there was a significant increase in lamina neuronal number **(Figure 5.2)**. This suggests that

the overexpression of spz3FL promoted the survival of more Toll-8 expressing neurons and from connections in the lamina that were distant to die in normal circumstances.

These findings suggest that the interaction between a ligand and a receptor operates together to regulate these events during development, where the presence of extra spz3 molecules rescues the Toll-8 expressing neurons that would normally be culled by apoptosis, meaning that neurotrophism is involved in connectivity between neurons.

The promotion of cell survival to establish synaptic connectivity in the visual system has also been reported to operate via different mechanisms such in cell adhesion molecules. It was shown that the interactions between the immunoglobulin superfamily CAM DIP-α and its binding partners, Dpr10 and Dpr6, control synaptic targeting and survival in the optic lobe of medulla Dm12, Dm4 neurons (Xu et al., 2022). The postsynaptic partner to these medulla neurons are the lamina L3 neurons in medulla layer M3 that express the binding partners Dpr10 and Dpr6. Loss of interactions between the CAM and its partners causes the death of Dm4 and Dm12 neurons and mistargeting of Dm12 from M3 layer to M8 (Xu et al., 2018).

Although this study provides strong evidence supporting the synaptic matching hypothesis, they also argue that connectivity is regulated by trophic support as well (Xu et al., 2018). The interaction between DIP-α and Dpr10 is shown to activate a trophic support pathway that prevents cell death (Xu et al., 2018). This happens when DIP-α on Dm4 neurons interacts with Dpr10, it triggers the pathway that promotes cell survival of these neurons preventing them from undergoing apoptosis (Xu et al., 2018). However, the specificity of DIP-α and Dpr10 interactions provides evidence supporting the chemoaffinty hypothesis. It demonstrates that by arguing that the correct pairing of these molecules are vital for proper synaptic targeting. Any alteration in these interactions can lead to changes in connectivity and circuit formation (Xu et al., 2022).

These findings suggest that it is possible that activating a trophic support pathway to regulate cell survival takes place in concert with the establishment of connectivity by a chemoaffinity mechanism operated by cell adhesion molecules rather than just one mechanism taking care of the entire process.

In my research, to overcome the limitations of tools used in this project shown in **Figure 5.3** I used a different approach to answer the connectivity question regarding the interactions between spz3 and Toll-8 in regulating Lawf1 neurons. The split Gal4 system was used to label Lawf1 neurons specifically and observe changes if any when manipulating spz3 and Toll-8. From my results section in Chapter 5, I could not conclude
whether these interactions influence the connectivity of Lawf1 neurons. This was due to the limitation of the tool where multiple neurons would be expressed besides Lawf1 limiting the accuracy of the analysis. This could be tackled by the use of different approaches such as MARCM clones or picking a different set of neurons that could be easily analysed.

My study provides evidence that the nervous system is a complicated system that does not operate via unique mechanisms. This may provide robustness, enabling compensating the loss of other pathways. One argument supporting both mechanisms operating in concert is that there is no evidence that any of these mechanisms alone would have enough information to establish correct connections between synaptic partners. Either way, my findings demonstrate that neurotrophism operates in the optic lobe of *Drosophila*. This adds further evidence that neurotrophism can operate across arthropods, not only vertebrates; therefore, it is a fundamental and universal principle of brain development.

6.3.2 Plasticity of lamina L1 dendrites

Dendrites in the *Drosophila* central nervous system are known to be plastic. It has been documented that dendrite plasticity play a major role in establishing neuronal circuits. It has been shown that the protein Fascin, plays a role in the dynamics and morphological changes of class III and class IV dendrites (Nagel et al., 2012). Loss of Fascin leads to partial conversion of class III dendrites to class IV dendrites. However, when overexpressing Fascin, it caused class IV dendrites to induce the formation of class III like spiked protrusions (Nagel et al., 2012). In a previous study, it has been reported that the dendrites of lamina neurons are plastic (Li et al., 2020). In the Hidalgo lab, it has been shown that loss of Toll-2 with MARCM clones caused cell death in lamina neurons and altered their connectivity (Li et al., 2020). These findings provide evidence that dendrites in the Drosophila

CNS are plastic and are vital for circuit formation.

In my research, the data shows that when altering DNT-2 or Toll-2, the dendrites of lamina L1 neurons changed size and morphology. When overexpressing UASDNT-2FL, there was a significant increase in dendrite size and branching. Also, when knocking down Toll-2 with RNAi, the dendrite size was significantly decreased.

Finally, I was able to rescue the phenotype of Toll-2 RNAi with epistasis while overexpressing UASDNT-2FL. These findings provide more evidence that there is a genetic interaction between DNT-2 and Toll-2 and that L1 dendrites are very plastic, changing their size and morphology depending on the circumstance they are under. In a previous study, it has been shown that lamina L3 dendrites are also plastic (Pecot et al., 2014). The study investigated the role of *Drosophila* Fezf, a conserved transcription factor that has a role in regulating layer specificity and affects the dendrites size of L3 neurons (Peng et al., 2018). Loss of dFezf with MARCM clones showed a significant decrease in dendrite size of lamina L3 neurons suggesting a role for dFezf in regulating dendrite size in the lamina. A different study showed that Toll-2 positive lamina neurons changed their size and morphology when altering Toll-2 with MARCM clones which affected the connectivity (Li et al., 2020). These Toll-2 positive neurons were later confirmed in my research to be the L1 lamina neurons (see Chapter 3). The modifications in the size and structure of the dendrites, which are regarded as secondary consequences of the modification in the cell density, are attributed to the specific, directed effects seen in the L1 neurons of my experiments. However, that was not the case when I overexpressed DNT-2 or knocked down Toll-2 as the dendritic structural changes that were observed were consistent with and specific to these genetic changes. This seems rather unlikely if number of cells alone was to be the major factor, as we would expect at least a more distributed pattern of variation in dendritic structure amongst a larger repertoire of

neurons rather than the finer changes that have been reported in this study. For instance, in a situation where DNT-2 is overexpressed, the size and the extent of branching of the dendrites significantly increase, whereas in the case where Toll-2 was knocked down the opposite was observed. These, therefore, appear to be the specific outcomes of the DNT-2/Toll-2 pathway through potential molecular interactions which regulate dendritic growth regardless of the cell number.

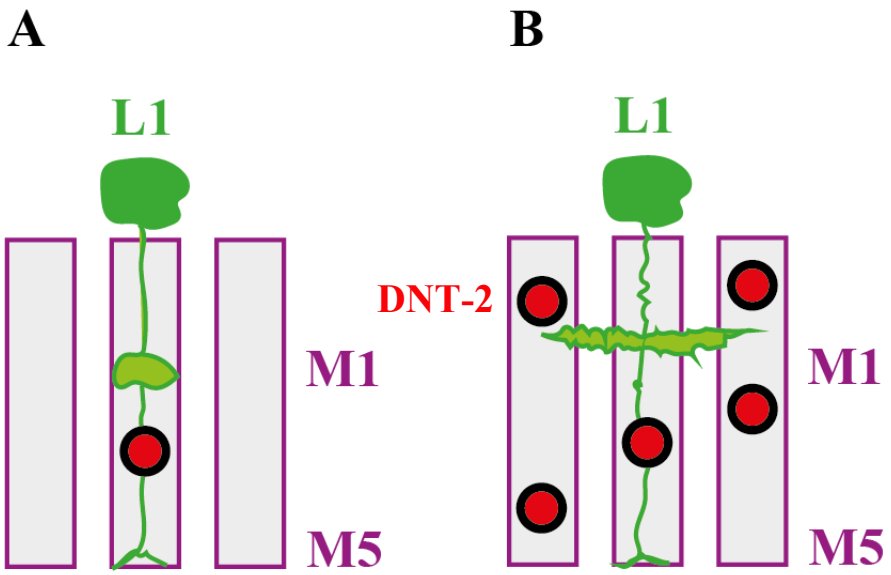
Moreover, the fact that the Toll-2 knockdown phenotype can be rescued by the simultaneous overexpression of DNT-2FL provides for further support that these effects are related not to fusion with, but to interactions of DNT-2 with Toll-2. This direct genetic interaction suggests that DNT-2, and indeed now also Toll-2, actually interact to influence dendritic architecture, in keeping with what other studies have demonstrated that Toll receptors are not only involved in modulating the cell's survival but also appear to be functionally involved at the dendrites and the cell's morphology and connectivity (Foldi et al., 2017; Li et al., 2020; McIlroy et al., 2013; Zhu et al., 2008). Therefore, these observations further support the argument that the dendritic changes seen in L1 neurons are probably caused by particular regulatory neurotrophic factors instead of merely reflecting an alteration in the number of cells.

6.3.3 Regulation of lamina L1 connectivity in the medulla

In Chapter 5, the data shows that when altering DNT-2 or Toll-2, L1 axonal terminals in the medulla are misrouted and are not restricted to a single column. In the Drosophila visual system, R7, R8, and L1-L5 neurons form connections in a single column within the medulla's layers, each containing one axon of each of these cells (Nériec and Desplan, 2016).

In a previous study, it has been shown that Dscam2, the immunoglobulin superfamily member, restricts the connections formed by L1 lamina neurons to columns in the medulla (Millard et al., 2007). They suggest that the Dscam2 homophilic interactions cause a repulsion between the L1 neurons, restricting them to a single column (Millard et al., 2007). Any alteration in Dscam2 has resulted in a misrouting of these L1 axonal terminals into nearby columns in the medulla (Millard et al., 2007). My data shows similar results to this study regarding the regulation of L1 neurons routing in the medulla layers, but the mechanism differs. Their conclusion is that the interaction between two Dscam2 molecules causes the repulsion to restrict the axonal terminals into their correct target to form functional circuits (Millard et al., 2007). This suggests a mechanism by which two cell adhesion molecules regulate these events. For example, in my case, it would be the equivalent of a Toll-2-Toll-2 interaction. However, my results suggest a different mechanism by which these L1 axonal terminals are restricted to a single column. The interaction between a ligand such as DNT-2 and a receptor such as Toll-2 is an example of neurotrophism. When overexpressing UASDNT-2FL, L1 axonal terminals cannot decided the correct amount of this trophic factor to take since trophic factors are limited in amounts, L1 axonal terminals compete for more of these trophic factors located in nearby columns which results in a misrouting phenotype **(Figure 6.2).** Moreover, altering Toll-2 with RNAi has resulted in a misrouting phenotype similar to the overexpression. This result could be because when there are no Toll-2 receptors for DNT-2 to bind to, L1 axonal terminals search for other ways to survive and establish connections by innervating nearby columns in the medulla that are not Toll-2 RNAi **(Figure 6.3)**. A previous study has reported similar outcomes when altering dFezf in L3 neurons. Loss of dFezf caused L3 neurons to show three different mistargeting phenotypes, where one phenotype shows the axonal terminals stopping before the M3 medulla layer, the final target for L3 neurons in wild-type animals (Peng et al., 2018).

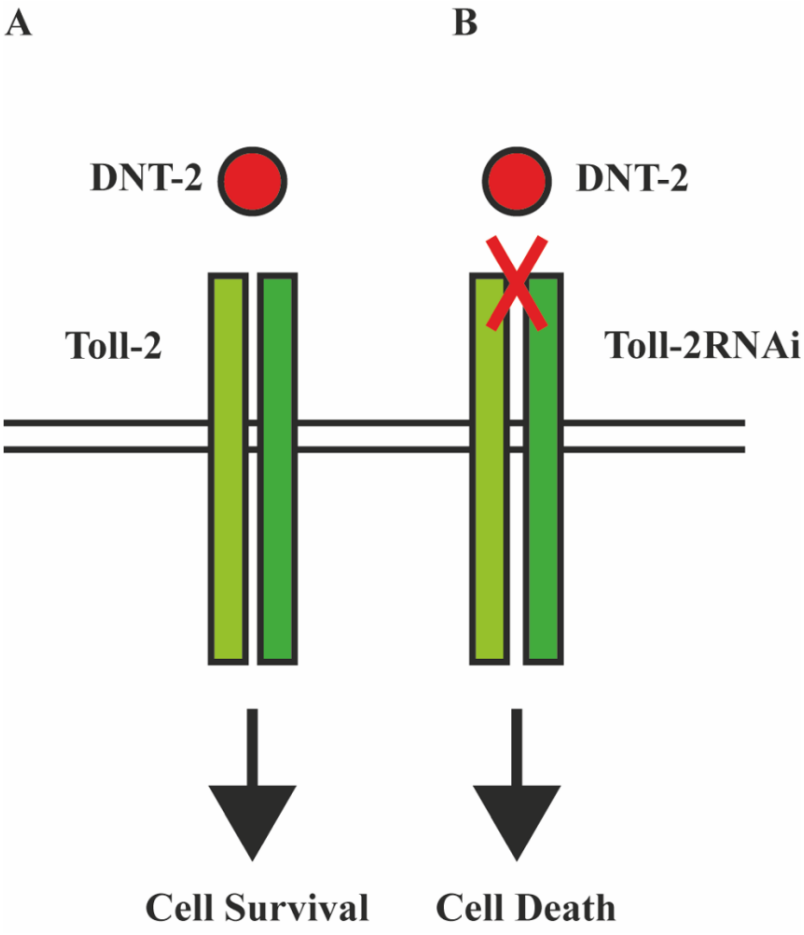
Figure 6.2 DNT-2 overexpression misroutes L1 axonal terminals in M1 layer



(A). L1 axonal terminals restricted to a single column in wild type animal

(B). L1 axonal terminals are misrouted when overexpressing DNT-2FL.

Figure 6.3 **Toll-2 KD causes L1 neuronal death**



(A). DNT-2 interacts with Toll-2 to promote cell survival

(B). Toll-2 KD with RNAi causes cell death in the lamina as well as in L1 lamina neurons.

Another phenotype was that L3 axonal terminals in the medulla misrouted and crossed to the nearby column in the medulla, similar to the phenotype shown in my results (Peng et al., 2018). The transcription factor dFezf is highly relevant to my findings on DNT-2 and Toll-2 in L1 neurons (Peng et al., 2018). DFezf plays a crucial role in regulating the targeting and layer specificity of L3 neurons in the *Drosophila* visual system, and its loss results in mistargeting phenotypes strikingly similar to what I observed with DNT-2 and Toll-2 alterations (Peng et al., 2018). Like the cell-autonomous function of dFezf in L3 neurons, DNT-2 and Toll-2 appear to regulate L1 axon targeting in my study. Interestingly, dFezf controls the expression of cell surface and secreted molecules, including dpr family members, which mediate interactions with medulla target cells (Peng et al., 2018). This mechanism parallels my proposed role for DNT-2 and Toll-2 in guiding L1 axons. While dFezf operates in L3 neurons and my study focuses on L1 neurons, the similarities in phenotypes and mechanisms suggest parallel systems regulating axon targeting in different neuron types in the *Drosophila* visual system. Furthermore, the broader effects of dFezf loss on circuit formation, including its impact on R8 axon targeting, highlight the importance of precise axon targeting in establishing proper neural circuits (Peng et al., 2018). Overall, the dFezf molecule and its role in L3 neuron targeting provide a valuable comparison to my findings on DNT-2 and Toll-2 in L1 neurons, contributing to our understanding of how complex neural circuits are formed in the *Drosophila* visual system.

Recent work by Hiesinger lab demonstrated a mechanism in which self-organization takes place in the developing visual system of Drosophila, where axonal growth cones self-pattern through a filopodial meshwork without depending on target-dependent guidance mechanism such as in neurotrophic (Agi et al., 2024). They show that in the absence of lamina neurons, PRs cells still organize and establish connections in their target areas without relying on cues

from the lamina neurons to guide them (Agi et al., 2024). While these findings show only the role of lamina neurons, they do not consider other cells, such as glial cells, that could potentially be a source in the lamina cortex for trophic and guidance cues that guide these PRs to their correct targets (Allen and Lyons, 2018). This study also provides evidence that circuit formation does not depend on a single mechanism; the collective work of multiple mechanisms, such as trophic factors or cell adhesion molecules, all together work to form a final functional nervous system.

6.4 Future work

In this project, I have provided more evidence that supports the idea of neurotrophism in the developing optic lobes that controls cell survival, cell number and connectivity.

In the future, I would like to retest the connectivity experiment with the Lawf1 lamina neuron using a different technique from the split GAL4, such as MARCM clones. This will allow me to visualize individual mutant Lawf1 neurons with greater accuracy. Testing other ligands, such as DNT-1 or spz1 in the developing optic lobe would be an interesting area to investigate. Focusing on DNT-1 and Spz1 as possible ligands leads to several observations regarding their importance in the developing optic lobe and their interaction with other neurotrophins and Toll receptors. First, it appears DNT-1 and Spz1, similar to DNT-2 and Spz3, are also ligands that bind Toll family receptors, which implicates them in vital functions of cell survival, growth, and connectivity in the visual system. There is evidence of the roles of these ligands in regulating cell survival and connectivity in different tissues of the nervous system in Drosophila (Foldi et al., 2017; Zhu et al., 2008). Experimenting in vitro with these ligands may help establish whether they exert similar neurotrophic effects as DNT-2 and Spz3, or if they influence different pathways or have different effects altogether,

thus adding to the complexities surrounding the molecular biology of neurotrophic regulation.

Moreover, the potential functional redundancy or DNT-1 and Spz1's interaction with other Toll receptors within the optic lobe could suggest an evolutionarily preserved pattern whereby a variety of different ligands bind a Toll receptor, or set of Toll receptors, to modulate different stages of development. It is known that DNT-1 is involved in neurogenesis and in the maintenance of neurons in other neural structures, and thus, it may also assist in the survival of cells in the optic lobe thus complementing the neurotrophic system (Zhu et al., 2008). Looking into Spz1, it belongs to a group of ligands whose characteristics are already well studied that are known to be involved in cellular signaling and immunity development (Sutcliffe et al., 2013). And so perhaps its investigation may shed light on uncharacterized aspects in the development of the visual system.

Studying DNT-1 and Spz1 also raises the question of whether several ligands could work together or add to each other's activity, which is consistent with the observation that complex multifactorial developmental systems are often robust and precise owing to many signaling molecules. Their examination from the perspective of memory, learning, and visual perception could help in their understanding of what other processes and processes these ligands could turn out to affect, which reaches the neurotrophic control of these higher cognitive functions.

Another idea that could be tested is whether these findings could be replicated in a different context. For example, instead of neurotrophism, it would be interesting to see whether Toll-Toll interactions could have similar consequences on cell death and connectivity. Also, when knocking down multiple Tolls, does that cause more severe phenotypes due to the lack of

DNTs binding to these receptors?

6.5 Conclusion

To conclude, my PhD research has shown that neurotrophism takes place in the Drosophila Visual system development ranges from regulating cell survival to regulating connectivity. Therefore, neurotrophism is a fundamental mechanism of development.

My work shows a conserved molecular pathway in which neurotrophins operate to form Functional nervous system. This would be interesting to test in higher animals, such as Humans whether an overexpression of trophic factors could reverse neurodegenerative Diseases such as Alzheimer's disease and Parkinson's disease, where neurons die and whether the trophic support could allow more survival in these neurons, reducing the severity of these diseases.

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