FUNCTIONAL ANALYSIS OF THE TOLL RECEPTOR PROTEIN FAMILY AND THEIR DOWNSTREAM SIGNALING PATHWAYS IN THE CENTRAL NERVOUS SYSTEM OF *DROSOPHILA*

By

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ABSTRACT

Cell number plasticity drives organismal growth, and is coupled in the central nervous system (CNS) to the emergence of neural circuits, ensuring appropriate function. In mammals, neurotrophins (NT) promote cell survival via Trk and $p75^{NTR}$ receptors or induce cell death via $p75^{NTR}$ and Sortilin. In *Drosophila* the DNTs bind Toll receptors to promote cell survival, but whether they can regulate cell death within the CNS remains unknown.

In this thesis, I show that Toll receptors have distinct and overlapping spatial and temporal expression and functions. Driving RNAi knockdown and over-expression of each Toll, I show that Toll-5 and -7 are required in glia for adult locomotion; Toll-3 is required in neurons for the regulation of ventral nerve cord size; and different Tolls can induce either cell survival or death in distinct contexts. By counting Dcp1+ dying cells and Eve+ neurons with DeadEasy imaging software, I show Toll-1 and -3 are pro-apoptotic in the VNC. However, Toll-2 and -9 are pro-apoptotic in the retina. I focused on the signalling mechanisms downstream of Toll-6. My data contribute to showing that DNT-Toll-6 signalling switches between promoting cell survival or death via NFkB, ERK, or JNK signalling. These outcomes depend on the cleavage state of the DNT, time and available downstream adaptors. Toll-6 induces cell survival via MyD88 and cell death via dSarm, and these alternative outcomes depend on Weckle.

Altogether, my data contribute to showing that the Toll receptors, DNTs and downstream signalling adaptors constitute a novel mechanism of cell number plasticity within the CNS.

For my husband Jarred

~ Who believes in me more than myself!

Mum and Neil

~ For supporting me through all of the "average" bits!

Dad

~ For always setting the bar high!

xxxxx

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LIST OF ABBREVIATIONS

18w	18 Wheeler
5-HT	5-Hydroxytryptamine
AMP	Antimicrobial Peptide
BDNF	Brain-Derived Neurotrophic Factor
CK	Cystine knot
CNS	Central nervous system
CRC	Cysteine-rich cluster
CTD	C-terminal domain
DAG	Diacylglycerol
DAMPs	Danger Associated Molecular Pattern
DD	Dark/Dark cycle
DM	Dorsal median
DNT	*Drosophila* Neurotrophin
Dpp	Decapentaplegic
EGF	Epidermal Growth Factor
ERK	Extracellular-Signal-Regulated Kinase
FasII	Fasciclin II
FasIII	Fasciclin III
Gcm	Glial cells missing
Gd	Gastrulation Defective
GDNF	Glial Cell-Derived Neurotrophic Factor
GFP	Green Fluorescent Protein
GNBP	Gram Negative Binding Protein
HMGB1	High-Mobility Group Protein B1
HRP	Horse Radish Peroxidase
Ig	Immunoglobulin
IMD	Immune Deficiency
IP_3	Inositol Trisphosphate
ISN	Intersegmental nerve
JNK	c-Jun N-terminal Kinase
LD	Light/Dark cycle
LIG	Leucine-rich repeat and Immunoglobulin domain
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTP	Long-term potentiation
MAP	Mitogen-Activated Protein
MMP	Matrix Metalloprotease
NGF	Nerve Growth Factor
NMJ	Neuromuscular junction
NPC	Neuronal progenitor cell
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PBTw	Phosphate-buffered saline, 0.1% Tween-20
PBTx	Phosphate-buffered saline, 0.1% Triton-X-100
PCR	Polymerase chain reaction

PDGF	Platelet-Derived Growth Factor
PGRP	Peptidoglycan Recognition Protein
PI3K	Phosphatidylinositol 3-Kinases PLC Phospholipase C
PNS	Peripheral nervous system
PRR	Pathogen recognition receptor
PtdIns	Phosphatidylinositol
Rel	Relish
Rho	Rhomboid
rpm	Revolutions per minute
SI	Survival index
Sim	Single-Minded
SN	Segmental nerve
Sog	Short Gastrulation
SP	Signal peptide
SPE	Spätzle Processing Enzyme
Spz	Spätzle
TEV	Tobacco etch virus
TGF	Transforming Growth Factor
TIR	Toll/Interleukin-1 Receptor
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
TNFRSF	Tumour Necrosis Factor Receptor superfamily
tPA	Tissue Plasminogen Activator
TRAF	Tumour Necrosis Factor Receptor-Associated Factor
TRPV	Transient Receptor Potential Vanilloid
VNC	Ventral nerve cord

CHAPTER 1

INTRODUCTION

In order to determine and characterize biological processes occurring within the brain we need to understand the link between structure and function. Levi-Montalcini, 1987 first described the Neurotrophic theory whereby during development neurons are produced in excess and only those that secure synaptic connections survive. Those neurons that fail to establish synaptic connections are eliminated through apoptosis. Only in the region of 20% of the original population survive, a number, which is not predetermined. During development neurons connect with target cells that secrete neurotrophins (NTs), which are the main class of molecules that underpin nervous system development and function. They regulate neuronal number, circuit formation, synaptic transmission and learning and memory. These events also take place in the brains of distant animals including the mollusc and within flies.

Neurotrophins promote neuronal survival and neuronal death, which they achieve via two distinct mechanisms. First, cleaved neurotrophins bind $p75^{NTR}$ receptors in order to activate NF-κB signalling to promote cell survival and synaptic plasticity. Cleaved neurotrophins can also bind Trk receptors to promote cell survival and synaptic plasticity via AKT, ERK, CREB, PLCγ or CaMKII signalling pathways. Secondly, pro-neurotophins bind $p75^{NTR}$ and Sortilin to activate JNK signalling to promote cell death and axonal degredation. Therefore a key aspect of structural plasticity is the ability to regulate both cell survival and death. If a cell lives or dies is dependent upon the state of the ligand, the receptor it binds and the downstream signalling mechanism. These receptors are the Toll-like receptors (TLRs), named due to their similarity to *Drosophila* Toll identified by Christine Nusslein-Volhard where she

is said to have exclaimed "Das ist ja toll!" the english translation of which is 'this is amazing/great' in response to the severe developmental defects in the *Drosophila* embryo patterning (Hanson and Edfeldt., 2005). TLRs are expressed within neurons where the function in the regulation of neurogenesis, apoptosis, neurite growth and collapse. These neuronal functions have only been minimally explored, and most strikingly the endogenous ligands coupled to these cell outcomes remain unknown.

For a long time it was thought that the CNS of flies was not plastic. The Hidalgo lab has found that *Drosophila* neurotrophins (DNTs) bind Toll receptors instead of $p75^{NTR}$ and Trk receptors in order to regulate neuronal survival, connectivity and behaviour. The aim of this thesis is to to investigate the function of the Toll receptors in the CNS. Firstly; to determine if they are expressed within the CNS. Secondly; if they function in a similar manner or if there are distinct groups between the Tolls in their ability to regulate function. Thirdly to investigate the signalling mechanisms downstream of Toll-6 in the regulation of cell death and/or survival in the CNS.

1.1 Vertebrate neurotrophins

In mammals, four NTs have been identified to date, including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), which shares structural and functional homology to NGF, neurotrophin 3 (NT3) and neurotrophin 4 (NT4), all of which are present in most vertebrates (levi-Montalacini and Hamburger, 1951; Barde *et al.,* 1982; Leibrock *et al.,* 1989; Ernfors *et al.,* 1990; Hohn *et al.,* 1990; Maisonpierre *et al.,* 1990; Rosenthal *et al.,* 1990; Hallbook *et al.,* 1991; Berkemeier *et al.,* 1991; Ip *et al.,* 1992). NT4 is not present in avian species and bony fish also have the additional NT-6/7, indicating gene loss and duplication events have taken place over time (Gotz *et al.,* 1994; Nilson *et al.,* 1998; Hallböök et al.,

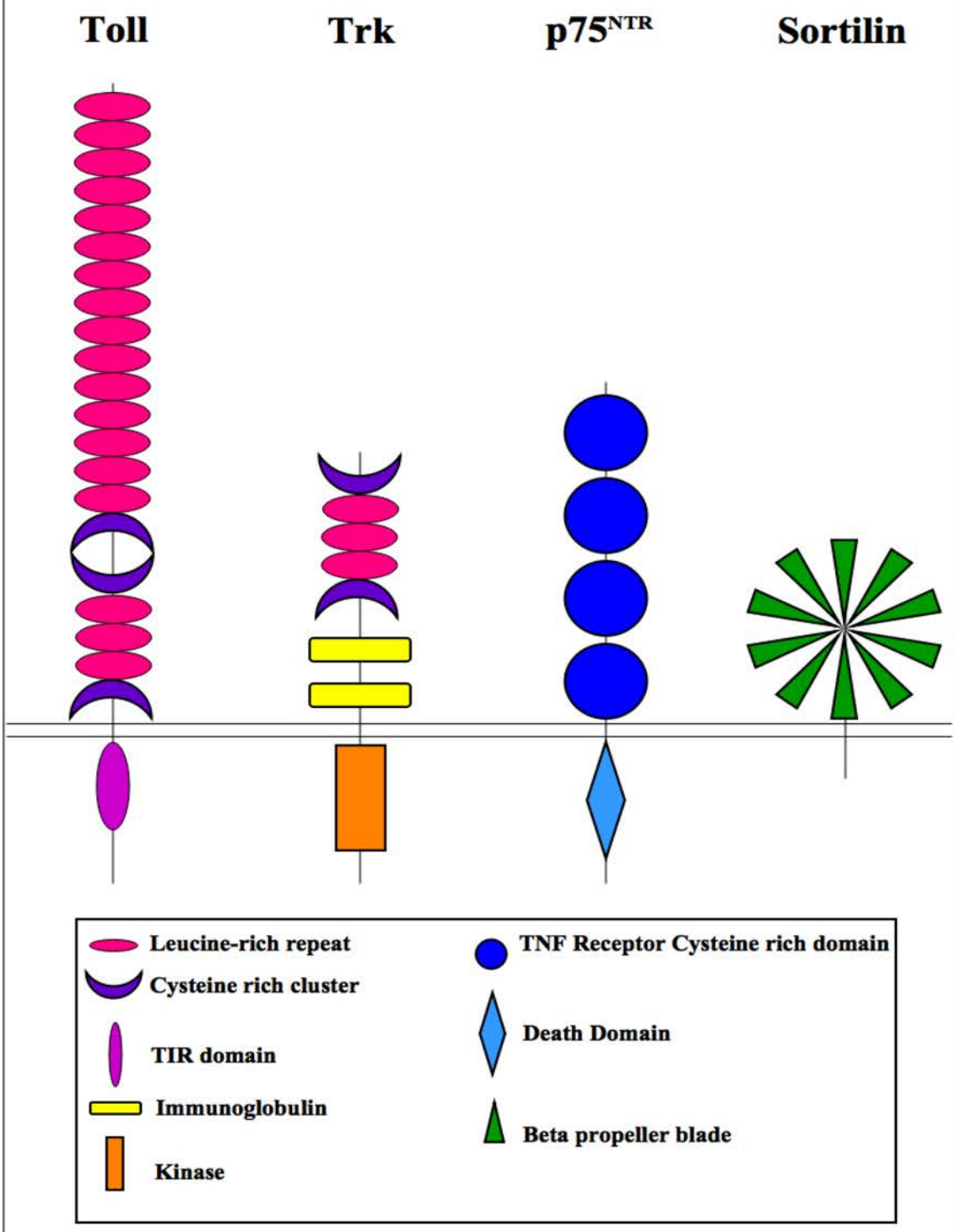
2006).

NTs are synthesized as pre-pro-neurotrophin precursors and processed to give rise to proneurotrophins, which are formed of an N-terminal pro-domain and a C-terminal Cys-Knot (Bibel and Barde., 2000). NTs can be secreted in their full-length form or as a cleaved NTs. Pro-neurotrophins are proteolytically cleaved by members of the pro-convertase family of serine proteases, (PC1 and PC2) as well as Furin to produce mature processed proteins (Roux and Barker., 2002). NGF can also be cleaved by a kallikrein-family protease (Seidah *et al*., 1996, Edwards *et al*., 1988). Following processing NTs form homodimers, whereby six conserved cysteine residues give rise to the cysteine knot form (McDonald and Hendrickson, 1993; Wiesmann and de Vos, 2001). These homodimers are then secreted through the constituitive secretory pathway or the regulated pathway (LessmaNn *et al.,* 2003).

NTs bind different types of plasma membrane receptors including Receptor Tyrosine Kinases including TrkA, TrkB, TrkC, the atypical TNFR superfamily member p75 pan-neurotrophin receptor ($p75^{NTR}$), Sortilin and Integrin $\alpha 9\beta 1$ (Kaplan and Miller., 2000; Dechant and Barde., 2002; Roux and Barker., 2002; Chao., 2003; Huang and Reichardt., 2003; Staniszewska *et al.,* 2008) (Figure 1.1). Originally it was believed that the pro-neurotrophins were limited to promoting folding of the mature domain (Suter *et al.,* 1991; Rattenholl *et al.,* 2001; Kolbeck *et al.,* 1994) or the sorting of mature NTs into either constiuitive or regulated secretory pathways (Farhadi *et al.,* 2000). However it is now known that both the pro-neurotrophin and mature neurotrophins can have different outcomes dependent upon their binding partners.

ProNGF and proBDNF can be cleaved *in vitro* by the extracellular proteases, MMP7 and plasmin, which is a protease known to contribute to synaptic plasticity, to form mature NGF and BDNF (Bruno and Cuello. 2006; Chen *et al.,* 2004; Lee *et al.,* 2001; Lou *et al.,* 2005;

Figure 1.1 Structural domains of vertebrate neurotrophin receptors



This figure displays the diversity amongst protein structures of the vertebrate neurotrophin receptors Trk, $p75^{NTR}$ and Sortilin. The Drosophila Toll receptor is shown for comparison.

Mowla *et al.,* 2001; Mowla *et al.,* 1999; Teng *et al.,* 2005). Pro-neurotrophins bind with high affinity to $p75^{NTR}$ to promote cell death. Binding of proNGF to $p75^{NTR}$ leads to an increase rate of apoptosis, in vascular smooth muscle cell lines that do not express any Trk receptors (Lee *et al.,* 2001). This apoptotic phenotype relies on the binding of proNGF to not only $p75^{NTR}$ but also to a co-receptor, the type 1 transmembrane receptor sortilin Vps10p (Nykjaer *et al.,* 2004). The ability of proNGF to promote cell death has been verified in numerous different cell types including, oligodendrocytes (Beattie *et al.,* 2002), corticospinal neurons (Harrington *et al.,* 2004) and photoreceptors (Srinivasan *et al.,* 2004).

In cultured sympathetic neurons binding of proBDNF to $p75^{NTR}$ also results in an increased rate of apoptosis. Furthermore by blocking the interaction of proBDNF and sortilin with neurotensin results in reduced apoptosis indicating that sortilin is a critical co-receptor for proBDNF, like proNGF (Teng *et al.,* 2005). A single nucleotide polymorphism, which replaces $valine^{66}$ with a methionine in the pro region of BDNF, leads to memory associated problems as well as abnormal hippocampal function. This Val/Met alteration leads to deleterious trafficking of BDNF to synapses, which results in a decline of overall BDNF release. (Chen *et al.,* 2004; Egan *et al.,* 2003).

Mature NTs can bind both $p75^{NTR}$ and Trk receptors. The NTs having specific affinity for different Trk receptors. NGF binds preferentially to TrkA, BDNF and NT4 to TrkB and NT3 to TrkC (although NT3 can also bind TrkA and TrkB at lower affinities) (Barbacid*.,* 1994). The binding of mature NTs to Trk receptors results in the dimerisation and autophosphorylation of tyrosine residues and subsequently leads to the activation of numerous signalling cascades. These pathways then go onto modulate differential gene expression in cell specific manners.

Promotion of cell survival can be mediated via the phosphatidylinositol 3-Kinase (PI3K) – Akt pathway whereby Akt phosphorylates a multitude of substrates including Forkhead (Brunet *et al.,* 1999), B-cell leukaemia/lymphoma 2-associated (BLC-2) death protein (BAD) (del Peso *et al.,* 1997; Datta *et al.,* 1997), Caspase 9 (Cardone *et al.,* 1998; Rohn *et al.,* 1998) and the Iκ□B kinase (Kane *et al.,* 1999) in a Ras-independent manner. Furthermore, Ras can interact directly with PI3-K, where Ras inhibition suppresses NGF activity (Kleese and Parada. 1998).

Cell surivival can also be mediated via the mitogen-activated protein kinase (MAPK)-MEK (MAPK/ERK (extracellular signal-regulated kinase) kinase signalling pathway. This cascade induces activity and/or expression of both BLC-2 (Aloyz *et al.,* 1998) and cyclic AMP responsive element binding protein (CREB) (Riccio *et al.,* 1999) both of which are anti-apoptotic proteins. The Trk receptors are also capable of phosphorylating PLCγ to create an active enzyme which has the ability of converting phosphatidyl inositides to inositol triphospate (IP_3) which in turn increases cytoplasmic Ca^{2+} and diacylglycerol (DAG) which activates protein kinase Cδ, a kinase which is involved in the ERK pathway (Vetter *et al.,* 1991; Corbit *et al.,* 1999).

Mature NTs also bind $p75^{NTR}$ in the absence of Trk receptors to mediate cell survival via NF-KB. In cultured cortical neurons expressing $p75^{NTR}$ but not TrkA, mature NGF is able to inhibit cell death caused by glutamate-induced cytotoxicity (Shimohama *et al.,* 1993; Klume *et al.,* 2000). Binding of NGF to $p75^{NT}$ also promotes cell survival in different cell lines and tissues including neocortical neurons (De Freitas *et al.,* 2001), sensory neurons (Hamanoue *et al.,* 1999) and human breast cancer cell lines (Descamps *et al.,* 2001). In contrast mature NTs can also bind to $p75^{NTR}$ in order to induce cell death via c-Jun amino-terminal kinase (JNK)

cascades, whereby dominant-negative JNK and inhibitors of JNK signalling are able to inhibit $p75^{NTR}$ induced death in hippocampal neurons and oligodendrocytes (Friedman, 2000; Harrington *et al.,* 2002).

Overall cell survival or cell death outcomes are dependent upon both the cellular context of the NT receptors as well as secretion of either a proNT or a mature NT. Generally mature NTs preferentially bind Trk receptors to mediate cell survival and pro-NTs bind $p75^{NTR}$ to mediate cell death, however this is not exclusive as mature NTs are also able to bind $p75^{NTR}$ regulating both survival and death.

1.2 Vertebrate TLRs

Vertebrate TLRs provide pathogen-detecting systems for both innate and adaptive immunity (Takeda *et al.* 2003). The functions of TLRs have primarily been investigated for their roles in immunity. However the CNS is not completely removed from pathogenic insult, TLRs are required during the regulation of neural innate immunity responses (Rivest, 2009), and found to be widespread in microglia (Jack *et al.,* 2005).

TLRs therefore can function in both immunity and developmental capacities within the CNS. TLRs are activated during CNS injury and disease (Hanisch *et al.* 2008; Salminen *et al.* 2009; Okun *et al.* 2009; Downes & Crack 2010), cell number regulation (Teng *et al.,* 2005; Bandtlow and Dechant*.,* 2004), neurite growth (Gentry *et al.,* 2004; Bandtlow and Dechant*.,* 2004; Barker*.,* 2004) and learning and memory (Peterson *et al.,* 1999; Wright *et al.,* 2004).

Microglia are widespread immune cells throughout the mammalian CNS, and through a variety of PRRs, including TLRs, can identify numerous pathogens (Lehnardt. 2009). All known TLRs are expressed within microglia (Kielian *et al.,* 2002; laflamme *et al.,* 2003;

Zekki *et al.,* 2002; Olson and Miller.2004; Rasley *et al.,* 2002; Kielian *et al.,* 2005; Bsibsi *et al.,* 2002; Laflamme *et al.,* 2001).

TLRs are type-1 integral membrane receptors that each contain a N-terminal recognition ectodomain, an extracellular LRR motif as well as a C-terminal signalling domain (Bell *et al.* 2003). Sequence homologies of the TLRs group them into 6 subfamilies (Matsushima *et al.* 2007). Not all vertebrates contain members of each subfamily. For example, human TLRs (hTLR) consist of 10 members, which fall into sub-families 1 to 5. Each subfamily contains a varying number of LRR motifs as well as variable extent of glycosylation of the N-terminus (Botos *et al.* 2011).

Group 1 constists of hTLR-1, hTLR-2, hTLR-6 and hTLR-10. They are found on plasma membranes and respond to PAMPs that contain lipids such as lipoteichoic acid. Signalling occurs via the formation of complexes with hTLR-1 and hTLR-6 (Takeda *et al.* 2003). The extracellular domains contain 19 LRR motifs and there are between 3 and 8 N-linked glycosylation sites (hToll-1: 4(7), hToll-2: 3(4), hToll-6: 8(9) and hToll-10: (8))(Botos *et al.* 2011). Using semi-quantative RT-PCR and flow cytometry techniques Kielian *et al.,* 2002 demonstrated that the expression of TLR-1, -2 and -6 increased in N9 microglia following *S.aureus* and PGN exposure.

Group 2 consists of hToll-3, which is found localized to endosomes and is activated via the detection of dsRNA produced by most viruses (Leonard *et al.* 2008). It contains 23 extracellular LRRs and at least 11 (predicted to be 15) N-linked glycosylation sites. Okun *et al.* 2010, demonstrated that TLR-3 could negatively regulate the proliferation of neural progenitor cells, affecting memory formation and behavior. Town *et al*, demonstrated that mice deficient of TLR-3 show reduced microglial activation following a challenge with west

nile virus. This retrovirus produces dsRNA, the authors then determined that primary murine microglia identify dsRNA via TLR3 (Town *et al.,* 2006).

Group 3 consists of hTLR-4, which has been extensively investigated due to its response to lipopolysaccharide (LPS), a component of the outer membranes of Gram$^-$ bacteria. It requires the co-receptors MD-1 and CD-14 to signal most effectively (Beutler & Rietschel 2003). hTLR-4 contains 21 extracellular LRRs as well as 5-10 N-linked glycosylation sites (Botos *et al.* 2011). TLR-4 has been shown to regulate cell death. Tang *et al,* documented that TLR-4 promotes cell death via a JNK dependent signalling cascade (Tang *et al.,* 2007). Schecter *et al,* provided evidence that TLR-4 in retinal progenitor cells prevents proliferation (Schecter *et al.,* 2008).

Group 4 contains hTLR-5, which responds to bacterial flagellin (Hayashi *et al.* 2001). This receptor is primarily found within the gut in lamina propria dendritic cells (Uematsu & Akira 2009). hTLR-5 contains 20 LRRs and a predicted 7 N-linked glycosylation sites (Botos *et al.* 2011).

Group 5 contains hTLR-7, hTLR-8 and h-TLR-9, which are localized to endosomes and recognize nucleic acids PAMPs (Bell *et al.* 2003). They have 25 extracellular LRRs and are heavily glycosylated with predicted N-linked glycosylation sites of 14 (hTLR-7) and 18 (hTLR-8 and hTLR-9). He *et al,* has demonstrated that TLR-9 is induced in microglia by morphine, and TLR9 deficiency results in the inhibition of apoptosis. They infer these results could point to the capability of TLR-9 inhibition leading to the prevention of brain damage following opioid use (He *et al.,* 2011).

Signalling occurs whereby (except hTLR-4) two extracellular domains are bound by a single ligand. In the case of hTLR-4, two MD-2 molecules bind a single receptor. In response, the

cytoplasmic TIR domain dimerises and activates downstream targets including MyD88, MAL, TRIF and TRAM leading to further downstream signalling events (Palson-McDermott & O'Neill 2007). Once activated the intracellular processes are homologous to those already described for *Drosophila* Tolls and culminate in the expression of inflammatory cytokines (Gay & Gangloff 2007, Medzhitov *et al.* 1997). In some circumstances such as necrotic processes endogenous ligands such as hyaluronan also have the capability of activating TLRs without the requirement of PAMPs (Sloane *et al.* 2010).

Some of the 10 hTLRs have been detected within neurons, and evidence shows that they play a role in neuronal development utilizing non-canonical signalling pathways for spatially and temporally distinct neuronal cell types (Zhou *et al.* 2009 and Okun *et al.* 2011). For instance in dendrites TLR4 activation signals via MyD88 dependent pathway to activate NFκB and produce cytokines or TRIF, which produces STAT-1/2. In comparison, astrocytic TLR4 activate the MyD88 dependent pathway but not a TRIF-dependent pathway (Jung *et al.,* 2005). The downstream signalling mechanisms have yet to be completely identified (Rolls *et al.,* 2007).

Neuronal functions of the TLRs are increasingly being investigated. TLR-8 is expressed along axonal tracts initially, becoming more widespread throughout neuronal soma indicating changing functions during development. TLR-8 activation (via R-848) results in a reduction of length of primary neurites and independently, neuronal death. TLR-8 inhibition can rescue these phenotypes (Ma *et al.,* 2006; Ma *et al.,* 2007). During the development of neuronal circuits, neuronal TLR-3 activation via poly 1:C of DAMP mRNA leads to growth cone collapse and inhibition of neurite growth, independently of NF-κB. This occurs without an increase in cell death, in both chick DRG neurons and E14 mouse embryonic brains

(Cameron *et al.,* 2007). This demonstrates that the signalling outcomes of the different TLRs in different tissues/cells can be markedly different, highlighting the possibility that other signalling pathways are involved during development.

Some TLRs have been shown to be involved in cognition. In mice, loss of TLR-3 resulted in both defective working memory formation and retention (Okun *et al.,* 2010) and increased anxiety responses (Sloan *et al.,* 2010). Furthermore, TLR-9 loss of function (by CpG DNA) results in decreased learning and memory retention (Tauber *et al.,* 2009).

Following injury, such as ischemic injury (stroke), activation of microglial TLR-2 and TLR-4 contribute to neuronal damage and promote cell death mediated by the JNK signalling pathway and transcription factor AP-1. Levels of both are significantly increased in cerebral cortical neurons, and mice deficient for either TLR-2 or TLR-4 show a substantial increase in their ability to recover neurologically (Tang *et al.,* 2007). In contrast these phenotypes were not seen with either TLR-3 or TLR-9 (Hyakkoku *et al.,* 2010).

1.3 Evolution of *Drosophila* Toll and Mammalian Toll Like Receptors

Genes of the Toll superfamily are present in most eumetazoans (except platyhelminths) with their origin preceding the separation of bilaterians and cnidarians over 600 million years ago (mya). However, as Toll superfamily molecules have been discovered in sponges and more divergent cnidarian species it is likely that these genes first emerged in the common ancestor of animals greater than 700mya (Leulier & Lemaitre 2008). The *Drosophila* Toll protein family consists of nine members (Toll-1 - Toll-9). All of the *Drosophila* Tolls with the exception of Toll-9 (a sccTLR) are phylogenetically distinct from known vertebrate TLR counterparts (also sccTLRs) forming two separate clades (Leulier & Lemaitre, 2008 and Imler & Zheng, 2003).

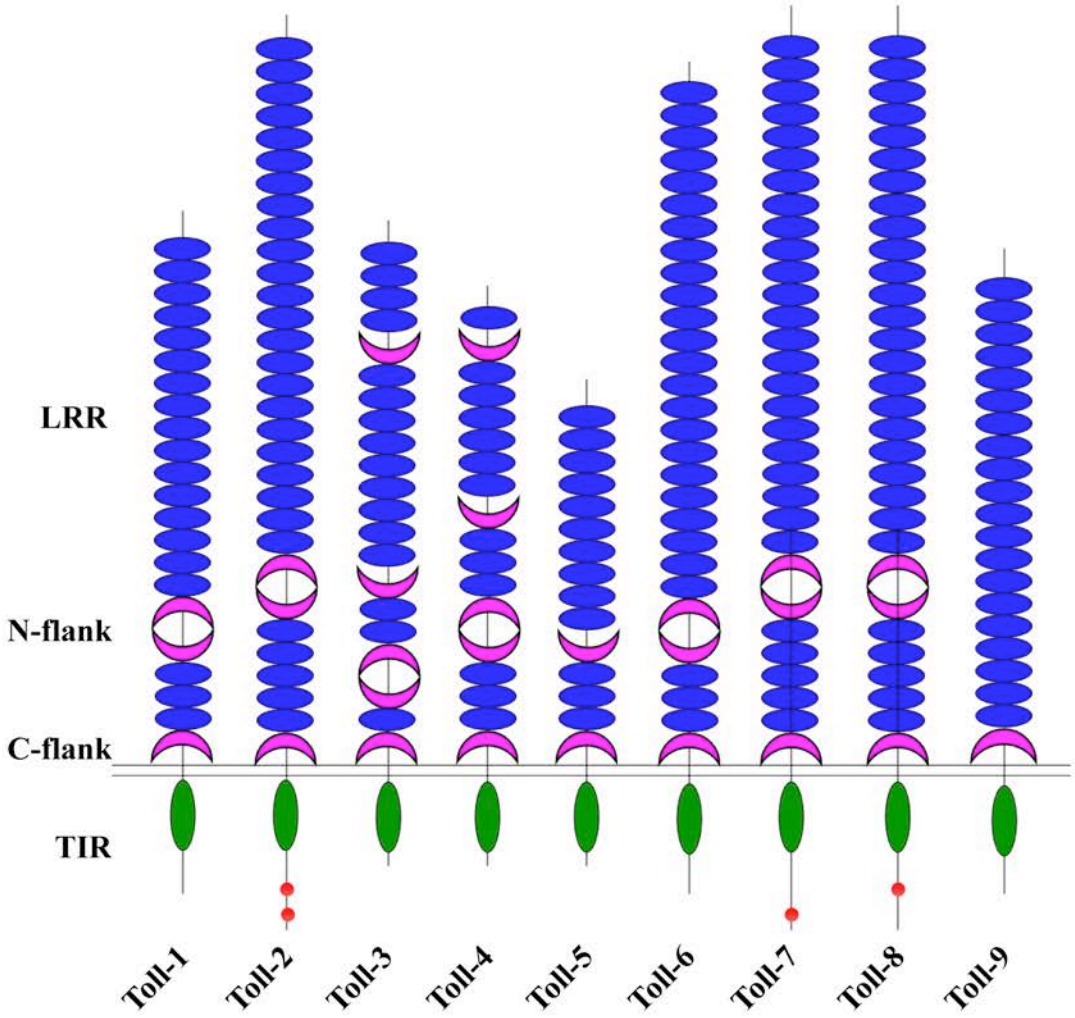
There are two major structural variations of the ectodomains of TLRs (Imler & Zheng 2003). Most deuterostomes, as well as *Drosophila* Toll-9 have a domain structure with a single cysteine cluster (sccTLRs) at the C-terminal end of the LRR (CF motif). Whereas, most proteostomes have a domain structure with multiple cysteine clusters (mccTLRs) at the N-terminal end of the LRR (NF motif).

Drosophila Tolls are transmembrane proteins containing a single membrane-spanning domain, with an extracellular N terminal domain and an intracellular C terminal domain (Figure 1.2). The ectodomain is comprised of 2 sections. The first contains a repertoire of Leucine rich repeats (LRRs), which is flanked at its C terminal by a cysteine rich cluster (CRC). The second contains LRRs flanked at both its N and C terminals by a CRC (Hashimoto *et al.,* 1988, Imler and Hoffman. 2001).

The cytoplasmic intracellular region of *Drosophila* Tolls share signalling similarities to the mammalian Interleukin-1 receptor and was thus named the Toll-Interleukin receptor (TIR) (O'Neill *et al.,* 2003) This conserved intracellular TIR domain functions to induce intracellular signalling via the interaction and recruitment of adaptor proteins (Schneider *et al.,* 1991; Gay & keith 1991; Imler and Hoffman. 2001). *Drosophila* Toll-1, -2, -6, -7 and -8 contain an intracytoplasmic C-terminal extension following the TIR domain, with Toll-2, -7 and -8 containing polyglutamine stretches. Furthermore, similar to mammalian TLRs, *Drosophila* Toll-3, -4 and Toll-5 have no C-terminal extension with a stop codon a few residues after the TIR domain (Tauszig *et al.,* 2000).

There are facets of Toll signalling that are similar between *Drosophila* and mammals, particularly in the context of immunity. All mammalian TLRs function within the immune system, but this is not known for all *Drosophila* Tolls. Similarities include the induction of

Figure 1.2: Structure of *Drosophila* Toll receptors



This diagram represents the protein structure of the nine *Drosophila* Toll family members. The ectodomain is composed of leucine-rich repeats (LRR) flanked by cysteine-rich motifs (C and N-flanks). All, with the exception of Toll-9 (which is similar to mammalian TLRs) contain a N-flanking region . The intracytoplamic domain is comprised of Toll/IL-1R homology (TIR) domains, and is a shared domain with members of the IL-1R family and the adaptor protein MyD88.

Drosomycin and *Metchnikowin* expression by Toll-5 (Tauszig *et al.,* 2000; Imler and Hoffman. 2000; Luo *et al.,* 2001), induction of Dorsal (Dorsal also has immunity independent functions) dependent transcription via the interaction between Toll-1, Pelle and Toll-5 (Luo *et al.,* 2001). Furthermore; similar to Toll-5, Toll-9 is able to activate *Drosomycin* expression (Ooi *et al.,* 2001) a process which may be dependent upon other Toll signalling components (Bettencourt *et al.,* 2004).

Drosophila Toll-1 and mammalian TLRs interact with MyD88 via TIR domains in order to activate their respective protein kinases Pelle (*Drosophila*) and IRAK (mammals) (Imler and Hoffman 2001). Towb *et al.,* 2009 aligned the human IRAK-1 and IRAK-4 death domains against the TTLK proteins of eight arthropods. Using a blast search, the authors identified 49 identical positions. This demonstrated that *Drosophila* Tube and Pelle are an orthologous pair to IRAK4 (with 47% conserved residues) and IRAK1 (with 22% conserved residues) respectively.

Some downstream signalling components are conserved between *Drosophila* Tolls and mammalian TLRs. *Drosophila* Toll-1 via MyD88 and downstream effectors lead to cactus degradation and nuclear translocation of NF-KB transcription factors Dif and Dorsal (Wu *et al.,* 1998). Similar mechanisms are seen in mammals, where most mammalian TLRs are able to activate numerous signalling cascades including NF-KB signalling, possibly representing an evolutionary conserved suite of signalling mechanism between flies and mammals (Medzhitov *et al.,* 1997; Muzio *et al.,* 1998; Aliprantis *et al.,* 1999).

1.4 *Drosophila* Toll-1

Through the use of *in situ* hybridization it was established that during early stages of dorso-ventral pattern establishment, maternal *toll* transcripts are distributed throughout the embryo.

Trans-locating towards the embryonic plasma membrane during the syncytial blastoderm stage, following which expression vastly decreases during cellularisation (Gerttula *et al.,* 1988; Hashimoto *et al.,* 1991). During gastrulation Toll is expressed at anterior and posterior ends forming the midgut as well as within mesodermal invaginated cells of the ventral furrow (Gerttula *et al.,* 1988).

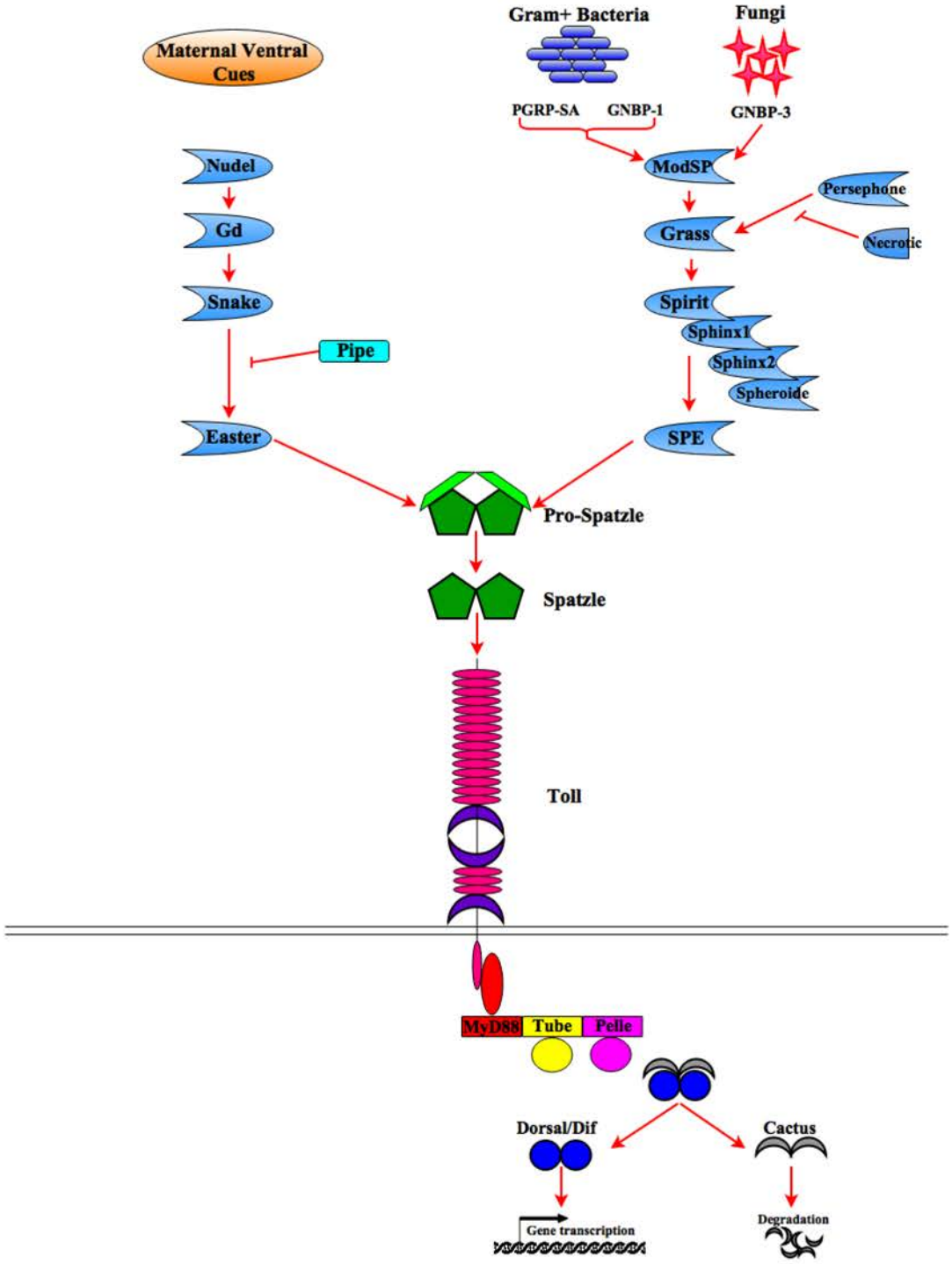
Using antibodies against the cytoplasmic domain of Toll-1, and *in situ* hybridisation it was shown that transcription of zygotic *toll-1* occurs around the time of germ band extension. During embryonic development Toll-1 is expressed in the posterior and anterior midgut, salivary glands and epidermis (Gerttula *et al.,* 1988). Toll-1 is expressed in regions where there are high levels of invagination, and following germ band shortening in many embryonic tissues including epidermis and muscle attachment sites, at the dorsal midline and segment borders (Gerttula *et al.,* 1988; Hashimoto et al., 1991; Halfon *et al.,* 1995). Following differentiation during late embryogenesis Toll-1 decreases significantly (Gerttula *et al.,* 1988).

Toll-1 is also observed in embryonic neurons. Furthermore *Toll-Gal4>UAS-GAP-GFP* labels the larval and adult neurons of the CNS (Zhu *et al.,* 2008; Sutcliffe B PhD), and is located within muscle fibers, their pre-cursors, midline glia and dorsal median cells of the larval CNS (Nose *et al.,* 1992; Wharton and Crews *et al.,* 1993). Northern blot and RT-PCR analysis show that Toll-1 is expressed during the pupal stage at high levels (Tauszig *et al.,* 2000).

1.4.1 *Drosophila* Toll-1 in early development

Toll is a member of a group of maternal effect genes, now known as the 'dorsal' group and the *toll* signalling cascade in development has since been elucidated via the molecular characterization of numerous dorso-ventral patterning genes (Figure 1.3). During early stages

Figure 1.3 *Drosophila* Toll signaling pathways



This diagram shows the differing Toll signaling pathways during development and innate immune functions. During embryogenesis maternal ventral cues activate a protease signaling cascade leading to the cleavage and activation of Spatzle. During immune responses the sensing of pathogen associated molecules also results in the activation of a protease signaling cascade to activate Spatzle. Cleavage of spatzle leads to the activation of Toll and initiates downstream signaling of adaptor proteins.

of embryogenesis dorso-ventral patterning is determined via the localization of *Toll* at the ventral side of the embryo (Anderson *et al*., 1985).

Within the maternal egg chamber *nudel* is uniformly expressed. Positional cues originating during oogenesis in the follicle cell layer lead to the sequential activation of the zymogens *Gastrulation defective (gd), Snake* and *Easter* within the perivitelline space. The sulfotransferase *Pipe* is located ventrally on the follicular epithelium restricting *snake* activity to the ventral region of embryos. *Snake* activates *Easter*, which then processes the cysteine knot molecule Pro-*Spatzle (Spz)* in a graded manner along the ventral axis of the embryo (Schneider *et al.,* 1994, DeLotto & DeLotto 1998).

The inactive prepro-protein *Spz* ligand binds to the first 10 LRRs of Toll with high affinity ($K_d < 0.4$ nM) via its C-terminal fragment (Gangloff *et al*., 2008, Gay *et al.,* 2014). Following which *Spz* is cleaved into its mature form and becomes functional ligand for Toll. Dimerization of the Toll receptor operates as a scaffold for downstream adaptor proteins that associate via TIR-TIR interactions (Gay *et al.,* 2014). Toll interacts with the TIR domain of the signalling adaptor Myeloid Differentiation primary response protein 88 (dMyD88) (Tauszig *et al.,* 2002) As well as containing a TIR domain dMyD88 also contains a death domain (DD) and is localized at the plasma membrane via a ~100 amino acid C-terminal extension (CTE) (Kambris *et al.,* 2003).

Signalling occurs via the association of the DD of dMyD88 and the adaptor protein Tube, which contains a DD but does not contain a TIR domain. The serine/threonine protein kinase Pelle is then recruited, creating a submembranous signalling complex (Towb *et al.,* 1998, Sun *et al.,* 2002, Kambris *et al.,*2003, Sun *et al*., 2004). Pelle autophosphorylates and dissociates from the complex (Shen *et al*., 2001) interacting with dTRAF2. Downstream, Cactus (Ik-B-

like protein) is subsequently degradaded and Dorsal (NF-kB transcription factor) is translocated to the nucleus (Hoffman & Reichhart, 2002). This nuclear translocation and increasing ventral Dorsal expression leads to activation of the zygotic genes *twist* and *snail* as well as the reduction of *zerknullt* and *decapentaplegic* due to reduced Dorsal protein in dorsal regions (Ray *et al.,* 1991).

1.4.2 *Drosophila* Toll-1 in immunity

Subsequent studies revealed Toll-1 to be an essential receptor acting within innate immunity. Specifically in the recognition of some gram-positive bacteria and fungi. It was this unearthing that paved the way for the identification of vertebrate TLRs and established the Toll pathway as an evolutionary conserved signalling pathway (Valane *et al*, 2011). In *Drosophila* the extracellular machinery required for the activation of the Toll pathway differs between developmental and immune requirements (Figure 1.3).

In mammals TLRs are also primarily known for their functions in immunity. They function as pattern recognition receptors (PRRs) in order to directly combat invading microorganisms. It has been demonstrated that the resident macrophages of the mammalian nervous system; microglia, express TLRs (Rock *et al* 2004). Unlike TLRs however, *Drosophila* Toll-1 is activated by Spz and does not directly bind to the pathogens.

In order to control and clear pathogens PRRs recognize specific conserved pathogen associated molecular patterns (PAMPs). PAMPS only associate with the pathogen or specific virulence factors, allowing the recognition of non-self from self (Janeway 1989). Acting upstream of the Toll pathway as PRRs are members of the peptidoglycan recognition protein (PGRP) as well as gram-negative binding proteins (GNBP) (Kurata 2010). GNBPs form part of the B-glucan-binding proteins (Ochiai & Ashida 1999, Werner *et al.,* 2000). PGRP genes

are separated into two classes dependent upon the length of their transcripts, PGRP-LB and PGRP-LC form one class (long transcripts) whilst PGRP-SA and PGRP-SD form the other (short transcripts) (Bischoff *et al.,* 2004).

There are multiple ways in which the PRRs work independently or cooperate in order to detect PAMPs. PGRP-SA participates with GNBP1 and GNBP2 in order to recognize some Gram-positive bacterial cell wall components, for example lysine containing PGN (Lys-type-PGN) (Bischoff *et al.,* 2004, Gobert *et al.,* 2003). On the other hand the PRR GNBP3 recognises fungal determinants (mostly the b-glucans), all of which activate the Toll pathway (Gottar *et al.,* 2006).

Binding of PAMPs by the soluble PRRs induces the differential activation of a protease cascade whereby ModSP integrates signals from upstream PRRs and activates Gram-positive specific serine protease (Grass). The protease Spatzle processing enzyme is then activated (SPE) via several serine proteases including the Clip-serine protease Persephone (involved in a separate proteolytic cascade detecting virulence factors along with the serine protease inhibitor Necrotic), Sphinx1/2, Spirit and Spheroide (Levashina *et al.,* 1999, Ligoxygakis *et al.,* 2002, Kambris *et al.,* 2006, Cahmy *et al.,* 2008).

SPE is the functional equivalent of Easter sharing 44% amino acid similarity as well as sharing the same active site upon Spatzle. Once activated SPE cleaves Spz, culminating in the generation of a fully functional Toll ligand (Jang *et al*., 2006). Once bound, the Toll pathway leads to the degradation of cactus and nuclear localization of the NF-kB transcription factors Dorsal and Dorsal-related Immune Factor (DIF). Subsequent transcription of antimicrobial peptides (AMPs) such as Drosomycin occurs which can then combat invading pathogens (Manfruelli *et al.,* 1999).

1.4.3 *Drosophila* Toll-1 during development

Toll-1 has been documented as being involved in many complex processes such as development of the nervous system, epidermis and muscles. Much of the insight into the role it plays is accounted for by loss of function (LOF) experiments, in particular within muscles. Halfon & Keshishian 1998, demonstrated that similar to mutations in *spz*, Tube and Pelle, epidermal loss of Toll-1 leads to disordering muscle development.

This disruption in development can lead to the malformation of synapses for example, as demonstrated by Suzuki *et al.* 2000 where they showed that a reduction of muscular Toll-1 prevents interactions required between muscles and growth cones for correct synapse formation. Halfon *et al.* 1995, presented evidence that Toll-1 is expressed within motor-neurons and mutations of the Toll-1 gene lead to irregularities of muscles associated primarily with motor axon misrouting as well as the loss of motor-neurons.

Targeting defects are also established in Toll-1 mutant embryos. Loss of Toll-1 in muscles 15 and 17 lead to inappropriate axon targeting (Rose *et al.* 1997). Zhu *et al*. 2008 also showed that Toll-1 is required for cell survival in the embryonic CNS and that Toll-1 mutants showed an increase in apoptosis as well as the over-expression of Toll-1 in neurons can rescue naturally occurring cell death in embryos.

1.5 Other *Drosophila* Toll receptors

1.5.1 *Drosophila* Toll-2

Some of the Tolls are highly expressed during embryogenesis and metamorphosis (Tauzsig *et*

al. 2000). Toll-2 is expressed in the early embryo, at the cellular blastoderm stage as 9 circular stripes that reach the ventral side of the embryo. With the exception of a single anterior most dorsal band (Eldon *et al.,* 1994). These bands are immediately posterior and adjacent to eve patterning. At germ band extension secondary stripes appear and form a group of 18 that overlap wingless expression (Eldon *et al.,* 1994; Kambris *et al.,* 2002). During early gastrulation these stripes widen and a further two become visible within the head region. Lateral expression in each then declines and a cluster of cells becomes apparent along the ventral midline during germ band elongation. During this time there is also accumulation of transcipts around the tracheal pits, salivary glands and presumptive head regions. During germ band retraction expression becomes more restricted to these regions as well as along the dorsal midline, cells that are involved in development of the dorsal vessel.

In situ hybridization, northern blot and RT-PCR analysis revealed that Toll-2 is expressed in the fat body (localized to the plasma membrane and within the cytoplasm), within blood cells and in the lymph gland of larvae (Williams *et al.,* 1997;Kambris *et al.,* 2002) and during the pupal stage of development (Tauszig *et al.,* 2000). Similar to Toll-1, Toll-2 has been shown to have some influence over the innate immune response. As well as being located in immune response tissues, Toll-2 is induced following bacterial challenge with *Escherichia coli* (Williams *et al.,* 1997). Furthermore, Toll-2 mutants have decreased viability when challenged with *Escherichia coli* or *Enterobacter cloacae.* They also have reduced ability of synthesizing the anti-microbial peptide (AMP) genes *attacin* and *cecropin* in response to an insult of gram-negative infection (Williams *et al.,* 1997). Chimeric constructs with the ectodomain of constitutively active Toll-1 fused to trans- and intracytoplasmic domains of Toll-2 however failed to induce *drosomycin* or AMP genes (Tauszig *et al.,* 2000)

The immune functions of Toll-2 may be restricted to the fat body, the primary site of AMP production and secretion. A lacZ reporter was constructed with an ecdysone responsive fragment attached to a fat body specific gene (Fbp1). When ecdysone is generated in fat body cells it results in β-gal transcription, which can be monitored. Lixoxygakis *et al.,* 2002, determined that wild type flies express β-gal normally around 108 hours post egg laying. However Toll-2 mutant do not express β-gal and therefore these mutants do not have normal fat body development.

Furthermore, mutations in Toll-2 result in larval lethality and delayed adult development followed by early death (Eldon et al 1994) and therefore it is likely that Toll-2 functions during development. Toll-2 may act as a heterophilic cell adhesion molecule and facilitate the movement of cells. During morphogenesis as the over-expression of Toll-2 in S2 cells form aggregates within 2-4 hours post induction (Eldon *et al.,* 1994). Furthermore, mutations of Toll-2 lead to delayed migration of ovarian follicular cells (Kleve *et al.* 2006). Toll-2 mutant escapers showed significant incidences of morphological defects of the legs, wings and antennae. This is further indicative of eversion of imaginal discs, a process that requires extensive cell migration (Eldon et al 1994). Toll-2 mutants also display embryonic salivary gland invagination defects, similar to the defects of embryos lacking Rho-GTPase activity (Kolesnikov & Beckendorf 2007). It appears as though Toll-2 has a diverse set of functions, however the role of Toll-2 within the CNS still remains to be investigated.

1.5.2. *Drosophila* Toll-3 and Toll-4

Toll-3 and Toll-4 share 79% structural similarity to each other in respect to their TIR domains. Both proteins are shorter than other Toll members as they lack C-terminal extensions, instead containing a stop codon only a few residues after the TIR domain (Tauszig *et al.,* 2000). Both have very limited expression. Toll-3 transcripts are expressed at very low levels, detected by RT-PCR. Toll-4 is located in lymph gland precursor cells in late stage embryos (*in situ* hybridization) and within the fat body and lymph gland cells (RT-PCR) (Tauszig *et al.,* 2000; Kambris *et al.,* 2002). Furthermore, chimeric constructs with the ectodomain of constitutively active Toll-1 fused to trans- and intracytoplasmic domains of Toll-3 and Toll-4 could not induce AMP genes (Tauszig *et al.,* 2000). Transgenic expression assays using a suite of different ubiquitous and specific Gal4 (particularly in wing discs) drivers resulted in no lethality or phenotypic manifestations (Yagi *et al.,* 2010).

1.5.3 *Drosophila* Toll-5

As the closest homologue of Toll-1, Toll-5 and Toll-1 share a 61% identity within their intracellular domains. However, structural differences include Toll-5 lacking a C-terminal extension, instead containing a stop codon close to its TIR domain (Tauszig *et al.,* 2000). During embryonic development stage 10, zygotic Toll-5 is distributed along the ventral midline and in clusters of muscle progenitors. By stage 13 Toll-5 protein is located within some ventral muscles and the dorsal vessel. Toll-5 is also located throughout regions that express Toll-1 in abundance including the salivary glands, fat body and midgut, however Toll-5 is not present in the epidermis (Kambris *et al.,* 2002). Furthermore, Toll-5 protein was detected in ovaries and throughout all developmental stages via western blotting with Toll-5 peptide antibodies (Luo *et al.,* 2001).

A chimera construct consisting of the ectodomain of constitutively active Toll-1 fused to trans- and intracytoplasmic domains of Toll-5 led to efficient induction of the *drosomycin* promoter in S2 cells, indicating Toll-5 may play some role in host defense (Tauszig *et al.,* 2000). Further to this there was an increase in the level of Toll-5 protein *in vivo* when adult flies were challenged with both gram negative and gram positive bacteria, as well as when S2 cells where challenged with LPS (Luo *et al.,* 2001). A micro-array of AMP genes and the over-expression of Toll-5 led to an up regulation of both *drosomycin* and *metchenikowin* (Luo *et al.,* 2001) indicating further Toll-5 involvement in immune regulation.

Not only can Toll-5 initiate immune responses in a similar manner to Toll-1 it may also use the same signalling mechanisms. When S2 cells were transfected with differing concentrations of Toll-5, there was a significant increase in Dorsal and this was even furthered with the co-expression of Toll-5 and Pelle in SL2 cells. Therefore Toll-5 can independently activate Dorsal. Toll-5 can also physically interact with Pelle, to induce Dorsal activation in a manner similar to Toll-1 (Luo *et al.,* 2001). Furthermore, Toll-1 and Toll-5 intracellular domains can physically interact *in vitro*, and co-transfections of Toll-1 and Toll-5 vectors in S2 cells strongly activate Dorsal (Luo *et al.,* 2001). However, they may be structurally similar, and be able to interact, but the over-expression of Toll-5 with several Gal4 drivers resulted in no lethality or observable phenotype in contrast to Toll-1 (Yagi *et al.,* 2010) (Table 1.1).

1.5.4 *Drosophila* Toll-6

Toll-6 shares less structural similarities to the other *Drosophila* Toll TIR domains (Tauszig *et al.,* 2000). Transcription of Toll-6 starts during the cellular blastoderm stage. Several bands, from the anterior most regions, appear in altering intensity throughout germ band extension.

Table 1.1 Structure and Function of the Toll proteins up until 2011			
Gene/Protein	**Category**	**Result**	**Reference**
Toll-1	Expression	Maternal toll transcripts are distributed throughout the embryo. Trans-locating towards the embryonic plasma membrane during the syncytial blastoderm stage, following which expression vastly decreases during cellularisation	Gerttula et al., 1988; Hashimoto et al., 1991
Toll-1	Expression	During gastrulation Toll is expressed at anterior and posterior ends forming the midgut as well as within mesodermal invaginated cells of the ventral furrow	Gerttula et al., 1988
Toll-1	Expression	Transcription of zygotic toll-1 occurs around the time of germ band extension. During embryonic development Toll-1 is expressed in the posterior and anterior midgut, salivary glands and epidermis	Gerttula et al., 1988
Toll-1	Expression	Toll-1 is expressed in regions where there are high levels of invagination, and following germ band shortening in many embryonic tissues including epidermis and muscle attachment sites, at the dorsal midline and segment borders	Gerttula et al., 1988; Hashimoto et al., 1991; Halfon et al., 1995
Toll-1	Expression	Following differentiation during late embryogenesis Toll-1 decreases significantly	Gerttula et al., 1988
Toll-1	Expression	Toll-1 is also observed in embryonic neurons. Toll-Gal4>UAS-GAP-GFP labels the larval and adult neurons of the CNS	Zhu et al., 2008; Sutcliffe B PhD
Toll-1	Expression	located within muscle fibers, their pre-cursors, midline glia and dorsal median cells of the larval CNS	Nose et al., 1992; Wharton and Crews et al., 1993
Toll-1	Expression	Toll-1 is expressed during the pupal stage at high levels	Tauszig et al., 2000
Toll-1	Signalling	Essential receptor acting within innate immunity in the recognition of some gram-positive bacteria and fungi as well as having developmental functions.	
Toll-1	Development	Epidermal loss of Toll-1 leads to disordering muscle development	Halfon & Keshishian 1998
Toll-1	Development	Disruption in development can lead to the malformation of synapses, reduction of muscular Toll-1 prevents interactions required between muscles and growth cones for correct synapse formation	Suzuki et al. 2000
Toll-1	Development	Toll-1 is expressed within motorneurons and mutations of the Toll-1 gene lead to irregularities of muscles associated primarily with motor axon misrouting as well as the loss of motor-neurons	Halfon et al. 1995,
Toll-1	Development	Targeting defects in Toll-1 mutant embryos. Loss of Toll-1 in muscles 15 and 17 lead to inappropriate axon targeting	Rose et al. 1997
Toll-1	Development	Toll-1 is required for cell survival in the embryonic CNS	Zhu et al. 2008
Toll-2	Expression	Toll-2 is expressed in the early embryo, at the cellular blastoderm stage as 9 circular stripes that reach the ventral side of the embryo. With the exception of a single anterior most dorsal band. These bands are immediately posterior and adjacent to eve patterning.	Eldon et al., 1994
Toll-2	Expression	At germ band extension secondary stripes appear and form a group of 18 that overlap wingless expression	Eldon et al., 1994; Kambris et al., 2002
Toll-2	Expression	During early gastrulation stripes widen and a further two become visible within the head region. Lateral expression in each then declines and a cluster of cells becomes apparent along the ventral midline during germ band elongation. During this time there is also accumulation of transcipts around the tracheal pits, salivary glands and presumptive head regions. During germ band retraction expression becomes more restricted to these regions as well as along the dorsal midline, cells that are involved in development of the dorsal vessel	Williams et al., 1997;Kambris et al., 2002
Toll-2	Expression	Toll-2 is expressed in the fat body (localized to the plasma membrane and within the cytoplasm), within blood cells and in the lymph gland of larvae and during the pupal stage of development	Williams et al., 1997;Kambris et al., 2002; Tauszig et al., 2000
Toll-2	Immune response	Toll-2 mutants have decreased viability when challenged with Escherichia coli or Enterobacter cloacae. They also have reduced ability of synthesizing the anti-microbial peptide (AMP) genes attacin and cecropin in response to an insult of gram-negative infection	Williams et al., 1997
Toll-2	Immune response	Chimeric constructs with the ectodomain of constitutively active Toll-1 fused to trans- and intracytoplasmic domains of Toll-2 however failed to induce drosomycin or AMP genes	Tauszig et al., 2000
Toll-2	Immune response	The immune functions of Toll-2 may be restricted to the fat body, the primary site of AMP production and secretion. A lacZ reporter was constructed with an ecdysone responsive fragment attached to a fat body specific gene (Fbp1). When ecdysone is generated in fat body cells it results in β-gal transcription, which can be monitored. determined that wild type flies express β-gal normally around 108 hours post egg laying. However Toll-2 mutant do not express β-gal and therefore these mutants do not have normal fat body development.	Lixoxygakis et al., 2002
Toll-2	Development	Mutations in Toll-2 result in larval lethality and delayed adult development followed by early death and therefore it is likely that Toll-2 functions during development	Eldon et al 1994
Toll-2	Development	Toll-2 may act as a heterophilic cell adhesion molecule and facilitate the movement of cells. During morphogenesis as the over-expression of Toll-2 in S2 cells form aggregates within 2-4 hours post induction	Eldon et al 1994
Toll-2	Development	Mutations of Toll-2 lead to delayed migration of ovarian follicular cells	Kleve et al. 2006
Toll-2	Development	Toll-2 mutant escapers showed significant incidences of morphological defects of the legs, wings and antennae. This is further indicative of eversion of imaginal discs, a process that requires extensive cell migration	Eldon et al 1994

Toll-2	Development	Toll-2 mutants also display embryonic salivary gland invagination defects, similar to the defects of embryos lacking Rho-GTPase activity	Kolesnikov & Beckendorf 2007
Toll-3 & Toll-4	Structure	Toll-3 and Toll-4 share 79% structural similarity to each other in respect to their TIR domains. Both proteins are shorter than other Toll members as they lack C-terminal extensions, instead containing a stop codon only a few residues after the TIR domain	Tauszig et al., 2000
Toll-3 & Toll-4	Expression	Toll-3 transcripts are expressed at very low levels, detected by RT-PCR. Toll-4 is located in lymph gland precursor cells in late stage embryos (in situ hybridization) and within the fat body and lymph gland cells (RT-PCR)	Tauszig et al., 2000; Kambris et al., 2002
Toll-3 & Toll-4	Immune response	chimeric constructs with the ectodomain of constitutively active Toll-1 fused to trans- and intracytoplasmic domains of Toll-3 and Toll-4 could not induce AMP genes	Tauszig et al., 2000
Toll-3 & Toll-4	Development	Transgenic expression assays using a suite of different ubiquitous and specific Gal4 (particularly in wing discs) drivers resulted in no lethality or phenotypic manifestations	Yagi et al., 2010
Toll-5	Structure	As the closest homologue of Toll-1, Toll-5 and Toll-1 share a 61% identity within their intracellular domains. However, structural differences include Toll-5 lacking a C-terminal extension, instead containing a stop codon close to its TIR domain	Tauszig et al., 2000
Toll-5	Expression	During embryonic development stage 10, zygotic Toll-5 is distributed along the ventral midline and in clusters of muscle progenitors. By stage 13 Toll-5 protein is located within some ventral muscles and the dorsal vessel. Toll-5 is also located throughout regions that express Toll-1 in abundance including the salivary glands, fat body and midgut, however Toll-5 is not present in the epidermis	Kambris et al., 2002
Toll-5	Expression	Toll-5 protein was detected in ovaries and throughout all developmental stages via western blotting with Toll-5 peptide antibodies	Luo et al., 2001
Toll-5	Immune response	A chimerae construct consisting of the ectodomain of constitutively active Toll-1 fused to trans- and intracytoplasmic domains of Toll-5 led to efficient induction of the drosomycin promoter in S2 cells, indicating Toll-5 may plays some role in host defense	Tauszig et al., 2000
Toll-5	Immune response	Further to this there was an increase in the level of Tolll-5 protein in vivo when adult flies were challenged with both gram negative and gram positive bacteria, as well as when S2 cells where challenged with LPS	Luo et al., 2001
Toll-5	Immune response	A micro-array of AMP genes and the over-expression of Toll-5 led to an up regulation of both drosomycin and metchenikowin	Luo et al., 2001
Toll-5	Immune response	Not only can Toll-5 initiate immune responses in a similar manner to Toll-1 it may also use the same signaling mechanisms. When S2 cells were transfected with differing concentrations of Toll-5, there was a significant increase in Dorsal and this was even furthered with the coexpression of Toll-5 and Pelle in SL2 cells. Therefore Toll-5 can independently activate Dorsal. Toll-5 can also physically interact with Pelle, to induce Dorsal activation in a manner similar to Toll-1	Luo et al., 2001
Toll-5	Immune response	Toll-1 and Toll-5 intracellular domains can physically interact in vitro, and co-transfections of Toll-1 and Toll-5 vectors in S2 cells strongly activate Dorsal	Luo et al., 2001
Toll-5	Development	The over-expression of Toll-5 with several Gal4 drivers resulted in no lethality or observable phenotype in contrast to Toll-1	Yagi et al., 2010)
Toll-6	Expression	Toll-6 shares less structural similarities to the other Drosophila Toll TIR domains	Tauszig et al., 2000
Toll-6	Expression	Transcription of Toll-6 starts during the cellular blastoderm stage. Several bands, from the anterior most regions, appear in altering intensity throughout germ band extension. At the end of this developmental stage transcription reaches its maximal levels and is followed by a reduction of expression except for within the CNS	Kambris et al., 2002
Toll-6	Expression	Toll-6 is present within longitudinal interneuron axons, ventral HB9+ neurons and Eve+ motorneurons (except for RP2 neurons)	McIlroy et al., 2013
Toll-6	Expression	During larval development Toll-6 protein is located within the CNS along the VNC neuropile and within aCC motorneurons	McIlroy et al., 2013
Toll-6	Expression	Toll-6 transcripts are expressed during pupal stages, detected by RT-PCR	Tauszig et al., 2000
Toll-6	Expression	Within adults Toll-6 is present within dopaminergic neurons as well as within complimentary layers and rings of the fan shaped body (FSB) and ellipsoid body (EB) respectively, both sites of locomotor control	McIlroy et al., 2013
Toll-6	Development	Toll-6 (along with Toll-2, Toll-7 and Toll-8) is required for wing and leg imaginal disc development where it has a specific function in the formation of the anterior – posterior (A-P) boundaries. When over-expressed with decapentaplegic Gal4 (dppGal4) mild splitting of the A-P boundary occurs at the distal wing end due to abnormal folding	Yagi et al., 2010
Toll-6	Immune response	Toll-6 mutants induce AMP genes (AttacinA, Diptericin, Drosomycin or Metchnikowin) in a similar manner to the control following septic injury. Indicating Toll-6 may not play a significant role in antimicrobial immune responses	Yagi et al., 2010
Toll-6	Signalling	While DNT2;Toll-6 mutant embryos are viable, DNT1;Toll-6 mutants are semi-lethal. A phenotype that can be rescued by the over-expression of Toll-6 (and Toll-7). This indicates that Toll-6 acts downstream of DNT-2 and is likely its receptor, furthermore coimmunoprecipitations showed that Toll-6 can physically interact with DNT-2	McIlroy et al., 2013

Toll-6	Function	Toll-6 together with Toll-7 is required for normal locomotion as Toll-6; Toll-7 double mutant larvae crawl slower than controls.	McIlroy et al., 2013
Toll-6	Development	Toll-6 together with Toll-7 are required for correct motor axon targeting which was visualised via projections of FasII ISNb/d whereby both Toll-6 and Toll- 7 single and Toll-6; Toll-7 double mutants cause deficient targeting due to loss of projections and motoraxon misrouting in stage 17 embryos. Over-expression of both Toll-6 and Toll-7 in neurons also causes targeting defects	McIlroy et al., 2013
Toll-6	Function	Toll-6 and Toll-7 regulate neuronal survival as single and double mutants resulting in increased number of apoptotic HB9+ and Eve+ EL interneurons in the embryonic CNS	McIlroy et al., 2013
Toll-7	Structure	The TIR domain of Toll-7 shares 60% identity with Toll-2	Tauszig et al., 2000
Toll-7	Expression	Toll-7 expression is diverse throughout embryogenesis. Zygotic expression begins during germ band extension as a strong spot within the head region, with diffuse expression in the trunk. By the end of germ band extension, when CNS and PNS differentiation occurs, there are 14 well defined stripes that are in line with engrailed and posterior to wingless expression. At germ band retraction Toll-7 is located in leg imaginal disc precursors and epithelium of the large intestine	Kambris et al, 2002
Toll-7	Expression	Toll-7 is distributed throughout central HB9+ and Lim3+ RP motorneurons projecting along intersegmental nerve b/d which targets muscles 6, 7, 12, and 13, motor axons exiting CNS and interneuon axons that cross the midline	McIlroy et al., 2013
Toll-7	Expression	By stage 16, expression becomes weaker and restricted only to CNS tissues	Kambris et al, 2002
Toll-7	Expression	Toll-7 projections are visible along the three FasII and longitudinal fascicles	McIlroy et al., 2013
Toll-7	Expression	During larval stages Toll-7 is distributed along the VNC neuropile, and in the central complex of brains, similar to Toll-6, within complimentary layers and rings of the FSB and EB respectively	McIlroy et al., 2013
Toll-7	Expression	While structurally more similar to Toll-2, Toll-7 protein localisation is more similar to that of Toll-6.	McIlroy et al., 2013
Toll-7	Function	As highlighted previously Toll-7 has neurotrophic functions in the Drosophila CNS and is required in conjunction with Toll-6 for normal locomotion and motor axon targeting and to maintain neuronal survival	McIlroy et al., 2013
Toll-7	Signalling	Toll-7; DNT-2 mutants are semi lethal, and coimmunoprecipitations revealed physical interactions with both DNT-1 and DNT-2, therefore Toll-7 and Toll-6 may be promiscuous in its ligand binding	McIlroy et al., 2013
Toll-7	Development	Toll-7 mutants whilst viable display leg defects and Toll-7 is located around the wing pouch and hinge region of the wing disc, as well as along the A-P border from tarsal to tibia segments of leg discs. A transgenic expression assay identified Toll-7, Toll-2, Toll-6 and Toll-8 as required for the formation of the anterior – posterior (A-P) boundaries for wing and leg imaginal disc development, and identifies this group of Tolls as possibly being a subgroup	Yagi et al., 2010
Toll-7	Immune response	Mutants for Toll-6, -7 and -8 were subjected to septic injury, and displayed a similar increase in the levels of AMPs to the wild type control. Therefore Toll-6, -7 and -8 may not play a significant role in antimicrobial immune responses	Yagi et al., 2010
Toll-7	Immune response	Toll-7 is involved in anti-viral immune responses Drosophila cells were pre-treated with Toll-7 dsRNAs samples were challenged with vesicular stomatitis virus (VSV-GFP) and analysed for infection. Toll-7 viral load was increased by 26% in comparison to controls. Furthermore, in vivo Toll-7 RNAi adult flies displayed an increase in viral load 6 and 9 days post infection. Loss of function of Toll-7 also lead to increased mortality following VSV infection however it was unable to induce expression of Drosomycin, MyD88, Dif, Diptericin and vir-1 indicating that this anti-viral mechanism is not dependent upon canonical Toll signalling pathways. Antibodies raised for endogenous Toll-7 show that VSV and Toll-7 interact at the plasma membrane. Toll-7 precipitates with VSV, whereas Toll-1 and tubulin did not. Thus, it appears that Toll-7 is a PRR for VSV. In S2 cells Toll-7 is required for antiviral autophagy. Cells were transfected with GFP-LC3 reporter and treated with dsRNA against Toll-7 and infected with VSV, resulting in a reduction of VSV puncta. Furthermore, in vivo, Toll-7 loss of function flies were infected with VSV-GFP and fat bodies dissected three days later and stained with Lysotracker. There was a significant reduction of Lysotracker staining despite extensive viral infection, and through immunoblot assays determined that VSV autophagy is decreased in Toll-7 RNAi flies	Nakamoto et al., 2012
Toll-8	Expression	Toll-8 expression at the cellular blastoderm stage consists of 8 circular bands in the embryo overlaping Eve expression. These bands develop into 14 strong stripes during germ band extension and sit posterior to wingless expression. Furthermore, during this period Toll-8 is expressed in the head as well as in the presumptive neurogenic region. Following germ band retraction Toll-8 is located in both dorsal and ventral epidermis, in the pharynx, proventriculus and midgut regions	Kambris et al. 2002
Toll-8	Expression	Toll-8 mRNA is localised along the VNC and PNS at sites where neural differentiation occurs, due to localisation of neural precursor cells and ectoderm contact sites	Seppo et al., 2003
Toll-8	Expression	Overall Toll-8 expression is very similar, but doesn't overlap that of Toll-2 in the embryo. RT-PCR experiments show that Toll-8 transcripts are located in blood cells, lymph gland and the fat body	Kambris et al. 2002
Toll-8	Expression	Toll-8 transcripts have been detected by RT-PCR in all developmental stages, and northen blot analysis revealed that both the macrophage-like cell lines S2 and l(2)mbn cells express Toll-8	Tauszig et al., 2000
Toll-8	Expression	Toll-8 mRNA is highly enriched in the larval tracheal epithelium with an apical subcellular localisation	Akhouayri et al., 2011
Toll-8	Function	Toll-8 can function as a cell adhesion molecule via the induction of cell aggregations	Keith & Gay 1990; Eldon et al. 1994; Kim et al. 2006

Toll-8	Function	Toll-8 linked to glycosylation and neural patterning during embryogenesis	Ayyar et al. 2007 and Seppo et al. 2003
Toll-8	Function	Toll-8 is able to antagonize Dpp signaling in wing imaginal discs	Kim et al., 2006
Toll-8	Signalling	Furthermore, in imaginal disc epithelium Toll-8 interacts with Rel protein during sensory organ development, and plays a role in specification of neurons	Ayyar et al., 2007
Toll-8	Development	Toll-8 is required for the formation of the anterior – posterior (A-P) boundaries for wing and leg imaginal disc development and forms a functional subgroup with these other Tolls. In the wing disc both Toll-8 and Toll-2 were observed in the proximal regions and in leg discs they were observed in the anterior compartment	Yagi et al., 2010
Toll-8	Development	Whilst the Toll-8 single mutant was mostly viable, both Toll-2; Toll-8 as well as Toll-7; Toll-8 double mutants displayed increased mortality during late embryogenesis or early larval development suggesting some kind of functional redundancy between Toll-2 and Toll-8 and Toll-7 and Toll-8 during development	Yagi et al., 2010
Toll-8	Immune response	Toll-8 mutants (similar to Toll-6 and Toll-7) failed to induce AMP genes following septic injury and mortality were the same as in wild type flies, indicating that Toll-8 may not function in antibacterial immune responses	Yagi et al., 2010
Toll-8	Immune response	Toll-8 inhibition has also been shown to be a negative regulator of the IMD signaling pathway.	Akhouayri et al. 2011
Toll-9	Structure	Structurally the TIR domain of Toll-9 has closer homology to mammalian TLRs containing two introns. The extracellular domain is more significantly diverged from the other Drosophila Tolls where it does not contain a N-flanking CRC at the C-terminus	Bilak et al., 2003; Imler and Zheng, 2003
Toll-9	Expression	Toll-9 is located in vitellophages, cells which are involved in breaking down yolk during stage 5 of embryonic development, and this localisation increases during gastrulation. As germ band extension proceeds there is clear Toll-9 localisation in the head region where the specification of hemocyte progenitors occurs, thus Toll-9 may have some function in hematopoiesis. After this period Toll-9 is no longer detected within the embryo. In tissues implicated in immunity, Toll-9 is located in the larval fat body and lymph gland	Kambris et al. 2002
Toll-9	Expression	Toll-9 is present in S2 cells	Ooi et al., 2001
Toll-9	Immune response	In S2 cells, a naturally constituitively active Toll-9, is able to activate the Drosomycin gene reporter, increasing luciferase activity >150 fold. This result was similar to the activated form of Toll-1, which contains a cysteine residue outside the trans-membrane domain. Therefore the activity of Toll-9 is due to a loss of cysteine or gain of tyrosine residues in the same region. This was confirmed due to the introduction of a cysteine residue, forming an inactive form of Toll-9. In order to activate drosomycin, Toll-9 works via Pelle and/or Cactus, as mutations of both lead to the inhibition of Toll-9 mediated drosomycin activity	Ooi et al., 2001; Bilak et al., 2003
Toll-9	Immune response	Toll-9 may utilize components of the Toll signaling pathway. Both the dominant-negative form of MyD88 and Pelle can block Drosomycin activation by Toll-9. S2 cells transfected with Toll-9 vectors lacking LRRs, were able to activate drosomycin. However this activation is inhibited by both dominant-negative Pelle and dMyD88, similar to what is known of Toll-1, therefore they may function by the same means	Bilak et al., 2003
Toll-9	Immune response	With regards to in vivo functions Toll-9 mutant flies appear to lack any form of phenotype. They generated a null allele for Toll-9 and measured transcript levels in whole larvae and adult flies and more localised gut tissues. Transcript levels for Toll-9 between normal conditions, flies reared in axenic conditions or infected with Ecc showed no differences between wild type and Toll-9 mutants. Furthermore levels of Drosomycin, Defensin, Diptericin and Drosomycin3 remained the same as wild type during these conditions and following infection. Therefore Toll-9 in vivo may not regulate levels of AMP genes within either normal or immune induced conditions	Narbonne-Reveau et al., 2011
Toll-9	Immune response	However, RNAi isolation and microarray assays have shown that some genes activated by Toll10b, are not activated by Toll-9 and vice versa	Bettencourt et al., 2004
Toll-9	Development	Transgenic expression analysis also demonstrates that activated Toll-1 and Toll-9 may share functional similarities. They induce similar phenotypes including, lethality when overexpressed with numerous imaginal disc Gal-4 driver lines (32B-, 71B-, en- and ptc-Gal4) as well as causing a glazed eye phenotypes when over-expressed in eye imaginal discs (GMRand lz-Gal4) However; some differences did occur for instance, both Toll-9 and Pelle overexpression with dpp-Gal4 results in leg defects or thickening of anterior cross veins respectively whereas activate Toll-1 results in cross vein loss	Yagi et al., 2010

At the end of this developmental stage transcription reaches its maximal levels and is followed by a reduction of expression except for within the CNS (Kambris *et al.,* 2002). Toll-6 is present within longitudinal interneuron axons, ventral HB9+ neurons and Eve+ motorneurons (except for RP2 neurons) (McIlroy *et al.,* 2013).

During larval development Toll-6 protein is located within the CNS along the VNC neuropile and within aCC motorneurons (McIlroy *et al.,* 2013) and Toll-6 transcripts are expressed during pupal stages, detected by RT-PCR (Tauszig *et al.,* 2000). Within adults Toll-6 is present within dopaminergic neurons as well as within complimentary layers and rings of the fan shaped body (FSB) and ellipsoid body (EB) respectively, both sites of locomotor control (McIlroy *et al.,* 2013).

Toll-6 (along with Toll-2, Toll-7 and Toll-8) is required for wing and leg imaginal disc development where it has a specific function in the formation of the anterior – posterior (A-P) boundaries. When over-expressed with decapentaplegic Gal4 (dppGal4) mild splitting of the A-P boundary occurs at the distal wing end due to abnormal folding (Yagi *et al.,* 2010). Unlike some of the other Tolls, Toll-6 mutants induce AMP genes (*AttacinA, Diptericin, Drosomycin* or *Metchnikowin*) in a similar manner to the control following septic injury. Indicating Toll-6 may not play a significant role in antimicrobial immune responses (Yagi *et al.,* 2010).

While DNT2;Toll-6 mutant embryos are viable, DNT1;Toll-6 mutants are semi-lethal. A phenotype that can be rescued by the over-expression of Toll-6 (and Toll-7). This indicates that Toll-6 acts downstream of DNT-2 and is likely its receptor, furthermore co-immunoprecipitations showed that Toll-6 can physically interact with DNT-2 (McIlroy *et al.,* 2013).

Toll-6 together with Toll-7 is required for normal locomotion as Toll-6; Toll-7 double mutant larvae crawl slower than controls. Furthermore both are required for correct motor axon targeting which was visualised via projections of FasII ISNb/d whereby both Toll-6 and Toll-7 single and Toll-6; Toll-7 double mutants cause deficient targeting due to loss of projections and motoraxon misrouting in stage 17 embryos. Over-expression of both Toll-6 and Toll-7 in neurons also causes targeting defects (McIlroy *et al.,* 2013). Toll-6 and Toll-7 also regulate neuronal survival as single and double mutants resulting in increased number of apoptotic HB9+ and Eve+ EL interneurons in the embryonic CNS (McIlroy *et al.,* 2013).

1.5.5 *Drosophila* Toll-7

The TIR domain of Toll-7 shares 60% identity with Toll-2 (Tauzsig *et al.,* 2000). Toll-7 expression is diverse throughout embryogenesis. Zygotic expression begins during germ band extension as a strong spot within the head region, with diffuse expression in the trunk.

By the end of germ band extension, when CNS and PNS differentiation occurs, there are 14 well defined stripes that are in line with engrailed and posterior to wingless expression. At germ band retraction Toll-7 is located in leg imaginal disc precursors and epithelium of the large intestine (Kambris *et al*, 2002). Furthermore, Toll-7 is distributed throughout central HB9+ and Lim3+ RP motorneurons projecting along intersegmental nerve b/d which targets muscles 6, 7, 12, and 13, motor axons exiting CNS and interneuon axons that cross the midline (McIlroy *et al.,* 2013). By stage 16, expression becomes weaker and restricted only to CNS tissues (Kambris *et al*, 2002), and projections are visible along the three FasII and longitudinal fascicles (McIlroy *et al.,* 2013).

During larval stages Toll-7 is distributed along the VNC neuropile, and in the central complex of brains, similar to Toll-6, within complimentary layers and rings of the FSB and EB

respectively (McIlroy *et al.,* 2013). While structurally more similar to Toll-2, Toll-7 protein localisation is more similar to that of Toll-6.

As highlighted previously Toll-7 has neurotrophic functions in the *Drosophila* CNS and is required in conjunction with Toll-6 for normal locomotion and motor axon targeting and to maintain neuronal survival. Toll-7; DNT-2 mutants are semi lethal, and co-immunoprecipitations revealed physical interactions with both DNT-1 and DNT-2, therefore Toll-7 and Toll-6 may be promiscuous in its ligand binding (McIlroy *et al.,* 2013).

Toll-7 mutants whilst viable display leg defects and Toll-7 is located around the wing pouch and hinge region of the wing disc, as well as along the A-P border from tarsal to tibia segments of leg discs. A transgenic expression assay identified Toll-7, Toll-2, Toll-6 and Toll-8 as required for the formation of the anterior – posterior (A-P) boundaries for wing and leg imaginal disc development, and identifies this group of Tolls as possibly being a subgroup (Yagi *et al.,* 2010).

Mutants for Toll-6, -7 and -8 were subjected to septic injury, and displayed a similar increase in the levels of AMPs to the wild type control. Therefore Toll-6, -7 and -8 may not play a significant role in antimicrobial immune responses (Yagi *et al.,* 2010). However, Toll-7 is involved in anti-viral immune responses (Nakamoto *et al.,* 2012).

Drosophila cells were pre-treated with Toll-7 dsRNAs samples were challenged with vesicular stomatitis virus (VSV-GFP) and analysed for infection. Toll-7 viral load was increased by 26% in comparison to controls. Furthermore, *in vivo* Toll-7 RNAi adult flies displayed an increase in viral load 6 and 9 days post infection. Loss of function of Toll-7 also lead to increased mortality following VSV infection however it was unable to induce expression of *Drosomycin*, MyD88, Dif, *Diptericin* and *vir-1* indicating that this anti-viral

mechanism is not dependent upon canonical Toll signalling pathways. Antibodies raised for endogenous Toll-7 show that VSV and Toll-7 interact at the plasma membrane. Toll-7 precipitates with VSV, whereas Toll-1 and tubulin did not. Thus, it appears that Toll-7 is a PRR for VSV. In S2 cells Toll-7 is required for antiviral autophagy. Cells were transfected with GFP-LC3 reporter and treated with dsRNA against Toll-7 and infected with VSV, resulting in a reduction of VSV puncta. Furthermore, *in vivo*, Toll-7 loss of function flies were infected with VSV-GFP and fat bodies dissected three days later and stained with Lysotracker. There was a significant reduction of Lysotracker staining despite extensive viral infection, and through immunoblot assays determined that VSV autophagy is decreased in Toll-7 RNAi flies (Nakamoto *et al.,* 2012).

1.5.6 *Drosophila* Toll-8

Toll-8 expression at the cellular blastoderm stage consists of 8 circular bands in the embryo overlaping *Eve* expression. These bands develop into 14 strong stripes during germ band extension and sit posterior to *wingless* expression. Furthermore, during this period Toll-8 is expressed in the head as well as in the presumptive neurogenic region. Following germ band retraction Toll-8 is located in both dorsal and ventral epidermis, in the pharynx, proventriculus and midgut regions (Kambris *et al.* 2002). Toll-8 mRNA is localised along the VNC and PNS at sites where neural differentiation occurs, due to localisation of neural precursor cells and ectoderm contact sites (Seppo *et al.,* 2003).

Overall Toll-8 expression is very similar, but doesn't overlap that of Toll-2 in the embryo. RT-PCR experiments show that Toll-8 transcripts are located in blood cells, lymph gland and the fat body (Kambris *et al.* 2002). Toll-8 transcripts have been detected by RT-PCR in all developmental stages, and northen blot analysis revealed that both the macrophage-like cell

lines S2 and l(2)mbn cells express Toll-8 (Tauszig *et al.,* 2000). Furthermore Toll-8 mRNA is highly enriched in the larval tracheal epithelium with an apical subcellular localisation (Akhouayri *et al.,* 2011).

From the location and distribution of Toll-8 it could be perceived that it may function in both development and immunity. As previously described for Toll-1 and Toll-2, Toll-8 over-expression can function as a cell adhesion molecule via the induction of cell aggregations (Keith & Gay 1990; Eldon et al. 1994; Kim et al. 2006). Toll-8 has also been linked to glycosylation and neural patterning during embryogenesis (Ayyar *et al.* 2007 and Seppo *et al.* 2003), and is able to antagonize Dpp signalling in wing imaginal discs (Kim *et al.,* 2006). Furthermore, in imaginal disc epithelium Toll-8 interacts with Rel protein during sensory organ development, and plays a role in specification of neurons (Ayyar *et al.,* 2007).

As has been previously described for Toll-2, Toll-6 and Toll-7, Toll-8 is required for the formation of the anterior – posterior (A-P) boundaries for wing and leg imaginal disc development and forms a functional subgroup with these other Tolls. In the wing disc both Toll-8 and Toll-2 were observed in the proximal regions and in leg discs they were observed in the anterior compartment (Yagi *et al.,* 2010). Whilst the Toll-8 single mutant was mostly viable, both Toll-2; Toll-8 as well as Toll-7; Toll-8 double mutants displayed increased mortality during late embryogenesis or early larval development suggesting some kind of functional redundancy between Toll-2 and Toll-8 and Toll-7 and Toll-8 during development (Yagi *et al.,* 2010).

A previous report identified Toll-8 has a critical function within the CNS. The loss of Horse Radish Peroxidase (HRP) in the TM3 chromosome and 71C deficiency was rescued by Toll-8 expression (Seppo *et al*., 2003). However Toll-8 null mutant embryos express normal HRP

patterns in the CNS (Yagi *et al.,* 2010). Furthermore Toll-8 mutants (similar to Toll-6 and Toll-7) failed to induce AMP genes following septic injury and mortality were the same as in wild type flies, indicating that Toll-8 may not function in antibacterial immune responses (Yagi *et al.,* 2010).

Toll-8 inhibition has also been shown to be a negative regulator of the IMD signalling pathway. Immune responses following bacterial infection, triggered AMP expression in the trachea of Toll-8 mutants and q-RT-PCR shows *Drosomycin, Drosocin* and *Attacin* mRNA levels are significantly increased in comparison to wild type controls. Using *Dipt-cherry* reporter constructs and q-RT-PCR, Toll-8 was shown not to be required in both the fat-body and gut following septic injury or oral ingestion of *Erwinia carotovora carotovora (Ecc).* Furthermore, Toll-8 mutants were still able to activate *toll* signalling following gram^{+} bacterial immune responses. Therefore it is likely that Toll-8 is required to reduce IMD pathway responses in trachea following infection (Akhouayri *et al*. 2011).

This mechanism Akhouayri *et al*. suggested work via the adaptor protein dSarm. dSarm mutants showed an increase of *Drosomycin* activation in the trachea following infection, a phenotype that was not observed in MyD88 mutants. Mutants of DNT-1 also phenocopy responses of Toll-8 and therefore it may be the ligand required for this immune response. Overall DNT-1, Toll-8 and dSarm may work together to down-regulate the IMD pathway in trachea following infection and immune responses (Akhouayri *et al*. 2011).

1.5.7 *Drosophila* Toll-9

Structurally the TIR domain of Toll-9 has closer homology to mammalian TLRs containing two introns. The extracellular domain is more significantly diverged from the other *Drosophila* Tolls where it does not contain a N-flanking CRC at the C-terminus (Bilak *et al.,*

2003; Imler and Zheng, 2003). Toll-9 is located in vitellophages, cells which are involved in breaking down yolk during stage 5 of embryonic development, and this localisation increases during gastrulation. As germ band extension proceeds there is clear Toll-9 localisation in the head region where the specification of hemocyte progenitors occurs, thus Toll-9 may have some function in hematopoiesis. After this period Toll-9 is no longer detected within the embryo. In tissues implicated in immunity, Toll-9 is located in the larval fat body and lymph gland (Kambris *et al.* 2002) and is present in S2 cells (Ooi *et al.*, 2001).

In S2 cells, a naturally constituitively active Toll-9, is able to activate the *Drosomycin* gene reporter, increasing luciferase activity >150 fold. This result was similar to the activated form of Toll-1, which contains a cysteine residue outside the trans-membrane domain. Therefore the activity of Toll-9 is due to a loss of cysteine or gain of tyrosine residues in the same region. This was confirmed due to the introduction of a cysteine residue, forming an inactive form of Toll-9. In order to activate *drosomycin*, Toll-9 works via Pelle and/or Cactus, as mutations of both lead to the inhibition of Toll-9 mediated *drosomycin* activity (Ooi *et al.,* 2001; Bilak *et al.,* 2003).

In order to function in this manner, Toll-9 may utilize components of the Toll signalling pathway. Both the dominant-negative form of MyD88 and Pelle can block Drosomycin activation by Toll-9. S2 cells transfected with Toll-9 vectors lacking LRRs, were able to activate *drosomycin*. However this activation is inhibited by both dominant-negative Pelle and dMyD88, similar to what is known of Toll-1, therefore they may function by the same means (Bilak *et al.,* 2003).

However Narbonne-Reveau *et al*. call this finding into question. With regards to *in vivo*

functions Toll-9 mutant flies appear to lack any form of phenotype. They generated a null allele for Toll-9 and measured transcript levels in whole larvae and adult flies and more localised gut tissues. Transcript levels for Toll-9 between normal conditions, flies reared in axenic conditions or infected with *Ecc* showed no differences between wild type and Toll-9 mutants. Furthermore levels of *Drosomycin, Defensin, Diptericin and Drosomycin3* remained the same as wild type during these conditions and following infection. Therefore Toll-9 *in vivo* may not regulate levels of AMP genes within either normal or immune induced conditions (Narbonne-Reveau *et al*., 2011). However, RNAi isolation and microarray assays have shown that some genes activated by $Toll^{10b}$, are not activated by Toll-9 and vice versa (Bettencourt *et al.,* 2004).

Transgenic expression analysis also demonstrates that activated Toll-1 and Toll-9 may share functional similarities. They induce similar phenotypes including, lethality when over-expressed with numerous imaginal disc Gal-4 driver lines (32B-, 71B-, *en*- and *ptc*-Gal4) as well as causing a glazed eye phenotypes when over-expressed in eye imaginal discs (GMR- and *lz*-Gal4). However; some differences did occur for instance, both Toll-9 and Pelle over-expression with *dpp*-Gal4 results in leg defects or thickening of anterior cross veins respectively whereas activate Toll-1 results in cross vein loss (Yagi *et al.,* 2010).

1.6 *Drosophila* ligands for Toll receptors belong to the Neurotrophin superfamily

Spz is the well-known ligand of Toll-1, initially discovered for inducing dorsal-ventral (DV) polarity in early *Drosophila* embryos (Anderson and Nusslein-Volhard., 1984; Morisato and Anderson., 1994; Schneider *et al.,* 1994; Stein and Nusslein-Volhard., 1992). Later Spz was identified via predicted structural and biochemical analysis as a secreted protein containing an

NGF domain (De lotto and De Lotto., 1998; Gay and Gangloff., 2007; Mizuguchi *et al.,* 1998). At this time though, Spz was thought to be more similar to horseshoe crab coagulogen. Coagulogen is involved in blood-clotting cascades, and this presumption led to Spz being overlooked as an NGF related protein (Mizuguchi *et al.,* 1998).

The idea that *Drosophila* expressed any neurotrophic type molecules was widely dismissed. This was in part due to a lack of neurotrophin homologues being identified in the *Drosophila* genome once it was published (Adams *et al.,* 2000). Furthermore, in the *drosophila* CNS cell death was thought to be strictly programmed, a fixed mechanism with no regulation of cell number (Truman.1984; Barde., 1994; White *et al.,* 1994; Jaaro *et al*., 2001).

DNT1 was identified as related to BDNF during a search of the *Drosophila* genome using vertebrate neurotrophin sequences. DNT1 was identified as *spz2*, a paralog of spz (Parker *et al.,* 2001; Zhu *et al.,* 2008). Structural prediction then identified that both DNT1 and DNT2 are more similar in structure to the mammalian NTs than either are to Spz, and FUGUE analysis revealed that both are more closely related to the NTs than coagulogen, which had previously been thought to resemble Spz (Zhu *et al.,* 2008).

Subsequently Spz was crystallised. The confirmed structure of NGF was superimposed upon the crystal structure of the cysteine knot domain of Spz confirming it as a member of the NT superfamily (Hoffman *et al.,* 2008; Hoffman *et al.,* 2008b; Arnot *et al.,* 2010; Weber *et al.,* 2010). Spz is secreted as a pro-protein and is extracellularly cleaved by either the serine protease Easter in DV patterning or SPE in immunity to release the active cysteine knot (Gay and Gangloff 2007).

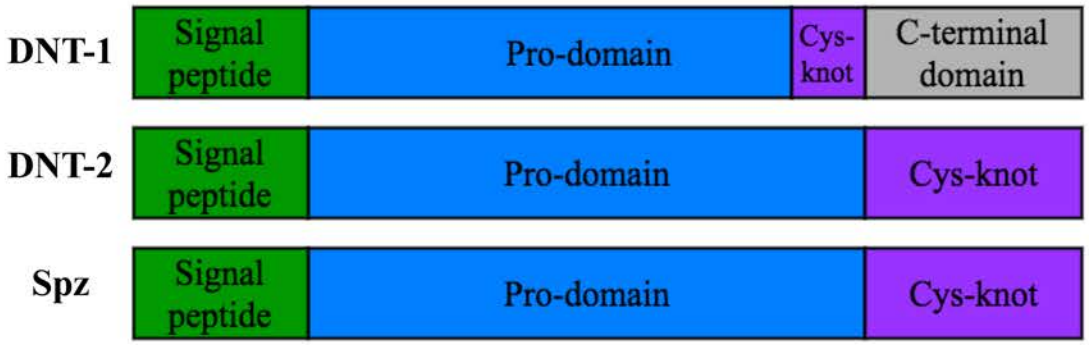
All three *Drosophila* NTs have the same protein structure as the other NT superfamily members which incorporates a pro-domain, signal peptide and a cystine-knot domain (Figure 1.4). This cystine-knot domain is highly conserved between members of the NT superfamily and is also different from other cystine-knots [De Lotto and De Lotto 1998; Mizugucgu *et al.,* 1998; Parker *et al.,* 2001; Zhu *et al.,* 2008; Weber *et al.,* 2007; Arnot *et al.,* 2010; Weber *et al.,* 2010].

Processing of Spz is similar to the cleavage of proBDNF at the synaptic cleft by plasmin (also a serine protease). Following proteolytic cleavage by the tissue plasminogen activator (tPA) under high frequency neuronal activity (Gualandris *et al.,* 1996). Dimerization of the DNTs occurs following cleavage, in a manner similar to mammalian NTs (Zhu *et al.,* 2008).

Now we know, three members of the *Drosophila* NT family are *spatzle* (Spz), *Drosophila neurotrophin-1* (*DNT1)* and *Drosophila neurotrophin-1 (*DNT2*)* (Parker *et al.,* 2001; Zhu *et al.,* 2008). *Drosophila* also has other neurotrophic factors including DmMANF, homologous to mammalian MANF and CDNF, as well as Netrins. DmMANF is produced by glial cells and maintains dopamine (DA) levels and protects dopaminergic neurons. Reductions in DmMANF levels result in decreased DA neurites and larval lethality (Palgi *et al.,* 2009; Lindholm and Saarma 2010). Homologues of mammalian NTs as well as *Drosophila* Spz have now also been characterised in other invertebrates including *Daphnia pulex* (Wilson., 2009).

During embryonic development DNT1, DNT2 and Spz are expressed in neuronal CNS target cells including *en-passant* midline (interneurons), muscles (motor-neurons) and optic lobes (photoreceptor neurons). These NTs are required for neuronal survival. Loss of function

Figure 1.4 Protein Structure of the *Drosophila* neurotrophins



All three Drosophila NTs including DNT-1, DNT-2 and Spz have the same protein structure as the mammalian NT superfamily members. They contain a signal peptide, pro-domain, and a conserved cystine-knot domain. This cystine-knot domain is highly conserved between members of the NT superfamily and is different from other proteins containing cystine-knots.

mutants of DNT-1, DNT-2 and Spz, resulted in increased cell death and Eve^{+} and $HB9^{+}$ neuron loss in the CNS, and whereas over-expression of the mature CK forms (DNT-1CK, DNT2-CK and spzCK) reduced apoptosis. DNTs also promote motor-axon targeting and connectivity. Loss of function of all three resulted in misrouting, mistargeting and sprouting defects in motor axon terminals. Functionally they may be similar, however there are some interesting differences. In embryonic muscles DNT-1, DNT-2 and spz are required by different neuronal types. DNT1;DNT2 double mutants affect the targeting of ISNb/d motoraxons, whereas spz mutants affect targeting of SNa motoraxons (Zhu *et al.,* 2008).

1.7 Downstream signalling events

Drosophila MyD88 is comprised of an N-terminal death domain, a TIR domain and a 150 amino acid C-terminal extension (Horng *et al.* 2001, Tauzsig-Dalamasure *et al* 2001; Charatsi 2003). Prior to Toll activation MyD88 requires to form a pre-signalling complex via death domain interactions with Tube (Sun *et al.* 2004, Towb *et al.* 1998).

Weckle (Wek) encodes a linking adaptor protein for Toll and MyD88 and is located dorsally at the plasma membrane of embryos. Wek is also localized to the nucleus and cytoplasm of fat body in larvae and adults. Localization is independent of the activation status of Toll. Chen *et al,* showed via genetic and S2 cell experiments that Wek is epistatic of Toll and acts upstream of both Dorsal and Cactus. They also established through yeast-2-Hybrid assays that Wek-FL self-associates, thus it is likely that it homodimerises and deletion mapping and co-immunoprecipitation studies provide evidence that WekN (N terminus of protein comprising amino acids 1-103) is the dimerization domain. Furthermor; co-immunoprecipitation studies using HA-tagged Wek transfected S2 cells interact with Flag-tagged Toll-1, Toll-9, Toll-5 and V5-tagged MyD88. All three domains of Wek (WekN, WekM amino acids 104-272 and

WekC C-terminus amino acids 273-470) are able to form stable complexes with Toll whereas only WekM and WekC can interact with MyD88 indicating that Wek is targeted to plasma membrane via the interaction with multiple sites of both Toll-1 and MyD88 in order to form a Toll-1/Wek/MyD88 complex (Chen *et al.,* 2006).

Chen *et al,* also showed that Wek is not required to activate a *drosomycin* reporter, as knocking down Wek mRNA levels with RNAi, had a negligible effect on the induction of *drosomycin* promoter by Toll-1. *In vivo* Wek heterozygotes and transheterozygotes (Wek^{lor}/cyo and Wek^{lor}/Wek^{EX14}) where exposed to $Gram^{+}$ bacteria and a fungus however the expression of *drosomycin* mRNA was not altered between them in northern blots. Conversely knocking down MyD88, depleted the levels of activity of the *drosomycin* reporter. Therefore it is unlikely that Wek is required for the induction of *drosomycin*, and thus is not involved in the humoral innate immune response.

Another Toll receptor signalling adaptor is *Drosophila* sterile alpha and Armadillo Motif (dSarm) was identified by Osterloch *et al.* 2012 via a forward genetic screen for loss of function mutants that are able to inhibit *Wallerian degeneration*. The dSarm gene encodes for a protein that contains an Armadillo/HEAT (ARM) domain, two sterile alpha motifs (SAM) as well as a Toll/Interleukin1 receptor homology (TIR) domain. Due to its combination of three protein-protein interacting domains it has the unique ability to interact with a large and varied range of molecules (Rodet *et al.* 2015).

In vertebrates SARM1 has shown to be influential in numerous processes including associating with mitochondria as well as microtubules in neurons and T cells (Zhou *et al.* 2013; Yuan *et al.* 2010). Furthermore it has also been shown that SARM1 has the ability to influence TLR signalling through interacting with Myd88, TRIF and TRAF6 (Zhou *et al.*

2013, Carty *et al.* 2006, Yang *et al.* 2010, Yuan *et al.* 2010).

Gerdts *et al,* cultured DRG neurons from SARM1 mutant mice and showed that when severed there is a delay in degeneration of the axons, a phenotype that is rescued by the over-expression of SARM1 by lentivirus. Furthermore, they demonstrated *in vivo* that severing SARM1 mutant sciatic nerves leads to only limited nerve loss in comparison to the control which by 7 days post injury resulted in complete nerve loss (Gerdts *et al.,* 2013). Osterloh *et al,* also determined that whilst wild type mice display a breakdown of axon and myelin sheaths as quick as three days post lesion, SARM1 mutants are protected for up to 14 days and furthermore axons and myelin sheaths are preserved (Osterloh *et al.,* 2012). These data indicate that SARM1 in mice is required for axonal degeneration in both sensory and motor fibres.

Gerdts *et al,* showed that SARM1 is required for non-apoptotic neuronal death as the expression of SARM1 mutant lacking amino acids 1-408 (SAM-TIR fragment) is capable of inducing axonal degeneration in the absence of injury, and apoptotic inhibitors do not inhibit this (Gerdts *et al.,* 2013). In *Drosophila* dSarm is widely expressed throughout the CNS and has been shown to be important in driving axon degeneration and the promotion of Wallerian degeneration. Osterloh *et al,* expressed full-length dSarm cDNA and OR22Gal4 in mutant clones and showed it suppressed the promotion of axonal degeneration seen in the dSarm mutant. They also show that over-expressing full-length dSarm by OR22a-Gal4 in a dSarm mutant background was able to rescue lethality and inhibition of wallerian degeneration seen in transheterozygotes. They conclude that dSarm is required in neurons to induce axonal degeneration (Osterloh *et al.,* 2012).

1.8 The use of *Drosophila* CNS as a Model System:

The *Drosophila* nervous system is a powerful model organism to investigate central nervous system development and function. In order to carry out complex functions, the *Drosophila* central nervous system requires being just as complex. As different functions are required during different developmental stages, the complexity of connections needs to reflect the requirements at that specific stage.

1.8.1 Cell number regulation

Organ growth is only finalised when the required amount of cells is reached, resulting in the cesation of cell division (Garcia-Bellido and Garcia-Bellido, 1998). The size an organ is determined by the proliferative state of the cell and regulated via local cell-to-cell interactions. The number of cell divisions is determined by its cell fate. Cells originate from neuroblast populations that are able to divide to generate precursor cells. The number of precursor cells is dependent upon two main factors. The first is the starting number of neuroblasts and the second is the neuroblast fate. Cell fate is whether or not a daughter cell retains stem cell properties and influences the outcome of becoming a precursor cell. Cell cycle exit and apoptosis of the neuroblasts and precursor cells determine the number of cell divisions that can occur.

Apoptosis can occur in a cell autonomous pre-determined cell fate to restrict cell number. Apoptosis can also occur non-autonomously following the incorrect promotion of cell survival from neighbouring cells. Neighbouring cells maintain survival via the production of pro-survival trophic factors (Raff, 1992; Raff *et al.,* 1993). In *Drosophila* and mammalian nervous systems approximately 50% of cells undergo programmed apoptosis, and to a greater extent, if non-autonomous disruption occurs (White *et al.,* 1994; Raff *et al.,* 1993).

Non-autonomous control of cell survival enables the adjustment of cell number for the correct

formation of tissues. For instance, more neurons are created than required. Only neurons that innervate the correct tissue, the source of trophic factors, will survive (Hamburger and Levi-Montalcini. 1949). CNS growth is thus dependent on a fine balance of cell cycle progression and survival/death. Cell cycle progression and cell survival are dependent upon correct nutrient uptake, temperature and growth factors. Growth factors work via the activation of insulin receptors that induce two signalling cascades. The RAS/MAPK kinase (Bergmann *et al.,* 2002) and PI3K kinase pathways (Fernandez *et al.,* 1995; Chen *et al.,* 1996; Brogiolo *et al.,* 2001).

In flies apoptosis is first seen during embryonic stage 11, after which it occurs throughout all development stages (Abrams *et al.,* 1993). There is a peak of apoptosis during metamorphosis where larval tissues that are no longer required for adult development are removed (Jiang *et al.,* 1997). Conservation of the main components involved in apoptosis occurs from mammals to flies, and involve numerous pro-apoptotic genes, initiator (in *Drosophila* these are Dronc, Dredd and Strica) and effector (in *Drosophila* these are drICE, dcp-1, decay and damm) caspases as well as inhibitor of apoptosis proteins (IAPs eg; Diap1 in *Drosophila*), which inhibit caspase activity (Xu *et al.,* 2009).

1.8.2 Structure of *Drosophila* CNS

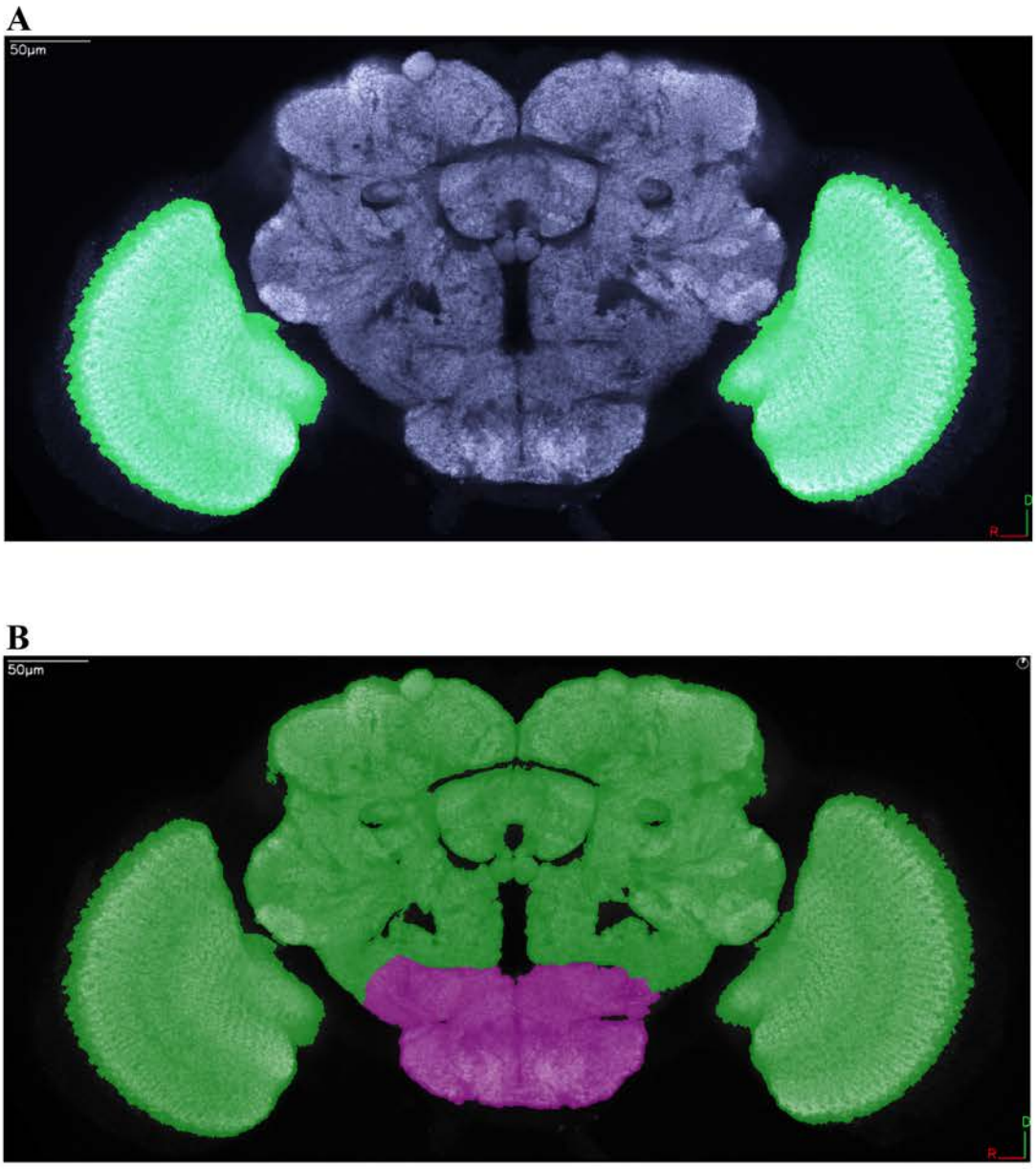
Precursors of the embryonic CNS (neuroblasts) derive from neurogenic regions of the ectoderm. Formation of neuroblasts is regulated by both proneural and neurogenic genes to promote or inhibit neuroblast formation respectively (Skeath and Carooll. 1994; Campos-Ortega 1995). Neuroblasts delaminate from the ectoderm in three waves starting during embryonic stage 9, continuing until stage 11 (Campos-Ortega and Hartenstein, 1985). Neuroblasts then progress through eight rounds of mitosis, forming ganglion mother cells

(GMC) which themselves then divide during stage 12 to create two neurons or glial cells (or a neuron and a glial cell) (Goodman and Doe, 1993). Commisural pioneer axons cross the midline of embryos in order to create two commissures (anterior and posterior). Longitudinal fibers form connectives for each segment (Hidalgo and Brand, 1997). It is the formation and anatomical positioning of these two commissures and their connection to longitudinal connectives that produce the laddered appearance of the embryonic neuropile (Goodman and Doe, 1993). Condensation of the VNC begins during stage 17 of embryo development.

During larval development the rate of CNS growth increases and the overall anatomy changes markedly in preparation for development into a fully formed adult. Neuroblasts derived from embryonic stages divide for a second time generating numerous immature, post-mitotic neurons (Prokop and Technau, 1991).

During metamorphosis interneurons extend both axons and dendrites and immature neurons differentiate sending out axons to target cells. Those cells that are no longer required or in abundance undergo programmed cell death in order to efficiently eliminate them (Truman, 1990). Optic lobes proliferate giving rise to both the outer and inner optic anlagen, which will go onto develop into the laminae, medulla, lobula and lobula plate (Hofbauer and Campos-Ortega 1990). Pupae undergo extensive remodeling with extensive programmed cell death and presumptive adult neurons differentiate. The optic lobes and some central brain compartments develop into the supraesophageal zone. A region of the adult brain that includes three fused neuromeres, the protocerebrum, deutocerebrum and tritocerebrum. Furthermore the VNC develop into the subesophageal ganglion. This region consists of fused ganglia of three gnathal segments the mandibular ganglion, maxillary ganglion and labial ganglion (Hartenstein, 1993) (Figure 1.5).

Figure 1.5: Regions of the adult *Drosophila* brain



The adult brain is comprised of two main compartments, the optic lobes (A: green) and the central brain (A: blue). During development the optic lobes and some central brain compartments of larvae develop into the supraesophageal zone. A region of the adult brain that includes three fused neuromeres, the protocerebrum, deutocerebrum and tritocerebrum. The larval VNC develop into the subesophageal ganglion (B:green). This region consists of fused ganglia of three gnathal segments the mandibular ganglion, maxillary ganglion and labial ganglion (B: purple). These schematic diagrams were generated using virtual fly brain; Janelia Adult Brain generator.

1.8.3 Circadian Rhythms

Circadian clocks regulate numerous rhythmic outputs including eclosion and locomotion. They do this through the involvement of numerous "clock" genes. The genes function to activate or repress transcription or modify stability, location, or the degradation of proteins (Hall, 2003). Transcriptional activators include Clock (Clk) and Cycle (Cyc) and Par domain protein 1ε (PDP1ε) (Rutila *et al.,* 1998; Allada *et al.,* 1998; Darlington *et al.,* 1998; Cyran *et al.,* 2003). Transcriptional repressors include Period (Per) and Timeless (Tim) that inhibit the functions of Clk-Cyc (; Darlington *et al.,* 1998; Cyran *et al.,* 2003). Proteins involved in stability and location include Doubletime (Dbt) (Price *et al.,* 1998; Kloss *et al.,* 1998), Casein kinase 2 (CK2) (Lin *et al.,* 2002; Akten *et al.,* 2003), Shaggy (Sgg) (Martinek *et al.,* 2001) and protein phosphatase 2a (PP2a) (Sathyanarayanan *et al.,* 2004). Proteins associated with Per degradation include Slimb (Slmb) (Ko *et al.,* 2002; Grima *et al.,* 2002).

Regulation of circadian rythyms is via two intracellular feedback mechanisms. The first is a Per/Tim loop and the second is a Clk loop (Hardin *et al.,* 1990; Glossop *et al.,* 1999). Transcription of associated clock genes is regulated via the protein products associated to each loop.

In the Per/Tim feedback mechanism, Clk-Cyc heterodimers activate transcription of *Per* and *Tim* as they bind to E-boxes from mid-day to early evening (Darlington *et al.,* 1998; Hao *et al.,* 1997; Wang *et al.,* 2001). Per is phosphorylated by Dbt and CK2 (Price *et al.,* 1998; Kloss *et al.,* 1998; Akten *et al.,* 2003; Nawathean and Rosbash, 2004). Tim binds to and stabalises the phosphorylated Per-Dbt complex. PP2a also works to stablises this Tim-Per-Dbt complex

(Lin *et al.,* 2002; Akten *et al.,* 2003; Martinek *et al.,* 2001). Sgg and CK2 phosphorylates this complex translocating it from cytoplasm to nucleus (Kloss *et al.,* 2001; Ashmore *et al.,* 2003; Shafer *et al.,* 2002). The complex then binds Clk-Cyc, subsequently inhibiting the transcription of Per and Tim (Lee *et al.,* 1999). Dbt phosphorylation destabilizes and degrades Per and Clk. Tim is degraded via tyrosine phosphorylation. Non-phosphorylated Clk and Cyc accumulate and trigger further cycles of Per and Tim transcription.

In the Clk feedback mechanism, during early evening Clk-Cyc heterodimers activate transcription of PDP1ε and Vri. Vri binds to V/P boxes to inhibit Clk transcription (Cyran *et al.,* 2003; Glossop *et al.,* 1999). PDP1ε accumulates during this time to remove Vri and activate Clk transcription (Cyran *et al.,* 2003). A clock independent activator (Act) activates Clk and potentiates accumulation of non-phosphorylated Clk, which again forms heterodimers with Cyc, and the cycle begins again (Cyran *et al.,* 2003; Glossop *et al.,* 1999). This loop also controls Cryptochrome (Cry) transcription, which encodes a photoreceptor that function in numerous tissues (Stanewsky *et al.,* 1998; Emery *et al.,* 1998; Krishnan *et al.,* 2001; Ivanchenko *et al.,* 2001).

Small ventral lateral neurons (sLNvs) are required for regular locomotor activity rhythms under dark/dark (DD) conditions. These sLNVs project into the dorsal brain, and contain the neuropeptide pigment-dispersing factor (PDF), which is pivotal in maintaining free running locomotor activity rhythms (Renn *et al.,* 1999; Park *et al.,* 2000). In light/dark (LD) conditions there are two main activity peaks, the first in the morning, and the next in the evening. Different oscillators differentially regulate the different peaks with morning activity driven by venterolateral neurons (LNVs) and the evening by dorsolateral neurons (LNDs)

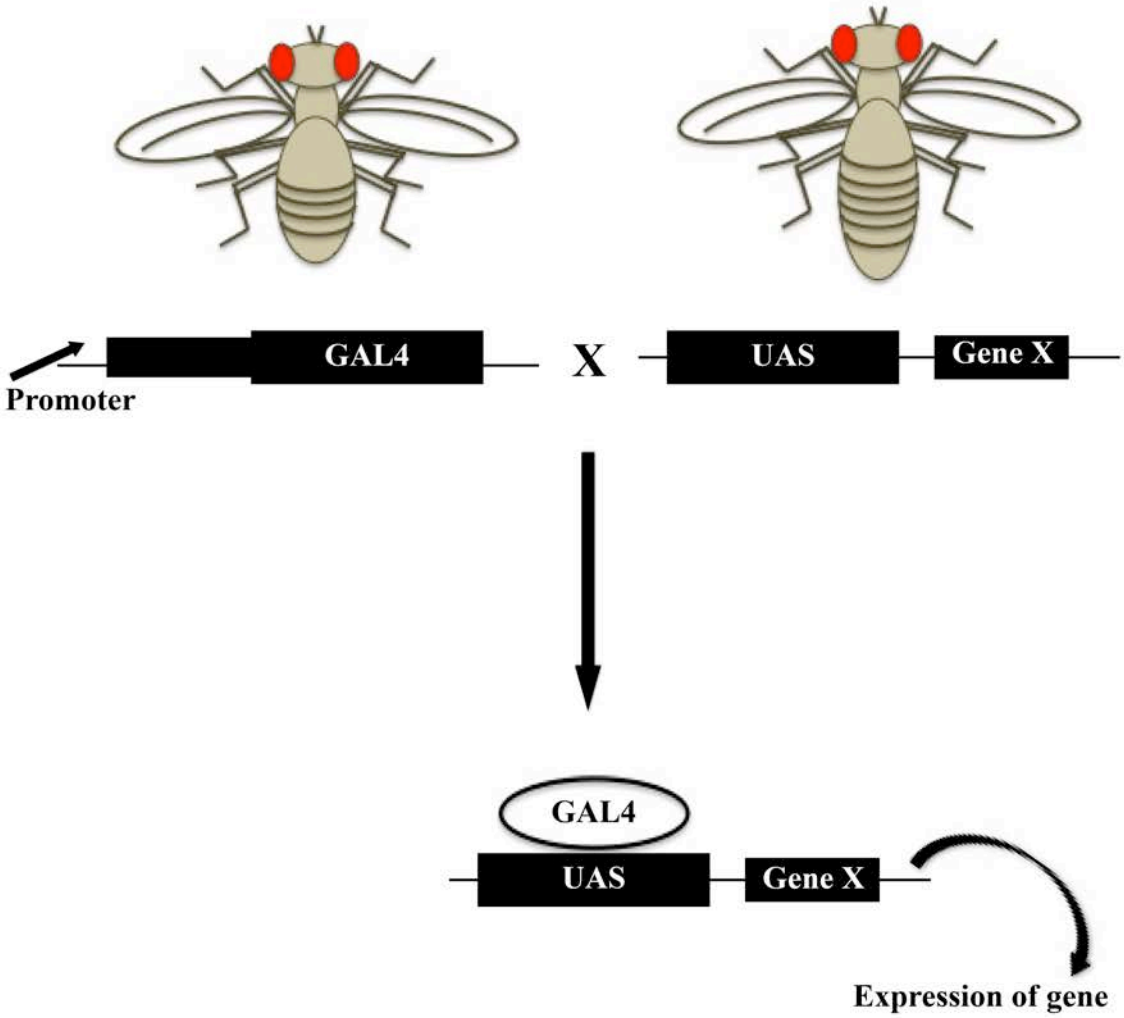
(Stoleru *et al.,* 2004; Grima *et al.,* 2004). In all there are in the region of 150 neurons that express "clock" genes dispersed throughout the adult brain (Nitabach and Taghert, 2008) that are activated/suppressed by Zeitgebers.

1.8.4 *Drosophila* Tools

Since the sequencing of the *Drosophila* genome in 2000 and the continuing development of different genetic tools there has been an increase in the ability to monitor or express an exogenous gene in a tightly regulated manner. Detection of gene enhancers were originally identified for their use in bacteria by Casadaban and Cohen 1979, who designed a vector, which was able to randomly integrate into the *Escherichia coli* chromosome, and carried a promoterless *lac* operon. The first gene enhancers for use in *Drosophila* were generated from transposable *P*-elements introducing the *lacZ* gene into the genome (O'Kane and Gehring, 1987). A P-element promoter then drives the gene and gene expression monitored via β-galactosidase activity (O'Kane and Gehring, 1987). Over time this gene enhancer has been developed further to incorporate mechanisms for their effective use such as carrying a dominant mini-*white* marker for increase genetic manipulation (Bier *et al.,* 1989).

One of the most widely used gene enhancer tools for targeted gene expression is the GAL4/UAS system (Figure 1.6). Originally identified in the yeast *Saccharomyces cerevisiae* as a potent gene regulator GAL4 encodes an 881 amino acid protein induced by galactose (Oshima *et al.,* 1982; Laughon *et al.,* 1984). In 1988, Fisher *et al.* demonstrated that the GAL4 transcriptional activator also functions in the *Drosophila* system where it is able to induce transcription of a specific reporter gene under Upstream Activating Sequences (UAS; series of GAL4 binding sites) control. The UAS/Gal4 system has become one of the most commonly, used *in vivo* tools in order to direct gene expression (Brand and Perrimon. 1993).

Figure 1.6: Schematic diagram of the GAL4/UAS System.



Schematic diagram of the GAL4/UAS System. The gene of interest (responder) is controlled by presence of UAS element, and its transcription is reliant upon the presence of GAL4 (driver), both of which are maintained in separate parental flies requiring mating for expression. The combination of the UAS responder and GAL4 driver results in offspring that express the gene of interest.

Furthermore there appears to be no deleterious phenotypic effects of this system, as the gene of interest (responder) is controlled by presence of UAS element, and its transcription is reliant upon the presence of GAL4 (driver), both of which are maintained in separate parental flies and thus requires mating for expression. This combination results in offspring, which express the responder wherever the GAL4 is driven (Duffy. 2002). The transgenes that were expressed in flies using the UAS/Gal4 system during this thesis are detailed in Table 2.1. A known limitation of GAL4 lines is that they may not always reflect endogenous expression of the gene of interest. This may be due to the lack a enhancer and/or repressor element required or the insert may affect the region into which it was inserted (Duffy. 2002). Furthermore the size, orientation and position (namely distance to promoter) have to be accurate for specific reflection of endogenous signal (Mayer *et al.,* 2013).

Minos Mediated Integrated Cassette (MiMIC) transposons were derived via TE mobilization using Mi{MIC} construct. This construct carries a gene trap cassette as well as a *yellow+* marker and a Avic\GFP fluorescent marker, all flanked by two inverted attP sites. These sites allow the conversion of a transposon sequence with any other via ϕC31 recombinase-mediated cassette exchange (RMCE) (Venken *et al.,* 2011). The transposon is exceptionally versatile as it can be modified due to the inversion of the attP sites allowing rapid change over of cassettes. For example, protein traps are achieved via the conversion of MiMIC insertions within coding introns into an artificial exon which encodes a protein tag e.g. GFP and thus creates protein fusion allowing the visualization of endogenous protein (Gnerer *et al.,* 2015).

Antibodies allow the detection of highly specific molecules within cells and tissues. They allow investigation into location and function of desired proteins, and when used correctly provide consistent results. Two antibodies that are used widely throughout this thesis include

anti-cleaved *Drosophila* death caspase-1 (Dcp-1) and anti-even skipped (Eve). Dcp-1 is one of seven known caspase genes (three initiator and four effector caspases). Dcp-1 is an effector caspase involved in the induction of apoptosis, and is therefore a very good readout of cell death (Steller *et al.,* 1994; Hay and Guo., 2006; Song *et al.,* 1997; Florentin and Arama., 2012). Eve is expressed in a small subset of neurons, which include qCC, pCC, RP2, CQ and EL clusters (Patel *et al.,* 1992), most of which also express Toll-6 receptors (Zhu *et al.,* 2008)

1.9 Aims of thesis

The aims of this thesis are to investigate if all of the Toll receptors are functionally equivalent in the *Drosophila* central nervous system and whether they can regulate cell number plasticity via the promotion of cell death and/or survival.

The specific research objectives are:

1. To visualize and test if all of the Toll receptors are expressed within the CNS of *Drosophila*
    a. This will be achieved through the use of immunohistochemical techniques and *Drosophila* genetic tools.
2. To test if all of the Toll receptors effect behaviour equally
    a. This will be achieved through the use of Trikinetics behavioural assays using over-expression and knockdown of the Toll receptors in neurons and glial cells.
3. To test if all of the Toll receptors affect CNS size, shape and cell number equally
    a. This will be achieved via the over-expression and knockdown of Toll receptors in neurons and glia following which VNC length and CNS area are measured. I will then test Toll receptors and DNTs ability to regulate cell survival and cell death using antibodies stainings and deadeasy software
4. To determine the signalling mechanisms downstream of Toll-6 that regulate both cell survival and death
    a. Test the effects of altering candidate downstream adaptors of Toll-6 using cell survival and cell death assays.

CHAPTER 2

MATERIALS AND METHODS

2.1 Genetics

2.1.1 Fly Stocks

Drosophila melanogaster was maintained on standard agar medium at 18 degrees for all stocks and at 25 degrees for all experimental flies along with a 12 hour light/dark cycle unless otherwise stated. Full lists of fly stocks used in experiments are shown in Table 2.1.

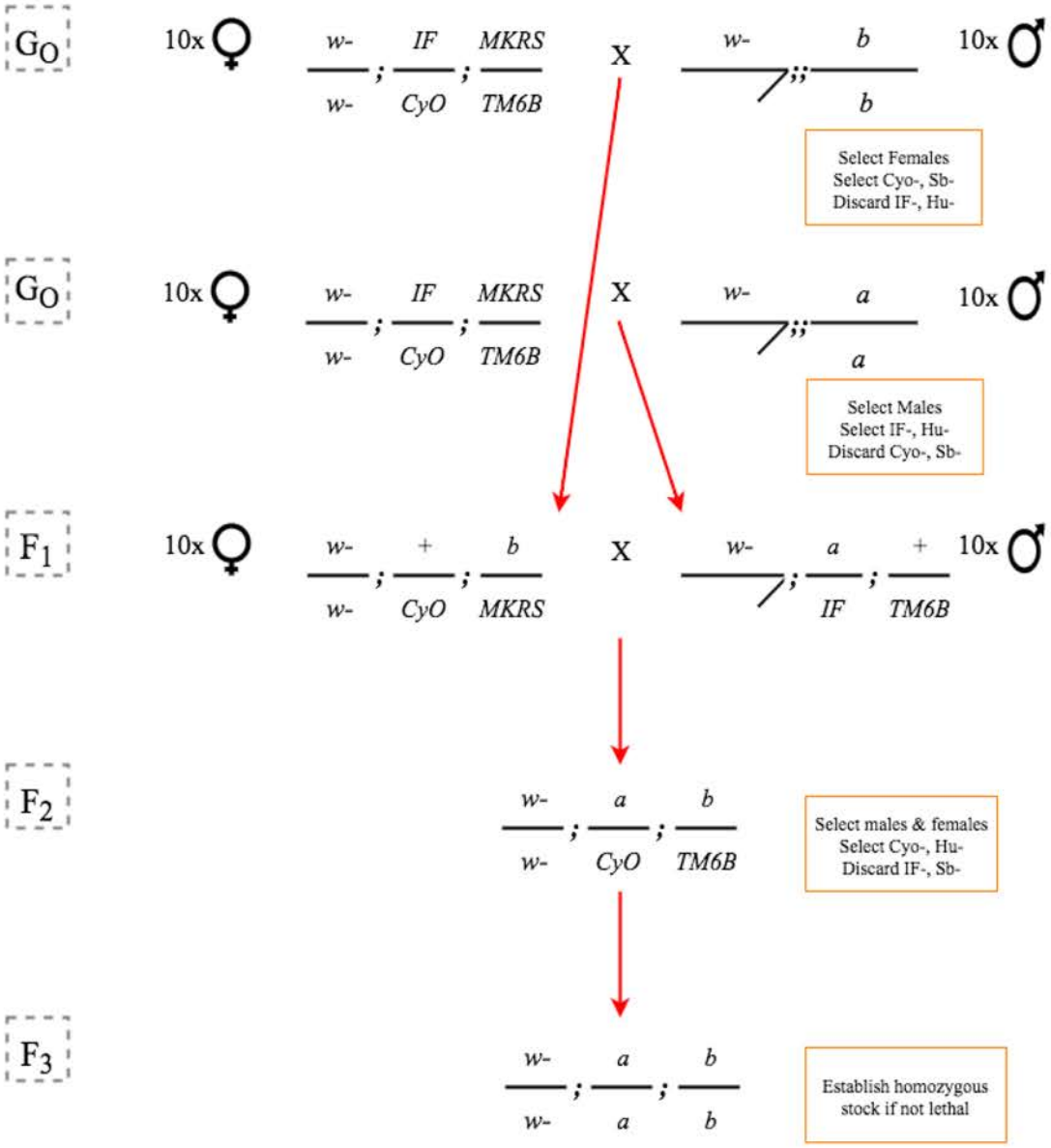
2.1.2 Genetic protocols

Drosophila is such a powerful model organism for use in biological research due in a large part to the conventional genetic techniques that can be utilised in order decipher different mechanisms which underpin fundamental biological processes. Throughout this thesis I have used a number of conventional genetic approaches to obtaining the desired fly lines for my research, including combinations of alleles on the second and third chromosomes (Figure 2.1) as well as recombination's on both the second (Figure 2.2) and the third chromosomes (Figure 2.3).

Drosophila has three pairs of autosomal chromosomes, each containing a left (L) and right (R) arm, as well as an X and Y chromosome. Each chromosome arm is assigned recombination units that allow the calculation of frequency of recombination between two genes that are located on the same chromosome arm. Recombination does not occur

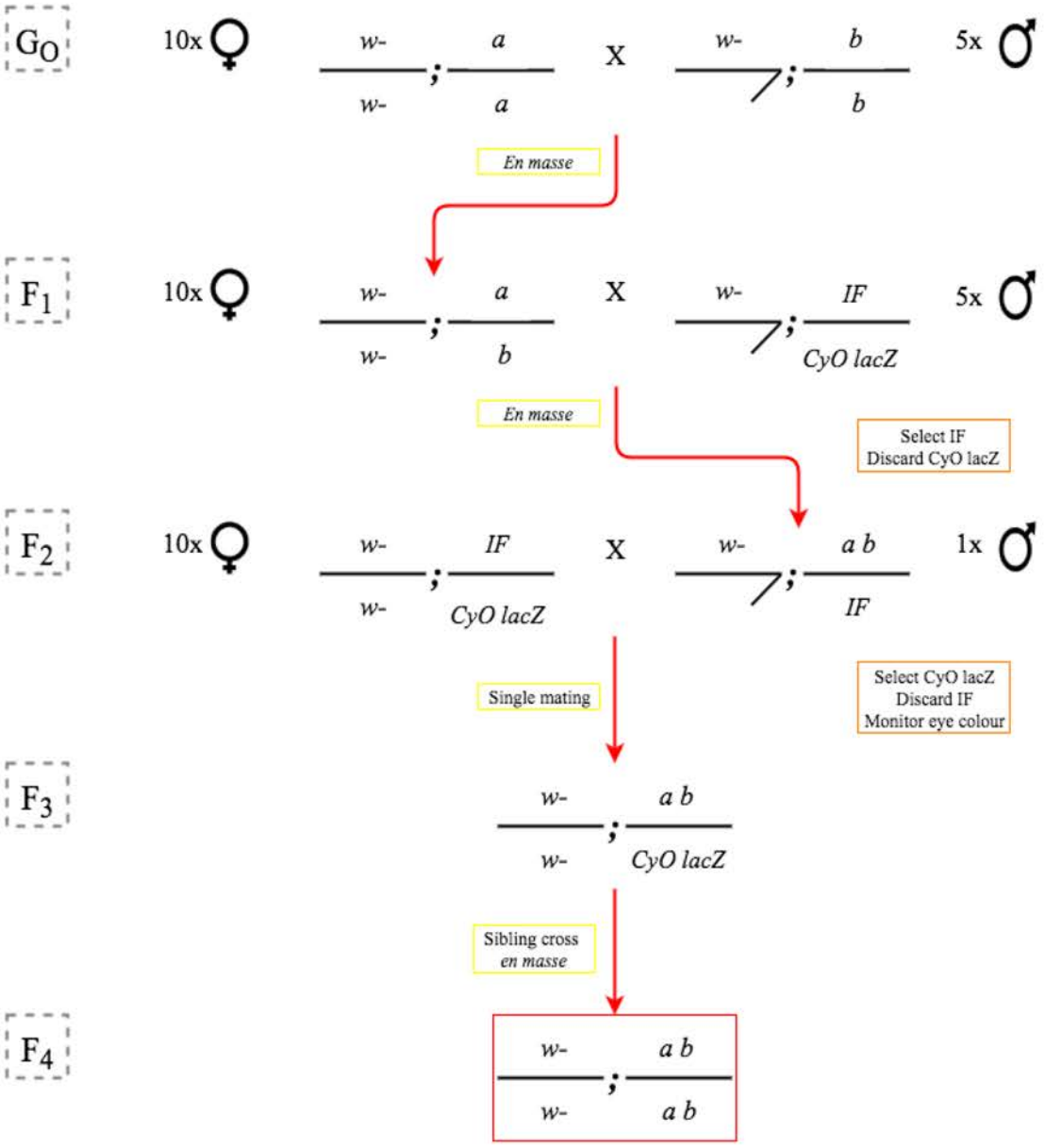
TABLE 2.1: FLY STOCK LIST		
Name as in thesis	**Genotype**	**Source**
YW		Hidalgo Lab Collection
Oregon R		Hidalgo Lab Collection
ElavGal4	w;+;ElavGal4	Hidalgo Lab Collection
RepoGal4	w;+;RepoGal4	Hidalgo Lab Collection
AlrmGal4	w;+;AlrmGal4	Hidalgo Lab Collection
c232Gal4	w;+;c232Gal4	Hidalgo Lab Collection
GMRGal4	w;GMRGal4/SM6aTm6B	Hidalgo Lab Collection
UAS-gcm		Hidalgo Lab Collection
myr-td-Tomato	10xUAS-myr-td-Tomato	Hidalgo Lab Collection
Toll-2^{K02701}	$y^{1}w^{67623}$(PlacW)$18w^{K02701}$	Bloomington
Toll-3MIMIC^{MIO2994}	y1w*;+;Mi{MIC}Mstprox$^{[MIO2994]}$	Bloomington
Toll-8Gal4^{MD806}	PGaw^{BMD806}/TM6B	Bloomington
UAS Toll-1	w;+;UASToll-1	Gift from Tony Ip
UAS Toll-2	w;UASToll-2;+	Gift from Tony Ip
UAS Toll-3	w;+;UASToll-3	Gift from Tony Ip
UAS Toll-4	w;UASToll-4;+	Gift from Tony Ip
UAS Toll-6	w;+;UASToll-6	Gift from Tony Ip
UAS Toll-6CY	w; UAS Toll-6[Cys-Tyr]; UAS Toll-6[Cys-Tyr]	Hidalgo Lab Collection
UAS Toll-7	w;UASToll-7;+	Gift from Tony Ip
UAS Toll-7CY	w; UAS Toll-7[Cys-Tyr] line 2-2M	Hidalgo Lab Collection
UAS Toll-8	w;+;UASToll-8	Gift from Tony Ip
UAS Toll-9	w;+;UASToll-9	Gift from Tony Ip
UAS Toll-1 RNAi	y[1]v[1]; UAS Toll-1-RNAi [P.TRiP.JF10491]	Bloomington
UAS Toll-2 RNAi	y1 sc* v1; P{TRiP.HM05241}attP2/TM3, Sb1	Bloomington
UAS Toll-3 RNAi	y1 v1; P{TRiP.HM05012}attP2	Bloomington
UAS Toll-4 RNAi	y1 v1; P{TRiP.HM05029}attP2	Bloomington
UAS Toll-5 RNAi	y1 v1; P{TRiP.HM05212}attP2	Bloomington
UAS Toll-6 RNAi	y1 v1; P{TRiP.HMS04251}attP2	Bloomington
UAS Toll-7 RNAi	y1 sc* v1; P{TRiP.HM05230}attP2	Bloomington
UAS Toll-8 RNAi	y1 v1; P{TRiP.HM05005}attP2	Bloomington
UAS Toll-9 RNAi	y1 sc* v1; P{TRiP.HM05227}attP2	Bloomington
UAS DNT1-FL	w;UASDNT1FL	Hidalgo Lab Collection
UAS DNT1-CK	UASDNT1ck3+ / UASDNT1ck3+ ; +/TM2	Removed 3xP3
UAS DNT2-FL	UASDNT2-FL-47C	Hidalgo Lab Collection
UAS DNT2-CK	UASDNT2CK	Removed 3xP3
MyD88^{NP6394}	y* w*; P{GawB}Myd88NP6394 / CyO, P{UAS-lacZ.UW14}UW14	Bloomington
UAS MyD88	w; UAS MyD88 FL/CyO	Gift fromJonathan Kagan
MyD88$^{cr2.8}$	w;MyD88$^{cr2.8}$	Hidalgo Lab- Neale H
DFBSC279	w1118; Df(2R)BSC279/SM6aTM6B	Bloomington
MyD88Kra56	MyD88Kra56/SM6aTM6B	This thesis
MyD88Kra56;UAS Toll-6	MyD88Kra56;UASToll6cy/SM6aTM6B	This thesis
ECT4MIO8854GFP	y1 w*; Mi{MIC}Ect4MI00854	Bloomington
EP3610	EP3610/TM6B	Bloomington
MyD88Kra56;ElavGal4	MyD88Kra56;ElavG4/SM6aTM6B	This thesis
MyD88Kra56;EP3610	MyD88Kra56;EP3610/SM6aTM6B	This thesis
EP3610;JNK RNAi	w:EP3610;JNK RNAi/SM6aTM6B	This thesis
MyD88Kra56;GMRGal4	MyD88Kra56;GMRG4/SM6aTM6B	This thesis
UASMyD88; ECT4MIO8854GFP	UASMyD88;Mi(MIC)ECT4Mio0854/TM6B	This thesis
DFBSC279; ECT4MIO8854GFP	DfBSC279;Mi(MIC)ECT4Mio0854/SM6aTM6B	This thesis
WekEX14	WekEX14FRT/SM6aTM6B	Gift from JeanLuc Imler
DFBSC690	w1118; Df(2L)BSC690, P+PBac{XP3.WH3}BSC690/CyO	Bloomington
UAS Wek	W;;UASWek-HA@86F	Removed 3xP3
WekEX14;ElavGal4	w;WekEX14;ElavGal4/SM6aTM6B	This thesis
dSarmRNAi;UAS Wek	W;dSarmRNAi;UAS Wek/SM6aTM6B	This thesis
WekEX14;EP3610	WekEX14;EP3610/SM6aTM6B	This thesis
WekEX14;UASToll-6cy	WekEX14;UASToll6cy/SM6aTM6B	This thesis
MyD88Gal4;UASHistoneYFP	w;MyD88Gal4;UASHistoneYFP	This thesis -Alicia
MyD88Kra56;UASWek	MyD88Kra56;UASWekHA/SM6aTM6B	This thesis
DfBSC690;ElavGal4	DfBSC690;ElavGal4/SM6aTM6B	This thesis
Spz6Gal4		Hidalgo Lab Collection
DfBSC279;UASToll6cy	DfBSC279;UASToll6cy/SM6aTM6B	This thesis
DfBSC279;UASWek	DfBSC279;UASWek/SM6aTM6B	This thesis

Figure 2.1 Combination of alleles on the 2ND and 3RD chromosome



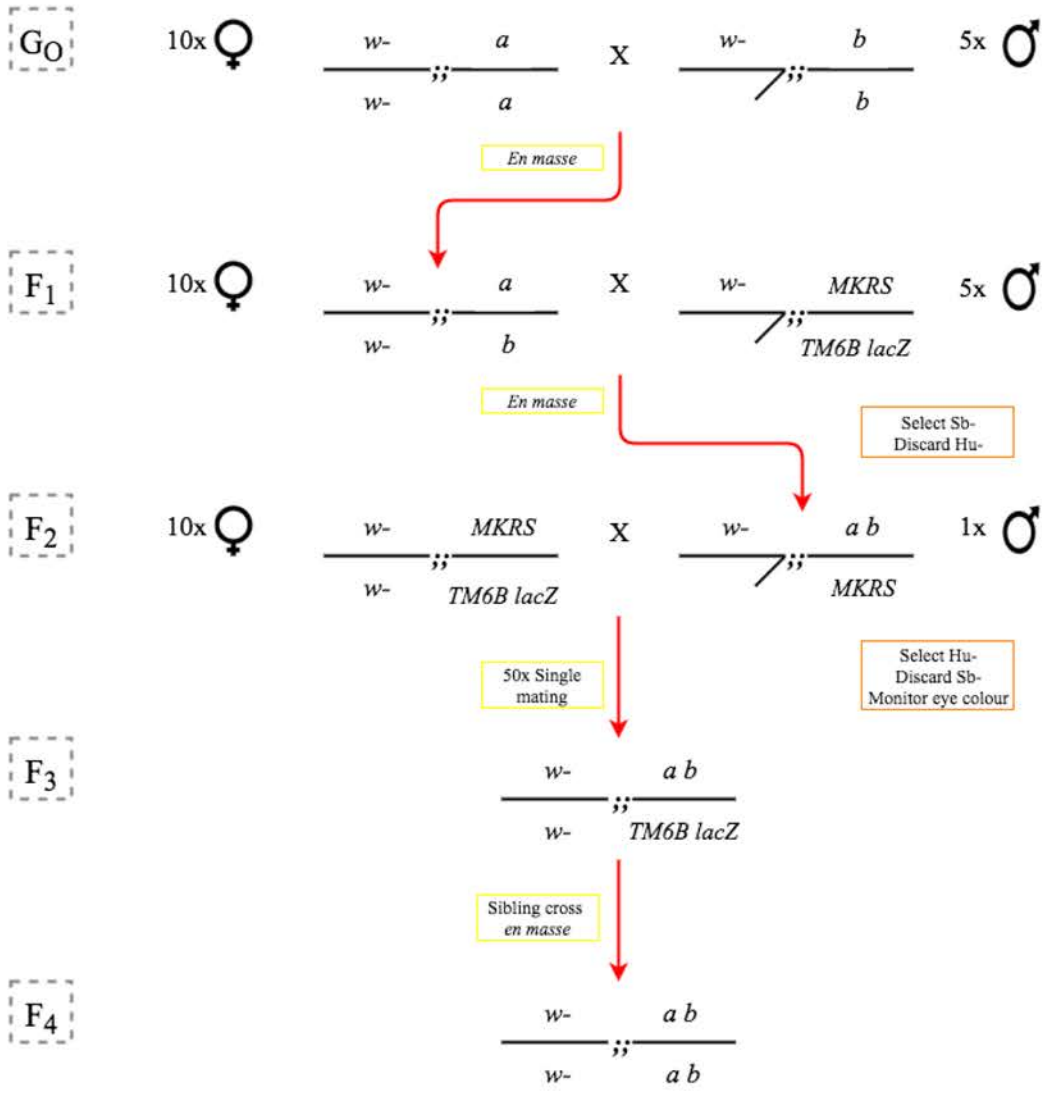
This protocol was used to combine alleles on both the 2ND and 3RD chromosome. All steps were completed *en masse* and multiple crosses set up to ensure correct progeny flies for F_2 were generated.

Figure 2.2 Recombination of alleles on the 2ND chromosome



This protocol was used to recombine two alleles on the 2ND chromosome. 50 single males were crossed to 10 females in F_2 in order to generate individual recombinant lines. The presence of the recombinant Gal4 line, was tested by crossing out to UAS GFP and examining fluorescence. Or if the recombinant was a UAS line, by examing eye colour and genomic PCR.

Figure 2.3 Recombination of alleles on the 3RD chromosome



This protocol was used to recombine two alleles on the 3RD chromosome. 50 single males were crossed to 10 females in F_2 in order to generate individual recombinant lines. The presence of the recombinant if a Gal4 line, was tested by crossing out to UAS GFP and examining fluorescence. Or if the recombinant was a UAS line, by examing eye colour and genomic PCR.

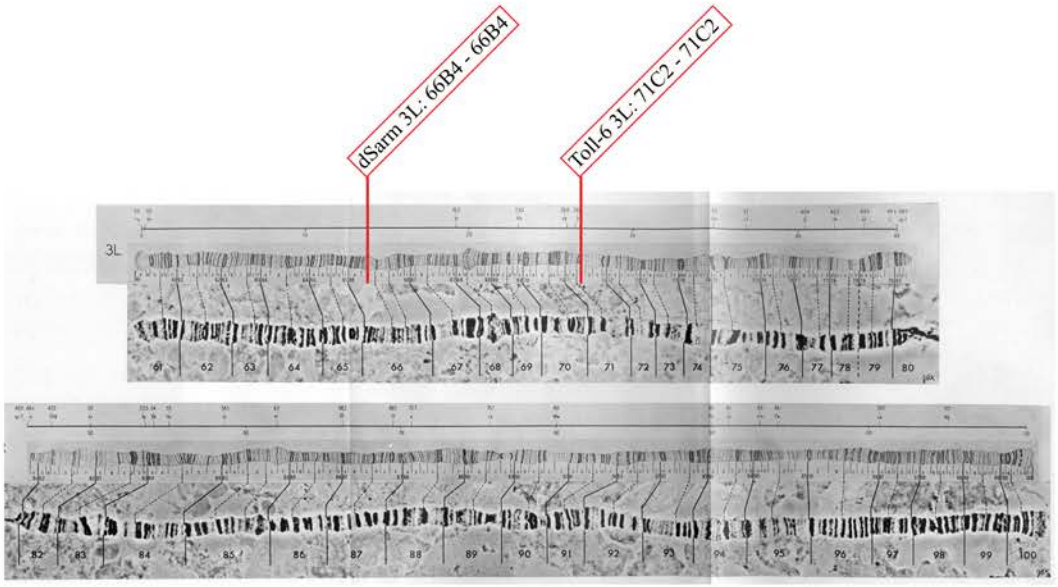
during every meiotic event, particularly in cases where the two gene positions are very close to each other on the chromosome. Thus the frequency with which recombination events take place is proportional to the distance between the two loci of interest along the chromosome. If these locations are known in relation to the cytogenetic map, their recombination frequency can be roughly estimated (Figure 2.4 for an example). If the positions of one or both are not known, estimations can not be made to determine recombination frequency and thus single mating's at F3 is usually set to 50 in the first instance to increase the likelihood of establishing a recombinant line.

Numerous transgenic lines contain the P{3xP3-EGFP) promoter which drives a 3xP3-RFP or GFP fluorescent phenotype as seen in Figure 3.13. 3xP3 was inserted using the Cre-LoxP system and therefore I was able to genetically remove this promoter with Cre-recombinase to ensure that any fluorescent phenotype that was visualised could be accurately attributed to the gene of interest and not the 3xP3 promoter (Figure 2.5).

2.1.3 CNS Area and VNC Length study

Driver strains included the post mitotic neuronal driver Elav-Gal4 and the pan-glial driver Repo-Gal4. Flies homozygous or balanced over SM6aTM6B for the UAS-RNAi (Knock-down) or UAS-Toll (over expression) were crossed to those carrying the above transgenic driver promoting UAS transgene expression. Control lines carrying the GAL4 transgene were crossed to YW for comparison. The CNS of wandering larvae were dissected and fixed for 50 minutes in 4% formaldehyde in PEM (0.1M PIPES, 2mM EGTA, 1mM $MgSO_4$ in water) then washed six times in 0.5% Triton-X-100 for

Figure 2.4 Calculating the frequency of recombination



The frequency of any recombination can be calculated when the location of two loci are known as the frequency is proportional to the distance between them. This cytogenetic map of chromosome 3L was published in Bridges, C.B. (1935).

An example of recombination frequency

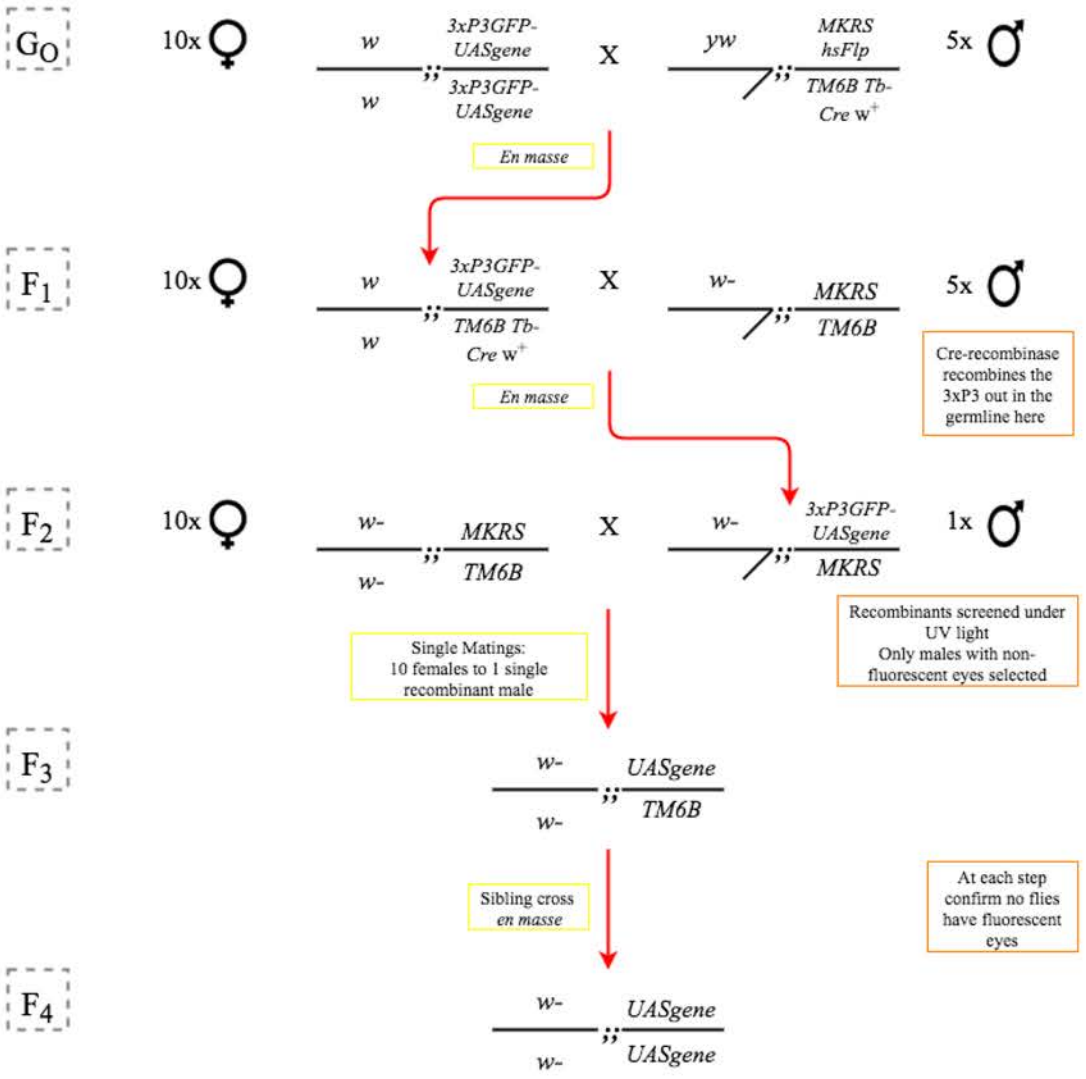
A) dSarm localises to cytogenetic map position 3L: 66B4 - 66B4

B) Toll-6 localises to cytogenetic map position 3L: 71C2 - 71C2

C) Distance between dSarm and Toll-6:
= 71.2 - 66.4
= 4.8cM

This indicates that less than 1 in 20 oocytes will carry the desired recombination event.

Figure 2.5 Genetic removal of P{3xP3-GFP)



This protocol was used to genetically remove the P{3xP3-EGFP) promoter from transgenic lines as it drives a fluorescent phenotype. P{3xP3-EGFP) was inserted using Cre-LoxP and therefore is able to be removed using Cre-recombinase. Between 20 - 35 indivdual single recombinant male crosses were set up to achieve desired fly line.

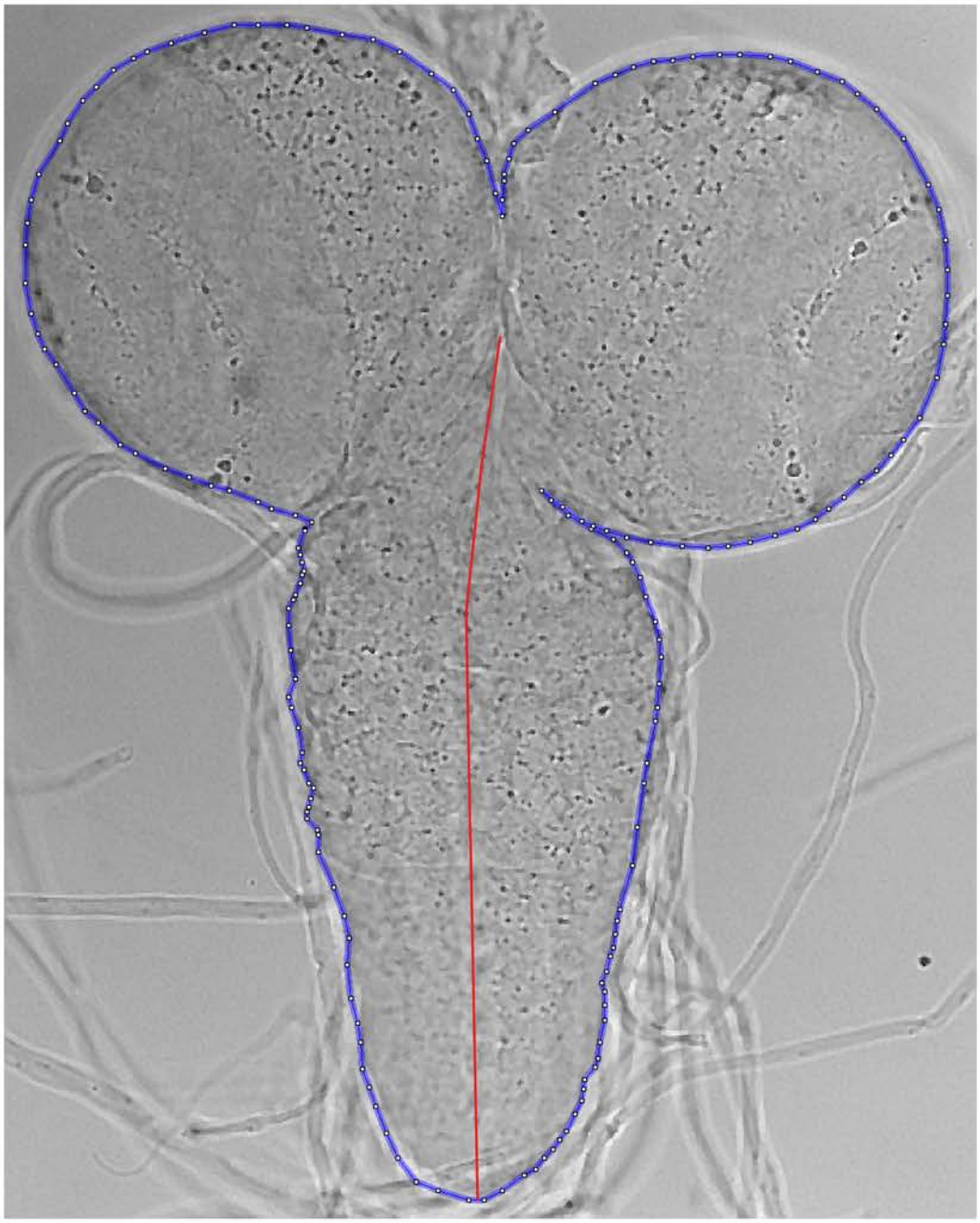
10 minutes. Brains were then stored in 80% glycerol until mounted onto glass slides. Bright field images were acquired using a Zeiss Axioplan 2 microscope and 40x objective. Image J was then used to measure both the CNS area and VNC size using a free hand area selection tool and a line selection tool respectively (Figure 2.6 for example and landmarks).

2.2 Behavioral Assays

2.2.1 Adult locomotion

Driver strains were crossed to flies homozygous or balanced UAS-RNAi (Knock-down) or UAS-Toll (over expression). Control lines carrying only the GAL4 transgene without the UAS-RNAi or UAS-Toll were used for comparison. Male flies between 1-3 days old were collected and separated as virgins and were used to monitor activity. Flies were monitored using Trikinetics DAM2 activity monitors. Thirty-two flies per genotype were individually analysed in activity assays tubes for 3 days in 12-h light and 12-h dark cycles, with a constant temperature of 25 °C settings of the incubator. Locomotor activity was determined as the average number of infrared beam crossings per 5-min bin spanning the full three-day period. Any fly that died during the experiment was excluded from the results. A behavioural analysis software package (BeFly!) was used to analyse all data recorded (Figure 2.7).

Figure 2.6 Determination of boundaries for VNC length and CNS area



The CNS area was measured by drawing around the optic lobes and VNC using the segemented line tool (Blue). The length of the VNC was measured by drawing a line between tip region where the optic lobes meet and the tip of the abdomen (Red).

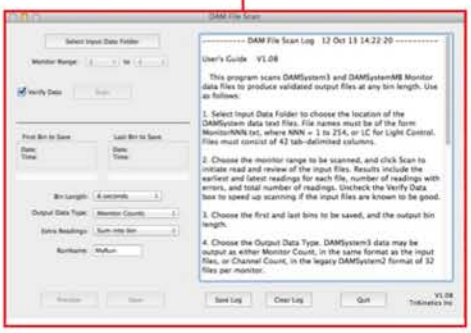
Figure 2.7 Schematic diagram of preparing Trikenetics Adult locomotion assays

32x 1-3 day old males loaded into assay tubes.

Assay tubes are loaded into DAM monitors O/N 25°C.

DAM monitors are loaded into incubators which are connected to main control PC.

Main control PC regulates temperature and light cycle: For my experiments these were set as 12hrL/D and 25°C for 36 hours.



Following 36 hours DAM monitors are removed, and text files are inported into BeFly software for analysis.

2.3 Molecular Biology

2.3.1 RT-PCR

Collection of embryos, dissection of second instar, third instar wandering larvae or pupae, and whole head preparations of Oregon-R (wild type) or ElavGal4>UASgcm (embryos only) were the starting materials. Total RNA was extracted from the starting samples by Trizol (Ambion) reagent. RNA integrity and concentration was confirmed via Nano-drop. RNA samples were DNase treated to remove residual genomic DNA contamination. 300ng of RNA was used for cDNA synthesis following GoScript™ Reverse Transcriptase methods. Samples were diluted 1:3 with Nuclease free H_20, and a no RT sample of 300ng of RNA made up to 60µl with Nuclease free H_20. Standard PCR reaction was performed to amplify each of the Toll receptor cDNA using GoTaq PCR protocol. PCR primers were designed using primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and 2uM of forward and reverse primers specific to each sample were used (See table 2.2 and 2.3 for primer list and PCR programme). GAPDH is a general housekeeping gene that was used as a positive control during every round of PCR. Gel electrophoresis for RT-PCR products were then carried out.

2.3.2 Immunohistochemistry

In order to ensure maximal stages were gathered for embryonic samples, embryos were collected from grape juice plates supplemented with yeast after 16.5 hours at 25°C. Embryos were then dechorionated (16% sodium hypochlorite), and fixed (4% formaldehyde) in PBS, and 3ml Heptane for 20 minutes at room temperature.

Table 2.2 List of Primers used for RT-PCR

Toll genes for RT-PCR			
Toll1-Fwd	Toll-1	CAACTGCCTACCAATCTCAC	with Toll1-Rev 2131-2422 of Toll CDS, 292 bp
Toll1-Rev	Toll-1	CTATGAACACGCCCTTTTCC	With Toll1-Fwd 2131-2422 of Toll CDS, 292 bp
Toll2-Fwd	Toll-2	GCAATATCGTCACAGCCTC	with Toll2-Rev 2718-3179 of 18-W CDS, 462 bp
Toll2-Rev	Toll-2	CACACAAACTCGTAGTCCTTC	with Toll2-Fwd 2718-3179 of 18-W CDS, 462 bp
Toll3-Fwd	Toll-3	AATCACCTTCCAGCGAAAC	with Toll3-Rev 1084-1346 of Toll-3 CDS, 263 bp
Toll3-Rev	Toll-3	CCCAAAAACTTCAAAAACCCC	with Toll3-Fwd 1084-1346 of Toll-3 CDS, 263 bp
Toll4-Fwd	Toll-4	CCTCATCTACTACACCTCCCTC	with Toll4-Rev 2766-3257 of Toll-4 CDS, 492 bp
Toll4-Rev	Toll-4	TACGCCCTCAACTCGCTATC	with Toll4-Fwd 2766-3257 of Toll-4 CDS, 492 bp
Toll5-Fwd	Toll-5	CTTAGCGACTTACTCAAGACC	with Toll5-Rev 313-670 of Toll-5 CDS, 358 bp
Toll5-Rev	Toll-5	TCCCGAATGACACTATACCC	with Toll5-Fwd 313-670 of Toll-5 CDS, 358 bp
Toll6-Fwd	Toll-6	CCTGAACGACAACCTGATAAC	with Toll6-Rev 2067-2449 of Toll-6 CDS, 383 bp
Toll6-Rev	Toll-6	ACTCACAGCAATGGCAAAC	with Toll6-Fwd 2067-2449 of Toll-6 CDS, 383 bp
Toll7-Fwd	Toll-7	CTCGCACAATCGCATCACAG	with Toll7-Rev 1914-2280 of Toll-7 CDS, 386 bp
Toll7-Rev	Toll-7	GACGCAGACCACTCAAAGGA	with Toll7-Fwd 1914-2280 of Toll-7 CDS, 386 bp
Toll8-Fwd	Toll-8	GCAGATCCTTAACCTGTCCC	with Toll8-Rev 1503-1946 of Toll-8 CDS, 444 bp
Toll8-Rev	Toll-8	TTCCTCACCAAATCCACCC	with Toll8-Fwd 1503-1946 of Toll-8 CDS, 444 bp
Toll9-Fwd	Toll-9	CCCCTACCTATCCTACAACATC	with Toll9-Rev 1239-1582 of Toll-9 CDS, 344 bp
Toll9-Rev	Toll-9	AATCCAATCGCTCAAAGTCC	with Toll9-Fwd 1239-1582 of Toll-9 CDS, 344 bp

Table 2.3 PCR Cycle

Stage of cycle	Temperature	Time	Repetition
Denature	95°C	2 minutes	
Denature	95°C	30 seconds	
Anneal	55°C	30 seconds	x37
Extension	72°C	1 minute/kb	
Final Extension	72°C	5 minutes	
Hold	4°C	Infinity	

Formaldehyde was removed and replaced with methanol to devitillinise embryos through gentle vortexing. Methanol was then used to wash the embryos, followed by washing with PBT for six times every 10 minutes. Embryos were then incubated with primary antibodies (Full list see table 2.4) at 4°C overnight, followed by washing steps to remove primary, and the addition of secondary antibodies which were incubated in the dark overnight at 4°C. Following a final round of washing embryos were allowed to settle in glycerol prior to mounting on glass slides and image analysis.

Larval, pupal or adult brains were dissected in cold PBS for no longer than 20 minutes. They were directly placed into a fixative solution for 50 minutes (5 minutes for anti-Eve staining) after which they were washed 5 x 10 minutes, allowed to rest in Normal Goat Serum for one hour and then transferred to primary antibodies, which were used no greater than five times. After incubation of the brains in the primary antibody at 4°C overnight, brains were washed in PBT, and secondary antibodies were added and incubated again at 4°C overnight. Following a final round of washing brains were allowed to settle in glycerol (generally 1-2 hours) before mounting on glass slides and image analysis.

Staining's were carried out on wandering third stage larvae, one day pupae for general antibody staining or when staining to detect apoptosis only pupae within the first 10 minutes were dissected to reduce any biological variety, and 1-5 day old adults. Staining was completed in batches of 5 to 10 brains under the same conditions for direct comparison experiments. Most often staining's were repeated on multiple occasions to ensure accuracy of results. Each tissue was mounted onto slides with a rectangular

Table2.4 List of Primary Antibodies

Antibody	Donor	Source	Catalogue Number	Dilution
Primary Antibody				
anti-GFP	Rabbit	Invitrogen	A11122	1:500 Larvae, Pupae & Adult
anti-βgal	Rabbit	Cappel		1:5000 Embryo 1:2500 larvae & Adult
anti-DsRed	Rabbit	Clontech	632496	1:100 L3 & Adult
anti-pJNK	Rabbit	Promega	V7931	1:200 Larvae
anti-Dcp1 (Cleaved Drosophila Dcp-1 (Asp216))	Rabbit	Cell Signalling	9578S	1:500 Larvae & Pupae
anti-Eve	Mouse	DSHB	3C10	1:20 Larvae & Pupae
anti-pERK	Mouse	Cell Signalling	9106	1:100 Larval Optic lobe 1:500 Larval Retina
Secondary Antibody				
anti-Rabbit-Alexa488		Invitrogen		1:250
anti-Rabbit-Alexa546		Invitrogen		1:250
anti-Rabbit-Alexa647		Invitrogen		1:250
anti-Mouse-Alexa488		Invitrogen		1:250
anti-Mouse-Alexa647		Invitrogen		1:250

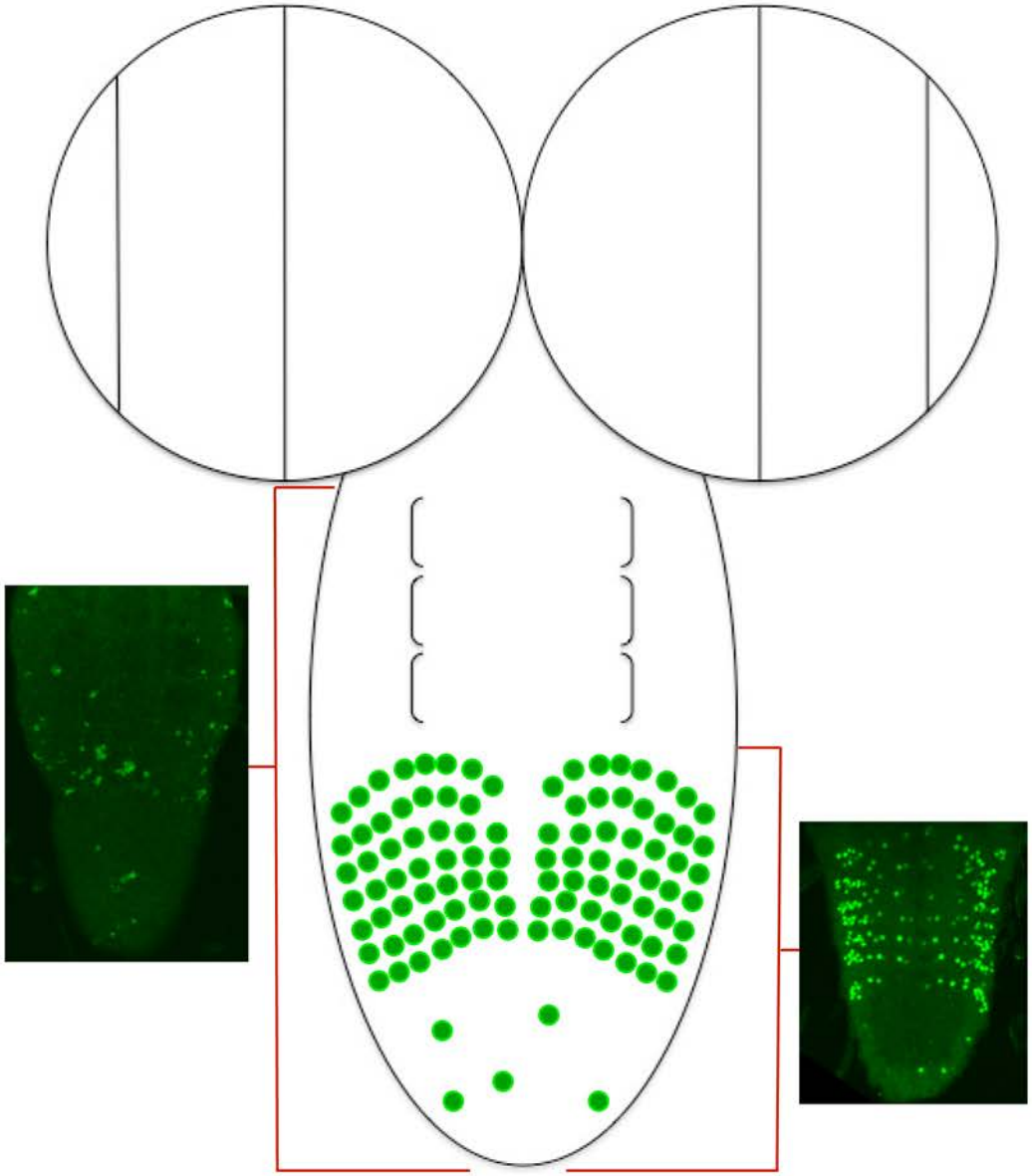
sections removed from two slices of sticky tape acting like a bridge. The area was then saturated in 70% glycerol and a cover slip gently placed on top ensuring that pressure was not applied, preventing any movement of the tissue.

2.3.3 Imaging

All fluorescent confocal scanning was carried out on a Leica SP2-AOBS and a 20x or 40x oil immersion lens at either 512:512 or 1024:1024 pixel resolution, with between a 0.5 > 1.0um step increase. Confocal stacks were then analysed and processed using Image J software. Adobe Photoshop and Illustrator were subsequently used to process the images in order to generate image plates.

Numerous image J plugins were used in order to quantify phenotypes. Retinal pJNK+ cells were counted manually from a complete confocal stack using the cell counter macro. DeadEasy Larval Caspase software (Forero *et al.* 2009; Kato *et al.,* 2011) was used to count Dcp1+ apoptotic cells in both complete stacks of the retina, as well as along the entire VNC of the pupal CNS. The full VNC was counted for apoptotic cells with boundaries specified as surrounding the VNC and up to the edges of optic lobes (Figure 2.8). DeadEasy larval glia software (Forero *et al.* 2012) counts nuclear stains and was used to quantify the number of Eve+ cells in the larval and pupal VNC. The boundaries for Eve+ staining included only abdominal segments (Figure 2.8).

Figure 2.8 Boundaries for anti-DCP1 staining and anti-Eve staining



Dcp1+ cells were counted throughout the whole VNC using DeadEasy Larval Caspase software (Forero et al. 2009; Kato et al., 2011) and Eve+ cells were counted in the abdominal regions of the VNC only using DeadEasy larval glia software (Forero et al. 2012)

2.4 Statistical Analysis

All data have been analysed using SPSS statistical software. Continuous data including counts of Dcp1+, pJNK+ and Eve+ cells were tested for normality by kurtosis and skewness, and a Levenes test to determine homogeneity of variance. If Leven's did not pass significance, data were analysed using a One Way ANOVA. If Leven's test was significant a Welch ANOVA was used. Post-Hoc testing compared multiple genotypes to a control via the Dunnett test, or when comparisons were made to each other via a Bonferroni test (For all statistical tests performed see Table 2.5).

Table 2.5 Table of statistical Analysis

TABLE 2.5 STATISTICS

	DATA	GENOTYPE OR EXPERIMENT TYPE	SAMPLE TYPE & N	COMPARISON	TEST	NORMALITY TESTS	HOMOGENEITY OF VARIANCE	DESCRIPTIVE STATISTICS	DF & TEST VALUE	p VALUE	MULTIPLE COMPARISONS & CORRECTION	
Figure 5.1		**Genotype**	**Number of VNCs**				**Levene's test**	**Means±d.**				
	VNC Length	Elav/Gal4>+	**9**		**One Way ANOVA**	kur: -.655 skew: .608		385.700 ± 26.106	F=10.293 *d.f.*=8 *d.f.*= 99	< 0.0001	**Dunnett post-hoc**	
		Elav/Gal4>Toll-1	**11**	Elav/Gal4>+ vs Elav/Gal4>Toll-1		kur: .655 skew: .334		413.257 ± 32.138			ns	
		Elav/Gal4>Toll-2	**18**	Elav/Gal4>+ vs Elav/Gal4>Toll-2		kur: 3.534 skew: 2.371		385.018 ± 41.797			ns	
		Elav/Gal4>Toll-3	**14**	Elav/Gal4>+ vs Elav/Gal4>Toll-3		kur: .352 skew: .672		338.642 ± 36.574			0.033	
		Elav/Gal4>Toll-4	**9**	Elav/Gal4>+ vs Elav/Gal4>Toll-4		kur: -1.111 skew: .563		409.717 ± 35.306			ns	
		Elav/Gal4>Toll-6	**14**	Elav/Gal4>+ vs Elav/Gal4>Toll-6		kur: -.610 skew: .602		385.153 ± 18.792			ns	
		Elav/Gal4>Toll-7	**12**	Elav/Gal4>+ vs Elav/Gal4>Toll-7		kur: -1.092 skew: .680		406.363 ± 51.699			ns	
		Elav/Gal4>Toll-8	**12**	Elav/Gal4>+ vs Elav/Gal4>Toll-8		kur: .335 skew: .180		364.958 ± 20.514			ns	
		Elav/Gal4>Toll-9	**9**	Elav/Gal4>+ vs Elav/Gal4>Toll-9		kur: .058 skew: .336		469.343 ± 57.698			<0.0001	
Figure 5.2		**Genotype**	**Number of VNCs**				**Levene's test**	**Means±d.**				
	VNC Length	Elav/Gal4>+	**9**		**One Way ANOVA**	kur: -.655 skew: .608		385.700 ± 26.106	F=6.803 *d.f.*=7 *d.f.*= 88	< 0.0001	**Dunnett post-hoc**	
		Elav/Gal4>Toll-1 RNAi	**15**	Elav/Gal4>+ vs Elav/Gal4>Toll-1 RNAi		kur: 1.388 skew: 1.034		410.352 ± 41.807			ns	
		Elav/Gal4>Toll-3 RNAi	**8**	Elav/Gal4>+ vs Elav/Gal4>Toll-3 RNAi		kur: .014 skew: .639		461.717 ± 53.458			<0.0001	
		Elav/Gal4>Toll-4 RNAi	**11**	Elav/Gal4>+ vs Elav/Gal4>Toll-4 RNAi		kur: .409 skew: -.302		398.819 ± 41.367			ns	
		Elav/Gal4>Toll-5 RNAi	**13**	Elav/Gal4>+ vs Elav/Gal4>Toll-4 RNAi		kur: -.725 skew: .486		395.819 ± 29.805			ns	
		Elav/Gal4>Toll-7 RNAi	**8**	Elav/Gal4>+ vs Elav/Gal4>Toll-7 RNAi		kur: 1.994 skew: 1.108		434.535 ± 40.413			<0.0001	
		Elav/Gal4>Toll-8 RNAi	**13**	Elav/Gal4>+ vs Elav/Gal4>Toll-8 RNAi		kur: 1.614 skew: .728		390.113 ± 26.467			ns	
		Elav/Gal4>Toll-9 RNAi	**19**	Elav/Gal4>+ vs Elav/Gal4>Toll-9 RNAi		kur: .128 skew: .664		382.731 ± 33.497			ns	
Figure 5.3		**Genotype**	**mber of hemigments**				**Levene's test**	**Means±d.**				
	VNC Length	Elav/Gal4>+	**9**			kur: -.655 skew: .608	0.159	385.700 ± 26.106	F=25.207 *d.f.*=2 *d.f.*= 28	< 0.0001	**Dunnett post-hoc**	
		Elav/Gal4>Toll-3	**14**	Elav/Gal4>+ vs Elav/Gal4>Toll-3		kur: .352 skew: .672		338.642 ± 36.574			0.022	
		Elav/Gal4>Toll-3 RNAi	**8**	Elav/Gal4>+ vs Elav/Gal4>Toll-3 RNAi		kur: .014 skew: .639		461.717 ± 53.458			<0.001	
Figure 5.4		**Genotype**	**Number of brains**				**Levene's test**	**Means±d.**				
	CNS Area	Elav/Gal4>+	**9**		**Welch ANOVA**	kur: -.323 skew: -.738	0	223685.41 ± 26600.109	F=17.506 *d.f.*=8 *d.f.*= 38.106	< 0.0001	**Dunnett post-hoc**	
		Elav/Gal4>Toll-1	**11**	Elav/Gal4>+ vs Elav/Gal4>Toll-1		kur: 2.935 skew: 1.143		231354.73 ± 29666.396			ns	
		Elav/Gal4>Toll-2	**18**	Elav/Gal4>+ vs Elav/Gal4>Toll-2		kur: .194 skew: -.736		293194.962 ± 44322.533			<0.001	
		Elav/Gal4>Toll-3	**14**	Elav/Gal4>+ vs Elav/Gal4>Toll-3		kur: .029 skew: .379		193610.492 ± 43754.185			ns	
		Elav/Gal4>Toll-4	**9**	Elav/Gal4>+ vs Elav/Gal4>Toll-4		kur: -.848 skew: -.382		298087.492 ± 50958.859			<0.01	
		Elav/Gal4>Toll-6	**14**	Elav/Gal4>+ vs Elav/Gal4>Toll-6		kur: -.048 skew: .703		232396.028 ± 29392.413			ns	
		Elav/Gal4>Toll-7	**12**	Elav/Gal4>+ vs Elav/Gal4>Toll-7		kur: -.404 skew: .615		246718.258 ± 20773.536			ns	
		Elav/Gal4>Toll-8	**12**	Elav/Gal4>+ vs Elav/Gal4>Toll-8		kur: 2.555 skew: -1.370		244796.701 ± 25927.169			ns	
		Elav/Gal4>Toll-9	**9**	Elav/Gal4>+ vs Elav/Gal4>Toll-9		kur: -1.306 skew: -.618		323412.743 ± 64924.515			<0.0001	
Figure 5.5		**Genotype**	**Number of brains**				**Levene's test**	**Means±d.**				
	CNS Area	Elav/Gal4>+	**9**		**Welch ANOVA**	kur: -.323 skew: -.738	0	223685.406 ± 26600.109	F=21.020 *d.f.*=7 *d.f.*= 32.5	< 0.0001	**Dunnett post-hoc**	
		Elav/Gal4>Toll-1 RNAi	**15**	Elav/Gal4>+ vs Elav/Gal4>Toll-1 RNAi		kur: .584 skew: -.637		331807.783 ± 41299.403			<0.0001	
		Elav/Gal4>Toll-3 RNAi	**8**	Elav/Gal4>+ vs Elav/Gal4>Toll-3 RNAi		kur: -1.786 skew: .092		137421.675 ± 63642.568			<0.0001	
		Elav/Gal4>Toll-4 RNAi	**11**	Elav/Gal4>+ vs Elav/Gal4>Toll-4 RNAi		kur: .855 skew: -.529		314858.621 ± 35743.919			<0.0001	
		Elav/Gal4>Toll-5 RNAi	**13**	Elav/Gal4>+ vs Elav/Gal4>Toll-4 RNAi		kur: -1.195 skew: .149		312872.280 ± 77262.345			<0.0001	
		Elav/Gal4>Toll-7 RNAi	**8**	Elav/Gal4>+ vs Elav/Gal4>Toll-7 RNAi		kur: -.193 skew: .962		402183.829 ± 47104.923			<0.0001	
		Elav/Gal4>Toll-8 RNAi	**13**	Elav/Gal4>+ vs Elav/Gal4>Toll-8 RNAi		kur: .244 skew: -.328		266483.094 ± 32800.029			ns	
		Elav/Gal4>Toll-9 RNAi	**19**	Elav/Gal4>+ vs Elav/Gal4>Toll-9 RNAi		kur: -.263 skew: .308		237430.883 ± 29575.758			ns	
Figure 5.6		**Genotype**	**Number of brains**				**Levene's test**	**Means±d.**				
	CNS Area	Elav/Gal4>+	**9**		**Welch ANOVA**	kur: -.323 skew: -.738	0.039	223685.406 ± 26600.109	F=25.431 *d.f.*=2 *d.f.*= 15.213	< 0.0001	**Dunnett post-hoc**	
		Elav/Gal4>Toll-3	**14**	Elav/Gal4>+ vs Elav/Gal4>Toll-3		kur: .029 skew: .379		193610.492 ± 43754.185			ns	
		Elav/Gal4>Toll-3 RNAi	**8**	Elav/Gal4>+ vs Elav/Gal4>Toll-3 RNAi		kur: -1.786 skew: -.092		137421.675 ± 63642.568			<0.0001	
Figure 5.7		**Genotype**	**Number of VNCs**				**Levene's test**	**Means±d.**				
	VNC Length	RepoGal4>+	**14**		**Welch ANOVA**	kur: -.655 skew: .608	**0**	356.67 ± 29.285	F=18.2718 *d.f.*=6 *d.f.*= 31.703	< 0.0001	**Dunnett post-hoc**	
		RepoGal4>Toll-3	**12**	RepoGal4>+ vs RepoGal4>Toll-3		kur: .352 skew: .672		338.642 ± 36.574			<0.001	
		RepoGal4>Toll-4	**10**	RepoGal4>+ vs RepoGal4>Toll-4		kur: -1.111 skew: -.563		409.717 ± 35.306			ns	
		RepoGal4>Toll-6	**10**	RepoGal4>+ vs RepoGal4>Toll-6		kur: -.610 skew: .602		385.153 ± 18.792			ns	
		RepoGal4>Toll-7	**11**	RepoGal4>+ vs RepoGal4>Toll-7		kur: -1.092 skew: .680		406.363 ± 51.699			<0.0001	
		RepoGal4>Toll-8	**11**	RepoGal4>+ vs RepoGal4>Toll-8		kur: .335 skew: -.180		364.958 ± 20.514			ns	
		RepoGal4>Toll-9	**12**	RepoGal4>+ vs RepoGal4>Toll-9		kur: -.058 skew: .336		469.343 ± 57.698			<0.0001	
Figure 5.8		**Genotype**	**Number of VNCs**				**Levene's test**	**Means±d.**				
	VNC Length	RepoGal4>+	**14**		**One Way ANOVA**	kur: -.573 skew: .437	0.08600	356.67 ± 29.285	F=9.773 *d.f.*=7 *d.f.*= 79	< 0.0001	**Dunnett post-hoc**	
		RepoGal4>Toll-1 RNAi	**14**	RepoGal4>+ vs RepoGal4>Toll-1 RNAi		kur: -.607 skew: .401		358.15 ± 33.127			ns	
		RepoGal4>Toll-3 RNAi	**11**	RepoGal4>+ vs RepoGal4>Toll-3 RNAi		kur: -.701 skew: .671		467.88 ± 67.476			<0.0001	
		RepoGal4>Toll-4 RNAi	**7**	RepoGal4>+ vs RepoGal4>Toll-4 RNAi		kur: 1.865 skew: 1.342		423.01 ± 48.784			0.01	
		RepoGal4>Toll-5 RNAi	**6**	RepoGal4>+ vs RepoGal4>Toll-5 RNAi		kur: .169 skew: 1.047		349.78 ± 44.466			ns	
		RepoGal4>Toll-7 RNAi	**11**	RepoGal4>+ vs RepoGal4>Toll-7 RNAi		kur: -.688 skew: .592		355.13 ± 40.155			ns	
		RepoGal4>Toll-8 RNAi	**10**	RepoGal4>+ vs RepoGal4>Toll-8 RNAi		kur: -.258 skew: 2.05		417.04 ± 48.070			0.011	
		RepoGal4>Toll-9 RNAi	**14**	RepoGal4>+ vs RepoGal4>Toll-9 RNAi		kur: -.294 skew: .597		371.31 ± 46.081			ns	
Figure 5.9		**Genotype**	**Number of brains**				**Levene's test**	**Means±d.**				
	CNS Area	RepoGal4>+	**14**		**Welch ANOVA**	kur: -.990 skew: .146	**0.002**	63554.374 ± 8858.904	F=27.202 *d.f.*=6 *d.f.*= 31.487	< 0.0001	**Dunnett post-hoc**	
		RepoGal4>Toll-3	**12**	RepoGal4>+ vs RepoGal4>Toll-3		kur: -.855 skew: .440		84024.691 ± 17852.749			<0.001	
		RepoGal4>Toll-4	**10**	RepoGal4>+ vs RepoGal4>Toll-4		kur: 2.334 skew: -1.450		56150.171 ± 4947.250			ns	
		RepoGal4>Toll-6	**10**	RepoGal4>+ vs RepoGal4>Toll-6		kur: .577 skew: 1.173		86946.184 ± 13574.606			<0.0001	
		RepoGal4>Toll-7	**11**	RepoGal4>+ vs RepoGal4>Toll-7		kur: -1.085 skew: .437		106879.436 ± 17490.231			<0.0001	
		RepoGal4>Toll-8	**11**	RepoGal4>+ vs RepoGal4>Toll-8		kur: .940 skew: .961		63495.067 ± 5501.702			ns	
		RepoGal4>Toll-9	**12**	RepoGal4>+ vs RepoGal4>Toll-9		kur: 5.980 skew: -1.982		92494.225 ± 13471.422			<0.0001	
Figure 5.10		**Genotype**	**Number of brains**				**Levene's test**	**Means±d.**				
	CNS Area	RepoGal4>+	**14**		**Welch ANOVA**	kur: -.990 skew: .146	0.00000	63554.374 ± 8858.904	F=9.337 *d.f.*=7 *d.f.*= 27.968	< 0.0001	**Dunnett post-hoc**	
		RepoGal4>Toll-1 RNAi	**14**	RepoGal4>+ vs RepoGal4>Toll-1 RNAi		kur: 2.191 skew: 1.227		65149.909 ± 7084.450			ns	
		RepoGal4>Toll-3 RNAi	**11**	RepoGal4>+ vs RepoGal4>Toll-3 RNAi		kur: -1.550 skew: -.148		129137.776 ± 35834.802			<0.0001	
		RepoGal4>Toll-4 RNAi	**7**	RepoGal4>+ vs RepoGal4>Toll-4 RNAi		kur: .657 skew: 1.078		94505.232 ± 17648.399			0.004	
		RepoGal4>Toll-5 RNAi	**6**	RepoGal4>+ vs RepoGal4>Toll-5 RNAi		kur: .885 skew: .331		61587.465 ± 14862.815			ns	
		RepoGal4>Toll-7 RNAi	**11**	RepoGal4>+ vs RepoGal4>Toll-7 RNAi		kur: -1.142 skew: .233		70258.215 ± 16430.403			ns	
		RepoGal4>Toll-8 RNAi	**10**	RepoGal4>+ vs RepoGal4>Toll-8 RNAi		kur: -.752 skew: .335		94820.700 ± 17861.146			<0.001	
		RepoGal4>Toll-9 RNAi	**14**	RepoGal4>+ vs RepoGal4>Toll-9 RNAi		kur: -1.378 skew: -.202		76203.871 ± 11711.531			ns	
Figure 5.11		**Genotype**	**Number of VNCs**				**Levene's test**	**Means±d.**				
	Number of Eve cells	Elav/Gal4>+			**One Way ANOVA**	kur: 1.401 skew: .998	**0.378**	192.50 ± 10.296	F=43.819 *d.f.*=8 *d.f.*= 75	< 0.0001	**Dunnett post-hoc**	
		Elav/Gal4>Toll-1		Elav/Gal4>+ vs Toll-1		kur: .812 skew: .903		188.40 ± 13.492			ns	
		Elav/Gal4>Toll-2		Elav/Gal4>+ vs Toll-2		kur: .049 skew: .623		299.40 ± 10.814			<0.0001	
		Elav/Gal4>Toll-3		Elav/Gal4>+ vs Toll-3		kur: -1.152 skew: .452		180.55 ± 7.866			ns	
		Elav/Gal4>Toll-4		Elav/Gal4>+ vs Toll-4		kur: .706 skew: .811		248.14 ± 14.334			<0.0001	
		Elav/Gal4>Toll-6		Elav/Gal4>+ vs Toll-6		kur: .184 skew: .124		210.18 ± 8.892			<0.001	
		Elav/Gal4>Toll-7		Elav/Gal4>+ vs Toll-7		kur: 4.664 skew: -2.141		229.60 ± 5.415			<0.0001	
		Elav/Gal4>Toll-8		Elav/Gal4>+ vs Toll-8		kur: .968 skew: -1.045		232.75 ± 13.874			<0.0001	
		Elav/Gal4>Toll-9		Elav/Gal4>+ vs Toll-9		kur: 2.532 skew: 1.089		226.17 ± 8.266			<0.0001	
Figure 5.12		**Genotype**	**Number of Retina**				**Levene's test**	**Means±d.**				
	Number of Dcp1+ cells	GMR0Gal4>+	**9**		**One Way ANOVA**	kur: -.193 skew: .469	**0.752**	147.44 ± 7.299	F=149.208 *d.f.*=8 *d.f.*= 69	< 0.0001	**Dunnett post-hoc**	
		GMR0Gal4>Toll-1	**8**	GMR0Gal4> + GMR0Gal4>Toll-1		kur: 1.213 skew: .385		133.13 ± 8.254			0.051	
		GMR0Gal4>Toll-2	**12**	GMR0Gal4> + GMR0Gal4>Toll-2		kur: -1.585 skew: .008		160.83 ± 7.907			0.042	
		GMR0Gal4>Toll-3	**13**	GMR0Gal4> + GMR0Gal4>Toll-3		kur: 9.919 skew: -2.999		97.31 ± 16.869			<0.0001	
		GMR0Gal4>Toll-4	**8**	GMR0Gal4> + GMR0Gal4>Toll-4		kur: 1.140 skew: .062		104.38 ± 7.090			<0.0001	
		GMR0Gal4>Toll-6	**10**	GMR0Gal4> + GMR0Gal4>Toll-6		kur: .837 skew: 1.339		46.40 ± 5.502			<0.0001	
		GMR0Gal4>Toll-7	**7**	GMR0Gal4> + GMR0Gal4>Toll-7		kur: 4.148 skew: 1.897		111.14 ± 9.206			<0.0001	
		GMR0Gal4>Toll-8	**7**	GMR0Gal4> + GMR0Gal4>Toll-8		kur: 3.346 skew: .070		167.86 ± 10.915			<0.001	
		GMR0Gal4>Toll-9	**4**	GMR0Gal4> + GMR0Gal4>Toll-9		kur: 2.120 skew: 1.463		212.00 ± 13.317			<0.0001	
Figure 5.13		**Genotype**	**Number of Retina**				**Levene's test**	**Means±d.**				
	Number of Dcp1+ cells	GMR0Gal4>+	**9**		**One Way ANOVA**	kur: -.193 skew: .469	**0.25**	147.44 ± 7.299	F=441.837 *d.f.*=9 *d.f.*= 75	< 0.0001	**Dunnett post-hoc**	
		GMR0Gal4>Toll-1 RNAi	**9**	GMR0Gal4> + GMR0Gal4>Toll-1 RNAi		kur: .481 skew: .819		124.67 ± 6.344			<0.0001	
		GMR0Gal4>Toll-2 RNAi	**10**	GMR0Gal4> + GMR0Gal4>Toll-2 RNAi		kur: -1.396 skew: .186		69.40 ± 6.328			<0.0001	
		GMR0Gal4>Toll-3 RNAi	**9**	GMR0Gal4> + GMR0Gal4>Toll-3 RNAi		kur: .674 skew: -.344		92.78 ± 5.069			<0.0001	
		GMR0Gal4>Toll-4 RNAi	**10**	GMR0Gal4> + GMR0Gal4>Toll-4 RNAi		kur: -1.026 skew: -.123		70.50 ± 4.813			<0.0001	
		GMR0Gal4>Toll-5 RNAi	**10**	GMR0Gal4> + GMR0Gal4>Toll-5 RNAi		kur: 1.751 skew: 1.224		234.50 ± 10.501			<0.0001	
		GMR0Gal4>Toll-6 RNAi	**9**	GMR0Gal4> + GMR0Gal4>Toll-6 RNAi		kur: -.981 skew: -.028		234.11 ± 9.880			<0.0001	
		GMR0Gal4>Toll-7 RNAi	**5**	GMR0Gal4> + GMR0Gal4>Toll-7 RNAi		kur: 1.875 skew: 1.346		100.00 ± 17.819			<0.0001	
		GMR0Gal4>Toll-8 RNAi	**6**	GMR0Gal4> + GMR0Gal4>Toll-8 RNAi		kur: -1.089 skew: .348		225.50 ± 12.470			<0.0001	
		GMR0Gal4>Toll-9 RNAi	**8**	GMR0Gal4> + GMR0Gal4>Toll-9 RNAi		kur: 2.158 skew: 1.444		91 ± 14.422			<0.0001	
Figure 5.13		**Genotype**	**Number of Retina**				**Levene's test**	**Means±d.**				
	Number of Dcp1+ cells	GMR0Gal4>+	**9**		**One Way ANOVA**	kur: -.193 skew: .469	**0.688**	147.44 ± 7.299	F=481.162 *d.f.*=2 *d.f.*= 28	< 0.0001	**Dunnett post-hoc**	**Bonferoni post-hoc**
		GMR0Gal4>Toll-2	**12**	GMR0Gal4> + GMR0Gal4>Toll-2		kur: -1.585 skew: .008		160.83 ± 7.907			<0.0001	
		GMR0Gal4>Toll-2 RNAi	**12**	GMR0Gal4> + GMR0Gal4>Toll-2 RNAi		kur: -1.396 skew: .186		69.40 ± 6.328			<0.0001	
				GMR0Gal4>Toll-2 > GMR0Gal4>Toll-2 RNAi								<0.0001
		GMR0Gal4>+	**9**		**One Way ANOVA**	kur: -.193 skew: .469	**0.25**	147.44 ± 7.299	F=441.837 *d.f.*=9 *d.f.*= 75		**Dunnett post-hoc**	**Bonferoni post-hoc**
		GMR0Gal4>Toll-9	**4**	GMR0Gal4> + GMR0Gal4>Toll-9		kur: 2.120 skew: 1.463		212.00 ± 13.317			<0.0001	
		GMR0Gal4>Toll-9 RNAi	**8**	GMR0Gal4> + GMR0Gal4>Toll-9 RNAi		kur: 2.158 skew: 1.444		91 ± 14.422			<0.0001	
				GMR0Gal4>Toll-9 > GMR0Gal4>Toll-9 RNAi								<0.0001
Figure 5.14		**Genotype**	**Number of Retina**				**Levene's test**	**Means±d.**				
	Number of Dcp1+ cells	GMR0Gal4>+	**9**		**One Way ANOVA**	kur: -.193 skew: .469	**0.688**	147.44 ± 7.299	F=481.162 *d.f.*=2 *d.f.*= 28	< 0.0001	**Dunnett post-hoc**	**Bonferoni post-hoc**
		GMR0Gal4>Toll-6	**10**	GMR0Gal4> + GMR0Gal4>Toll-6		kur: .837 skew: 1.339		46.40 ± 5.502			<0.0001	
		GMR0Gal4>Toll-6 RNAi	**9**	GMR0Gal4> + GMR0Gal4>Toll-6 RNAi		kur: -.981 skew: -.028		234.11 ± 9.880			<0.0001	
				GMR0Gal4>Toll-6 > (GMR0Gal4>Toll-6 RNAi)								<0.0001

TABLE 2.5 STATISTICS

Fig.4.1		**Genotype**	**Number of retinas**			**Kurtosis & Skewness**	**Levene's test**	**Means±s.d.**			
	Counts of pJNK+ cells	yw ; GMR1a4-t+	**9**	All together	**One Way ANOVA**	kurt:-0.515 skew:-0.621	0.322	69.89 ± 8.724	$F=63.291$ $df1:4$; $df2:54$	<0.001	**Dunnett post-hoc**
	in retina	yw ; GMR1a4-t > DNT1-FL	**14**	GMR1a4-t+ vs GMR1a4-t > DNT1-FL		kurt:-0.518 skew:-0.086		96.57 ± 11.752			<0.001
		yw ; GMR1a4-t > DNT1-CKCTD	**14**	GMR1a4-t+ vs GMR1a4-t > DNT1-CKCTD		kurt:-1.185 skew:-0.381		41.93 ± 10.491			<0.001
		yw ; GMR1a4-t > DNT2-FL	**11**	GMR1a4-t+ vs GMR1a4-t > DNT2-FL		kurt:-0.235 skew:0.645		51.73 ± 6.796			0.001
		yw ; GMR1a4-t > DNT2-CK	**11**	GMR1a4-t+ vs GMR1a4-t > DNT2-CK		kurt:-1.407 skew:-0.231		49.27 ± 11.046			<0.001
Fig.4.4		**Genotype**	**Number of VNC**				**Levene's test**	**Means±s.d.**			
	Counts of Evo+ neurons in	yw	**10**	yw xx w ; Elav|GAL4-t|-UASMyD88	**Student t-test**	kurt:-1.401 skew:.687	0.783	192.70±10.296	$F=0.078$ $df1:1$; $df2:20$	<0.0001	N/A
	Larva L3 abdominal VNCs	w ; Elav|GAL4-t|-UASMyD88	**12**			kurt: .445 skew:-1.232		219.67±9.717			
Fig.4.5		**Genotype**	**Number of VNC**				**Levene's test**	**Means±s.d.**			
	Counts of Evo+ neurons	yw ; Elav|GAL4-t	**12**	All together	**Welch ANOVA**	kurt: .688 skew: -.121	0.007	251.17±6.939	$F=8.017$ $df1:2$; $df2:13.313$	0.005	**Dunnett post-hoc**
	in pupal VNCs	MyD88c2.8/DfBSC 279	**8**	yw ; Elav|GAL4-t+ vs MyD88c2.8/DfBSC 279		kurt: -.962 skew: -.534		232.13±19.261			0.008
		w ; Elav|GAL4-t|-MyD88	**11**	yw ; Elav|GAL4-t+ vs w ; Elav|GAL4-t|-MyD88		kurt: -.523 skew: .096		263.64±13.522			0.061
Fig.4.6		**Genotype**	**Number of VNC**				**Levene's test**	**Means±s.d.**			
	Counts of DCP1+ cells	yw/+;Elav|Gal4-t+	**16**	All together	**Welch ANOVA**	kurt:-0.196 skew:-0.850	0	195.50 ± 13.317	$F=39.184$ $df1:3$; $df2:27$	<0.0001	**Dunnett post-hoc**
	in white pupae (10')	YW	**14**	yw/+;Elav|Gal4-t+ vs YW		kurt:-1.795 skew:-0.063		171.79 ± 32.209			0.010
		MyD88^{c4}	**6**	yw/+;Elav|Gal4-t+ vs MyD88^{c4}		kurt:-0.592 skew:0.313		196.83 ± 6.824			0.927
		MyD88^{c4}/DfBSC 279	**10**	yw/+;Elav|Gal4-t+ vs MyD88^{c4}/DfBSC 279		kurt:-0.973 skew:-0.491		240.00 ± 15.635			<0.001
		MyD88c2.8/DfBSC 279	**5**	yw/+;Elav|Gal4-t+ vs MyD88c2.8/DfBSC 279		kurt:-0.521 skew:-0.191		235.20 ± 5.167			0.001
		Elav|gal4-UASMyD88	**9**	yw/+;Elav|Gal4-t+ vs Elav|gal4-UASMyD88		kurt:-0.006 skew:0.821		247.56 ± 21.732			<0.001
		Elav|Gal4-UASTollrnex	**11**	yw/+;Elav|Gal4-t+ vs Elav|Gal4-UASTollrnex		kurt:-1.669 skew:-0.548		223.45 ± 13.931			0.002
		MyD88^{c4}/Elav|Gal4-UASTollrnex	**14**	yw/+;Elav|Gal4-t+ vs MyD88^{c4}/Elav|Gal4-UASTollrnex		kurt: 1.344 skew:0.048		241.86 ± 20.979			<0.001
Fig.4.8		**Genotype**	**Number of VNC**				**Levene's test**	**Means±s.d.**			
	Counts of DCP1+ cells	yw/+;Elav|Gal4-t+	**16**	All together	**One Way ANOVA**	kurt:-0.196 skew:-0.850	0.53	195.50 ± 13.317	$F=355.120$ $df1:3$; $df2:42$	<0.0001	**Dunnett post-hoc**
	in white pupae (10')	Elav|Gal4-EP3610	**10**	yw/+;Elav|Gal4-t+ vs Elav|Gal4-EP3610		kurt: 0.309 skew: -0.235		290.80 ± 16.006			<0.001
		MyD88^{c4}/Elav|Gal4-EP3610	**10**	yw/+;Elav|Gal4-t+ vs MyD88^{c4}/Elav|Gal4-EP3610		kurt: -1.131 skew: -0.019		370.30 ± 16.432			<0.001
		Elav|Gal4-EP3610, UASJNKRNAi	**10**	yw/+;Elav|Gal4-t+ vs Elav|Gal4-EP3610, UASJNKRNAi		kurt: -1.366 skew: 0.230		237.00 ± 4.876			<0.001
				Elav|Gal4-EP3610 vs MyD88scn36d.Elav|Gal4-EP3610							<0.001
				Elav|Gal4-EP3610 vs Elav|Gal4-EP3610, UASJNKRNAi							<0.01
Fig.4.9		**Genotype**	**Number of retinas**			**Kurtosis & Skewness**	**Levene's test**	**Means±s.d.**			
	Counts of pJNK+ cells	GMR1a4-t+	**9**	All together	**One Way ANOVA**	kurt:-0.515 skew:-0.621	0.214	69.89 ± 8.724	$F=9.315$ $df1:3$; $df2:36$	<0.001	**Dunnett post-hoc**
	in retina	MyD88^{c4}/DfBSC 279	**18**	GMR1a4-t+ vs MyD88^{c4}/DfBSC 279		kurt:-0.233 skew:-0.027		64.11 ± 20.642			0.734
		GMR1a4-t > EP3610	**9**	GMR1a4-t+ vs GMR1a4-t > EP3610		kurt:0.747 skew:0.766		82.67 ± 13.285			0.262
		MyD88^{c4}/GMR1a4-t/DfBSC 279.EP3610	**4**	GMR1a4-t+ vs MyD88^{c4}/GMR1a4-t/DfBSC 279.EP3610		kurt:2.928 skew:1.683		110.0 ± 15.769			0.001
Fig.4.10		**Genotype**	**Number of VNC**				**Levene's test**	**Means±s.d.**			
	Counts of Evo+ cells	yw/+;Elav|Gal4-t+	**12**	All together	**One Way ANOVA**	kurt: -0.688 skew: -0.123	0.112	251.17 ± 6.939	$F=59.151$ $df1:2$; $df2:30$	<0.0001	**Dunnett post-hoc**
	in day 1 pupae	MyD88^{c4}/Elav|Gal4-EP3610	**12**	yw/+;Elav|Gal4-t+ vs MyD88^{c4}/Elav|Gal4-EP3610		kurt: -0.883 skew: -0.599		220.50 ± 6.431			<0.001
		Elav|Gal4-EP3610	**9**	yw/+;Elav|Gal4-t+ vs Elav|Gal4-EP3610		kurt: -1.043 skew: 0.111		216.44 ± 11.566			<0.001
Fig.4.11		**Genotype**	**Number of VNCs**			**Kurtosis & Skewness**	**Levene's test**	**Means±s.d.**			
	Counts of DCP1+ cells	yw/+;Elav|Gal4-t+	**16**	All together	**Welch ANOVA**	kurt:-0.196 skew:-0.850	0.039	195.50 ± 13.317	$F=50.483$ $df1:3$; $df2:29$	<0.001	**Dunnett post-hoc**
		Wek^{c4}/DfBSC 690	**11**	yw/+;Elav|Gal4-t+ vs WekEX14/DfBSC690		kurt:-0.493 skew:-0.467		173.55 ± 11.544			0.002
		Elav|GAL4-UASwekHA	**8**	yw/+;Elav|Gal4-t+ vs Elav|GAL4-UASwekHA		kurt:2.564 skew:-0.494		253.50 ± 11.110			<0.001
		DfBSC 690/wekEX14; Elav|GAL4-UASwekHA	**9**	yw/+;Elav|Gal4-t+ vs DfBSC690/wekEX14; Elav|GAL4-UASwekHA		kurt:-0.875 skew:0.086		216.89 ± 8.038			0.002
		Elav|Gal4-UASTollrnex	**11**	yw/+;Elav|Gal4-t+ vs Elav|Gal4-UASTollrnex		kurt:-1.669 skew:-0.548		223.45 ± 13.931			<0.001
		Wek^{c4}/DfBSC 690.Elav|Gal4-UASTollrnex	**11**	yw/+;Elav|Gal4-t+ vs Wek^{c4}/DfBSC 690.Elav|Gal4-UASTollrnex		kurt:-1.407 skew:-0.640		197.00 ± 12.822			ns
		Elav|Gal4-dSpatzRNAi;UASWek	**8**	yw/+;Elav|Gal4-t+ vs Elav|Gal4-dSpatzRNAi;UASWek		kurt: 261 skew: 0.833		182.75 ± 13.688			ns
		Elav|Gal4-EP3610	**10**	yw/+;Elav|Gal4-t+ vs Elav|Gal4-EP3610		kurt:0.309 skew:-0.235		290.80 ± 16.006			<0.001
		Wek^{c4}/DfBSC 690.Elav|Gal4-EP3610	**8**	yw/+;Elav|Gal4-t+ vs WekEX14/DfBSC 690.Elav|Gal4-EP3610		kurt:1.901 skew:-0.745		249.00 ± 6.653			<0.001
				Elav|GAL4-UASwekHA vs DfBSC 690/wekEX14; Elav|GAL4-UASwekHA							<0.0001
				Elav|GAL4-UASwekHA vs Elav|Gal4-dSpatzRNAi;UASWek							<0.0001
				Elav|Gal4-UASTollrnex vs WekEX14/DfBSC690.Elav|Gal4-UASTollrnex							<0.0001
				Elav|Gal4-EP3610 vs WekEX14/DfBSC 690.Elav|Gal4-EP3610							1 n.s.
Fig.4.12		**Genotype**	**Number of VNCs**			**Kurtosis & Skewness**	**Levene's test**	**Means±s.d.**			
	Counts of Evo+	yw/+;Elav|Gal4-t+	**12**	All together	**One Way ANOVA**	kurt:-0.688 skew:-0.121	0.83	251.17 ± 6.939	$F=28.003$ $df1:2$; $df2:27$	<0.001	**Dunnett post-hoc**
	neurons	Wek^{c4}/DfBSC 690	**5**	yw/+;Elav|Gal4-t+ vs WekEX14/DfBSC690		kurt:0.296 skew:-0.816		248.80 ± 6.535			ns
		Elav|GAL4-UASwekHA	**13**	yw/+;Elav|Gal4-t+ vs Elav|Gal4-UASWekHA		kurt:-1.637 skew:-0.325		233.54 ± 5.222			<0.001
		MyD88c2.8/Elav|Gal4-Wek	**10**	yw/+;Elav|Gal4-t+ vsMyD88c2.8/Elav|Gal4-Wek		kurt: -.628 skew:-0.376		227.70 ± 8.338			<0.001
Fig.4.13		**Genotype**	**Number of VNCs**			**Kurtosis & Skewness**	**Levene's test**	**Means±s.d.**			
	Counts of Histone	MyD88|GAL4-UASHistoneYFP	**9**	All together	**Welch ANOVA**	kurt:-01.545 skew:-0.130	0.003	456.44 ± 5.325	$F=1187.475$ $df1:2$; $df2:7.184$	<0.001	**Dunnett post-hoc**
	YFP+ cells	MyD88|GAL4-UASHisYFP, UASwek	**6**	MyD88|GAL4-UASHistoneYFP vs MyD88|GAL4-UASHisYFP, UASwek		kurt: -2.166 skew:-0.441		431.00 ± 18.879			0.002
		MyD88|GAL4-UASHisYFP.EP3610	**5**	MyD88|GAL4-UASHistoneYFP vs MyD88|GAL4-UASHisYFP.EP3610		kurt:-0.486 skew:-0.757		211.40 ± 9.940			<0.001
Fig.4.14		**Genotype**	**Number of VNCs**			**Kurtosis & Skewness**	**Levene's test**	**Means±s.d.**			
	Counts of Evo+ neurons	MyD88|GAL4-UASHistoneYFP	**9**	All together	**One Way ANOVA**	kurt:0.459 skew:-1.210	0.39	260.56 ± 6.307	$F=76.350$ $df1:2$; $df2:18$	<0.001	**Dunnett post-hoc**
		MyD88|GAL4-UASHisYFP, UASwek	**6**	MyD88|GAL4-UASHistoneYFP vs MyD88|GAL4-UASHisYFP, UASwek		kurt:1.262 skew:-0.974		234.67 ± 21.232			0.003
		MyD88|GAL4-UASHisYFP.EP3610	**6**	MyD88|GAL4-UASHistoneYFP vs MyD88|GAL4-UASHisYFP.EP3610		kurt:0.274 skew:-1.026		175.67 ± 9.993			<0.001

CHAPTER 3

TOLLS ARE EXPRESSED WITHIN THE CNS OF *DROSOPHILA* AT DIFFERING TIMES

3.1 INTRODUCTION

It has been reported previously that not all of the Toll receptors are involved in immunity related functions. Therefore they may function within the nervous system. In order to characterize the functions of the different Toll receptors, it was pivotal in the first instance to investigate where they are expressed. The aim of this chapter was therefore to determine a spatial and temporal profile of Toll receptor expression within the CNS. RT-PCR was utilized to determine the stages of development that each of the Tolls is expressed, in wild type CNS. Using a combination of genetics and RT-PCR allowed the determination of the Tolls within glial and/or neuronal cells.

Immunohistochemistry was then utilised to further characterize the expression profiles of three of the Toll receptors (Toll-2, Toll-3 and Toll-8). The expression of Toll-1, Toll-6 and Toll-7 in the CNS has already been documented (McIlroy *et al.,* 2013; Sutcliffe B PhD Thesis; Lim A PhD Thesis, and M.P.Nallasivan unpublished data). Genetic tools were not available for Toll-4 and Toll-5. Toll-9Gal4 flies became available, however unfortunately it contains the P{3xP3-EGFP) promoter (Figure 3.14) which is expressed within the central nervous system. I have genetically removed this promoter from this line however time limitations prevented the expression profile from being characterised. These data show that

Figure 3.14: The Curse of P{3XP3-EGFP}

P{3XP3-EGFP} is a transgenic transposon which is widely used as a vector to integrate transgenes into the genome. However, expression is throughout the embryonic, larval and pupal CNS tissues. It was only after viewing similar phenotypes for a number of different genotypes that it was determined there was something other than the genotype creating the staining. It was determined that the P{3XP3-EGFP} landing site was the culprit and had to be removed genetically from numerous different fly lines (See protocol 2.5, materials and methods).

some of the Toll receptors are expressed to different levels during different developmental stages.

3.2 RESULTS

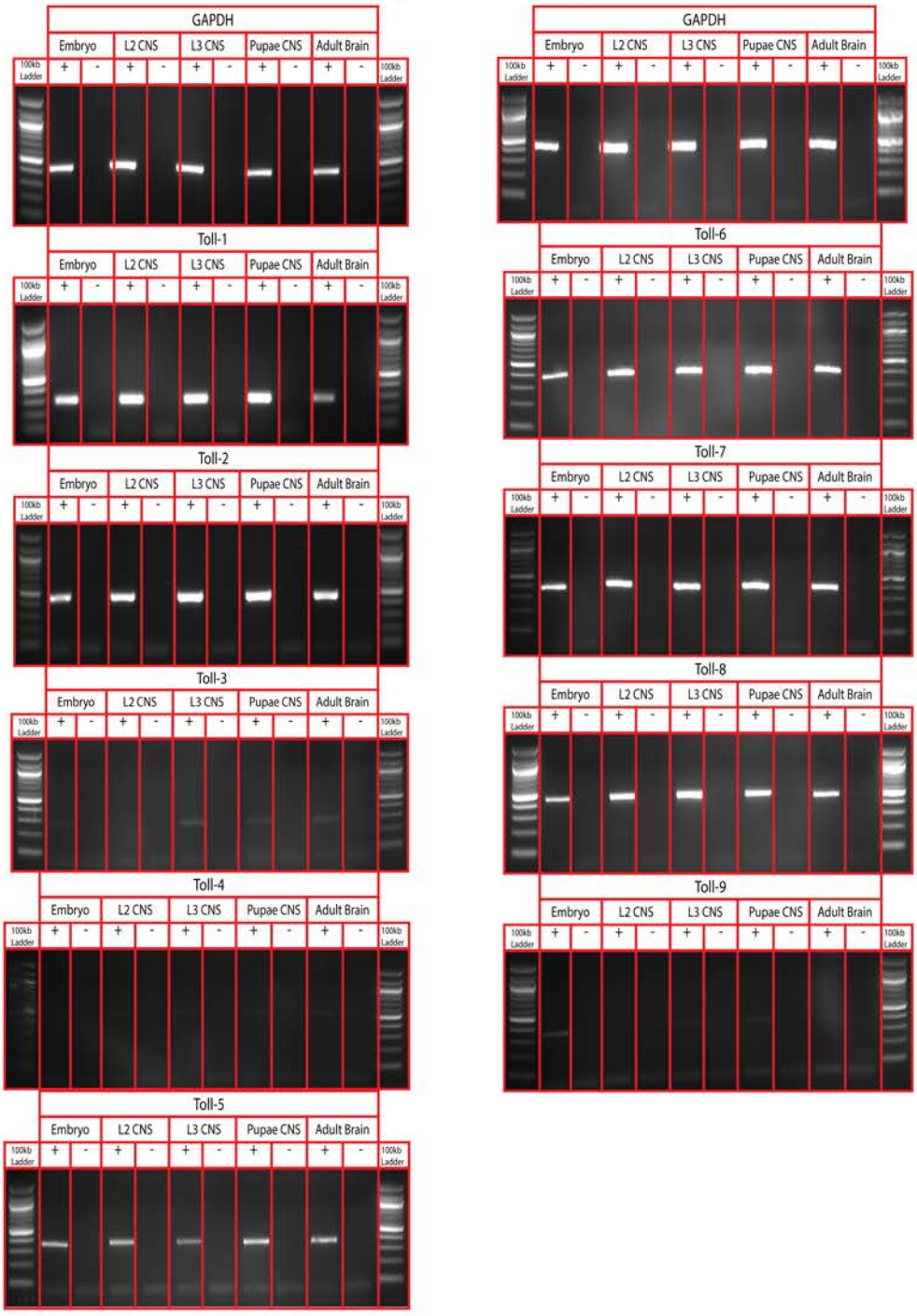
3.2.1 Toll receptors are expressed throughout development in neurons and glia

To determine if any of the Toll receptors are expressed within the CNS, RT-PCR was carried out during differing developmental time points. Collection of embryos, dissection of second instar, third instar wandering larvae or pupae brains, and whole head preparations of Oregon-R (wild type) or ElavGal4>UASgcm (embryos only) were the starting materials. Total RNA was extracted from the starting samples by Trizol (Ambion) reagent. RNA integrity and concentration was confirmed via Nano-drop. RNA samples were DNase treated to remove residual genomic DNA contamination. 300ng of RNA was used for cDNA synthesis following GoScript™ Reverse Transcriptase methods. Samples were diluted 1:3 with Nuclease free H_20, and a no RT sample of 300ng of RNA made up to 60ul with Nuclease free H_20. Standard PCR reaction was performed to amplify each of the Toll receptor cDNA using GoTaq PCR protocol. 2uM of forward and reverse primers were designed specific to each Toll gene of interest (See table 2.2 and 2.3 for primer sequences and PCR programme). GAPDH is a general housekeeping gene that was used as a positive control during every round of PCR. Gel electrophoresis for RT-PCR products were then carried out.

Toll-1, Toll-2, Toll-5, Toll-6, Toll-7 and Toll-8 are expressed in abundance during all developmental stages. Toll-4 and Toll-9 are also expressed during all developmental stages. Toll-3 is slightly different again to the other Tolls as there is no detectable expression during second instar larvae, and only low expression during all other time points (Figure 3.1).

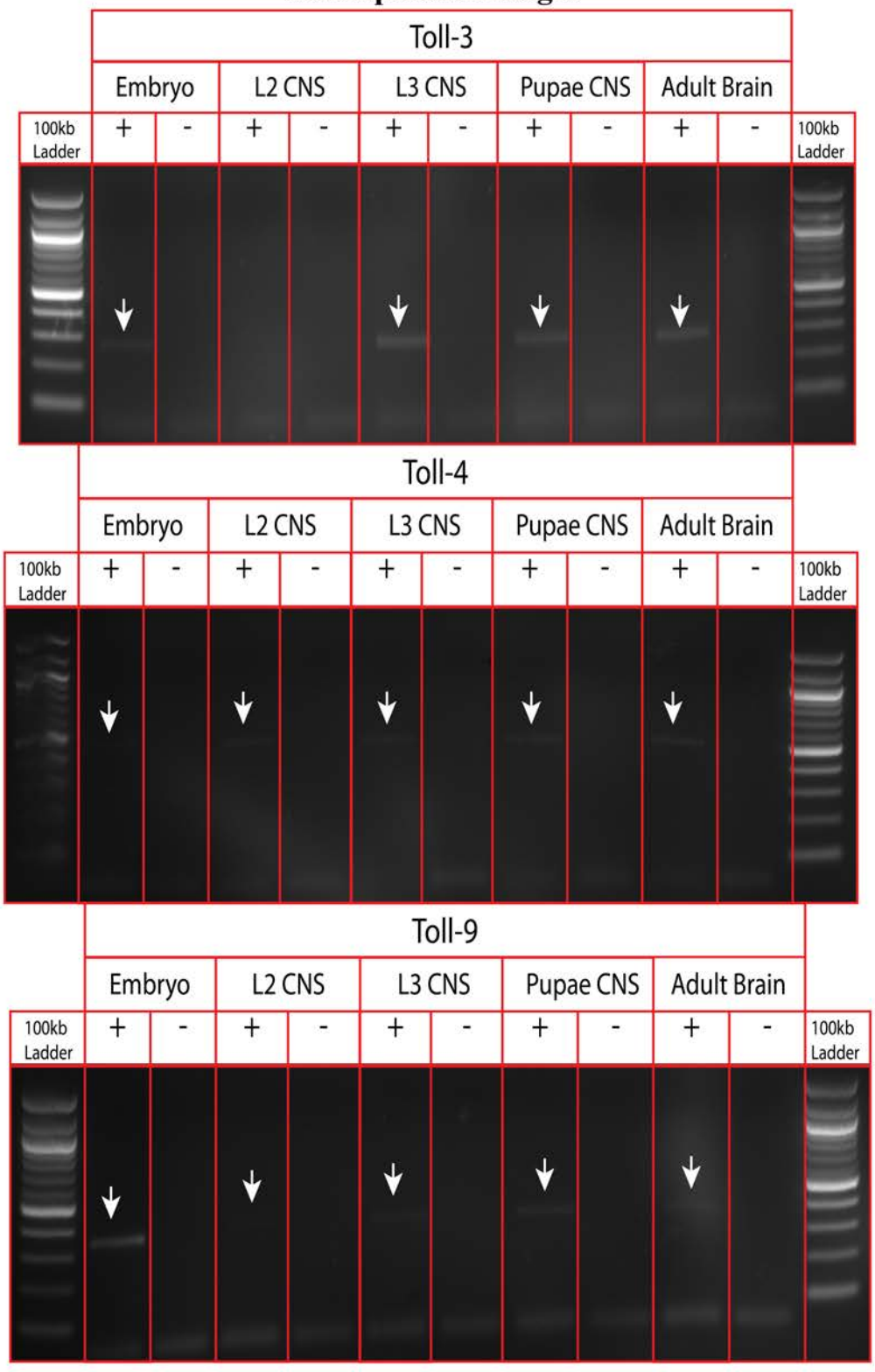
Transcriptional regulation of neuronal and glial fates is controlled by the expression of glial

Figure 3.1 Toll receptors are expressed during different developmental stages



Gel electrophoresis pictures of RT-PCR products for Tolls 1-9 during different developmental time points (Entire embryo, Larval stage 2 CNS, Larval stage 3 CNS, Pupal CNS, Adult head) with sample (+), and No-RT control sample (-) of Oregon R generated with specific forward and reverse primers for each of the Toll receptors (See table 2.2). Experiment was preformed in triplicate along with a housekeeping gene GAPDH as positive contol.

Figure 3.1b Toll receptors are expressed during different developmental stages



Enlarged gel electrophoresis pictures of RT-PCR products for Tolls -3, -4, -9.

cell missing (gcm) transcription factors. Gcm functions via downstream transcription factors in order to maintain glial gene expression. To determine if the Toll receptors are expressed within neurons or glial cells I changed cell fate to a glial cell fate and tested if there were changes in expression of the Toll receptors. ElavGAL4, a neuronal and post-miotic driver line, was crossed to UASgcm. This cross leads to the expression of gcm in all neurons, and thus all cells ultimately have a glial fate.

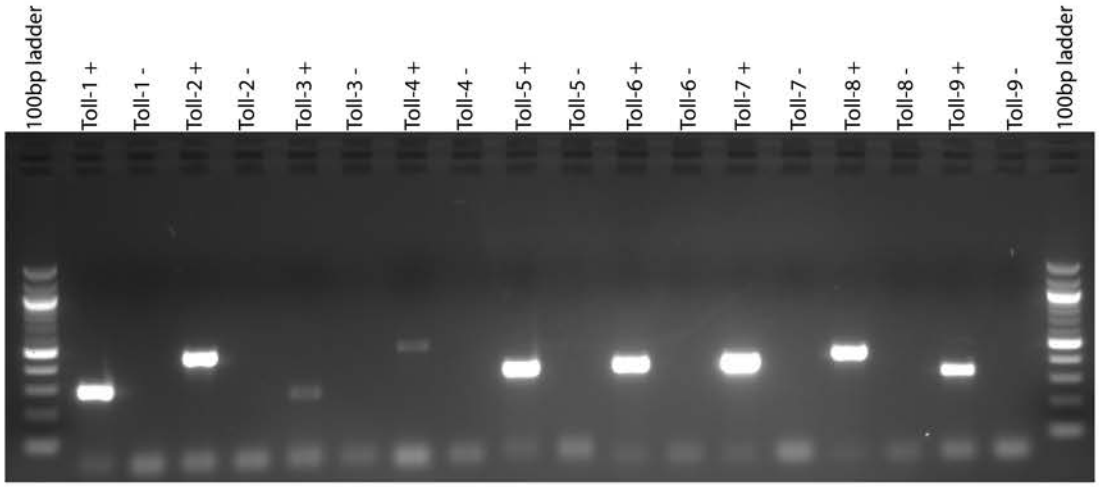
In Elavgal4>UASgcm embryos, all of the Tolls are detected during the embryonic stage (Figure 3.2). This includes those Tolls (Toll-3, Toll-4 and Toll-9), which were expressed much lower levels (Figure 3.1). This suggests that some or all of the Tolls are expressed in glia or that the expression of gcm somehow regulates the Toll receptors.

3.2.2 Toll-2 is expressed in the CNS during all developmental stages

Toll-2 (also known as 18w) expression was visualized using a lacZ enhancer trap line $y^{1}w^{67623}$(PlacW)$18w^{K02701}$ (here after named Toll-2^{K02701}) and anti-βgal antibodies (Figures 3.3 to 3.5). The lacZ insertion is located 4bp upstream of the Toll-2 transcriptional start point, and is in the correct orientation. Toll-2^{K02701} is distributed throughout the stage16/17 embryo (Figure 3.3). Toll-2^{K02701} is located in the head region (Figure 3.3A arrow 1), origin of heamocytes, the procephalic mesoderm (Figure 3.3A arrow 2), along the ventral midline (Figure 3.3A arrow 4) and within the ventral muscles (Figure 3.3A arrow 3).

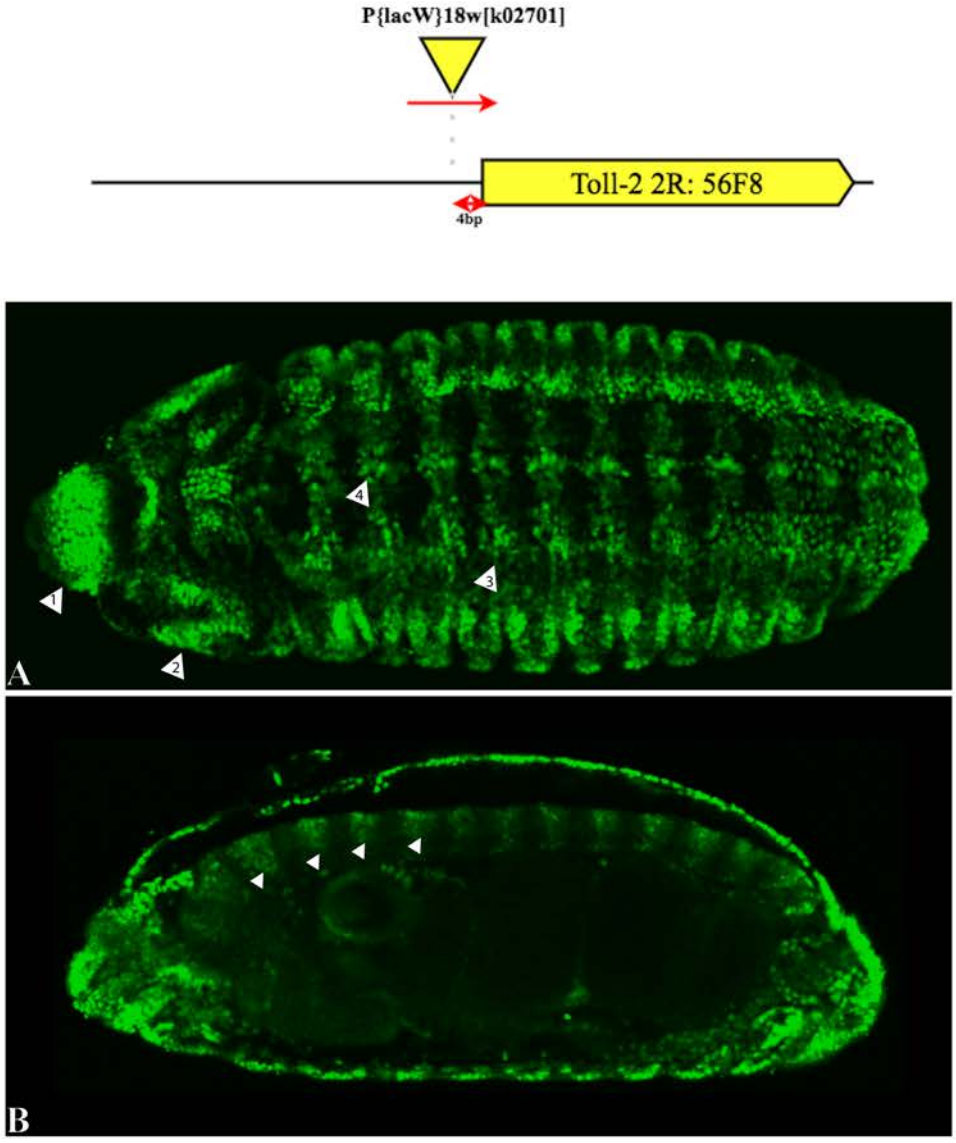
In third star wandering larvae, Toll-2^{K02701} is predominantly distributed throughout the optic lobes. There is expression of Toll-2^{K02701} within cells of the laminae that innervate the medullae and a few cells that are located within the central brain region. Toll-2^{K02701} is also

Figure 3.2 Toll receptors are expressed in ElavGal4>UASgcm embryos



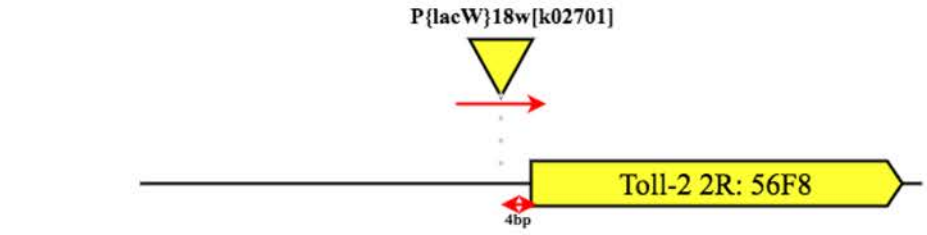
Gel electrophoresis picture of RT-PCR products for Tolls 1-9 during embryonic development where gcm is expressed in all neurons (ElavGal4>UASGcm;UASGcm2), within sample (+), and No-RT control sample (-). Specific forward and reverse primers were used for each of the Toll receptors (See table 2.2) and experiments were performed in triplicate.

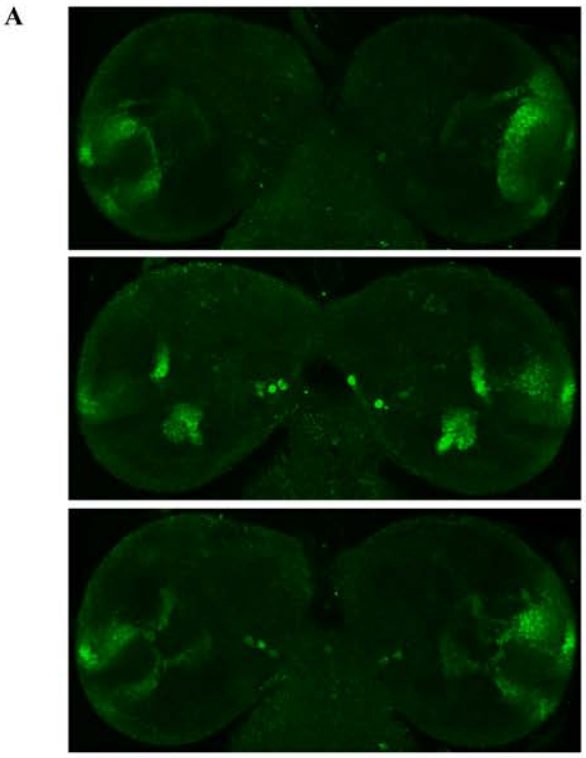
Figure 3.3: Expression of Toll 2 in stage 16/17 Embryo

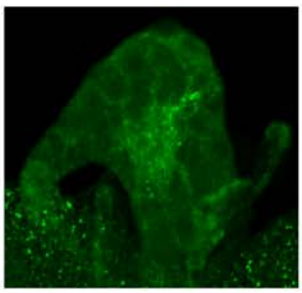


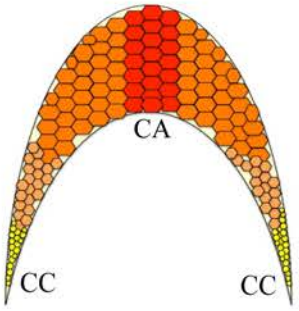
These images show the expression of Toll-2^{K02701} in stage 16/17 Embryo visualised with anti-βgal. The lacZ insertion is located 4bp upstream of Toll-2 in the correct orientation.
In panel A expression of Toll-2^{K02701} is located in the head region (arrow 1), origin of haemocytes (arrow 2), along ventral muscles (arrow 3) and within the ventral midline (arrow 4).
In panel B arrows highlight segmental expression of Toll-2^{K02701} along the VNC.

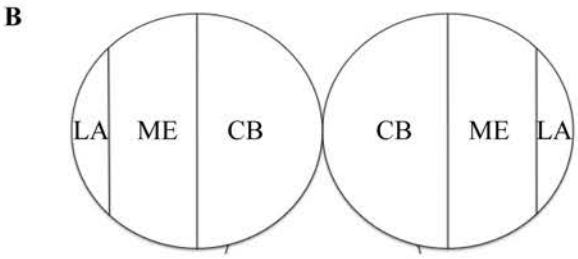
Figure 3.4: Expression of Toll 2 in wandering larval brain





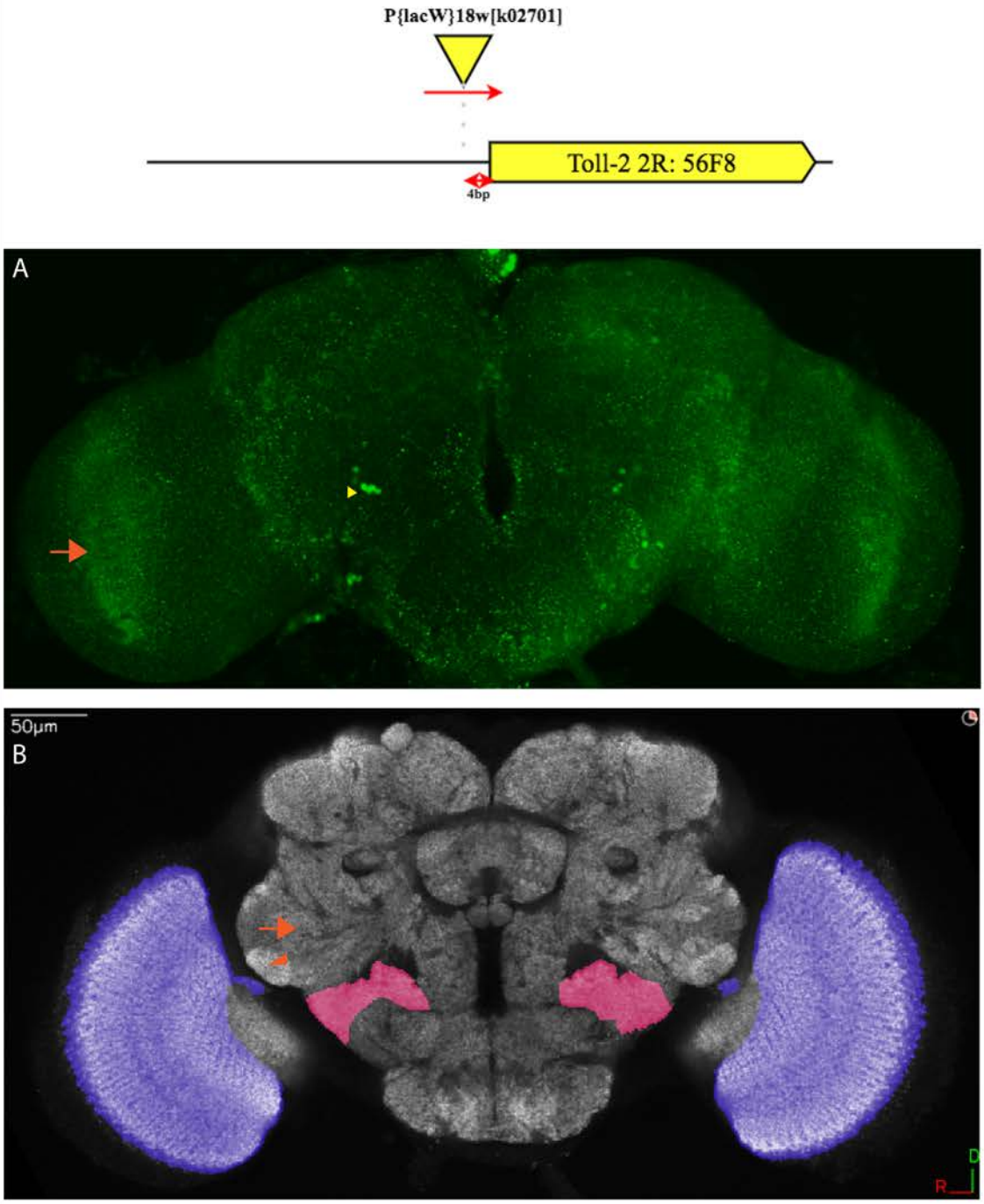






These images show the expression of Toll-2^{K02701} in third star wandering larvae visualised with anti-βgal. The lacZ insertion is located 4bp upstream of Toll-2 in the correct orientation. Panel A shows the expression of Toll-2^{K02701} is located throughout optic lobes, the lamina (LA), medulla (ME) and a few cells within the central brain (CB).
Panel B represents the areas detailed in panel A in a schematic diagram.
Panel C also highlights that expression of Toll-2^{K02701} is located within the corpus allatum (CA) of the ring gland. Panel D shows regions including the CA and corpus cardiaca (CC) demonstrated in a schematic diagram of the ring gland.

Figure 3.5: Expression of Toll 2 in the adult brain



These images show the expression of Toll-2^{K02701} in the adult brain visualised with anti-βgal. The lacZ insertion is located 4bp upstream of Toll-2 in the correct orientation. In panel A expression of Toll-2^{K02701} is located throughout medulla (Orange arrow) and a few cells within the central brain near or in the wedge region (yellow arrow head). The schematic diagram in panel B was generated using virtual fly brain; Janelia Adult Brain generator to highlight the medulla region (blue) and the wedge region (pink).

located within the corpus allatum of the ring gland (Figure 3.4). In the Adult brain Toll-2^{K02701} is distributed throughout the medulla. There are a couple of individual cells within the central brain region in close proximity to the wedge region (Figure 3.5).

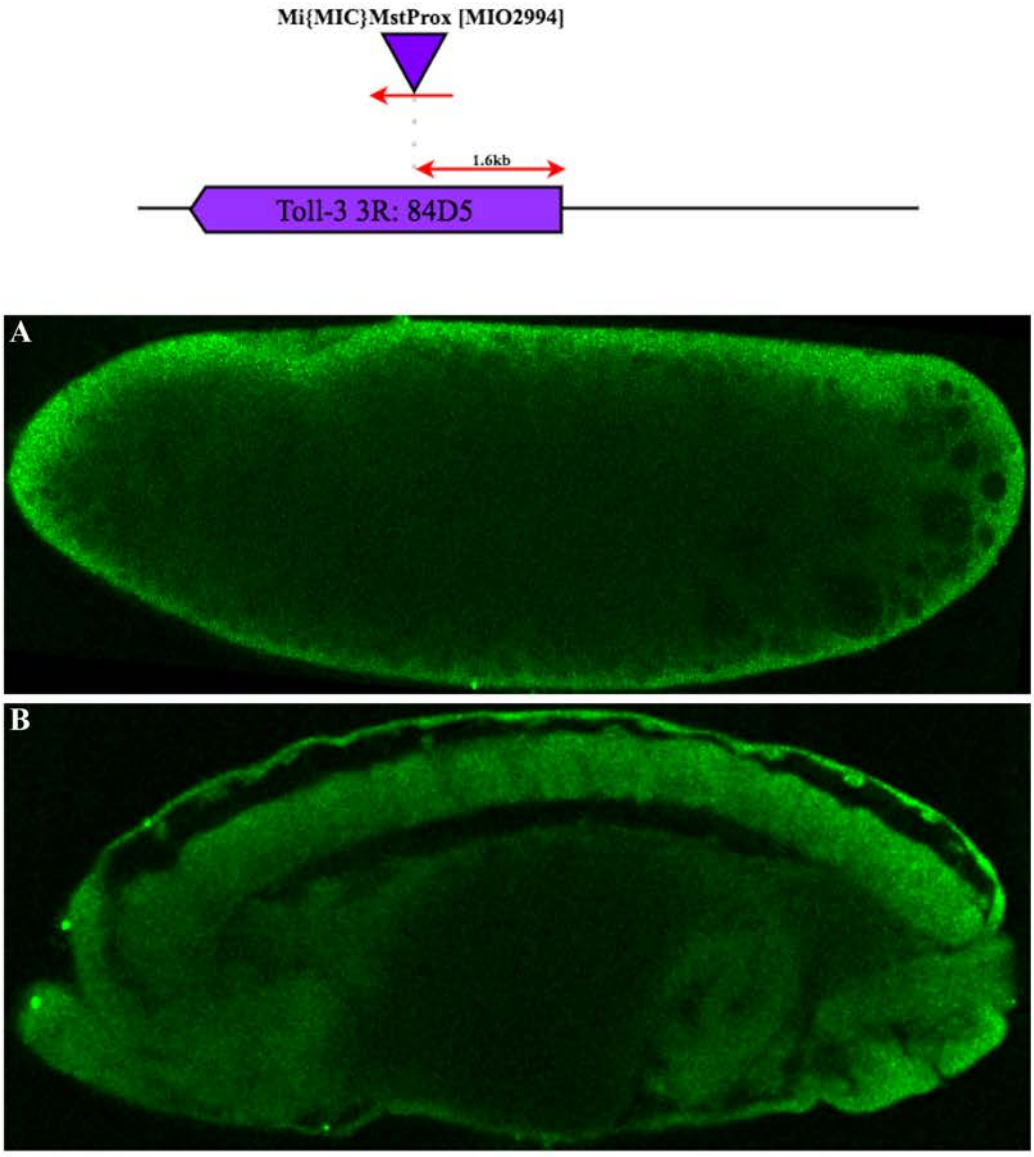
3.2.3 Toll-3 is expressed at low levels in the larvae increasing in the adult stage

Toll-3 (also known as Mstprox) expression was visualized using Toll-3MIMIC^{MIO2994} flies bearing a *MIMIC- GFP* insertion 1.6kb into the *Toll-3* locus in the correct orientation. There was no expression of Toll-3MIMIC^{MIO2994} in either early stage or late stage embryos (Figure 3.6). In the larval CNS Toll-3MIMIC^{MIO2994} was distributed throughout the VNC at very low levels along the neuropile. Within the optic lobes there is expression of Toll-3MIMIC^{MIO2994} in the lateral most regions, possibly close to the developing laminae. Furthermore there is some expression located within the central brain (Figure 3.7). In the adult brain Toll-3MIMIC^{MIO2994} is distributed throughout the outer medulla and a small group of highly expressed cells located in the region of the gnathal ganglion (Figure 3.8Ai & B). Toll-3MIMIC^{MIO2994} is also distributed within the fan shaped body and the mushroom bodies (Figure 3.8Ai, Aii & B).

3.2.4 Toll-8 is widely distributed throughout the wandering L3 and adult CNS.

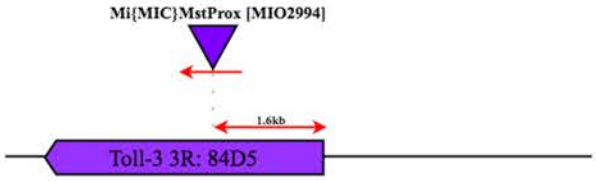
Toll-8 (also known as Tollo) expression was visualized using Toll-8Gal4^{MD806} flies bearing a GAL4 enhancer located 180bp upstream of Toll-8 transcriptional start site. These flies were used to drive the expression of membrane-tethered *10xUAS-myr-td-Tomato*, in conjunction with anti-DsRed antibodies. Toll-8Gal4^{MD806}>*myr-td-Tomato* is distributed widely within the VNC in a number of thoracic interneurons; abdominal neurons as well as being present within

Figure 3.6: Expression of Toll 3 in early and late stage Embryo

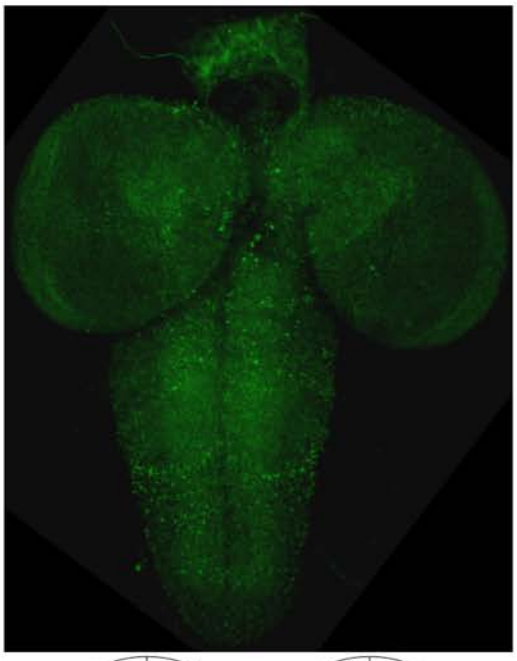


These images show the expression of Toll-3MIMIC^{MIO2994} in the the embryo visualised with anti-GFP. The MIMIC insertion is located 1.6kb within the Toll-3 locus in the correct orientation. Expression of Toll-3MIMIC^{MIO2994} could not be detected in early stage (A) or late stage embryos (B).

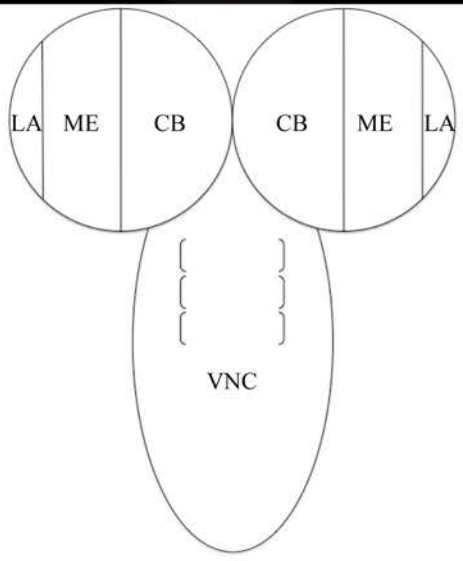
Figure 3.7: Expression of Toll 3 in wandering larval brain



A

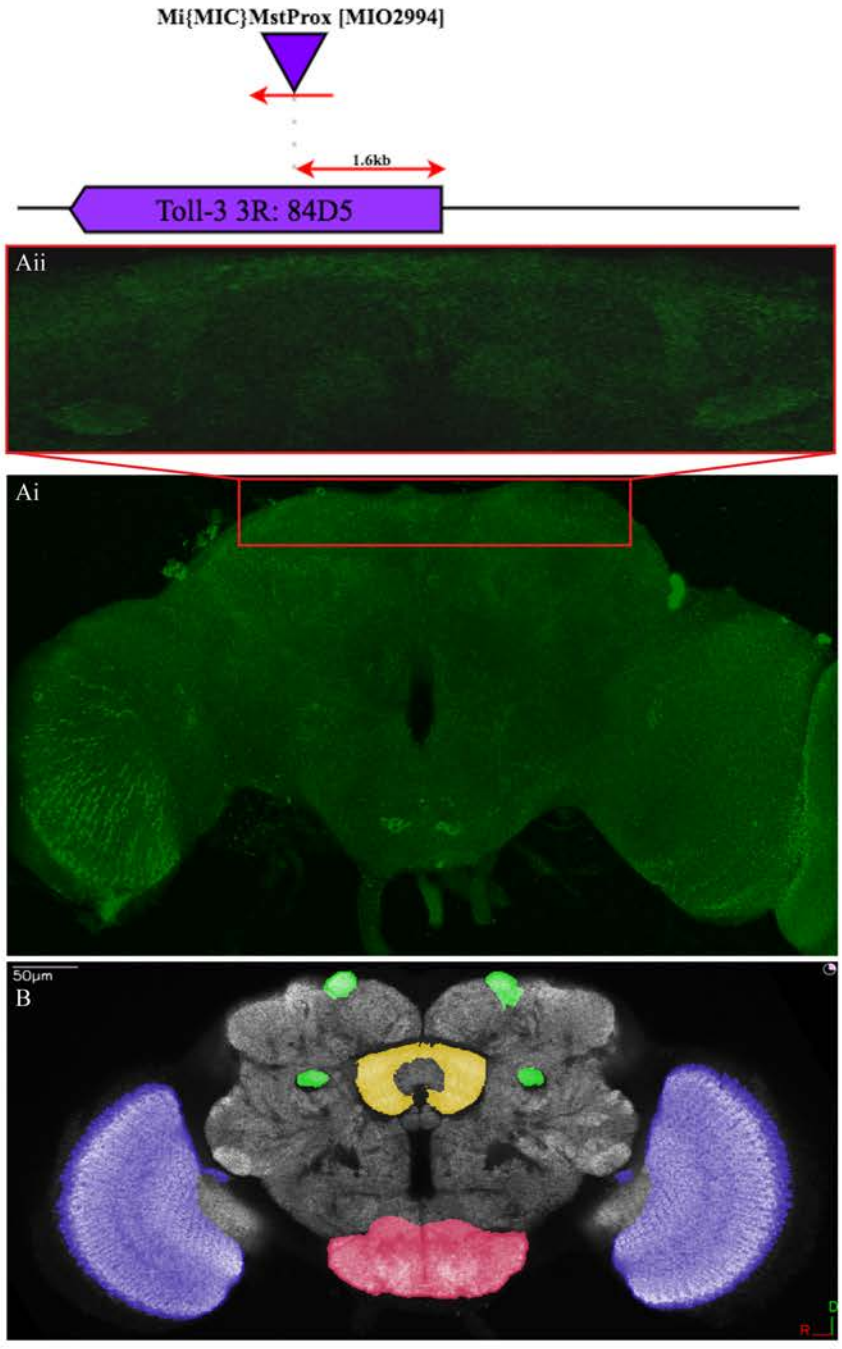


B



These images show the expression of Toll-3MIMIC^{MIO2994} in the wandering larval brain visualised with anti-GFP. The MIMIC insertion is located 1.6kb within the Toll-3 locus in the correct orientation. In panel A expression of Toll-3MIMIC^{MIO2994} is located throughout the neuropile of the VNC, the outermost regions of the laminae (LA) and within the central brain (CB). Panel B shows a representation of these areas in a schematic diagram.

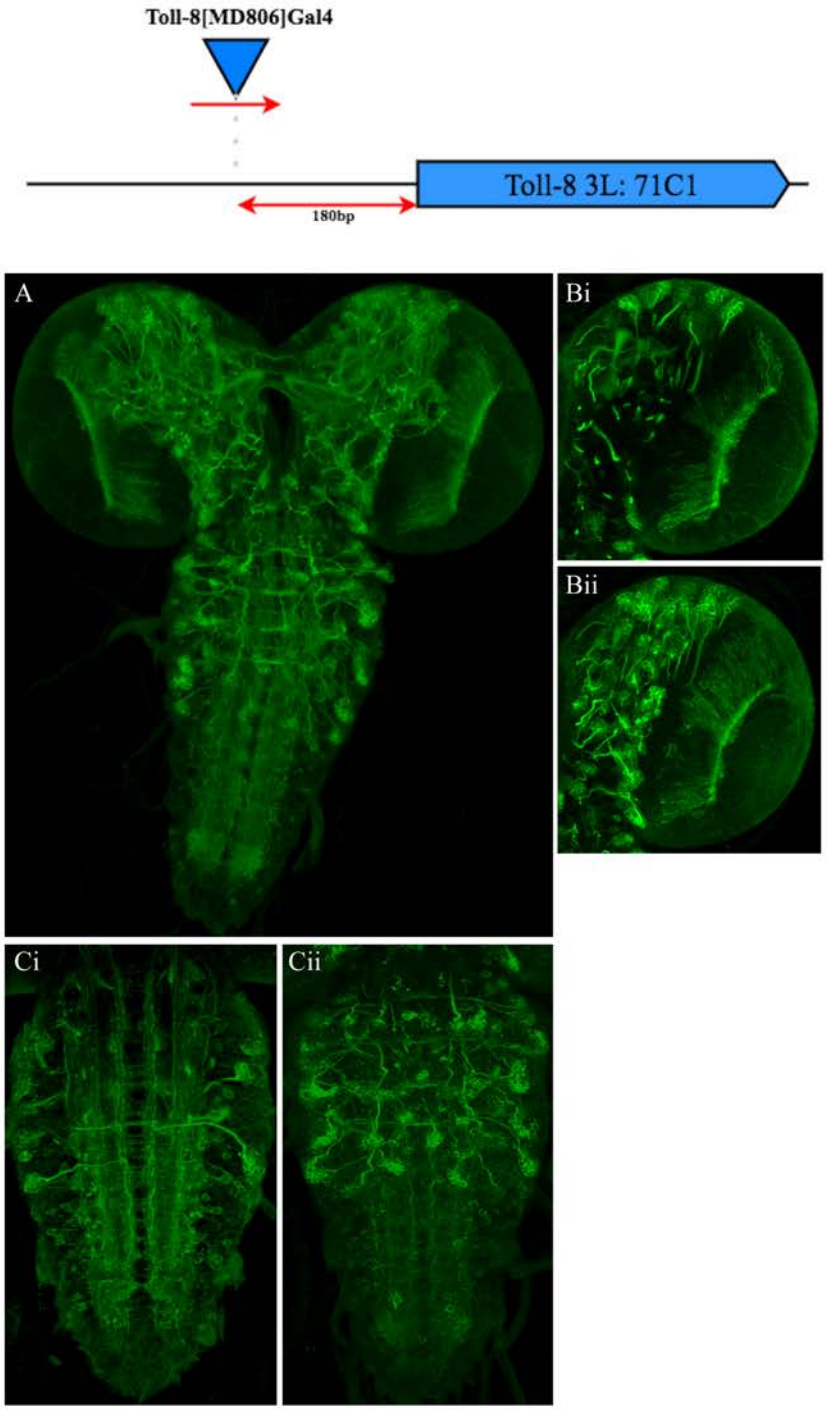
Figure 3.8: Expression of Toll 3 in the adult CNS



These images show the expression of Toll-3MIMIC^{MIO2994} in the CNS of the adult brain visualised with anti-GFP. The MIMIC insertion is located 1.6kb within the Toll-3 locus in the correct orientation. Panel Ai shows the expression of Toll-3MIMIC^{MIO2994} is located throughout the medulla, a few cells within the gnathal ganglion, within the mushroom body, and within the fan shaped body. Panel Aii is a magnified view of the mushroom body and fan shaped body regions. Panel B was generated using virtual fly brain; Janelia Adult Brain generator and is a schematic representation of the location of the medulla (blue), gnathal ganglion (pink), mushroom body (green) and fan shaped body (yellow)

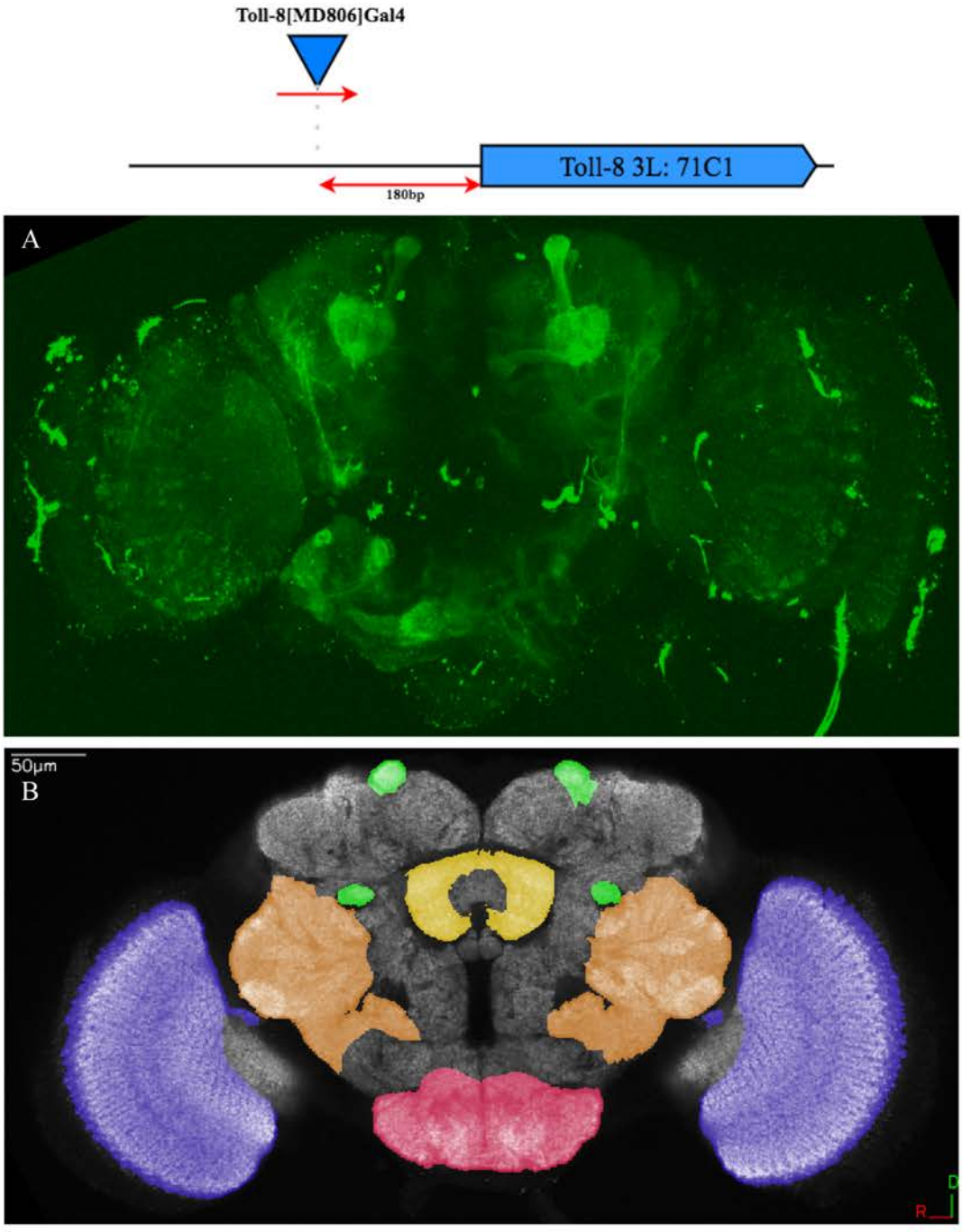
projections that innervate the CNS (Figure 3.9 Ci & Cii). Toll-8Gal4^{MD806}>*myr-td-Tomato* is widely distributed throughout the optic lobes in particular within the medullae and the central brain (Figure 3.9 Bi & Bii). In the adult brain, Toll-8Gal4^{MD806}>*myr-td-Tomato* is distributed throughout the outer medulla (Figure 3.10B: blue; Figure 3.12), fan shape body and mushroom bodies (Figure 3.10B: yellow and green; Figure 3.11), ventro lateral protocerebrum (Figure 3.10B: orange). Overall expression of Toll-8Gal4^{MD806}>*myr-td-Tomato* is widely distributed throughout both the optic lobes and central brain of the larvae and adult CNS (Figure 3.9; 3.10; 3.11 & 3.12).

Figure 3.9: Expression of Toll-8 in the larval CNS



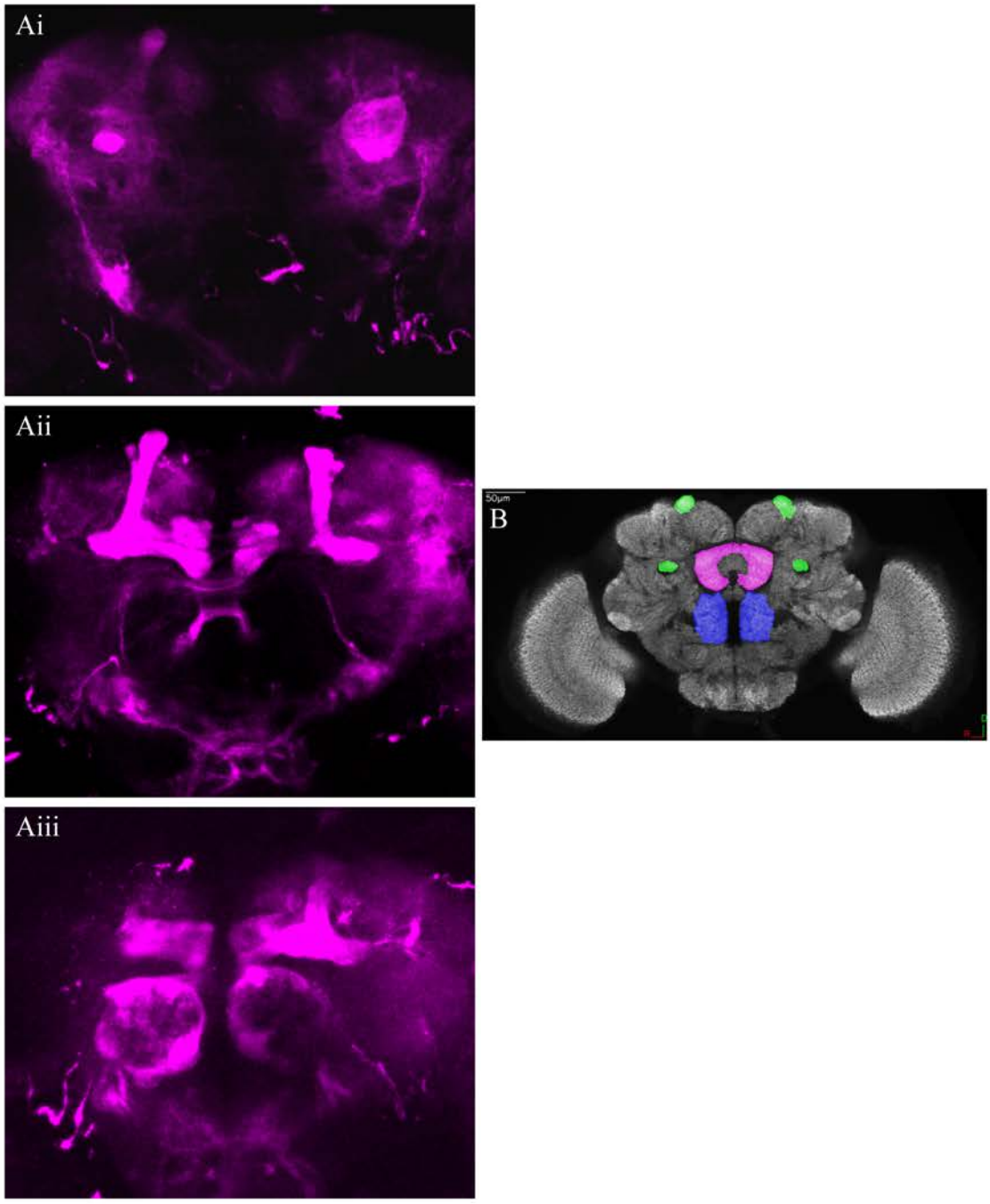
These images show the expression of Toll-8Gal4^{MD806} in the CNS of the larvae brain visualised with anti-DsRed. The Gal4 insertion is located 180bp upstream of Toll-8 in the correct orientation. In panel A expression of Toll-8^{MD806} is located throughout the optic lobes and VNC. Panel Bi & Bii show within the optic lobes there is expression throughout the medulla and central brain. Panel Ci & Cii shows that within the VNC there is expression in a number of thoracic interneurons, abdominal neurons and projections inervating the CNS.

Figure 3.10: Expression of Toll-8 in the adult brain



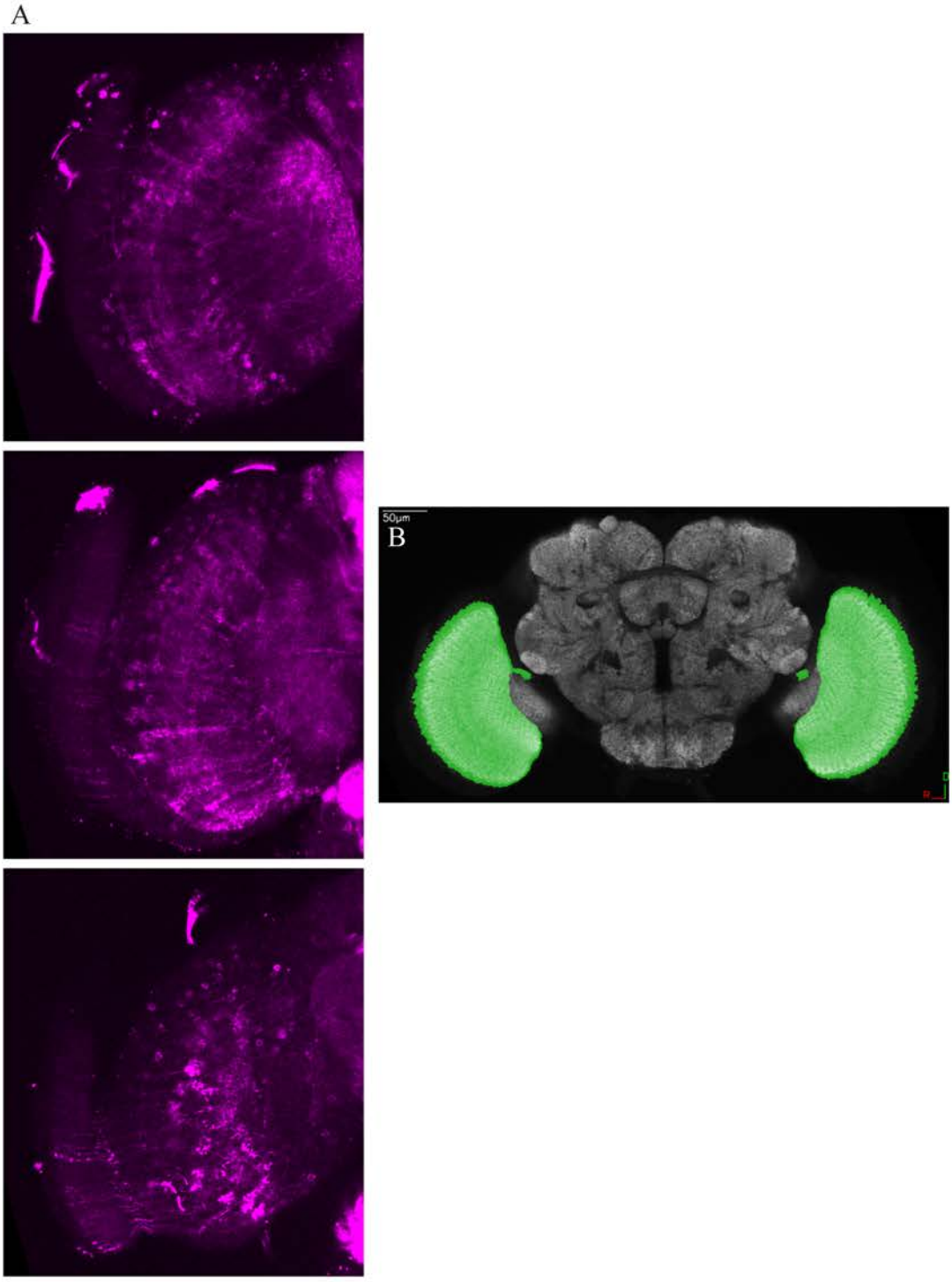
These images show the expression of Toll-8Gal4^{MD806} in the CNS of the adult visualised with anti-DsRed. The Gal4 insertion is located 180bp upstream of Toll-8 in the correct orientation. Panel A shows the expression of Toll-8^{MD806} is located throughout the medulla, mushroom bodies, fan shaped body, Saddle and along the ventrolateral protocerebrum. Panel B higlights these regions medulla (blue), mushroom bodies (green), fan shapped body (yellow), saddle (pink) and ventrolateral protocerebrum (orange) in a schematic diagram, which was generated using virtual fly brain; Janelia Adult Brain generator.

Figure 3.11: Expression of Toll-8 in the adult central brain



These magnified images show the expression of Toll-8Gal4^{MD806} in the adult central brain visualised with anti-DsRed. In panels Ai & Aii expression of Toll-8^{MD806} is located throughout mushroom bodies. Panel Aii also shows expression of Toll-8^{MD806} within the fan shaped body. Panel Aiii shows the expression of Toll-8^{MD806} within the vest. In Panel B these regions are highlighted in a schematic diagram, which was generated using virtual fly brain; Janelia Adult Brain generator.

Figure 3.12: Expression of Toll-8 in the adult optic lobe



These magnified images show the expression of Toll-8Gal4^{MD806} in the adult optic lobe visualised with anti-DsRed. In panel A expression of Toll-8^{MD806} is widely distributed throughout the medulla through all planes. In panel B this region is highlighted in a schematic diagram, which was generated using virtual fly brain; Janelia Adult Brain generator.

3.3 SUMMARY

In this chapter I have shown (Figure 3.13 and Table 3.1):

1) Most of the Tolls are expressed during all developmental stages
2) All of the Tolls are either expressed in glia or are regulated by gcm
3) *Toll-2*^{K02701} is widely distributed throughout the embryo, and is predominantly confined to the optic lobes in both larval and adult stages.
4) *Toll-3*MIMIC^{MIO2994} is not expressed during embryonic development. There are very low levels of expression within the VNC and optic lobes during larval stages and within the optic lobes of the adult.
5) *Toll-8*Gal4^{MD806} is very widely distributed throughout the entire CNS in both larval and adult stages.

I have used a combination of genetic tools, including lacZ, MiMIC and Gal4 fly lines, and molecular techniques to determine if the Tolls are located within the CNS and in which cell types. I have established that whilst the Toll receptors belong to the same protein family, they are required in different areas of the CNS and in different cell types during development. The use of whole embryo and adult head instead of dissection of CNS could add robustness to this assay in the future. However as the data show no concerning results this work provides evidence that the Tolls are likely to be expressed within the CNS at these stages.

Through the use of RT-PCR and antibody staining's I have shown that Toll-2^{K02701} is expressed during all developmental stages. In later developmental periods including the larval and adult stages expression is mainly confined to the optic regions.

Similarly *Toll-3*MIMIC^{MIO2994} was shown to be restricted in both the larval and to greater extent to the adult optic regions only. There was no detectable expression of *Toll-3*MIMIC^{MIO2994} during embryonic stages via immune-histochemical means. This may be due to the fact that Toll-3 is simply not expressed or that the endogenous expression levels are so low or restricted to only a few cells, possibly outwith the CNS. RT-PCR results also confirm the finding that Toll-3 is likely to be expressed at low levels or not at all as in early larval stages. Contrastingly, *Toll-8*^{MD806} was detected in abundance throughout the CNS in both the larval and adult stages. These findings were confirmed by RT-PCR data.

The use of molecular tools in order to determine the patterns of gene expression is a sound starting point for any analysis. The use of Gal4 lines can identify cell morphology normally expressing the gene of interest (in this case Toll-8). Furthermore the amplification effect can greatly enhance detection levels. However these tools also have certain limitations, as they are crucial during the interpretation of any result. For instance, perdurance of the gene product (e.g. B-galactosidase) following endogenous gene switch off resulting in longer temporal expression than the endogenous protein. The location of the reporter related to the gene of interest is also an important consideration. For instance if only a part of an upstream intergenic region is cloned into the vector, regulatory elements may not be incorporated into the fragment to be amplified. Furthermore dependent upon where the construct lands within the genome following transformation, influences positional effects of either enhancers or repressors.

Furthermore the location of endogenous genes both spatially and temporally does not always relate to function. To be more confident in regards to detecting the cellular distribution of my

Toll genes, *In situ* hybridisation to detect specific mRNA would be beneficial. However, a draw back of in-situ is the difficulty of obtaining reliable data. Poor resolution during this process means that you are unable to determine what cell type the gene of interest is located in, and you can thus only determine a rough spatial profile. For instance the resulting signal is restricted to mRNA location, in neurons this is typically the soma. Therefore in order to establish specific cell types double *in situ* staining's would be required.

All of the Toll receptors (with the exception of Toll-3 in second instar larvae) are expressed during all developmental stages. Toll-3, Toll-4 and Toll-9 are different to the others in that they are expressed at much lower levels. Indicating they are either more transient in nature or are expressed in fewer cells. For example Toll-9 in embryos is expressed only during stage 9 of embryonic development (Kambris *et al.*, 2002).

It is interesting to note that the entire Toll receptor group are expressed when gcm is over-expressed in all neurons. This indicates that all are expressed in glial cells or may be regulated by gcm. Interestingly even the Tolls that were expressed at lower levels (Toll3, Toll-4 and Toll-9) in wild type samples were detected when gcm is over-expressed in neurons. RT-PCR testing however is qualitative, and for quantitative results qRT-PCR would be required. Furthermore, the embryo's were full mount and adult was full head samples. Both of which would be more representative if they were only CNS samples, however; this would have been much more time consuming considering the number of samples required for each reaction.

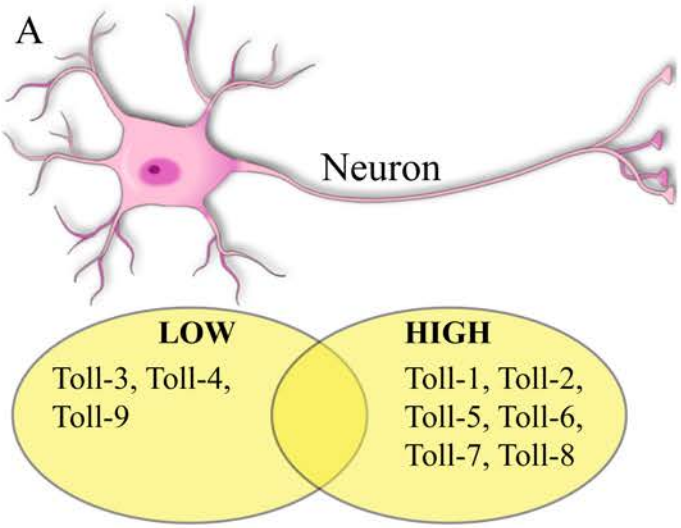
Furthermore gcm mutants may have been a better control than wild-type as mutant's lead to the failure of presumptive glial cell differentiation into glia. When over-expressing gcm in

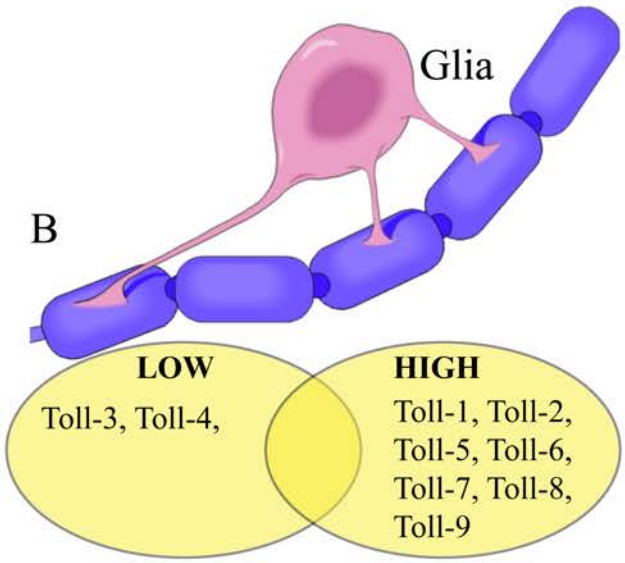
neurons, this results in lethality following the embryonic stage and therefore I was unable to test any of the later developmental time points. It may be possible to use the Gal80 system in order to only switch on UASgcm function when desired (e.g. L2, L3, Pupae and adult) to perform the same test and determine if any of the Tolls expressed in glia or regulated by gcm at later developmental time points.

Some early differences between the Toll receptors have been established. Using the RT-PCR data Toll-3, Toll-4 and Toll-9 may be expressed at lower levels within the CNS. Expression data via anti body staining shows that Toll-2 and Toll-3 are much lower expressed than Toll-8. Furthermore when over-expressing gcm in neurons, there is an increase in Toll-9 indicating it may be more glial than neuronal. Whereas Toll-3 and Toll-4 levels still remain relatively low. Therefore I initially propose that there are two groupings of the Toll receptors that are dependent upon the cell type involved (Figure 3.13 & Table 3.1).

In the next chapter I will investigate if the Toll receptors function in the same manner within the CNS. Using both knockdown and over-expression conditions I will test if all nine receptors affect behaviour equally. To do this I will use TriKinetics assays to monitor activity levels over a 72-hour period.

Figure 3.13: Possible grouping of Toll receptors in neurons or glia





These diagrams depict the proposed groupings of the Toll receptors in different cell types. Panel A shows the proposed groupings of the Toll receptors in neurons. Panel B shows the proposed groupings of the Toll receptors in glial cells. These have been grouped according to their expression profiles outlined in RT-PCR experiments from chapter 3.

Table 3.1: Table of Results from Chapter 3

	CHAPTER 3								
	Expression of the Toll receptors								
	wild type samples (RT-PCR)					ElavGal4>UASGcm (RT-PCR)	Immunohistochemistry		
	Embryo	L2 CNS	L3 CNS	Pupal CNS	Adult Head	Embryo	Embryo	Larval CNS	Adult CNS
Toll-1	Y	Y	Y	Y	Y	Y	Y	Y	Y
Toll-2	Y	Y	Y	Y	Y	Y	Y	Y	Y
Toll-3	Y	N	Y	Y	Y	Y	N	Y	Y
Toll-4	Y	Y	Y	Y	Y	Y	Y	?	?
Toll-5	Y	Y	Y	Y	Y	Y	Y	?	?
Toll-6	Y	Y	Y	Y	Y	Y	Y	Y	Y
Toll-7	Y	Y	Y	Y	Y	Y	Y	Y	Y
Toll-8	Y	Y	Y	Y	Y	Y	Y	Y	Y
Toll-9	Y	Y	Y	Y	Y	Y	Y	?	?

	Indicates work carried out during this thesis
	Indicates published work from Kambris *et al.,* 2002; McIlroy *et al.,* 2013; Sutclife B PhD and Lim A PhD
N	Indicates no expression pattern
?	Indicates not investigated to date

CHAPTER 4

TOLL RECEPTORS HAVE DISTINCT FUNCTIONS IN LOCOMOTION

4.1 INTRODUCTION

In the previous chapter I showed that all Toll receptors are expressed in the embryo, CNS of stage 2 larvae (except for Toll-3), stage 3 larvae and pupae, and in adult heads. *Toll-2*^{K02701}, *Toll-3*MIMIC^{MIO2994} and *Toll-8*Gal4^{MD806} are expressed within the CNS during larval and adult stages, particularly in the optic lobe regions. Toll-3, Toll-4 and Toll-9 are more transient in nature than the other Toll receptors and group together. Toll-9 in particular appears to be more glial than the other Tolls.

Overall data from chapter 3 show that the Tolls are not equal. The aim of this chapter therefore was to determine if there were any functional differences between the Toll receptors in the CNS. I used phenotypic assays to ask these questions. I asked whether all nine Toll receptors affected locomotion equally. Using over-expression and knockdown conditions, I tested their behaviour via their activity levels over a three-day period.

Drosophila behaviour is a very common tool to investigate nervous system function (Heisenberg, 1997). Circadian clocks regulate numerous rhythmic outputs including eclosion

and locomotion. They do this through the involvement of numerous “clock” genes. These genes function to activate or repress transcription or modify stability, location, or the degradation of proteins (Hall, 2003). Regulation of circadian rhythms are via transcriptional feedback mechanisms that result in the oscillation of numerous gene products. These feedback loops are the Per/Tim loop and the Clk loop (Hardin *et al.,* 1990; Glossop *et al.,* 1999). Importantly different neuronal types are required for the regulation of locomotion due to different environmental conditions (Renn *et al.,* 1999; Park *et al.,* 2000; Stoleru *et al.,* 2004; Grima *et al.,* 2004), and there are in the region of 150 neurons that express “clock” genes dispersed throughout the adult brain (Nitabach and Taghert, 2008).

These clock genes are activated/suppressed by Zeitgebers. Originally coined by Jurgen Aschoff, Zeitgebers are exogenous cues that influence timing of biological clocks. Zeitgebers include light, temperature, exercise, social cues, feeding behaviours and pharmacological manipulations (Rusak, 1981; Moore-Ede *et al.,* 1982; Roenneberg and Foster, 1997; Saunders, 2002; Dunlap *et al.,* 2003; Sharma. 2003). Circadian rhythms rely upon Zeitgebers in order to maintain desired routines. Zeitgeber time is now widely used in the study of circadian rythyms to depict the daily cycles that occur within a 24-hour period.

With the advent of TriKinetics systems, the ability to monitor and quantify the movement of multiple animals at any given time has benefited this area of exploration. Multiple behaviours can be simultaneously observed and measured including but not excluded to circadian rhythms, sleep and activity levels and thus these assays provide a invaluable tool in order to probe gene function (Pfeiffenberger *et al.,* 2010). Throughout this thesis ‘day’ is defined by

the 12 hour period where the incubator lights are switched on, and ‘night or evening’ when they are switched off. Temperature remains constant throughout experiments at 25°C.

I have shown that some of the Toll receptors are located throughout the optic regions and central brain of both larvae and adult flies. Furthermore it has been shown that all Toll receptors are not required in immunity, thus they may have other functions in the CNS. And I have now shown that they are not all equal in their expression. To determine if the Tolls are functionally equivalent I started by looking at the locomotor activity of adult flies.

4.2 RESULTS

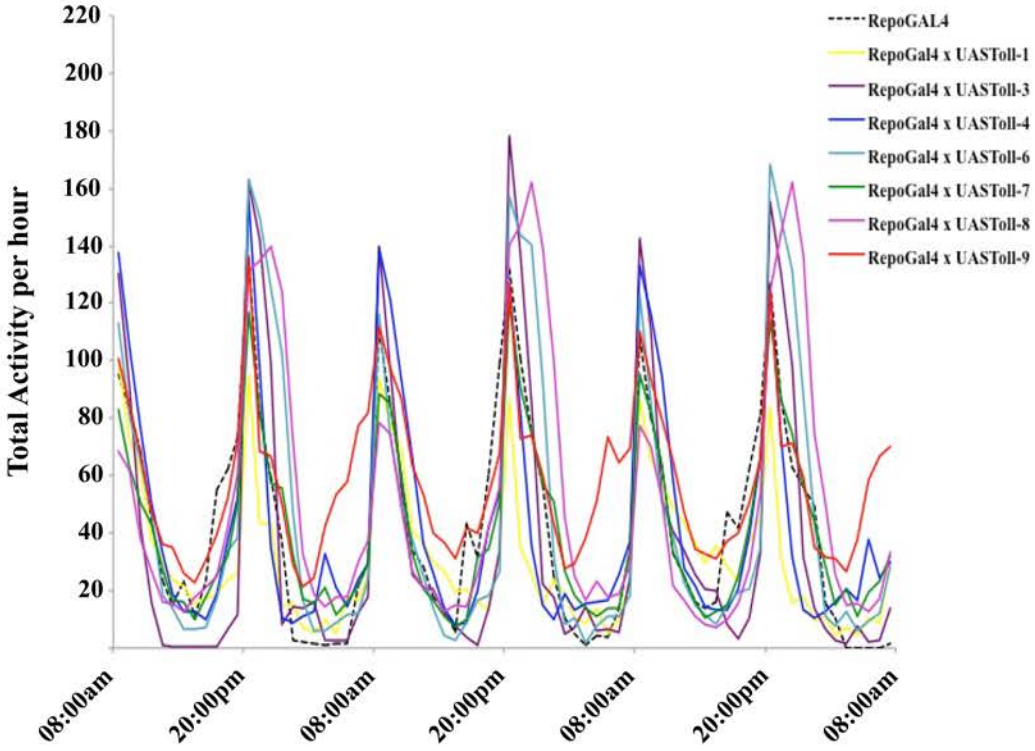
4.2.1 Different Tolls have different consequences in locomotion

Under laboratory light-dark (LD) conditions wild-type flies display a bimodal activity pattern (Dubruille and Emery, 2008) the first activity peak occurs just prior to dawn (morning peak) where there is anticipation of increasing light conditions. The second activity peak occurs just prior to dusk (evening peak) and anticipates lowering light conditions. This bimodal pattern does not solely rely on environmental conditions but is an intrinsic feature of internal clock whereby there is reciprocal synergy between many clock factors including several genes involved in regulatory feedback loops to initiate circadian cycling (Hall, 2003). McIlroy *et al* 2013, showed that both Toll-6 and Toll-7 are distributed throughout regions of locomotor control, and are required in larval locomotion (McIlroy *et al.,* 2013). To test if the nine Tolls had different or equal functions in locomotion, the activity of adult flies that either over-expressed each of the Tolls or had them knocked-down by RNAi, in neurons or glia, was monitored over a three-day period.

4.2.2 Different Tolls in glia have different consequences in locomotion

Over-expression of *Drosophila* Tolls in glia, with the exception of midline glia, using RepoGal4+ caused minor behavioural phenotypes with UAS Toll-3, UAS Toll-6 and UAS Toll-8 displaying hyperactive overshooting in both morning and evening activity peaks (Figure 4.1). Once the evening period had begun, they subsequently remained active for longer periods of time in comparison to the control and other Tolls (Figure 4.1).

Figure 4.1: Over-expression of Drosophila Tolls in RepoGal4+ glia cause behavioural phenotypes in total activity



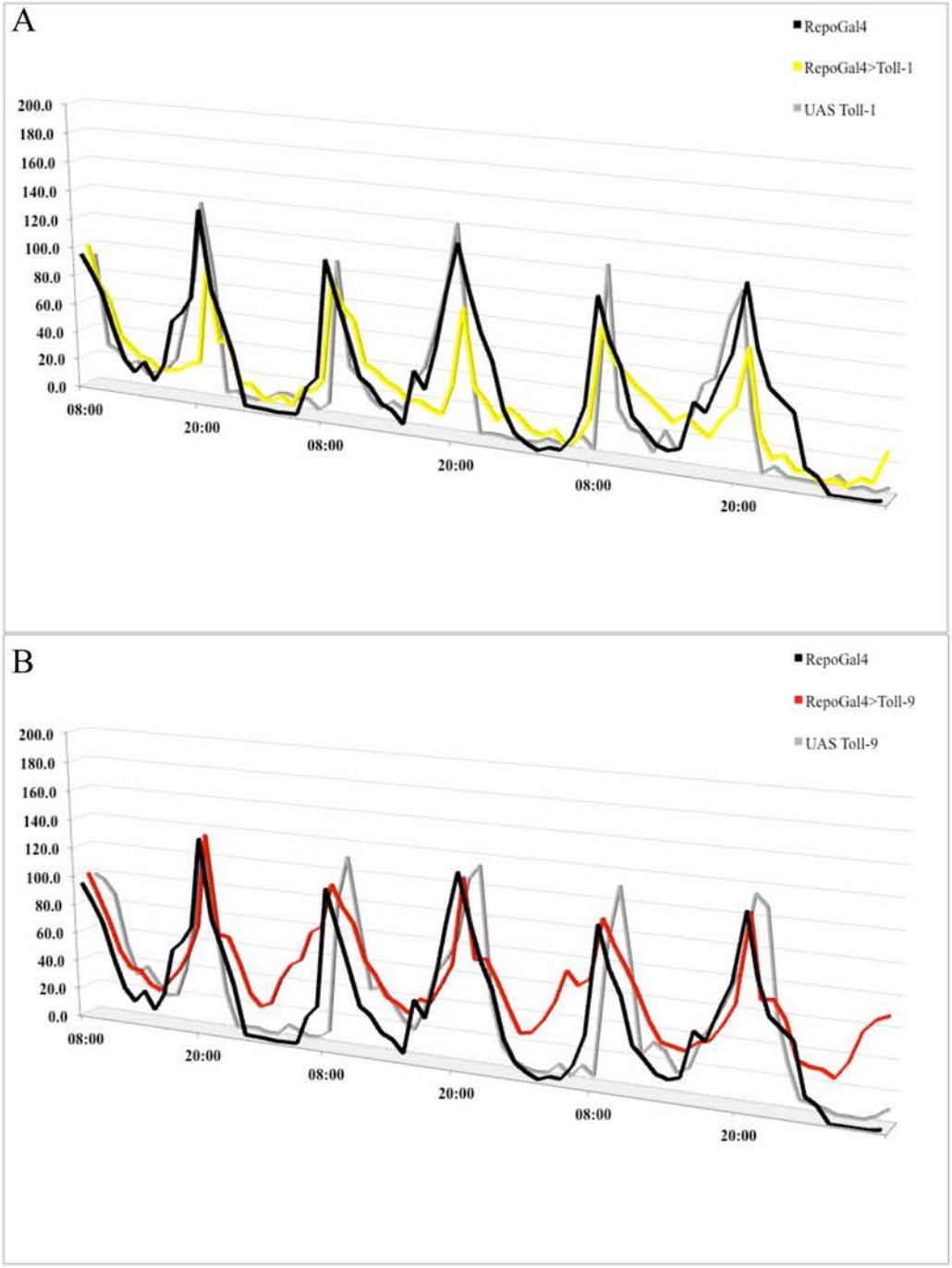
Graph showing the effect of over-expressing Drosophila Tolls in lateral glia cells on total activity per hour.

The most striking results however where seen with the over-expression of Toll-1 and Toll-9. Over-expression of UAS Toll-1 (RepoGAL4>Toll-1) (Figure 4.2A) resulted in relatively normal daytime activity followed by a phase shift during the evening. They fail to anticipate changes in light at the appropriate time; consequently failing to meet the activity levels seen in the control groups. Over-expression of UAS Toll-9 (Figure 4.2B) in glial cells leads to a greater incidence of day and night time activity with a bout of increase anticipatory response just prior to the normal morning peak.

Knockdown of several of the Tolls with RNAi (RepoGAL4>UASToll-RNAi) in glia resulted in disruption of normal waking activity levels (Figure 4.3). Toll-4 and Toll-9 displayed lower activity levels during the day whilst UAS Toll-1, UAS Toll-2 and UAS Toll-8 had increased activity levels during the evening period (Figure 4.3).

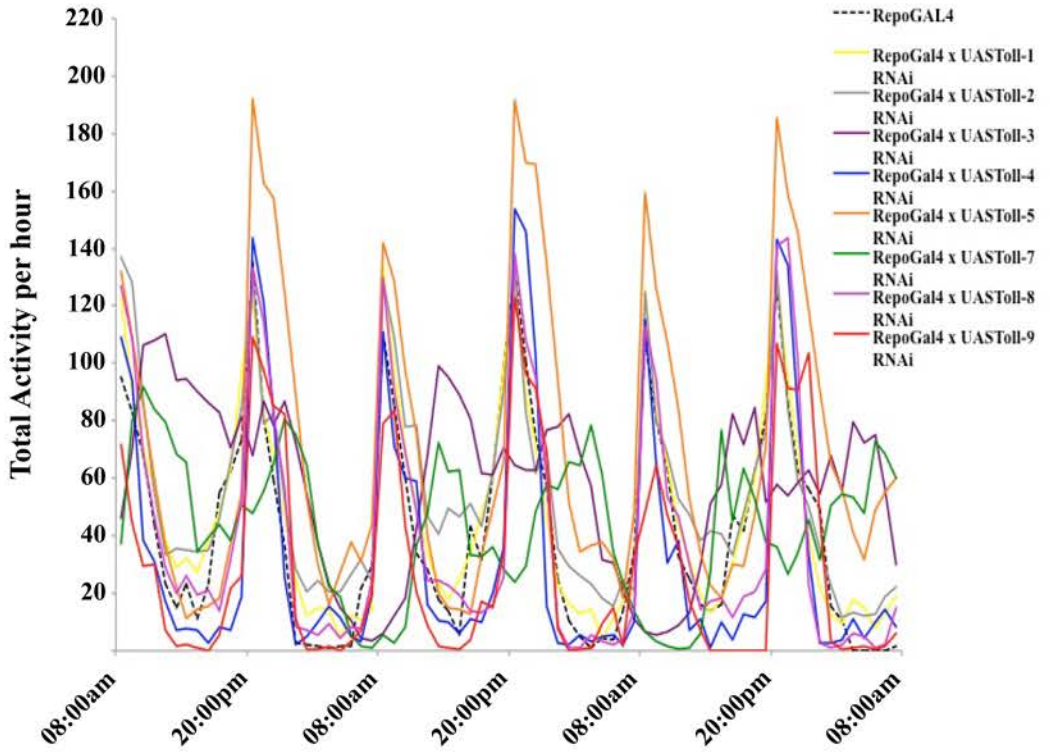
Knocking down Toll-5 in glia (RepoGAL4>UASToll-5RNAi) Figure 4.4A) displayed normal rhythmic patterns during the day. However they have a slower anticipatory response to the changing light conditions prior to dusk, followed by a substantial peak of hyperactivity at the onset of the evening period and greater activity levels throughout most of the evening hours. Contrastingly; knocking down Toll-7 (Figure 4.4 B) leads to visible arrhythmicity during both day and evening periods. Instead of presenting a bimodal activity pattern they display more crepuscular patterns. They are hyperactive later during the day and entering the evening period. This hyperactivity peak is subsequently followed by a crash during the later phase of evening and early morning periods.

Figure 4.2 - Over-expression of Drosophila Toll-1 and Toll-9 in RepoGal4+ glia cause alterations in activity levels



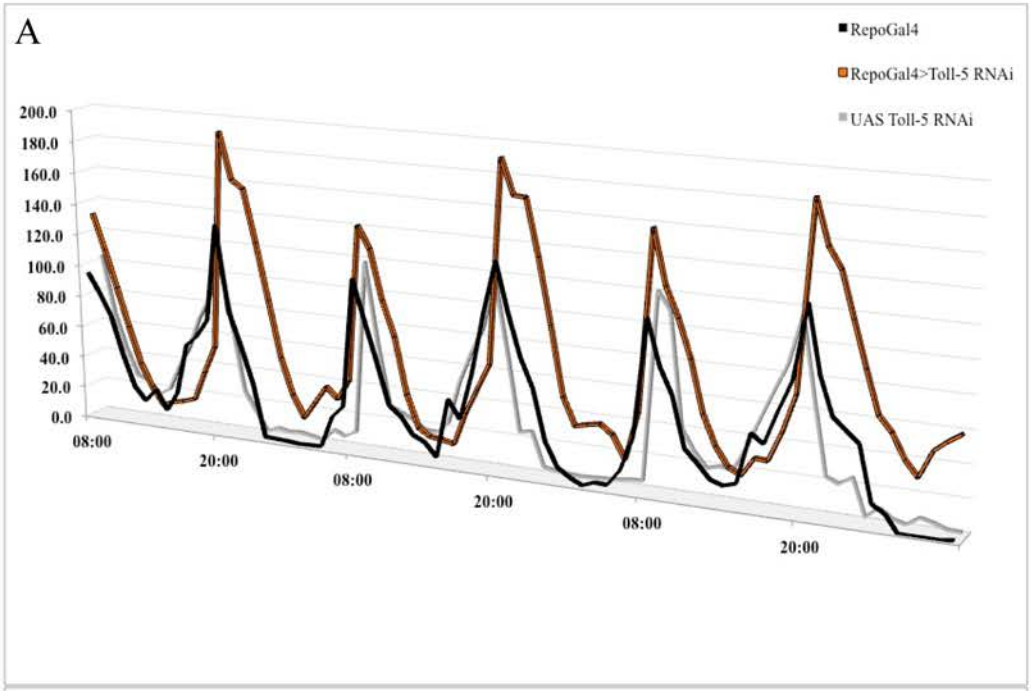
These graphs show the the over-expression of Toll-1 and Toll-9 in RepoGal4+ glia can cause alterations in activity. Panel A shows that the over-expression of Drosophila Toll-1 leads to a delayed evening peak and partial hyperactivity during later day periods. Panel B shows that the over-expression of Toll-9 leads to significantly greater hyperactivity periods during the day and evening periods.

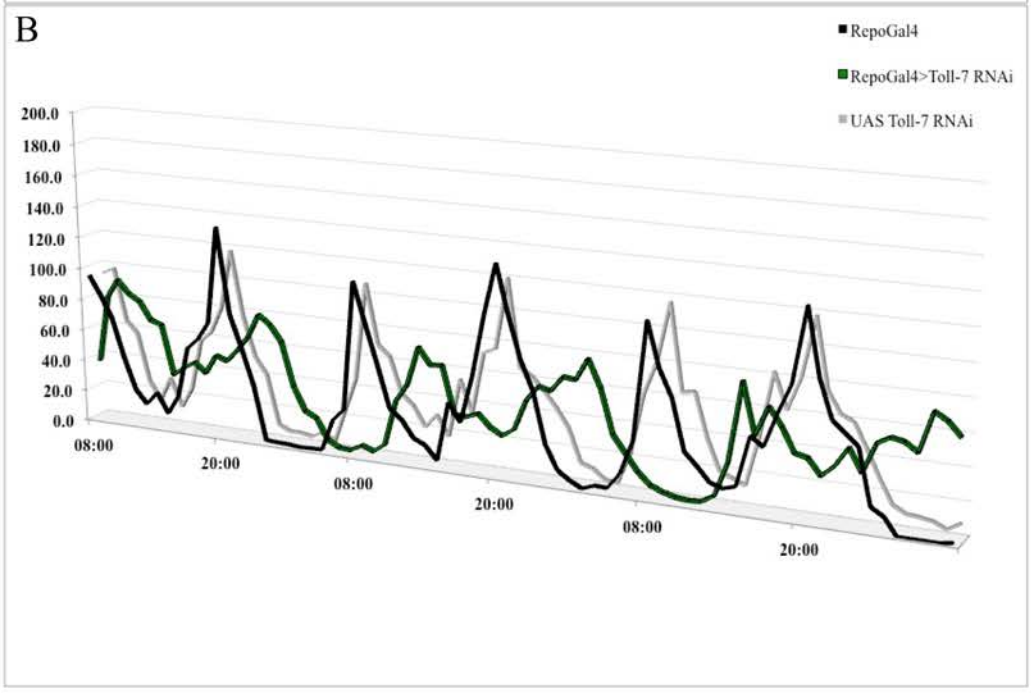
Figure 4.3: Knock-down of Drosophila Tolls in RepoGal4+ glia cause behavioural phenotypes in total activity



Graph showing the effect of knocking down Drosophila Tolls in lateral glia cells on total activity per hour.

Figure 4.4 - Knock-down of Drosophila Toll-5 and Toll-7 in RepoGal4+ glia cause alterations in activity levels





These graphs show that the loss of function of Drosophila Toll-5 and Toll-7 through RNAi knockdown lead to activity behavioural phenotypes. Panel A shows that knocking down Toll-5 leads to normal rythmic day periods followed by a peak of hyperactivity at the onset of the evening peak. Panel B shows that knocking down Toll-7 leads to arrhythmicity during both day and evening periods.

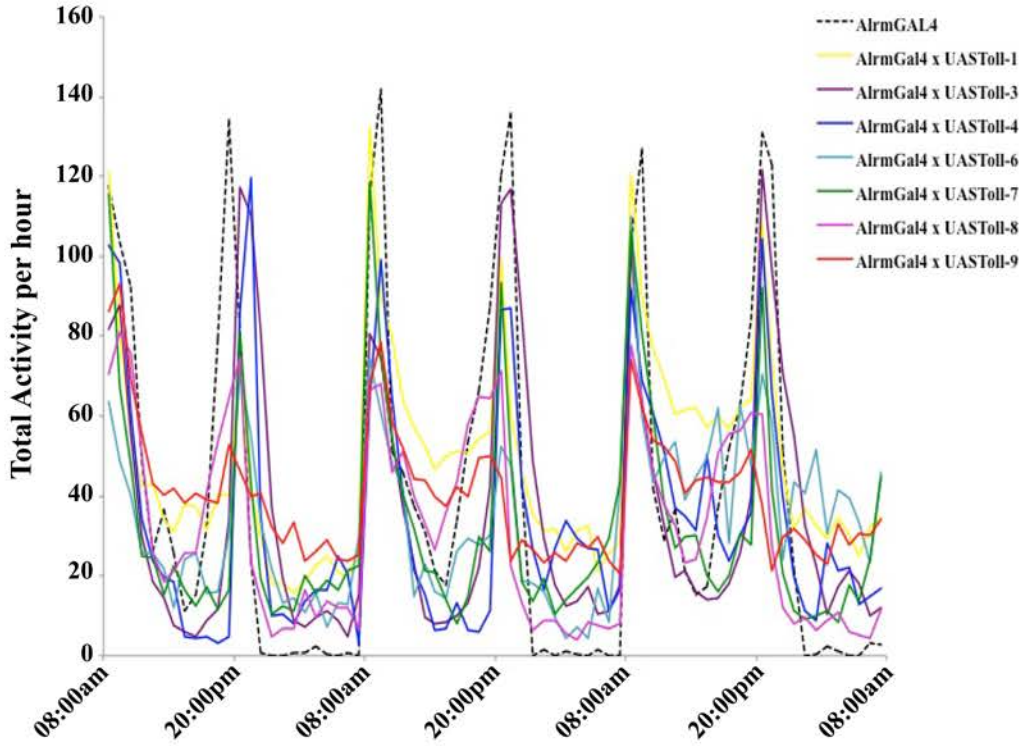
4.2.3 Different Tolls in neuropile glia have different consequences in locomotion

Since RepoGAL4 drives expression in all glia except midline glia, which includes glia forming the blood-brain barrier and enwrapping the CNS, I wondered whether the Tolls may have distinct functions in the neuropile glia, which are the glial class in contact with axons and synapses. Over-expression of the Tolls in neuropile glia with AlrmGal4 caused behavioural phenotypes in Activity (Figure 4.5): all of the Tolls were more active at night and their behaviour was more variable during the day. UAS Toll1, UAS Toll-6, UAS Toll-8 and UAS Toll-9 appear more active during the day whereas UAS Toll-3, UAS Toll-4 and UAS Toll-7 have lower activity levels (Figure 4.5).

Over-expression of UAS Toll-1 and UAS Toll-9 in neuropile glia results in arrhythmic activity levels (Figure 4.6 A & 4.6 B). During both the day and evening periods over-expression of both leads to higher levels of activity compared to the controls. UAS Toll-1 (Figure 4.6 A) are unable to anticipate the onset of dusk and fail to have a peak of activity entering the evening period, however; they return to control levels at the onset of dawn and have an increase in activity levels similar to the controls. A similar profile was found when UAS Toll-1 was over-expressed using RepoGal+ (Figure 4.2 A). AlrmGAL4>UAS Toll-9 (Figure 4.6 B) flies fail to anticipate lowering light levels during the dusk period altogether and only partially recover this anticipatory response at the onset of dawn (Figure 4.6 B).

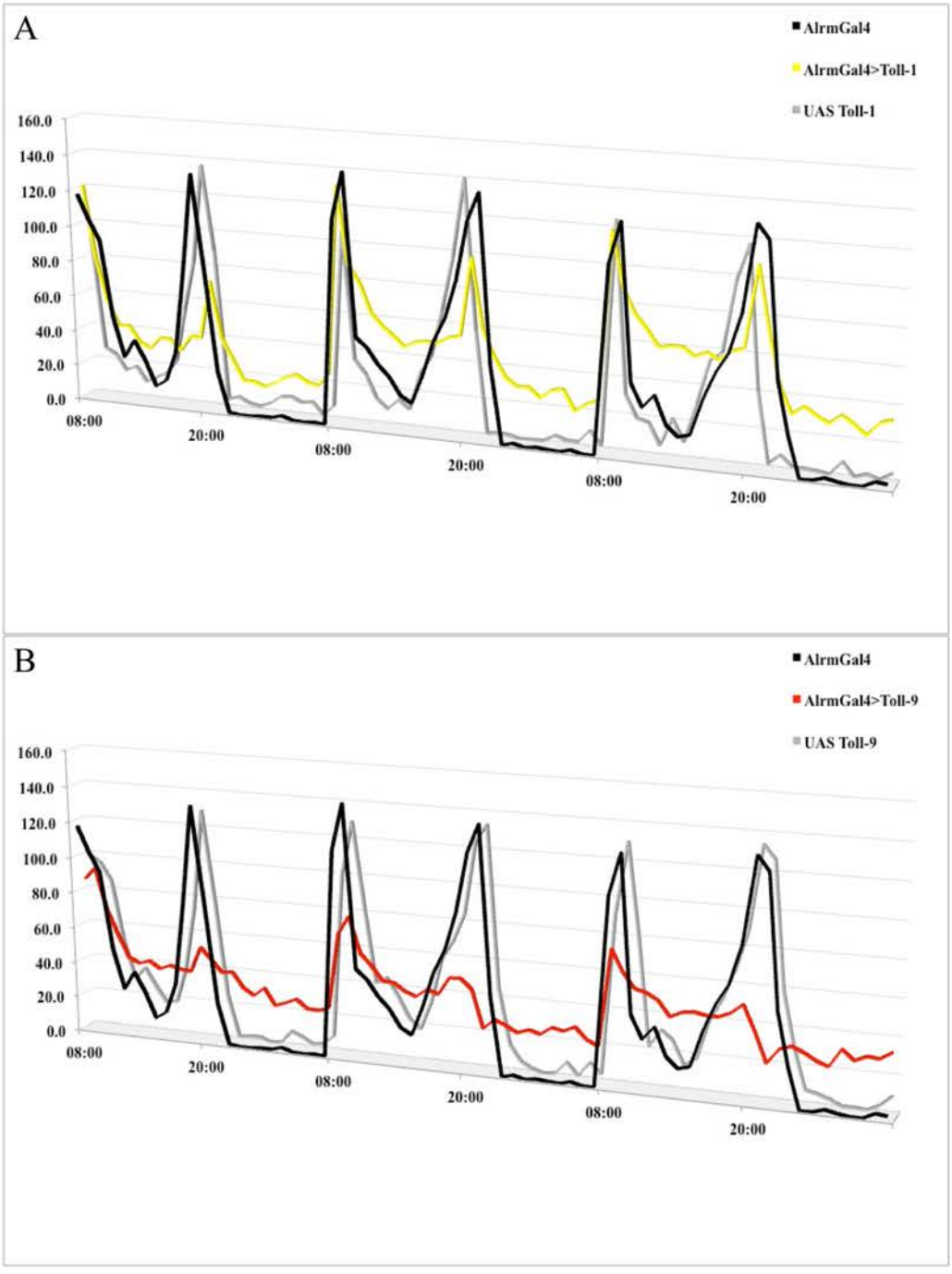
RNAi knockdown for all Tolls in neuropile glia with alrmGAL4 also resulted in variability of activity over the time course with all of the Tolls (Figure 4.7). Some of the Tolls show a

Figure 4.5: Over-expressing Drosophila Tolls in AlarmGal4+ glia cause behavioural phenotypes in total activity



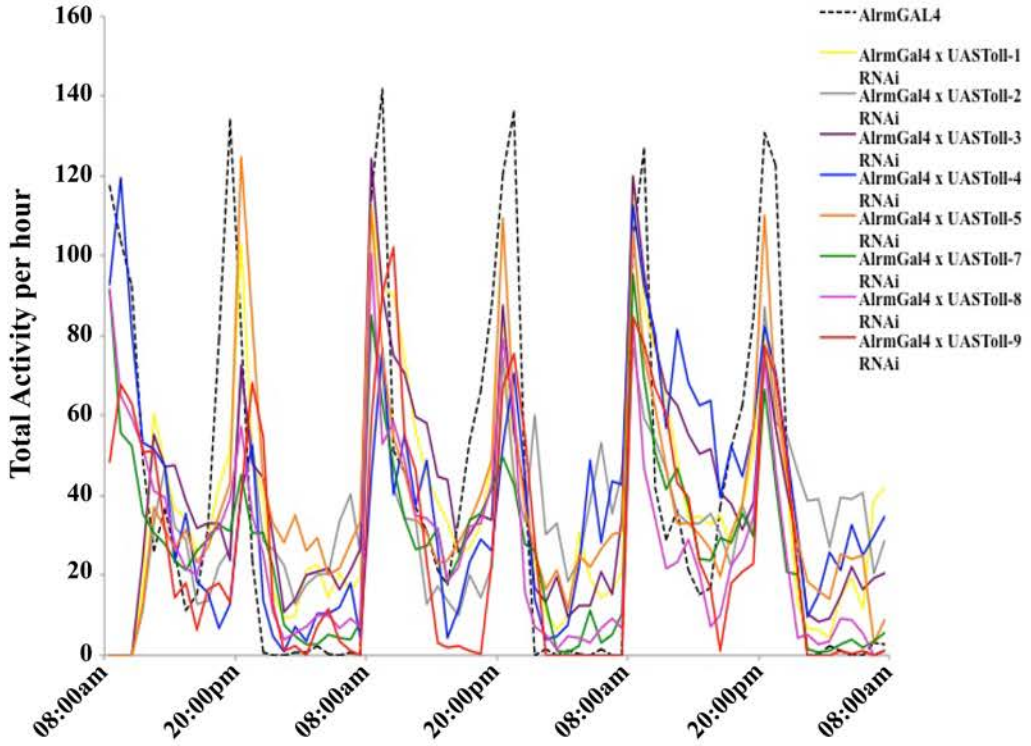
Graph showing the effect of over-expressing Drosophila Tolls in neuropile glia on total activity per hour.

Figure 4.6 - Over-expression of Drosophila Toll-1 and Toll-9 in AlrmGal4+ glia cause alterations in activity levels



These graphs showing that the over-expression of Drosophila Toll-1 and Toll-9 in neuropile glia lead to alterations in activity phenotypes. In panel A the over-expression of Toll-1 leads to increased day and evening activity and they fail to have significant evening peaks in comparison to the controls. Panel B shows that by over-expressing Toll-9 there is complete arrythmycity.

Figure 4.7: Knocking down Drosophila Tolls in AlarmGal4+ glia cause behavioural phenotypes in total activity



Graph showing the effect of knocking down Drosophila Tolls in neuropile glia on total activity per hour.

phenotype that is un-reproducible over the time course. Therefore I am unable to draw any conclusions from this data set.

4.2.4 Toll-8 in neurons has different consequences to the other Toll receptors in locomotion

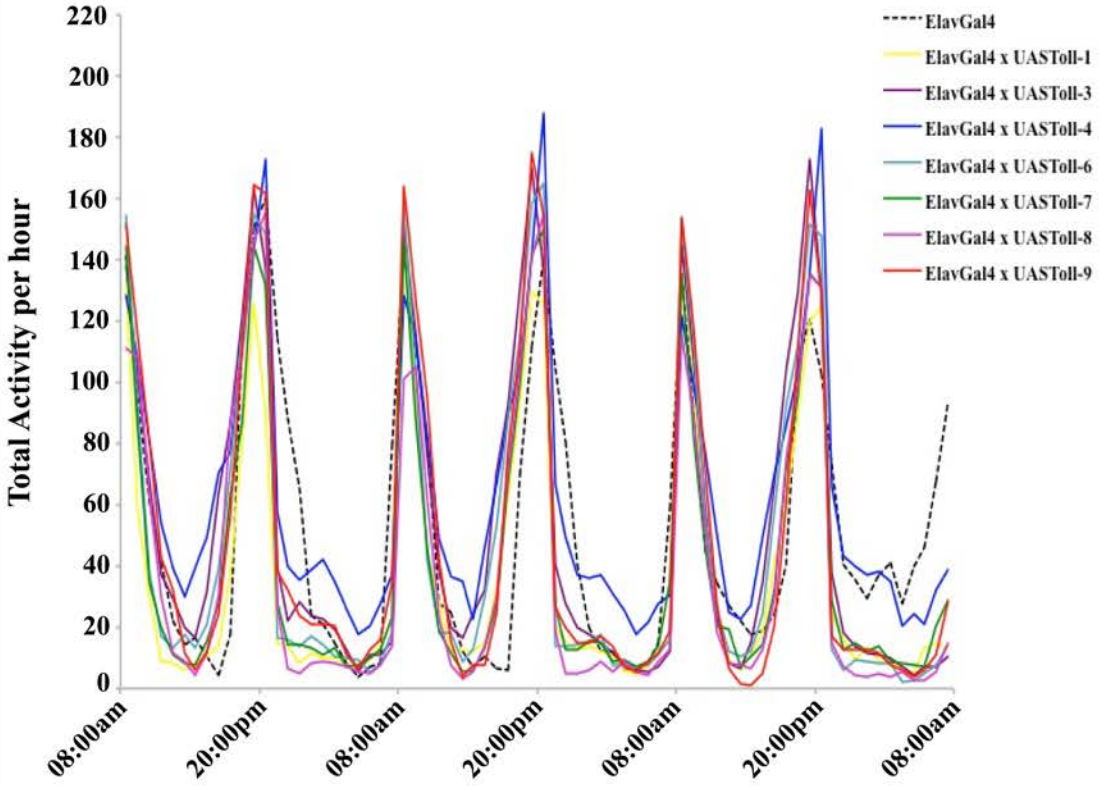
All Overexpression of *Drosophila* Toll receptors in ElavGal4+ (all subsets of neurons) did not cause behavioural phenotypes in activity (Figure 4.8). All of the distributions are very similar to the control, with any differences being too subtle and too variable. Therefore I do not see any phenotype with this assay.

Knockdown of Tolls in neurons (ElavGAL4>Toll-RNAi) caused activity phenotypes (Figure 4.9), whereby the majority of the Tolls caused a mild effect at different periods of the night and/or day periods. The most consistent of the Tolls was knocking down Toll-8 (ElavGAL4>UASToll-8RNAi), as there was a dramatic increase in daytime activity (Figure 4.10).

4.2.5 Different Tolls in ellipsoid body neurons do not cause locomotion phenotypes

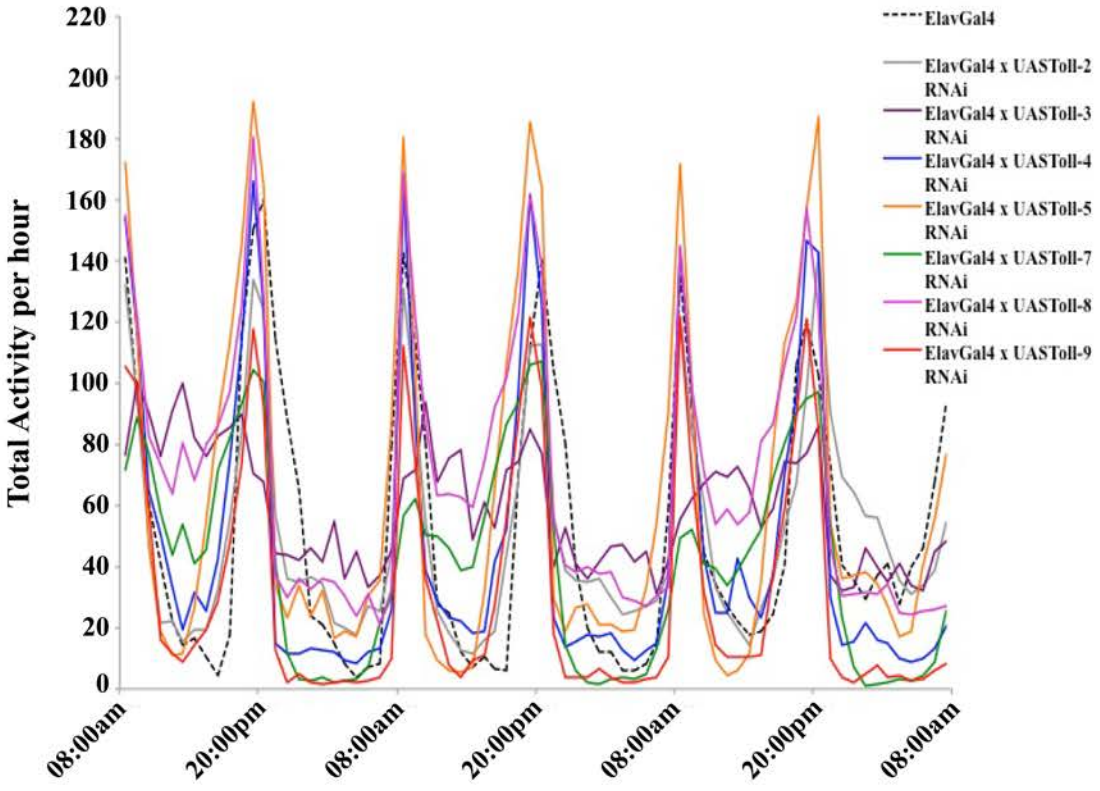
Over-expression and knockdown of *Drosophila* Toll receptors in c232Gal4+ (ellipsoid body neurons) do not cause behavioural phenotypes in Activity (Figures 4.11 and 4.12). The majority of the Tolls display control levels of activity. Any mild effect in activity was not reproducible over the full three days and therefore were not analysed further.

Figure 4.8: Over-expression of Drosophila Tolls in ElavGal4+ neurons cause behavioural phenotypes in total activity



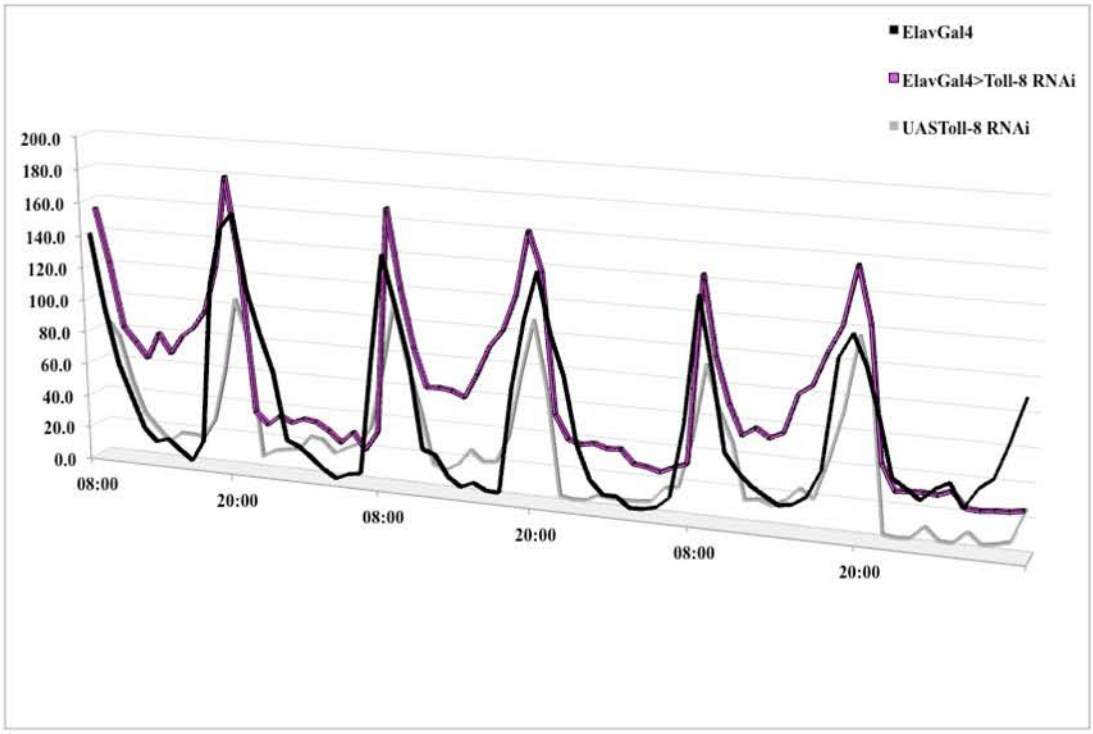
Graph showing there is no effect of over-expressing Drosophila Tolls in neurons on total activity per hour.

Figure 4.9: Knock-down of Drosophila Tolls in ElavGal4+ neurons cause behavioural phenotypes in total activity



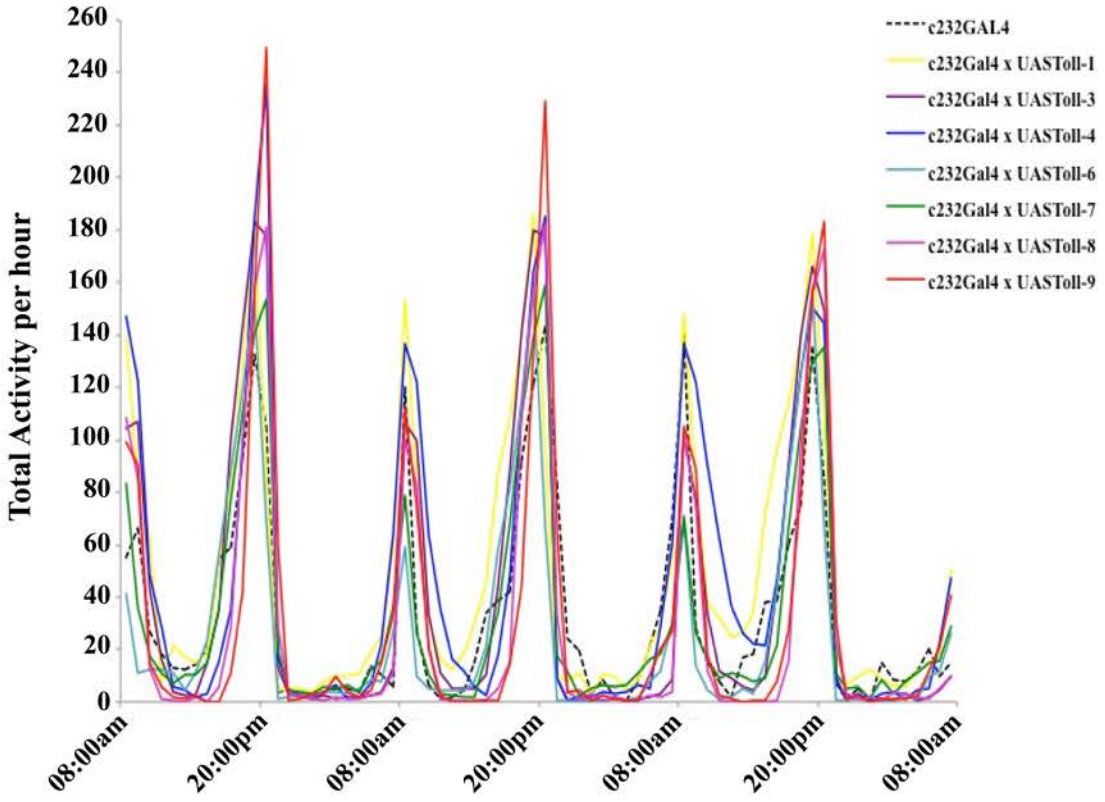
Graph showing the effect of knocking down Drosophila Tolls in neurons on total activity per hour.

Figure 4.10 - Knock-down of Drosophila Toll-8 in ElavGal4+ neurons cause alterations in activity levels



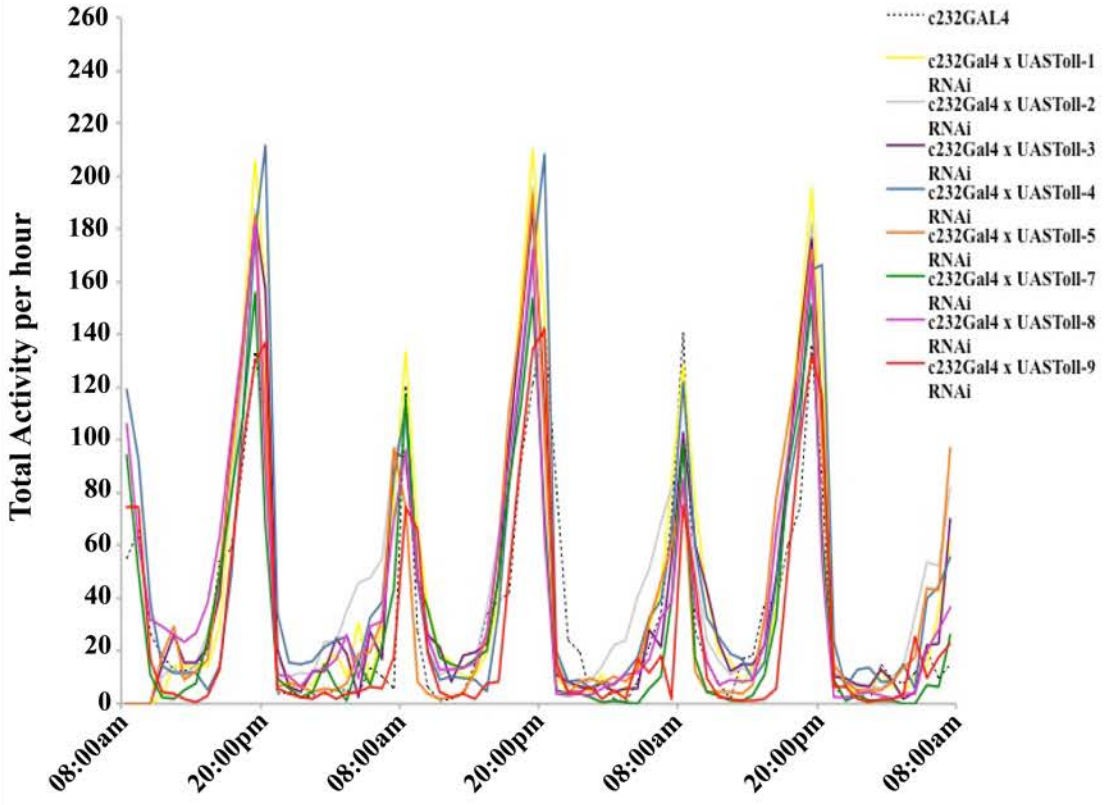
Graphs showing that the loss of function of Drosophila Toll-8 (ElavGal4>Toll-8 RNAi) leads to increased day and night activity with bursts of hyperactivity at the peak of these times.

Figure 4.11: Over-expression of Drosophila Tolls in c232Gal4+ neurons cause behavioural phenotypes in total activity



Graph showing the effect of over-expressing Drosophila Tolls in ellipsoid body neurons on total activity per hour.

Figure 4.12: Knocking down of Drosophila Tolls in c232Gal4+ neurons



Graph showing the effect of knocking down Drosophila Tolls in ellipsoid body neurons on total activity per hour.

4.3 SUMMARY

In this chapter, through the use of an adult locomotion assay I have shown (Table 4.1):

1) Over-expression of Toll-1 and Toll-9 particularly in neuropile glia can influence activity
2) Knocking-down Toll-5 and Toll-7 in glia (except midline and neuropile glia) indicates that both Toll-5 and Toll-7 are required for locomotion phenotypes
3) Toll-8 is required in neurons for locomotion

When over-expressed in both RepoGal4+ (glia, except midline glia) and AlrmGal4+ (neuropile glia) both Toll-1 and Toll-9 can influence locomotion phenotypes. This is consistent with data from chapter 3, whereby Toll-1 and Toll-9 group together (along with other Tolls) in glial cells. It is interesting to note that the locomotion phenotypes are more severe when over-expressed in neuropile glia. RepoGal4>UASToll-1 flies display a lower night-time peak, due to failing to anticipate lights off. These flies then return to wild type behaviours. However AlrmGal4>UASToll-1 flies also fail to anticipate lights off. Following which they remain more active throughout the rest of the evening and the following day. RepoGal4>UASToll-9 flies anticipate lights off and have a normal evening peak but remain more active during the evening. Whereas, when over-expressed in neuropile glia Toll-9 flies no longer have an evening peak, remaining arrhythmic during both the day and particularly during the evening periods.

Neuropile glia are found in association with axons and synapses. They extend along outer surfaces to ensheath axon bundles in order to isolate and protect neurons. Or they can extend

Table 4.1: Table of results from Chapter 4

	CHAPTER 4							
	Locomotion							
	RepoGal4> OE	RepoGal4> KD	AlrmGal4> OE	AlrmGal4> KD	ElavGal4> OE	ElavGal4> KD	c232Gal4> OE	c232Gal4> KD
	Adult flies	Adult flies	Adult flies	Adult flies	Adult flies	Adult flies	Adult flies	Adult flies
Toll-1	Y	N	Y	N	N	N	N	N
Toll-2	N	N	N	N	N	N	N	N
Toll-3	N	N	N	N	N	N	N	N
Toll-4	N	N	N	N	N	N	N	N
Toll-5	N	Y	N	N	N	N	N	N
Toll-6	N	N	N	N	N	N	N	N
Toll-7	N	Y	N	N	N	N	N	N
Toll-8	N	N	N	N	N	Y	N	N
Toll-9	Y	N	Y	N	N	N	N	N

OE	Represents the over-expression of UAS Toll 1-9
KD	Represents the knock down of UAS Toll 1-9 RNAi

RepoGal4	Expression in glial cells (except midline glia)
AlrmGal4	Expression in neuropile glial cells
ElavGal4	Expression in neurons
c232Gal4	Expression in ellipsoid body neurons

into the neuropile to function in the modulation of neuronal connections (Awasaki *et al.,* 2008). It is therefore likely that ectopic expression of both Toll-1 and Toll-9 is somehow involved in the modulation of locomotion activities. This may be dependent upon the proximity of these neuropile glia expressing these two receptors to the neurons that control locomotor function.

Contrastingly, knocking down Toll-5 and Toll-7 in RepoGal4+ glial cells resulted in altered locomotion. This indicates that these genes are normally required in glia for normal function of this behaviour. I previously showed that Toll-5 and Toll-7 group together and are expressed in glial cells during embryonic development. However, as this locomotion alteration was not seen when knocking down Toll-5 and Toll-7 in neuropile glia, they are likely to be required in a different subset of glial cells. McIlroy *et al.,* has previously reported that Toll-6 and Toll-7 are required in the locomotion of larvae. Double mutants of both Toll-6 (Toll-6^{26} /Toll-6^{31}) and Toll-7 (Toll-7^{P8}/Toll-7^{P114}) crawl much slower than control animals. Furthermore both of these mutants result in deficient targeting and axonal misrouting in embryos (McIlroy *et al.,* 2013). Therefore it is interesting that whilst Toll-7 retains behavioural abnormalities from embryo through larvae and into adult, that Toll-6 does not.

Toll-8 is the only Toll receptor that is required in neurons for locomotion. It is interesting that this result was not replicated when knocking down Toll-8 in the ellipsoid body. The ellipsoid body is one of the four synaptic neuropile domains of the adult brain. The other three regions include the fan shaped body, paired nodulli and Protocerebral Bridge. In chapter 3 I showed that Toll-8^{MD806} is highly expressed within the fan shaped body. This central complex is involved in locomtor control with both the fan shaped body and ellipsoid body are known to be involved in visual pattern memory (Liu *et al.,* 2006; Pan *et al.,* 2008; Wang *et al.,* 2009). It

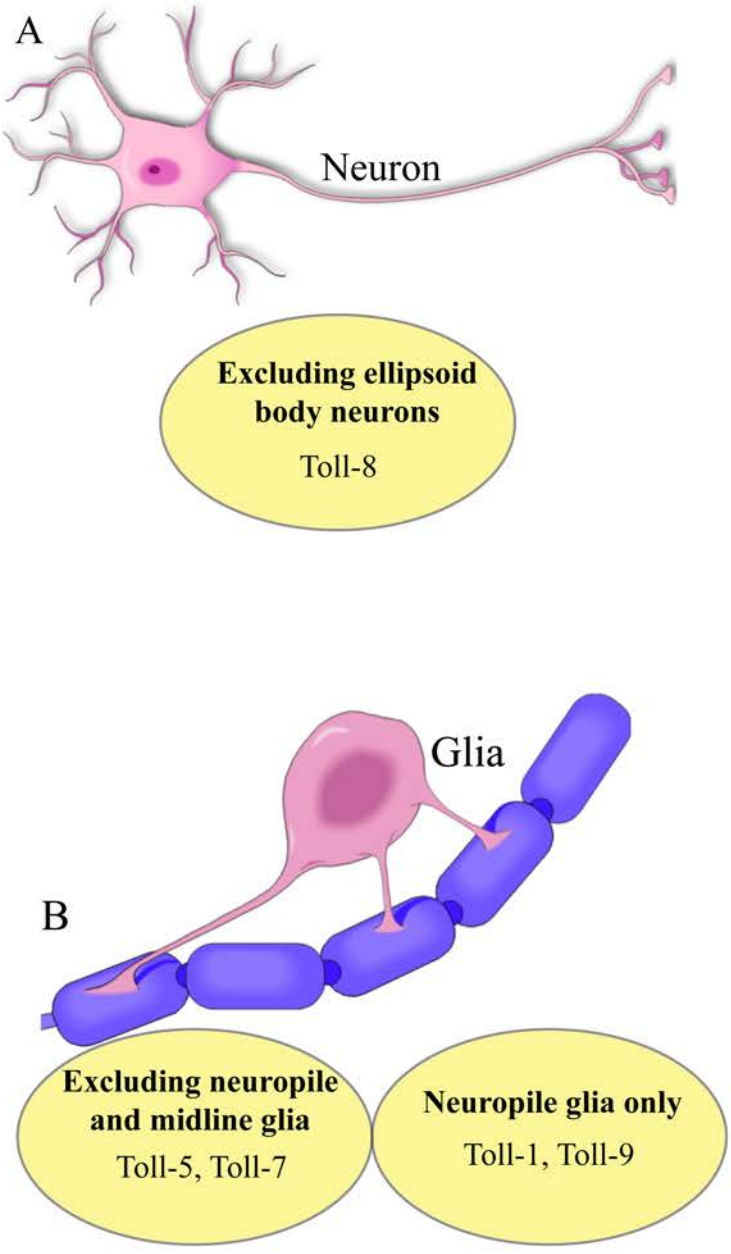
would be interesting to use other more neuronal specific Gal4 lines to see if knocking down Toll-8 in a certain subset of neurons impacts locomotion. Furthermore it would also be interesting to see if Toll-8 is expressed in any neurons in which clock factors such as PDF are also expressed.

Overall these results show that there are functional groupings within the Toll receptor family (Figure 4.13). Toll-1 and Toll-9 can influence activity in neuropile glia. Toll-5 and Toll-7 are required by glia, but not neuropile or midline glia. Comparing these results to the data obtained by RT-PCR, Toll-1, Toll-5, Toll-7 and Toll-9 group together as being expressed highly in glial cells. Conversely, Toll-8 is the only Toll receptor required by neurons locomotion.

These conclusions have been based on the results that are reproducible over the entire time course. For instance genotypes, which display a phenotype appearing to be arrhythmic, but which were not consistent over the full time course were not included. This way I have eliminated any phenotype that may be due to other factors, including ageing or death of the flies. Subsequently only those results that are convincing have been analysed further. It would therefore be beneficial to re-test genotypes that were not included as they may provide important information on the genes in question and their locomotion functions.

Furthermore, TriKinetics assays allow the evaluation of continuous recordings of multiple flies across multiple behavioural phenotypes. This high-throughput assay can monitor activity levels, sleep patterns and circadian rhythms (Pfeiffenberger *et al*., 2010). The work completed here, which were to asses activity levels over a three day period in LD conditions, could be

Figure 4.13: Possible grouping of Toll receptors in neurons or glia



These diagrams depict the proposed groupings of the Toll receptors in different cell types. Panel A highlights Toll-8 function within neurons. Panel B highlights that Toll-5 and Toll-7 function within glial cells (with the exception of both neuropile and midline glia) whereas Toll-1 and Toll-9 function within neuropile glia. These proposed groupings have been proposed from their behavioural phenotypes detailed in chapter 4.

expanded upon to provide more data on both sleep and circadian rhythms. Assays based on LD conditions provide qualitative information regarding free running conditions including bouts of daily activity. Thus allowing the assessment of phase changes from wild type conditions. In the future it would be beneficial to test those flies with a phenotype following a LD entrainment period by a constant darkness condition (DD) (LD DD). This would provide insight regarding internal clock state and the ability of these clocks to drive rhythmic outputs.

In the next chapter I will continue to use phenotypic assays in order to investigate if there is any functional differences within the CNS between the Toll receptors. I will ask if all Toll receptors affect the maintenance of cell number within the CNS. I will test these behaviours through a variety of measures. By over-expressing and knocking down the Toll receptors I will measure CNS area and VNC size. I will then test their ability to promote neuronal survival by over-expressing the Tolls in neurons and quantifying the number of neurons using anti-Eve staining. Subsequently I will then test the Tolls ability to promote neuronal death by over-expressing and knocking down the Tolls in neurons and quantifying the number of dying cells using anti-Dcp1 staining.

CHAPTER 5

TOLL RECEPTORS HAVE DISTINCT FUNCTIONS IN THE MAINTENANCE OF LARVAL CNS SIZE AND IN THE REGULATION OF CELL NUMBER

5.1 INTRODUCTION

In the previous chapter I showed that the Tolls function in different cell types in order to influence activity levels. Toll-1 and Toll-9 function in neuropile glia, Toll-5 and Toll-7 in glia (but not including neuropile or midline) and Toll-8 in neurons. Consistent with the findings in chapter 3, it is apparent that all of the Toll receptors are not equal in function.

The size and shape of the *Drosophila* nervous system grows in a dynamic manner throughout the life of a fly in order to adjust to the developing nervous system. Remodelling depends on both internal and external factors and requires large amounts of cell number regulation in order to generate a fully functional nervous system. During development at the end of embryogenesis the VNC undergoes co-ordinated reduction in size (Poulson, 1965). During VNC condensation two pivotal processes are taking place at the same time. The first is extracellular matrix (ECM) deposition and the second is neural activity, both of which are

required for condensation (Olofsson and Page 2005). VNC condensation is impaired if there is a lack of haemocyte migration, as this prevents ECM components being deposited. Furthermore, VNC condensation is impaired if there is disruption of Rac1 function in neurons and glia which leads to incorrect cytoskeleton re-arrangement (Olofsson and Page 2005). When Rac1 is mutant in peripheral and longitudinal glia cellular extension, glial migration and axon ensheathment are disrupted and thus do not generate the required condensation force. In neurons, when Rac1 is mutant, there is axonal outgrowth defects (Olofsson and Page 2005). These defects prevent correct neurite extension and prevent the required anteroposterior condensing force (Luo *et al.*, 1994).

In conjunction with correct ECM deposition and neural activity, there is also another process that is pivotal in VNC condensation. During embryogenesis the CNS and epidermis originate from a common ectodermal layer. Proneural and neurogenic signalling mechanisms determine if cells are driven to neural or epidermal fates (Urbach and Technau, 2004). As VNC condensation occurs programmed cell death ensures correct separation of these two tissues (Page and Olofsson, 2008). It is a combination of all three processes that ensure the VNC is condensed by at least 25% prior to larval development (Olofsson and Page 2005; Page and Olofsson, 2008).

The aim of this chapter was to determine if there are functional differences between the Toll receptors in the CNS in the regulation of maintenance of CNS size, and during the regulation of cell number. I used phenotypic assays to ask these questions. Firstly, I asked whether gain or loss of function for each of the Tolls affects the size of the larval central nervous system.

Secondly, I tested their involvement in the maintenance in cell number regulation and death. I asked if all Toll receptors affect neuronal number equally and do they all promote apoptosis.

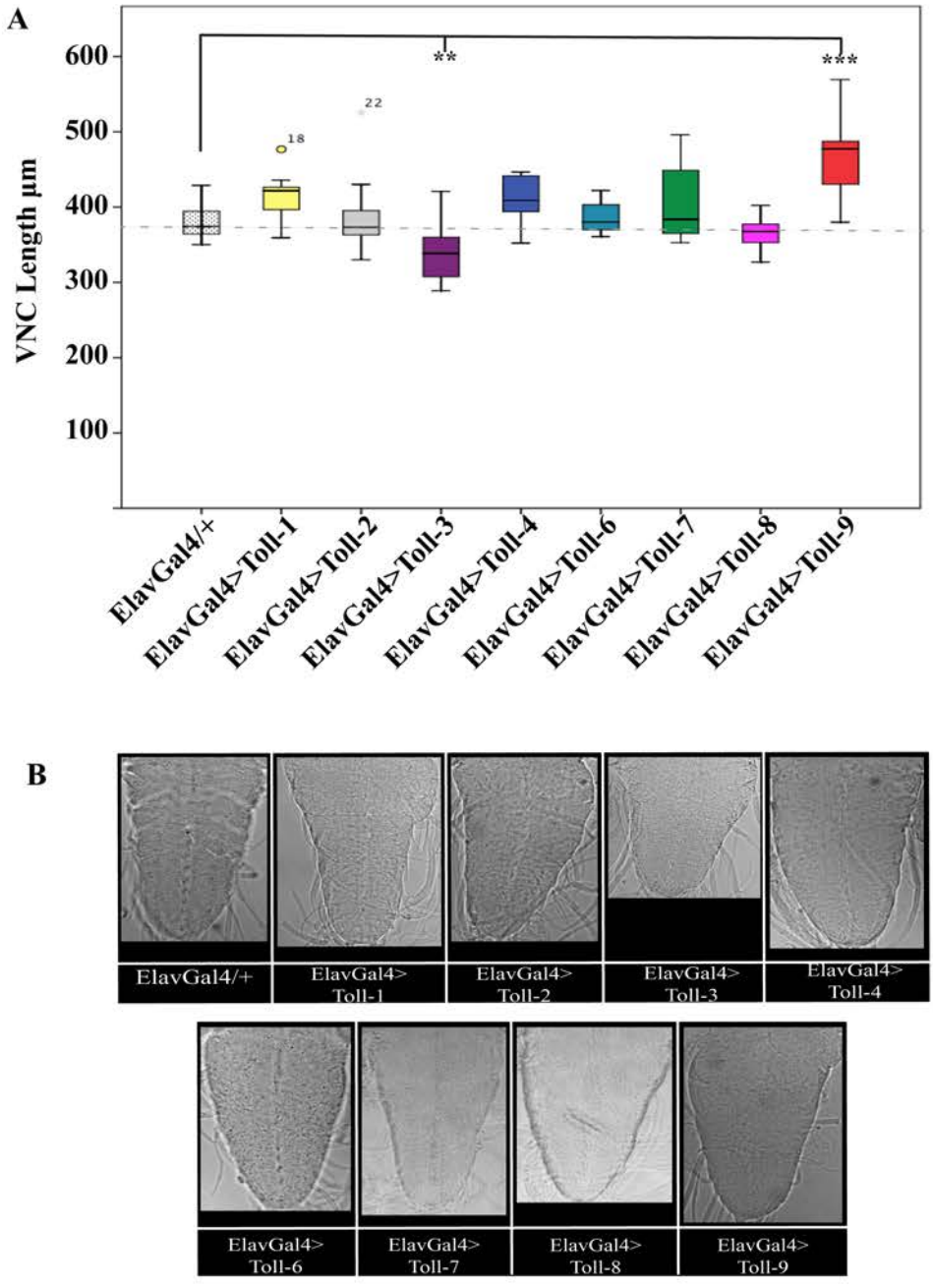
5.2 RESULTS

5.2.1 Regulation of VNC Length and CNS Area by Toll receptors in neurons

As another means to ask the question of whether the nine Tolls have equivalent functions in the CNS or not, I used the larval VNC as a context to test the effects of over-expressing or knocking down the Tolls in both neurons and glial cells. The total CNS area and VNC length were measured.

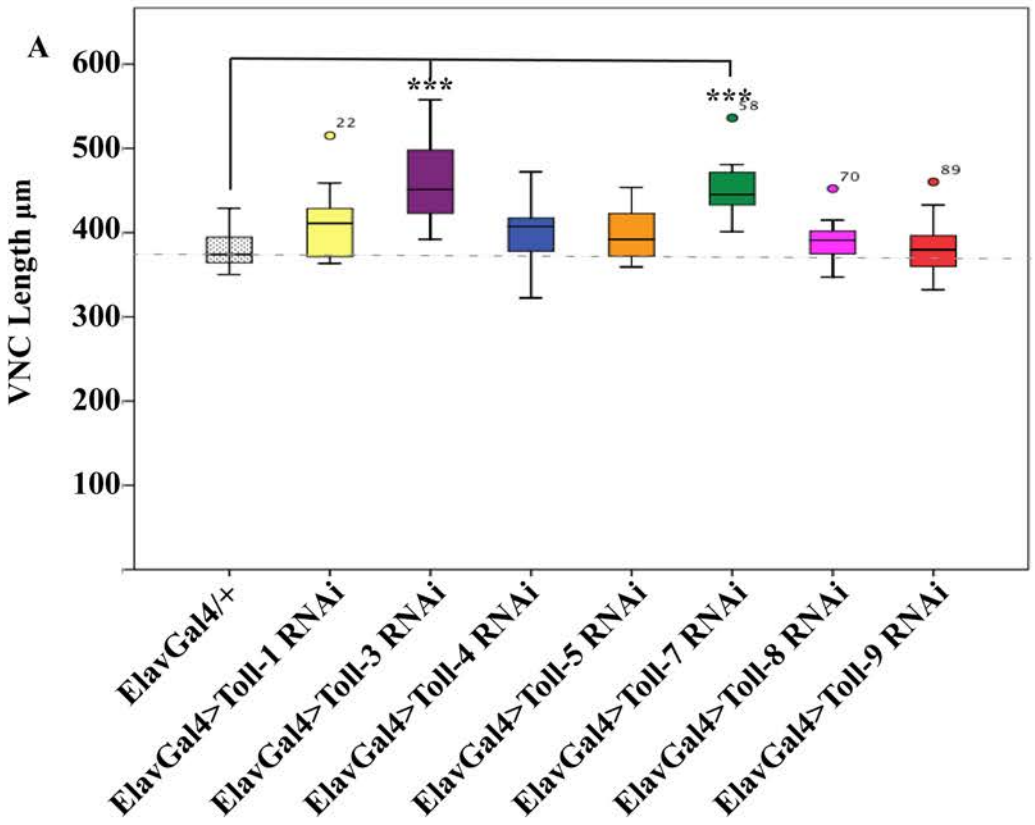
Over-expression of Toll-3 in neurons (ElavGAL4>UASToll-3) led to a reduced VNC length in comparison to controls (Figure 5.1). Toll-3 knock-down (ElavGAL4>UASToll-3RNAi) had the opposite effect causing an increase in VNC length in comparison to the control (Figure 5.2). The over-expression of Toll-9 in neurons (Elavgal4>UASToll-9) resulted in an increase in VNC length in comparison to controls (Figure 5.1). However when knocked-down (ElavGal4>UASToll-9RNAi) there was no phenotype, and VNC length was the same as the control (Figure 5.2). This was similar to the knockdown of Toll-7 in neurons (ElavGal4>UASToll-7RNAi) that resulted in an increase in VNC length (Figure 5.2). However when Toll-7 was over-expressed (ElavGal4>UASToll-7) there was no phenotype (Figure 5.1). Overall the most interesting candidate from these comparisons was Toll-3 (Figure 5.3). The over-expression and knockdown of Toll-3 resulted in opposite effect on VNC length.

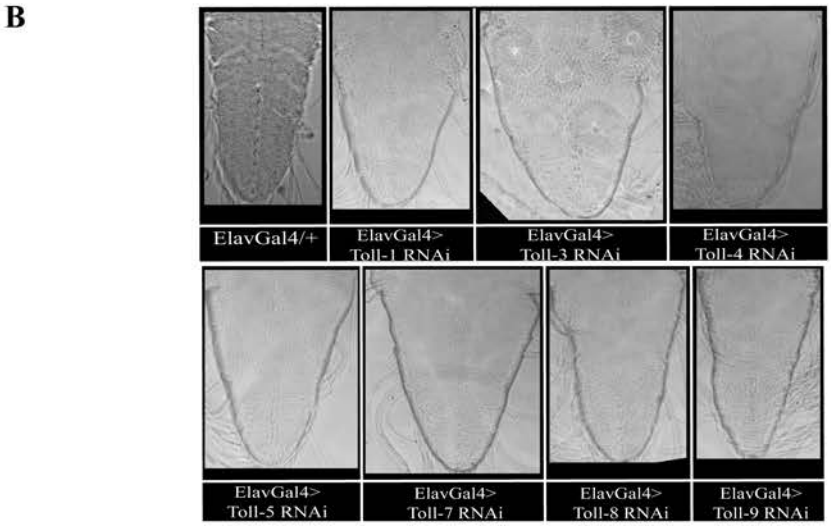
Figure 5.1: Ventral nerve cord length is regulated by the over-expression of *Drosophila* Toll receptors in neurons



Ventral nerve cord length is regulated via the over-expression of Toll receptors in neurons. The graph in panel A shows that ventral nerve cord length is regulated by the over-expression of Toll-3 and Toll-9 in neurons One Way ANOVA ($F(8,99)=10.293$, $p=0.000$). Panel B shows representative images of the over-expression of different Toll receptors.

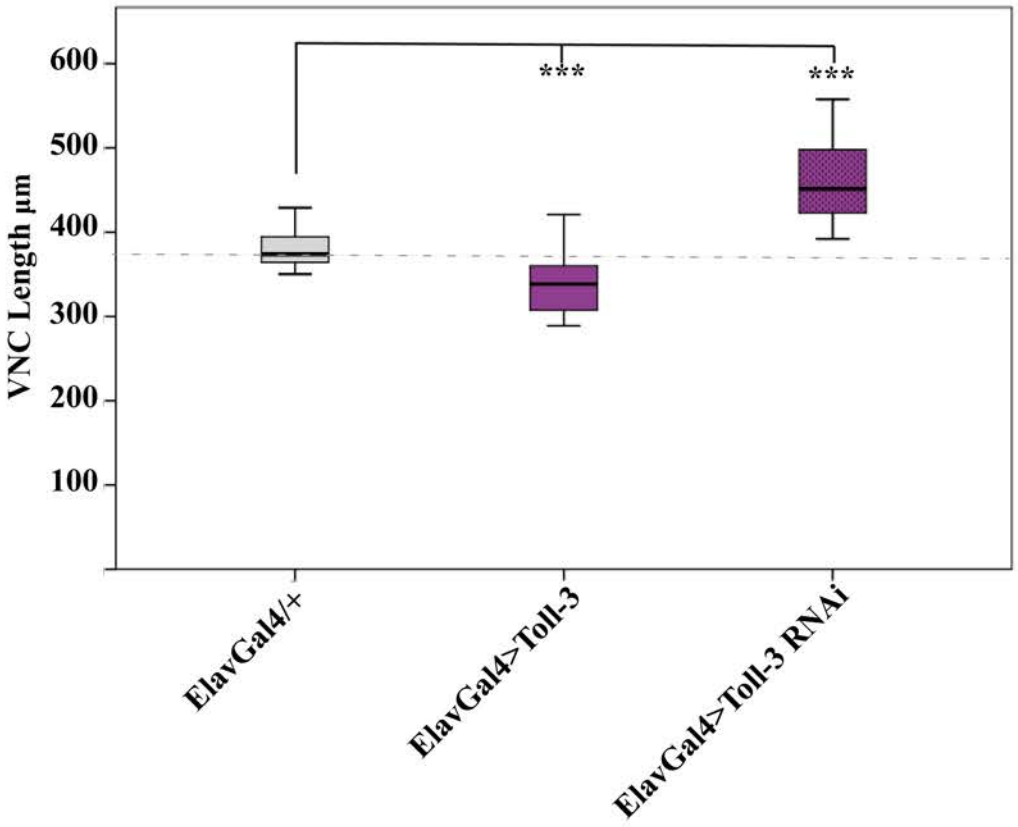
Figure 5.2: Ventral nerve cord length is regulated by the loss of function of *Drosophila* Tolls in neurons



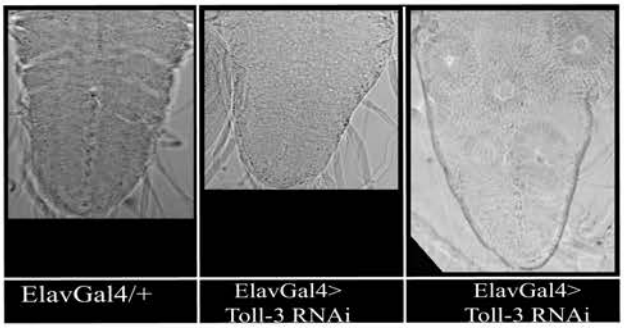


Ventral nerve cord length is regulated by the loss of function of Toll receptors via RNAi knockdown in neurons.The graph in panel A shows that VNC Length is regulated by the loss of function of Drosophila UAS Toll-3 and UAS Toll-7 in neurons One Way ANOVA ($F(7,88)=6.803$, $p=0.000$). Panel B shows representative images of the knock-down of the Toll receptors.

Figure 5.3: Ventral nerve cord length is regulated by the over-expression and loss of function of *Drosophila* Toll-3 in neurons



B



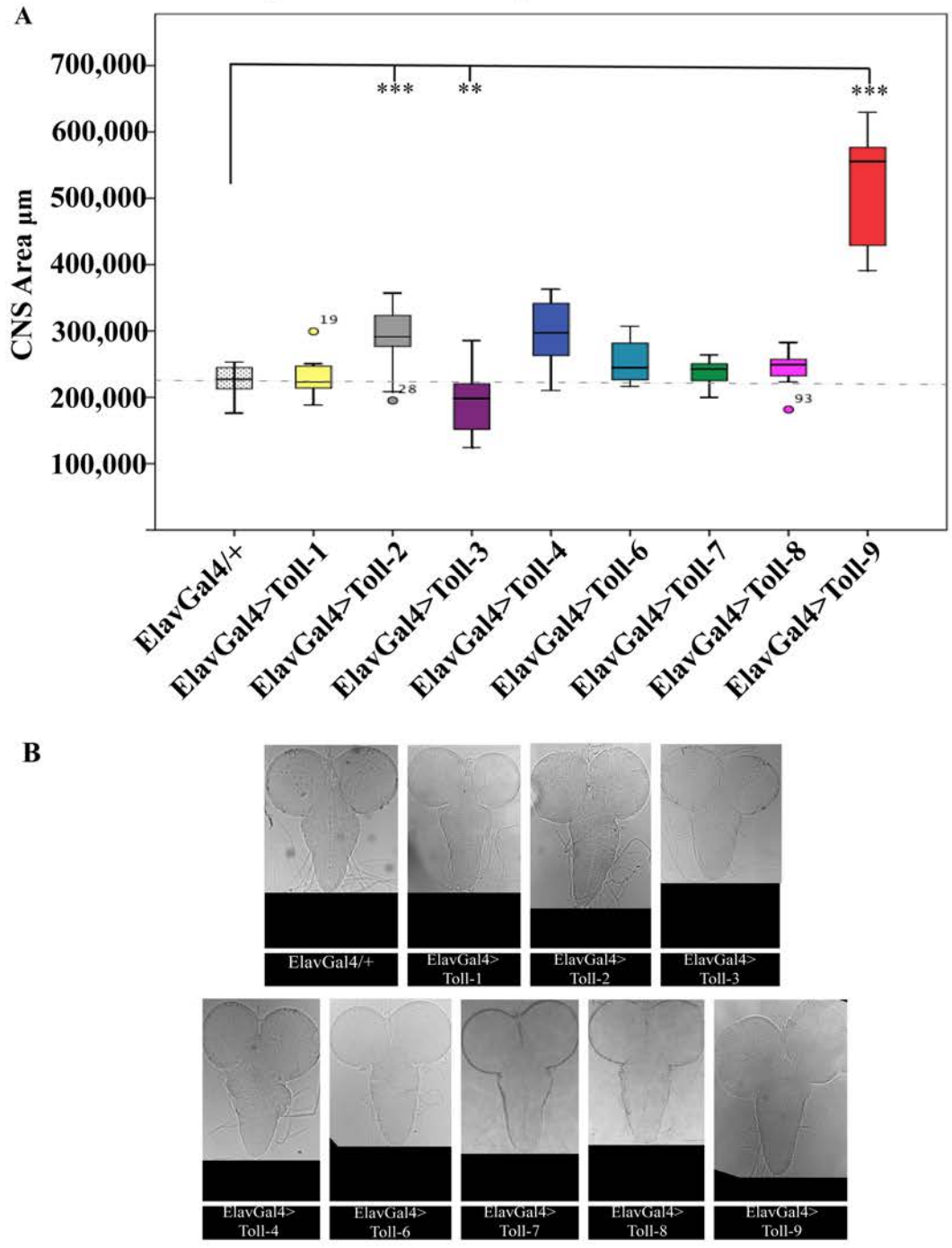
The graph in panel A shows that VNC Length is regulated by both the over-expression and loss of function of Drosophila UAS Toll-3 in neurons. Knocking down Toll-3 results in a smaller VNC length, conversley the over-expression of Toll-3 results in a longer VNC length. One Way ANOVA ($F(2,28)=25.207$, $p=0.000$). Panel B shows representative images of the loss of Toll-3 via RNAi knockdown and over-expression of Toll-3 in neurons.

When looking at the CNS area as a whole, the over-expression of Toll-3 in neurons (ElavGal4>UASToll-3) resulted in a much-reduced CNS area (Figure 5.4). Furthermore when Toll-3 is knocked down in neurons (ElavGal4>UASToll-3RNAi) there is an increase in CNS area (Figure 5.5). Similar to VNC length, over-expression of Toll-9 in neurons (ElavGal4>UASToll-9) resulted in a larger CNS area (Figure 5.4) but there was no effect when Toll-9 was knocked down (ElavGal4>UASToll-9RNAi) (Figure 5.5). Conversely, knocking down Toll-4 and Toll-7 (ElavGal4> UASToll4RNAi, -7RNAi) (Figure 5.5) resulted in an increase in CNS area. But there was no effect when over-expressed (ElavGal4> UASToll4, -7) (Figure 5.4). Again the most interesting candidate was that of Toll-3 (Figure 5.6) whereby the over-expression and knockdown resulted in opposite effects. This indicates that of all the Toll receptors Toll-3 in neurons is different from the rest. It is required for the maintenance of both CNS area and size.

5.2.2 Toll receptors in glia do not regulate VNC Length and CNS Area

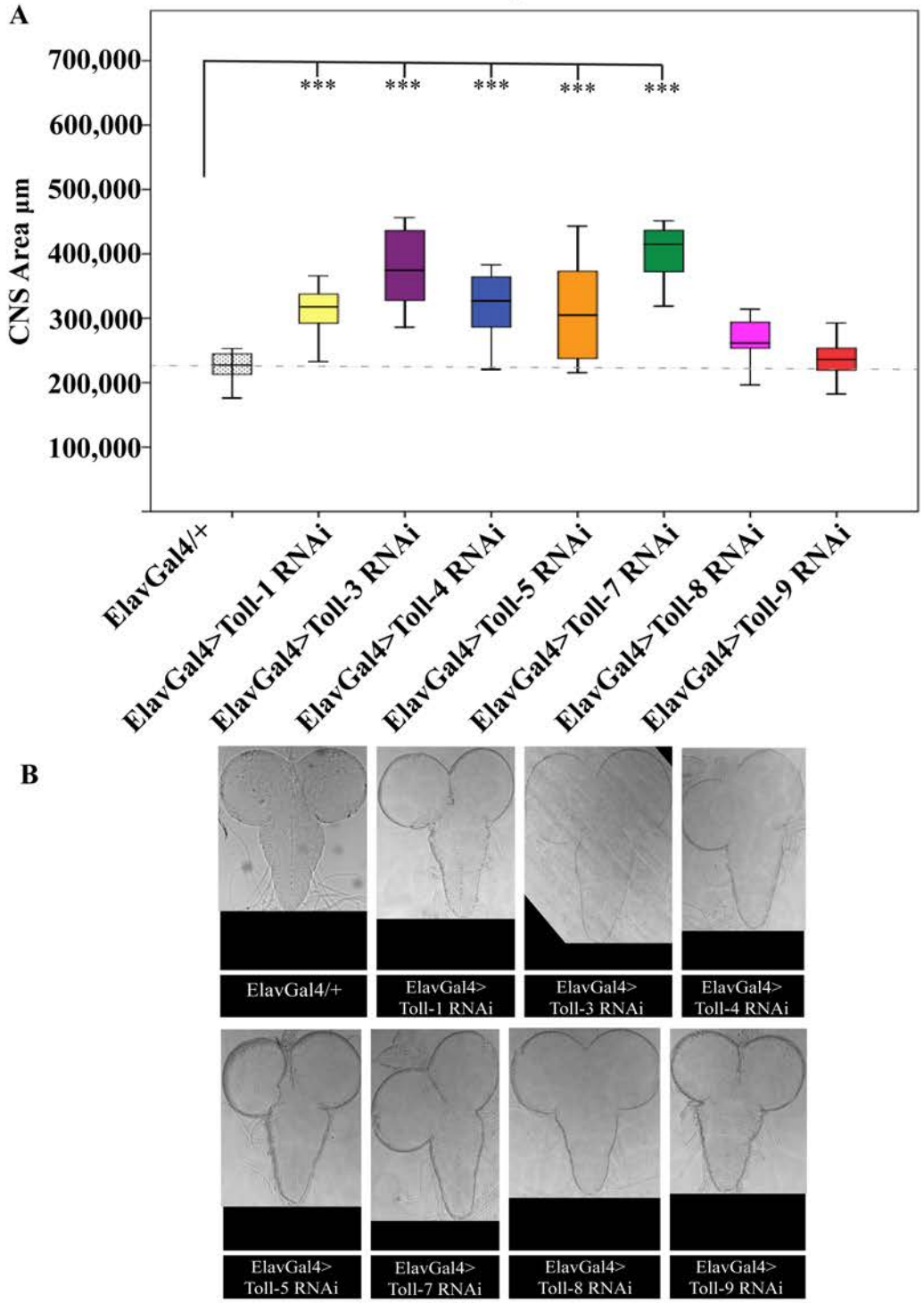
Over-expression of UASToll-7 and -9 in glia (RepoGal4>UASToll-7, -9) resulted in an increased VNC length (Figure 5.7). However when either were knocked-down (RepoGal4>UASToll-7RNAi, -9RNAi) there was no effect on VNC length (Figure 5.8) and therefore would not be investigated further. This was also the case for knocking down Toll-4 and -8 in glia (RepoGal4>UASToll-4RNAi, -8RNAi). These genotypes resulted in an increase in VNC length (Figure 5.8). But no observable phenotype was seen when either genotype was over-expressed (RepoGal4>UASToll-4, -8) (Figure 5.7). Furthermore the over-expression (RepoGal4>UASToll-3; Figure 5.7) and knockdown of Toll-3

Figure 5.4: The Area of the Central Nervous System is regulated by the over-expression of Drosophila Tolls in neurons



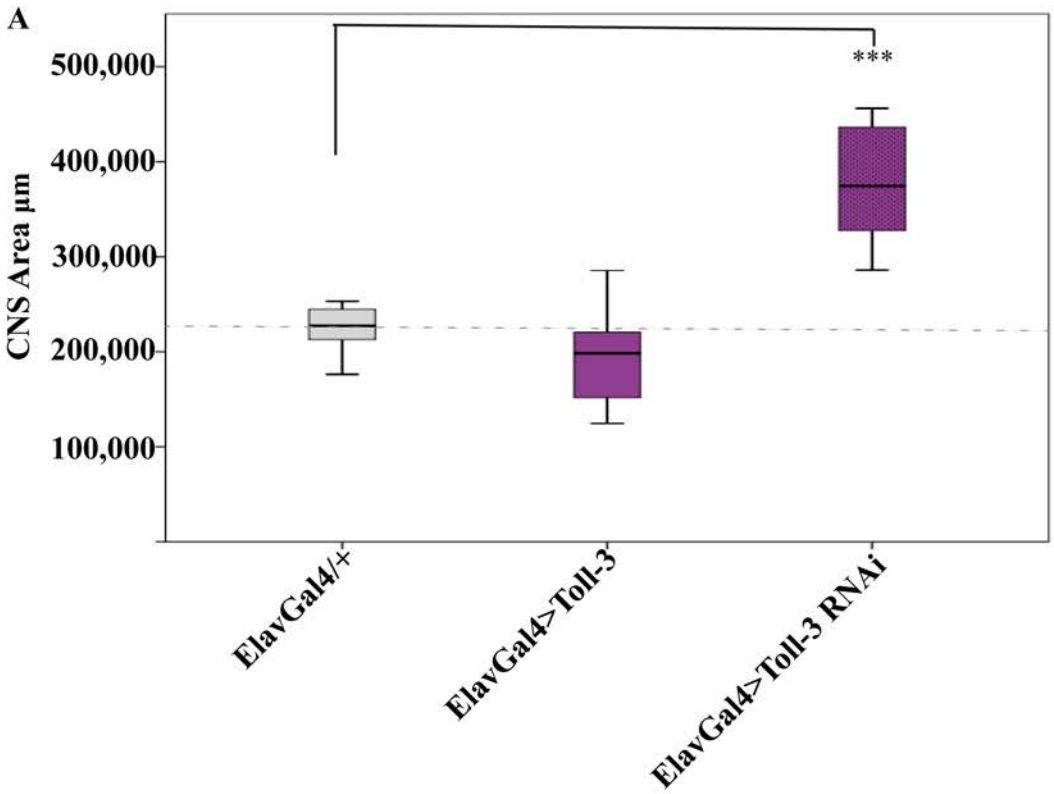
The area of the central nervous sytem is regulated by the over-expression of Toll receptors in neurons. The graph in panel A shows that CNS Area is regulated by the over-expression of UAS Toll-2, UAS Toll-3 and UAS Toll-9 in neurons. The over-expression of Toll-2 and Toll-9 result in larger CNS area, whereas the over-expression of Toll-3 results in reduced CNS area. One Way ANOVA ($F(8,38)=17.506$, $p=0.000$). Panel B shows representative images of the over-expression of Toll receptors in neurons.

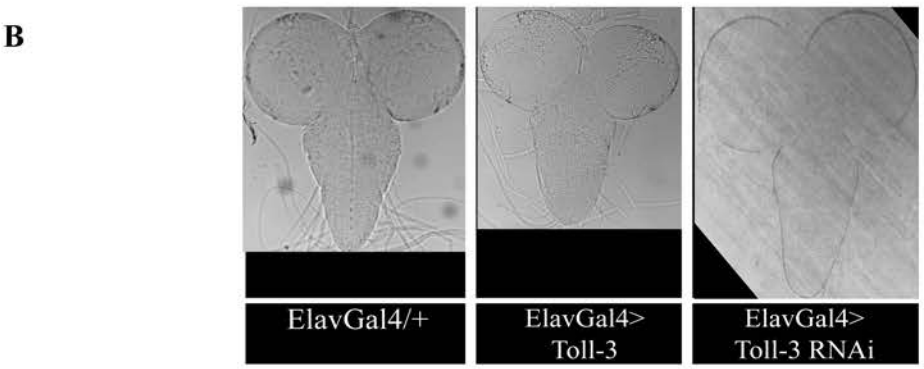
Figure 5.5: The Area of the Central Nervous System is regulated by the loss of function of Drosophila Tolls in neurons



Central nervous system area is regulated by the loss of function of Toll receptors via RNAi knockdown in neurons. The graph in panel A shows that CNS Area is regulated by the loss of function of Drosophila UAS Toll-1, UAS Toll-3, UAS Toll-4, UAS Toll-5 and UAS Toll-7 in neurons, all of which result in a larger CNS area. Welch ANOVA ($F(7,32.5)=21.020$, $p=0.000$). Panel B shows representative images of the different Toll receptors knocked down in neurons.

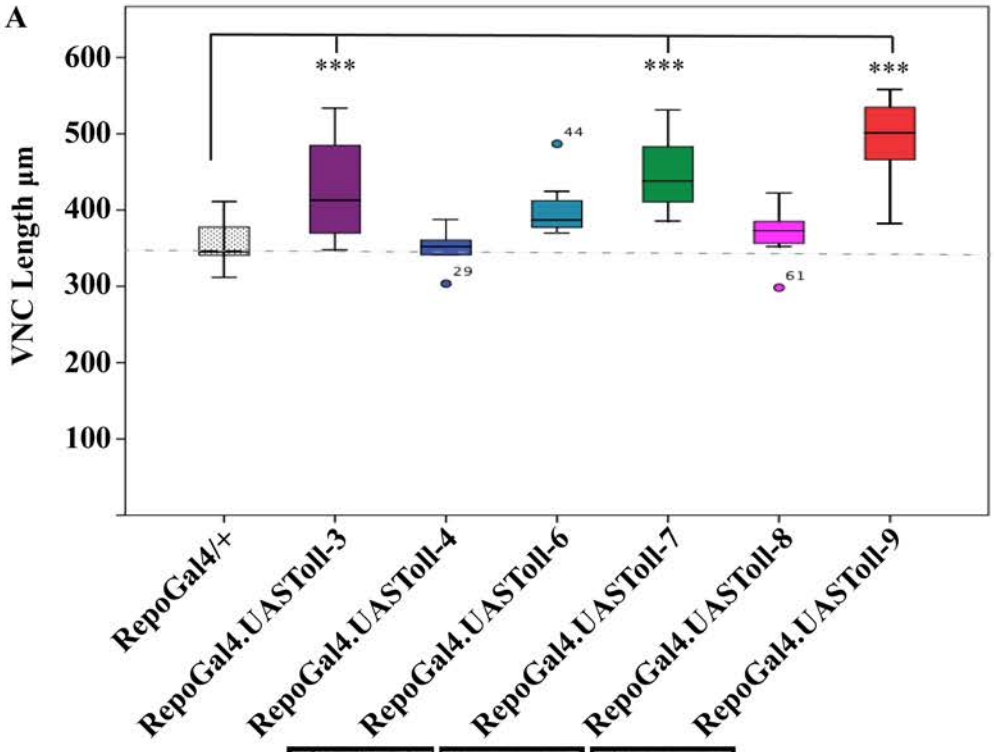
Figure 5.6: The Area of the CNS is regulated by the over-expression and loss of function of Drosophila Toll-3 in neurons

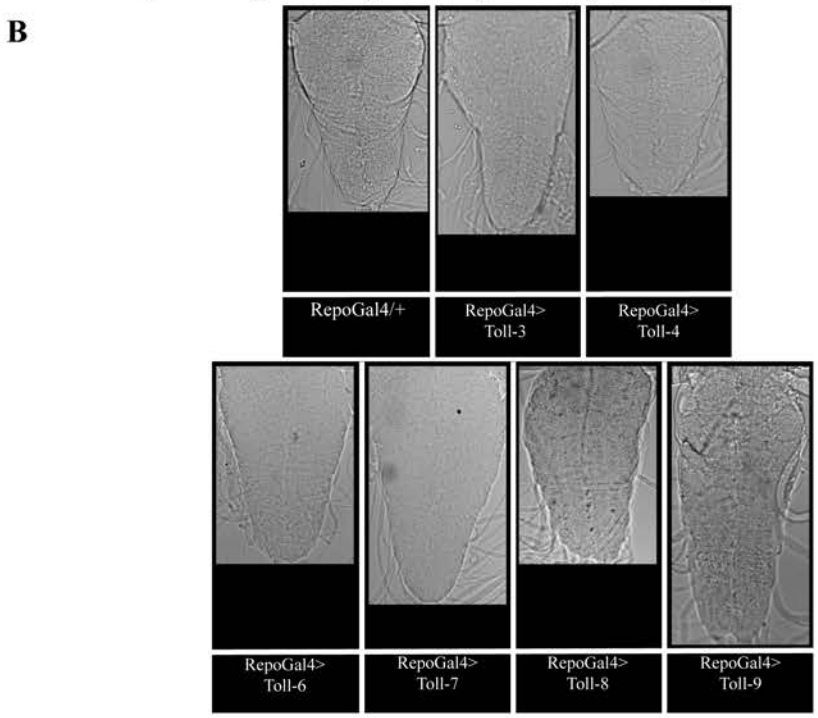




The graph in panel A shows that CNS Area is regulated by the over-expression and loss of function of Drosophila UAS Toll-3 in neurons. The over-expression of Toll-3 results in a smaller CNS area, whereas the loss of Toll-3 via RNAi knockdown results in a larger CNS area. Welch ANOVA ($F(2,15.213)=25.431$, $p=0.000$). The figures in Panel B are representative of the genotypes tested.

Figure 5.7: Ventral Nerve Cord length is regulated by the over-expression of Drosophila Tolls in lateral glia





Ventral nerve cord length is regulated by the over-expression of Toll receptors in lateral glia. The graph in panel A shows that Ventral Nerve Cord length is regulated by the over-expression of UAS Toll-3, UAS Toll-7 and UAS Toll-9. Over-expression of these receptors results in a larger VNC length. Welch ANOVA ($F(6,31.703)=18.218$, $p=0.000$). Panel B shows representative images of genotypes over-expressed in lateral glia.

(RepoGal4>UASToll-3RNAi; Figure 5.8) resulted in the same phenotype with an increase in VNC length.

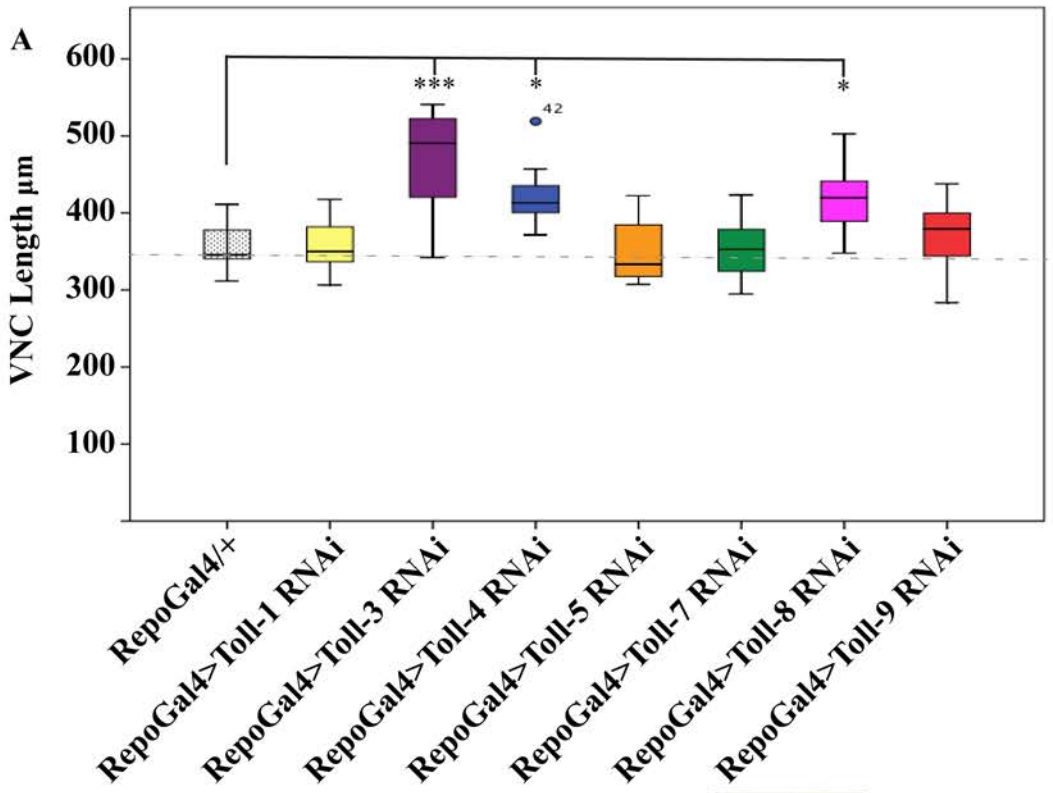
Similar results were obtained when measuring CNS area. The over-expression of Toll-3, -6, -7 and -9 (RepoGal4>UASToll-3, -6, -7, -9; Figure 5.9) resulted in increase CNS area. However Toll-3 knockdown (RepoGal4>UASToll-3RNAi) resulted in the same phenotype, whereas Toll-6 knockdown was not tested and Toll-7 and -9 (RepoGal4>UASToll-7RNAi, -9RNAi) there was no difference from the controls (Figure 5.10). Furthermore knocking down Toll-4 and Toll-8 (Repogal4>UASToll-4RNAi, -8RNAi; Figure 5.10) resulted in increase CNS area, but no observable phenotype when either was over-expressed (RepoGal4>UASToll-4, -8; Figure 5.9).

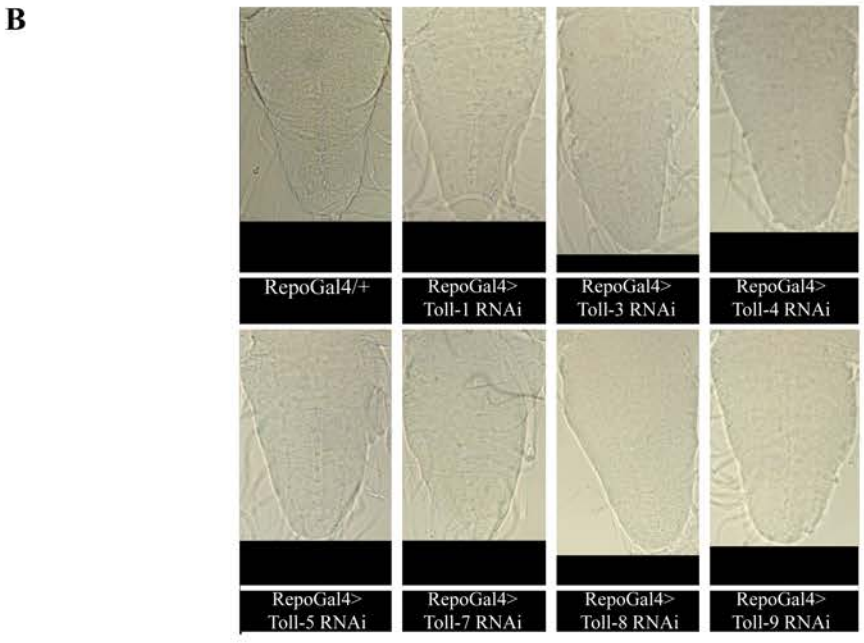
5.2.3 Toll receptors in the regulation of neuronal cell number

In order to establish if different Toll receptors had similar effects on neuronal number or not, I over-expressed them in neurons and tested whether or not they influenced Eve+ neuron number (Figure 5.11). To do this I used third instar larvae brains, stained with anti-eve antibodies and counted Eve+ cells in only the abdominal segments using DeadEasy software.

There was an increase in Eve+ cell number when I over-expressed UAS Toll-2, UAS Toll-4, UAS Toll-6, UAS Toll-7, UAS Toll-8 and UAS Toll-9 suggesting that these Toll receptors may play some function in promoting cell survival. The only members of the Toll family that failed to promote an increase in cell number were UAS Toll-1 and UAS-Toll-3. It is therefore

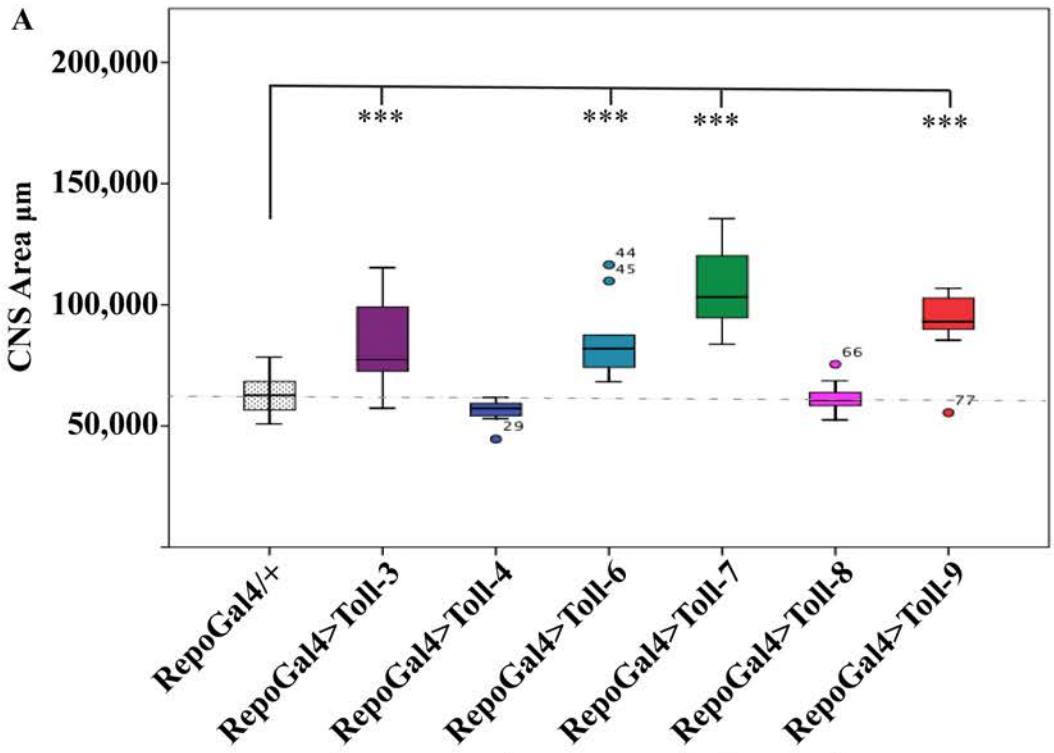
Figure 5.8: Ventral Nerve Cord length is regulated by the loss of function of Drosophila Tolls in lateral glia

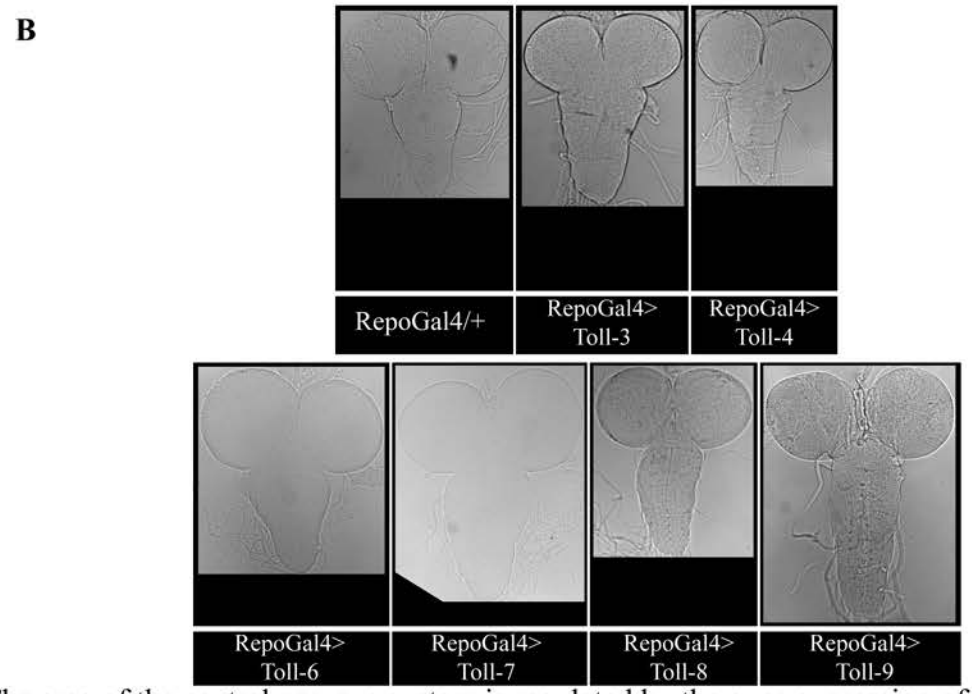




Ventral nerve cord length is regulated by the loss of function of Toll receptors in lateral glia. The graph in panel A show that Ventral Nerve Cord length is regulated by the loss of function of UAS Toll-3, UAS Toll-4 and UAS Toll-8. The knockdown of these receptors results in larger VNC length. One Way ANOVA ($F(7,79)=9.773$, $p=0.000$). Panel B shows representative images of genotypes knocked down in lateral glia.

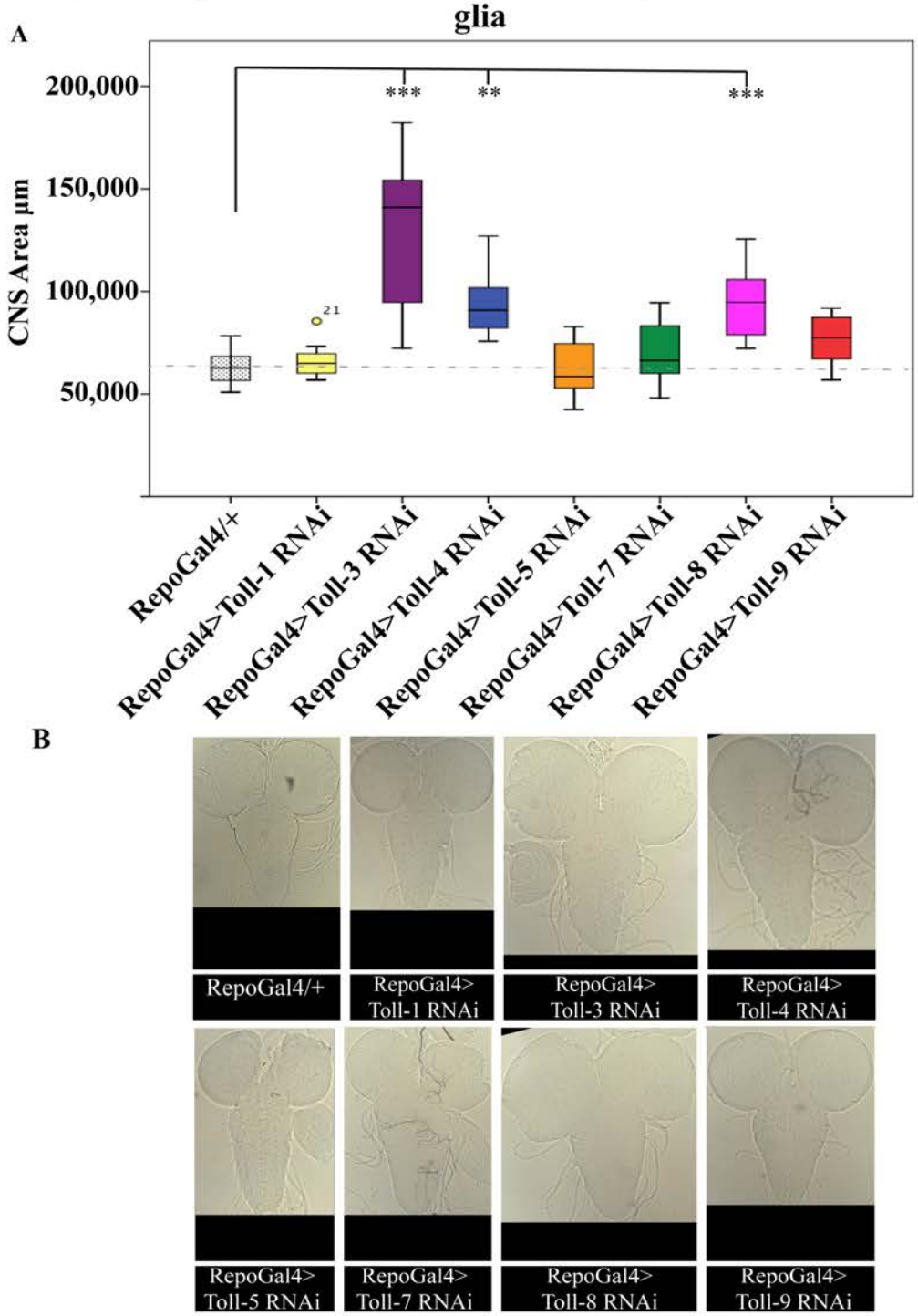
Figure 5.9: The Area of the Central Nervous System is regulated by the over-expression of Drosophila Tolls in lateral glia





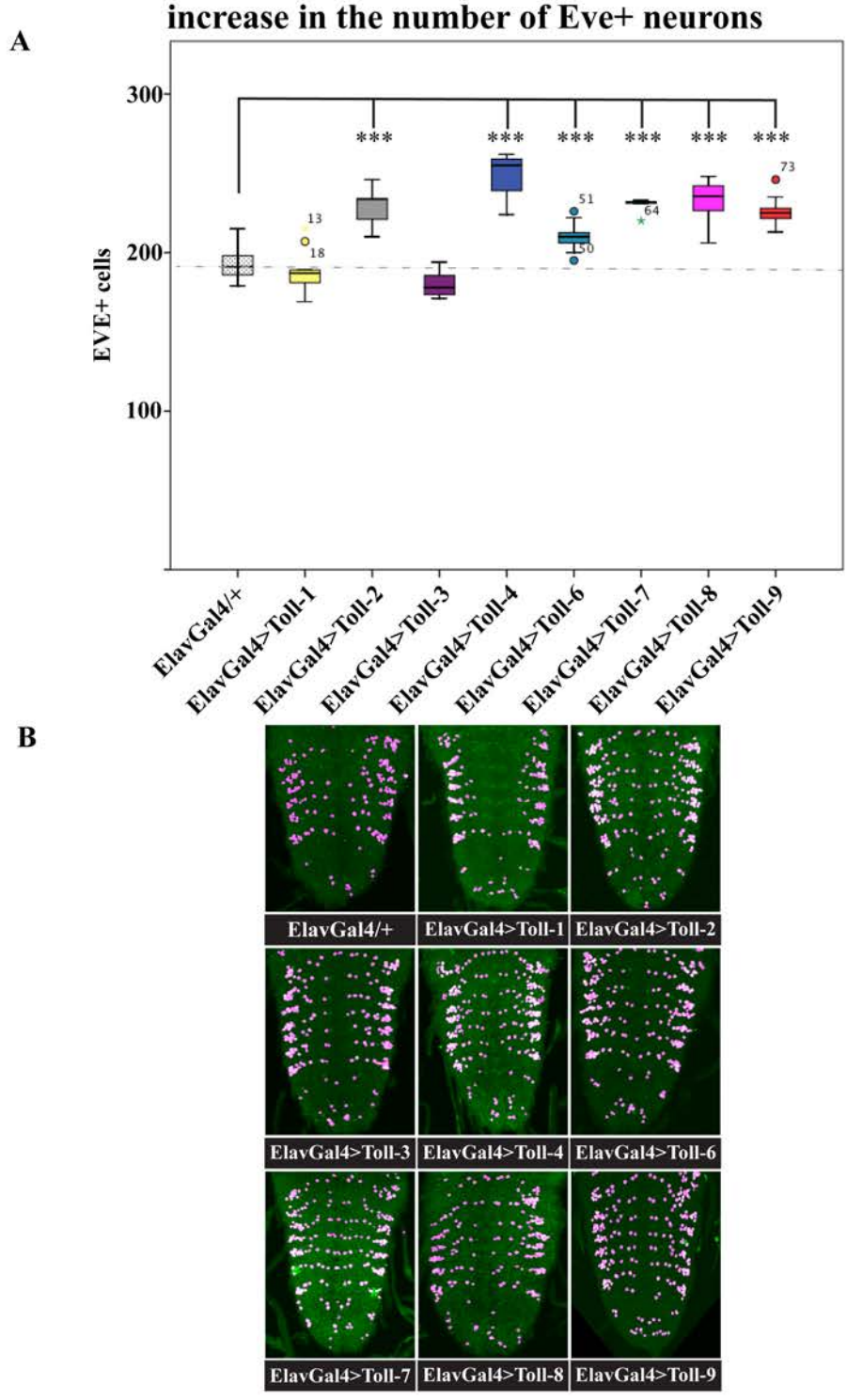
The area of the central nervous system is regulated by the over-expression of Toll receptors in lateral glia. The graph in panel A shows that CNS Area is regulated by the over-expression of UAS Toll-3, UAS Toll-6, UAS Toll-7 and UAS Toll-9. The over-expression of these Toll receptors results in larger CNS area. Welch ANOVA ($F(6,31.487)=27.202$, $p=0.000$). Panel B shows representative images of genotypes over-expressed in lateral glia.

Figure 5.10: The Area of the Central Nervous System is regulated by the loss of function of Drosophila Tolls in lateral glia



The area of the central nervous system is regulated by the loss of function of Toll receptors in lateral glia. The graph in panel A shows that CNS Area is regulated by the loss of function of UAS Toll-3, UAS Toll-4 and UAS Toll-8 in lateral glia. The knock-down of these Toll receptors results in a larger CNS area. Welch ANOVA ($F(7,27.968)=27.202$, $p=0.000$). Panel B shows representative images of genotypes knocked down in lateral glia.

Figure 5.11: Over-expression of Drosophila Tolls results in an increase in the number of Eve+ neurons



The over-expression of Toll receptors results in an increase in Eve+ neurons. The graph in panel A shows that the over-expression of Drosophila UAS Toll-2, UAS Toll-4, UAS Toll-6, UAS Toll-7, UAS Toll-8 and UAS Toll-9 results in an increase in the number of Eve+ neurons. One Way ANOVA ($F(8,75)=43.819$, $p=0.000$). Panel B shows representative images of genotypes over-expressed in neurons of the VNC and the Eve+ neurons counted by DeadEasy software.

possible that these Toll family members are not required in the regulation of cell survival in a similar manner to the other Toll receptors or they may be required to promote cell death.

5.2.4 Toll receptors in the regulation of cell death

In conjunction with correct ECM deposition and neural activity, there is also another process that is pivotal in VNC condensation. During embryogenesis the CNS and epidermis originate from a common ectodermal layer. Proneural and neurogenic signalling mechanisms determine if cells are driven to neural or epidermal fates (Urbach and Technau, 2004). As VNC condensation occurs programmed cell death ensures correct separation of these two tissues (Page and Olofsson, 2008). It is a combination of all three processes that ensure the VNC is condensed by at least 25% prior to larval development (Olofsson and Page 2005; Page and Olofsson, 2008).

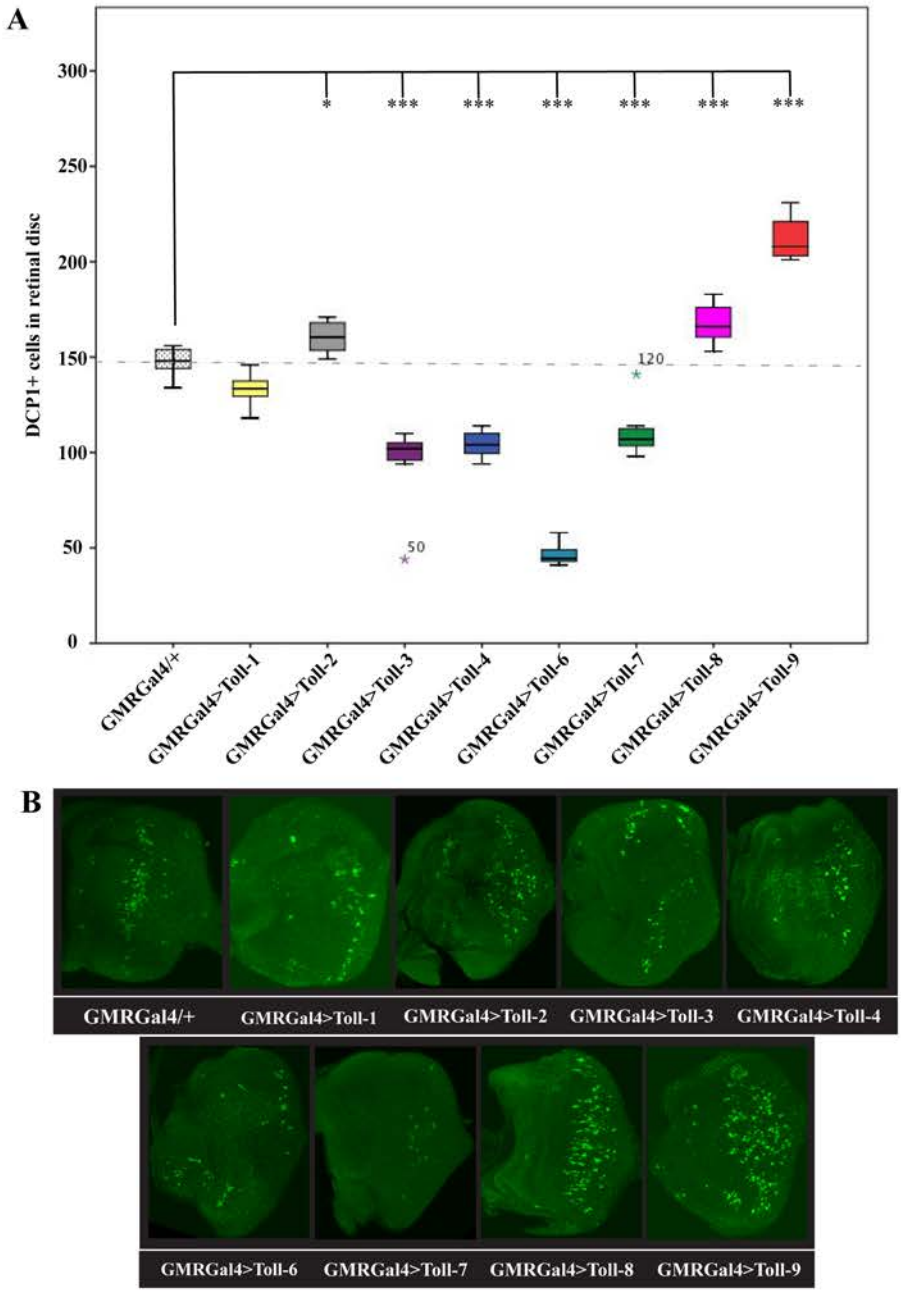
Therefore as another assay to ask the question of whether all Tolls are functionally equivalent or not, I tested how each of the Tolls regulated apoptosis in the CNS. Toll-6 and -7 have already been shown to promote neuronal survival in embryos (McIlroy *et al*., 2013). I over-expressed or knocked down all of the Toll receptors in the retina (as it is a much smaller tissue) using GMRGal4 and then tested if their expression increased the number of Anti Death Caspase 1+ (DCP1+) cells, a read out of apoptosis using the software DeadEasy Larval Caspase automatic quantification in 3D (Forero *et al.,* 2009; Kato *et al.*,2011; Forero *et al.,* 2012).

Over-expression of UAS Toll-2, UAS Toll-8 and UAS Toll-9 (GMRGal4>UASToll-2, -8, -9) resulted in increased rates of apoptosis in the retinal discs of wandering larvae. Whereas the over-expression of UAS Toll-3, UAS Toll-4, UAS Toll-6 and UAS Toll-7 (GMRGal4>UASToll-3, -4, -6, -7) lead to a decrease in the rates of apoptosis (Figure 5.12). Knockdown of UAS Toll-1, UAS Toll-2, UAS Toll-3, UAS Toll-4, UAS Toll-7 and UAS Toll-9 (GMRGal4>UASToll-1RNAi, -2RNAi, -3RNAi, -4RNAi, -7RNAi, -9RNAi) led to a decrease in the rate of apoptosis, whereas; knocking down UAS Toll-5, UAS Toll-6 and UAS Toll-8 (GMRGal4>UASToll-5RNAi, -6RNAi, -8RNAi) resulted in an increase in apoptosis in the retina (Figure 5.13).

The most interesting of these phenotypes are the effects seen with Toll-2, Toll-6 and Toll-9. Over-expression of both UAS Toll-2 and UAS Toll-9 (GMRGal4>UASToll-2, -9) resulted in an increase of apoptosis in the retina, whereas their knockdown (GMRGal4>UASToll2RNAi, -9RNAi) had the opposite effect decreasing the rate of apoptosis (Figure 5.14 A&B). Indicating that both Toll-2 and Toll-9 are more pro-apoptotic than the other Toll receptors. Interestingly I previously showed that the over-expression of UAS Toll-2 and UAS Toll-9 resulted in an increase in Eve+ cells suggesting their role in regulating cell survival. However these data suggests that these receptors may be involved in cell death pathways. It appears that for these receptors at least their role in survival or death is dependent upon the stage of development and the specific tissues they are expressed in.

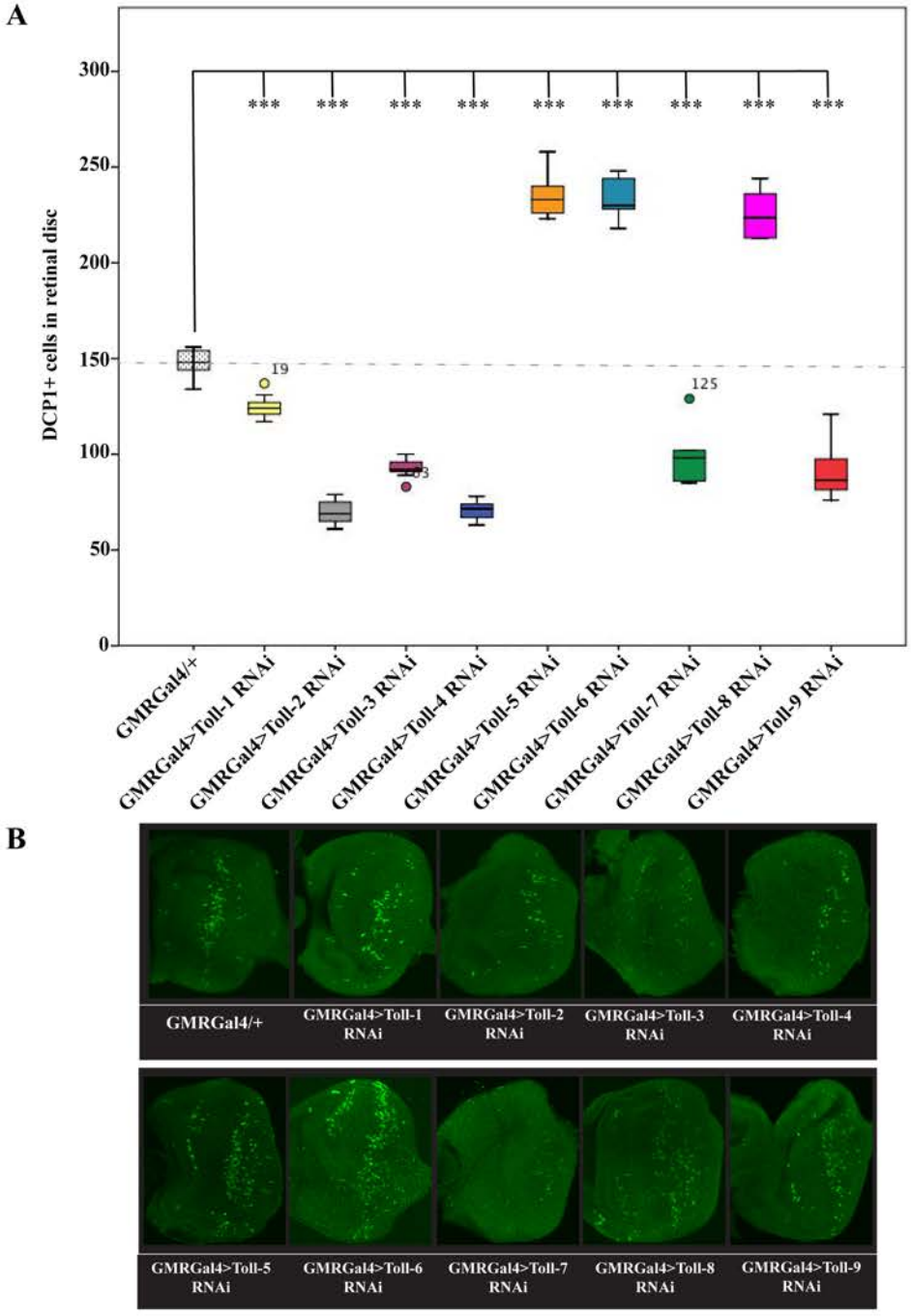
UAS Toll-6 on the other hand, when knocked down (GMRGal4>UASToll-6RNAi), results in an increase of cell death (Figure 5.15). When over-expressed in the retina

Figure 5.12: Over-expression of the different Tolls result in different Apoptotic levels in the larval retinal disc.



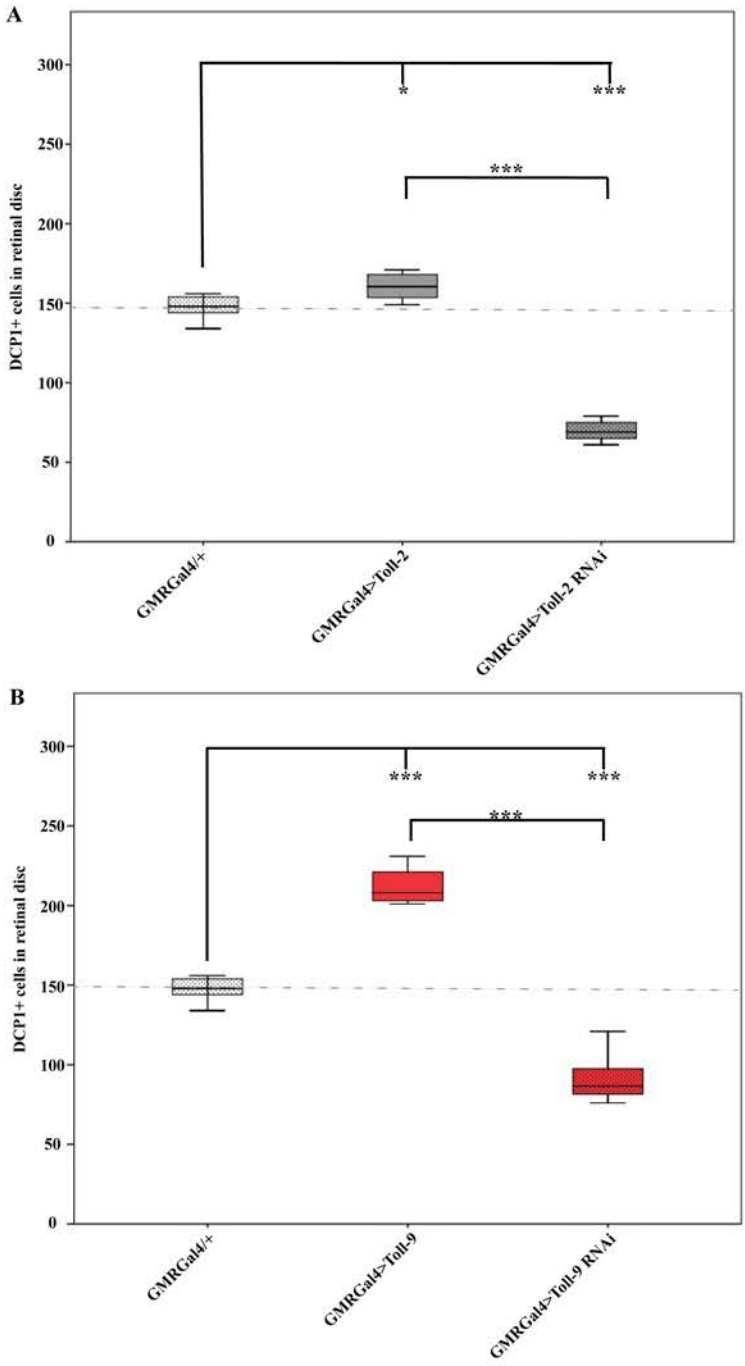
Over-expression of the Toll receptors in retinal discs leads to differing rate of apoptosis. The graph in panel A shows that the over-expression of Toll-2, Toll-8 and Toll-9 result in an increase in the rates of apoptosis in larval retinal discs. Conversley; over-expression of Toll-3, Tol-4, Toll-6 and Toll-7 lead to a reduction in the levels of apoptosis. The over-expression of Toll-1 has no effect upon levels of Apoptosis. One Way ANOVA ($F(8,69)=149.208$, $p=0.000$). Panel B shows representative images of the Toll receptors over-expressed in retinal discs with apoptotic cells stained using Dcp1 and counted manually via the cell counter plug in of ImageJ.

Figure 5.13: Knocking-down the different Tolls results in different Apoptotic levels in the larval retinal disc.



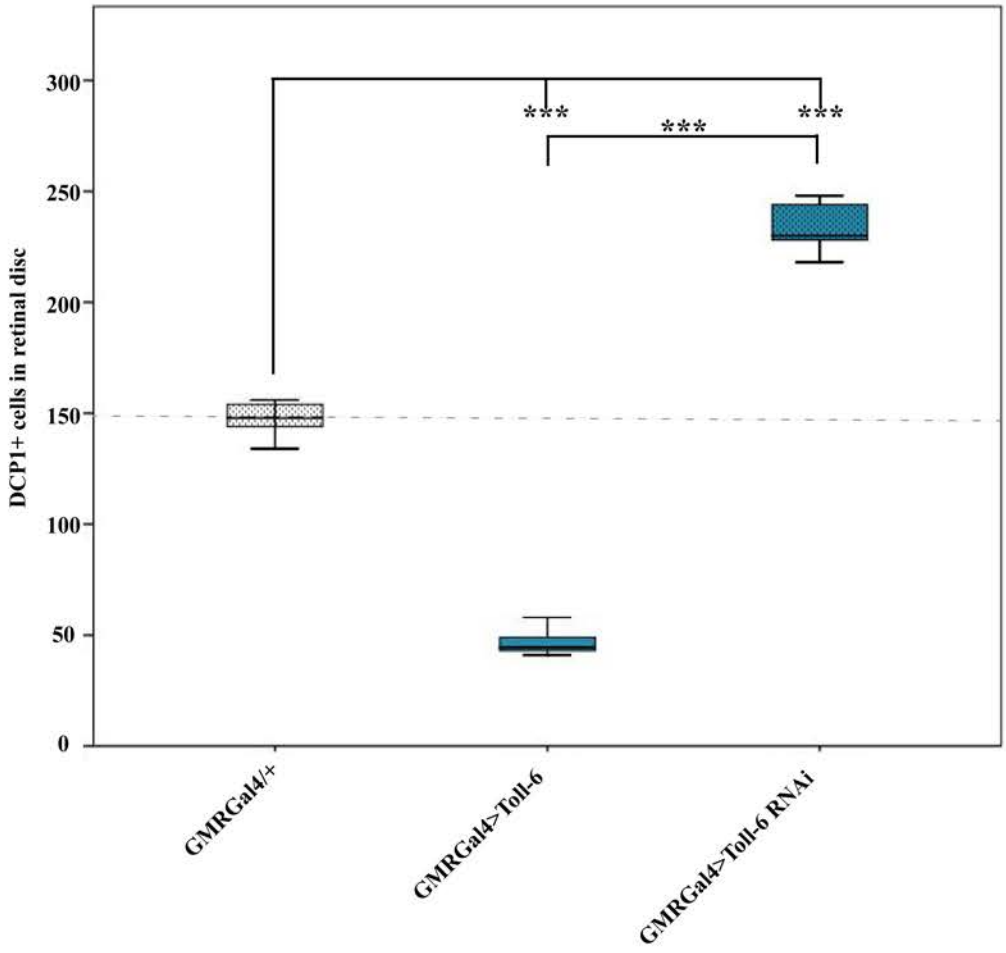
Knockdown via RNAi mediated knockdown of Toll receptors in larval retinal discs results in different levels of apoptosis. The graph in panel A shows that knocking down Toll-1, Toll-2, Toll-3, Toll-4, Toll-7 and Toll-9 result in an decrease in the rates of apoptosis in larval retinal discs. Conversley; knocking-down Toll-5, Toll-6 and Toll-8 lead to a increase in the levels of apoptosis. One Way ANOVA ($F(9,75)=441.837$, $p=0.000$). Panel B shows representative images of the Toll receptors knocked down in retinal discs with apoptotic cells stained using anti-Dcp1 and counted manually via the cell counter plug in of ImageJ.

Figure 5.14: Over-expression Drosophila Toll-2 and Toll-9 lead to an increase in the number of Apoptotic cells in larval retinal



The graph in panel A shows the over-expression of Toll-2 leads to an increase inapoptosis in larval retinal discs, and its knock down leads to a rescue and overall reduction in Apoptotic cells. One Way ANOVA $F(2,28)=481.162$, $p=0.000$). The graph in panel B shows the over-expression of Toll-9 leads to an increase inapoptosis in larval retinal discs, and its knock down leads to a rescue and overall reduction in Apoptotic cells.One Way ANOVA $F(2,18)=150.524$, $p=0.000$.

Figure 5.15: Knock-down and over-expression of Toll-6 leads to changes in the number of Apoptotic cells in larval retinal discs.



Graph displays that reduced levels of Toll-6 results in an increase in apoptosis in the larval retinal discs, and the over-expression of leads to a decrease in the rate of apoptosis. One Way ANOVA $F(2,25)=1410.10$, $p=0.000$

(GMRGal4>UASToll-6) there was an increase in cell survival. This further confirms that UAS Toll-6 in the larval VNC and retina is required for cell survival.

5.3 SUMMARY

Throughout this chapter I have shown (Figure 5.16 & Table 5.1):

1) Toll-3 is required in neurons to promote the maintenance of CNS area and VNC size
2) Over-expression of Toll-2, -4, -6, -7, -8 and -9 in neurons results in an increase in Eve+ neurons indicating that these Tolls may function in cell survival
3) Over-expression of Toll-1 and Toll-3 in neurons results in no change to the number of Eve+ neurons indicating that these Tolls may have no function in survival or death outcomes
4) Over-expression of Toll-2 and Toll-9 in retina results in an increase in apoptosis, indicating that these Tolls are pro-death in this tissue
5) Over-expression of Toll-6 in retina results in a decrease in apoptosis, indicating that it has pro-survival functions.

When Toll-3 is expressed within neurons it is able to regulate the maintenance of both VNC length and CNS area. This is interesting, as I have shown that this appears to be the only phenotype where Toll-3 has a role to play. In both neurons and glial cells, expression is low indicating that it may be restricted to only a few specific cells. Furthermore, anti-body staining has demonstrated that expression is mainly restricted to later developmental time points and within the visual system. There was no observable phenotype in locomotion assays, or in the regulation of cell survival or death. There was also no lethality or morphological defects when Toll-3 was over-expressed in a transgenic expression assay (Yagi *et al.*, 2010).

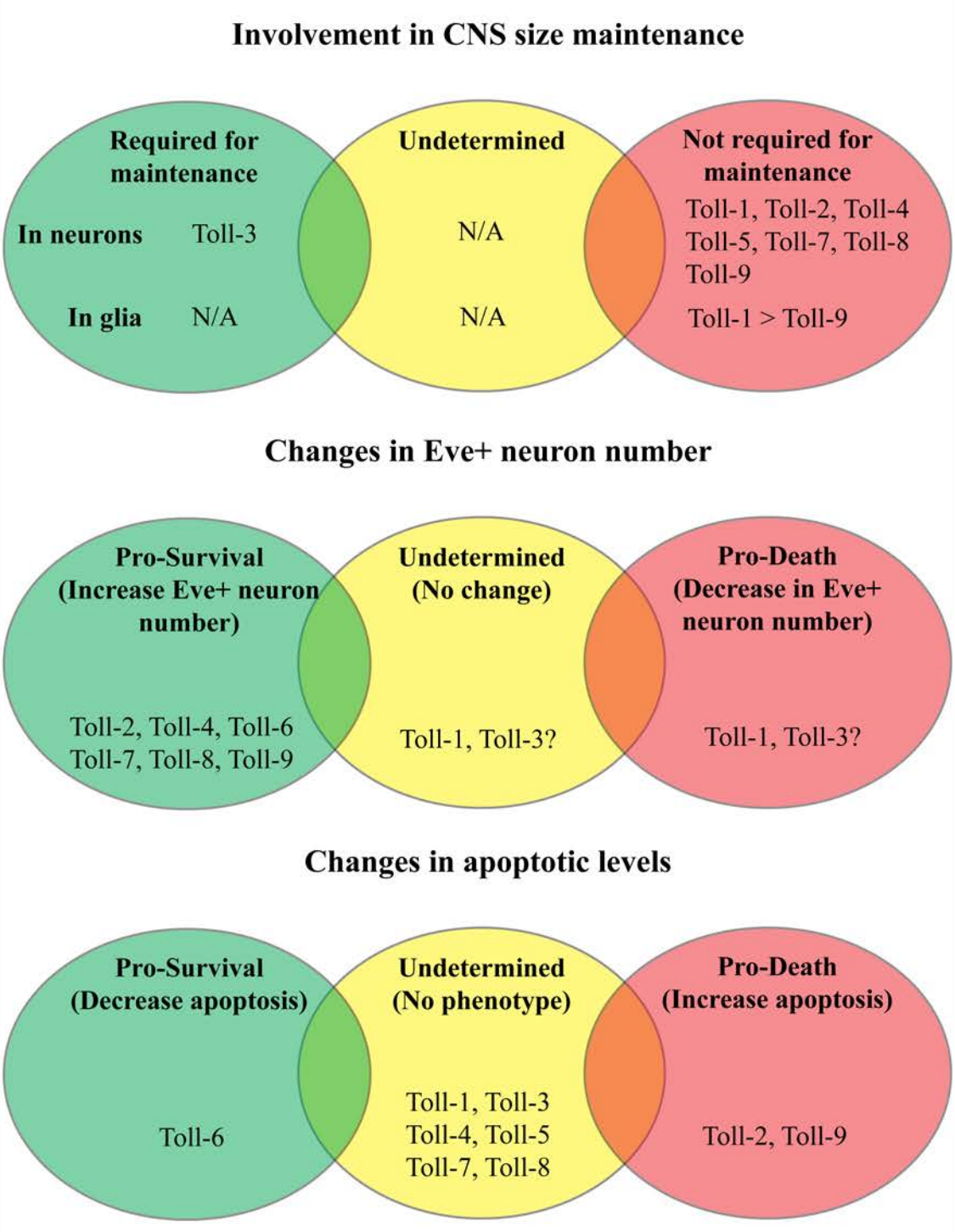
Table 5.1: Table of results from Chapter 5

	CHAPTER 5										
	CNS Size								Cell number regulation		
	VNC Length		CNS Area		VNC Length		CNS Area		Eve+ Neuron Number	Apoptosis in retinal discs (DCP1+)	
	ElavGal4> OE	ElavGal4> KD	ElavGal4> OE	ElavGal4> KD	RepoGal4> OE	RepoGal4> KD	RepoGal4> OE	RepoGal4> KD	ElavGal4> OE	GMRGal4> OE	GMRGal4> KD
	3* Larvae	3* Larvae	3* Larvae	3* Larvae	3* Larvae	3* Larvae	3* Larvae	3* Larvae	3* Larvae	3* Larvae	3* Larvae
Toll-1	N	N	N	Y	-	N	-	N	N	N	Y
Toll-2	N	-	Y	-	-	-	-	-	**Y**	**Y**	**Y**
Toll-3	**Y**	**Y**	**Y**	**Y**	Y	Y	Y	Y	N	Y	Y
Toll-4	N	N	N	Y	N	Y	N	Y	**Y**	Y	Y
Toll-5	-	N	-	Y	-	N	-	N	-	-	Y
Toll-6	N	-	N	-	N	-	Y	-	**Y**	**Y**	**Y**
Toll-7	N	Y	N	Y	Y	N	Y	N	**Y**	Y	Y
Toll-8	N	N	N	N	N	Y	N	Y	**Y**	Y	Y
Toll-9	Y	N	Y	N	Y	N	Y	N	**Y**	**Y**	**Y**

OE	Over-expression
KD	Knock Down
GMRGal4	Expression in retinal discs

	Genotype significiantly different from control in both OE & KD
Y	Genotype significiantly different from control in only OE or KD
N	Genotype not significiantly different from control
-	Not tested

Figure 5.16: Toll receptors in the regulation of CNS size, cell survival and/or death



These diagrams depict the proposed groupings of the Toll receptors in their involvement during the maintenance of CNS area and VNC length. Regulation of neuronal number and cell death.

Even though I have shown that by knocking down Toll-3 in neurons (ElavGal4>UASToll-3RNAi) results in a larger CNS area and VNC length the physical processes that are occurring remain elusive. There could be multiple processes that generate this phenotype. For instance there may be an increase in cell number, cell size or apoptosis levels. An increase in cell number does not seem likely as the over-expression of Toll-3 (ElavGal4>UASToll-3) resulted in a decrease in CNS size, but Eve+ neuron number in the VNC was the same as wild-type. Furthermore the over-expression of Toll-3 resulted in a reduction in apoptosis in the retina. This however is a different tissue and would need to be confirmed in the CNS. Cell size is an exciting avenue to explore as is the cell adhesion properties of Toll-3.

Some of the genotypes were missing from this assay due not having the appropriate flies at the time of the experiment. There is of course the possibility that the missing genotypes would provide essential data on their function in the maintenance of the CNS. For instance over-expression of Toll-2 in neurons (ElavGal4>UASToll-2) resulted in an increase in the CNS area, however the knock down of Toll-2 (ElavGal4>UASToll-2RNAi) was not tested. This was also the case for both the over-expression and knockdown of Toll-2 in glial cells. As Toll-2 is believed to function as a heterotypic cell adhesion molecule during development when cell movement is required (Eldon *et al.*, 1994), this would have been an ideal candidate to test. Therefore it is important that the missing genotypes, particularly Toll-2 are testing in the future.

In the regulation of neuronal cell number, all Tolls, with the exception of Toll-1 and Toll-3 lead to an increase in Eve+ neurons. Indicating that many of the Toll receptors could play important function in the regulation of neuronal survival. For Toll-7 this result is consistent

with findings from McIlroy *et al.* where they over-expressed the constitutivly active form of Toll-7 in neurons and found that it is able to rescue naturally occurring cell death (McIlroy *et al.,* 2013). However this is inconsistent with findings for two of the Toll family members. Over-expression of both Toll-2 and Toll-9 in retina resulted in an increase in the rate of apoptosis. This could be due to the fact that these assays were completed in two very different tissues. Neuron number was counted in the VNC and apoptosis in retina.

Programmed cell death (PCD) is not only essential for the removal of dying cells but also for tissue patterning and homeostatic maintenance. During larval development PCD is most evident throughout CNS tissues (Rusconi *et al.,* 2000), where apoptosis is controlled by the steroid hormone ecdysone (Baehrecke 2000). In the retina, there are two bands of apoptotic cells, the first in the posterior margin and the second anterior to the morphogenetic furrow (Wolff and Ready. 1991; Bonini *et al.,* 1999). The structure of this tissue is highly complex and exceptionally sensitive to perturbation and mutation (Tanenbaum *et al.,* 2000). Therefore the extensive rate of apoptosis may be due to the maintenance of the quality and number of ommatidial precursor cells (Werz *et al.,* 2005). Therefore it is possible that in different tissues these two Toll receptors perform opposing functions. Promoting neuronal survival in the VNC and cell death in the retina. It would therefore be beneficial to look at the rate of apoptosis in the VNC for these two receptors.

Toll-6 is different to the other receptors in that it is involved in the regulation of cell survival in both the VNC and retinal discs. This is consistent with the finding of McIlroy *et al.,* 2013 whereby there was an increase in apoptosis in Toll-6 double mutant embryos. Furthermore they show that the over-expression of the constitutively active form of Toll-6 in neurons is

able to rescue naturally occurring cell death. Both of these findings indicate that Toll-6 in both the embryonic and larval CNS is required for the promotion of cell survival (McIlroy *et al.*, 2013). It would therefore be interesting to determine the downstream signalling mechanisms that are mediating this pro-survival effect.

In the next chapter I will investigate the upstream and downstream signalling mechanisms of Toll-6. I hope to establish if the different forms of the neurotrophins (DNT-1 and DNT-2) are capable of mediating cell death and/or survival outcomes. I will then try to determine the downstream adaptors that regulate the function of Toll-6, including MyD88, dSarm and Wek.

CHAPTER 6

DOWNSTREAM SIGNALLING OF TOLL-6: REGULATION OF CELL SURVIVAL AND DEATH

6.1 INTRODUCTION

In the previous chapter I showed that different Tolls are able to regulate cell survival and/or cell death. And this function may change temporally and/or spatially. Toll-2 and Toll-9 mediate cell death in the retina, but survival in the VNC. Toll-6 however appears to be primarily pro-survival. These results again show that the Toll receptors appear to be able to mediate very different cellular events. It is therefore conceivable that ligand and receptor complexes work together to facilitate cell outcomes.

The aim of this chapter was to determine if the specific cleavage state of the NTs had differing effects in the regulation of downstream signalling outcomes. Furthermore I wanted to determine the downstream mechanisms that regulate cell number downstream of the Toll receptors (Toll-6).

Toll-1 (no other Toll receptor) has been shown to bind MyD88 (Tauszig *et al.,* 2000; Tauszig-Delamasure *et al.,* 2002) and activate pro-survival Nf-κB signalling (Carter *et al.,* 1996; Foehr *et al.,* 2000; Hoffman and Reichhart 2002). Furthermore Toll-6 and Toll-7 have been suggested to function via NF-κB (McIlroy *et al.,* 2013). However, other Toll receptors have been proposed to function in different roles independently of MyD88 function. For example Toll-2 is believed to work as a cell adhesion molecule, which does not require MyD88 function (Eldon *et al.,* 1994). Therefore the Toll receptors may function via two distinct mechanisms, the first a MyD88 dependent mechanism and the second in a MyD88 independent manner.

There are two main peaks of cell death during the development of the CNS. The first occurring during embryogenesis. The second during the pupal stage, where the CNS is restructured to give rise to an adult CNS. However the regulation of death by the Toll receptors remain uncharacterised. Sarm1 is known to promote neuronal apoptosis in mammals and inhibit MyD88 (Kaiser and Offermann, 2005; Carty *et al.,* 2006; Ma *et al.,* 2006; Kim *et al.,* 2007). In flies it is known to induce degeneration of axons following injury (Osterloch *et al.,* 2012), however if it is involved in the regulation of apoptosis remains to be determined. Another adaptor protein is Wek, which is epistatic of Toll and acts upstream of both Dorsal and Cactus. Furthermore Wek has been shown to interact with Toll-1, -5 and -9 as well as , Toll-5. Wek can also interact with MyD88 to form a Toll-Wek-MyD88 complex (Chen *et al.,* 2007).

6.2 RESULTS

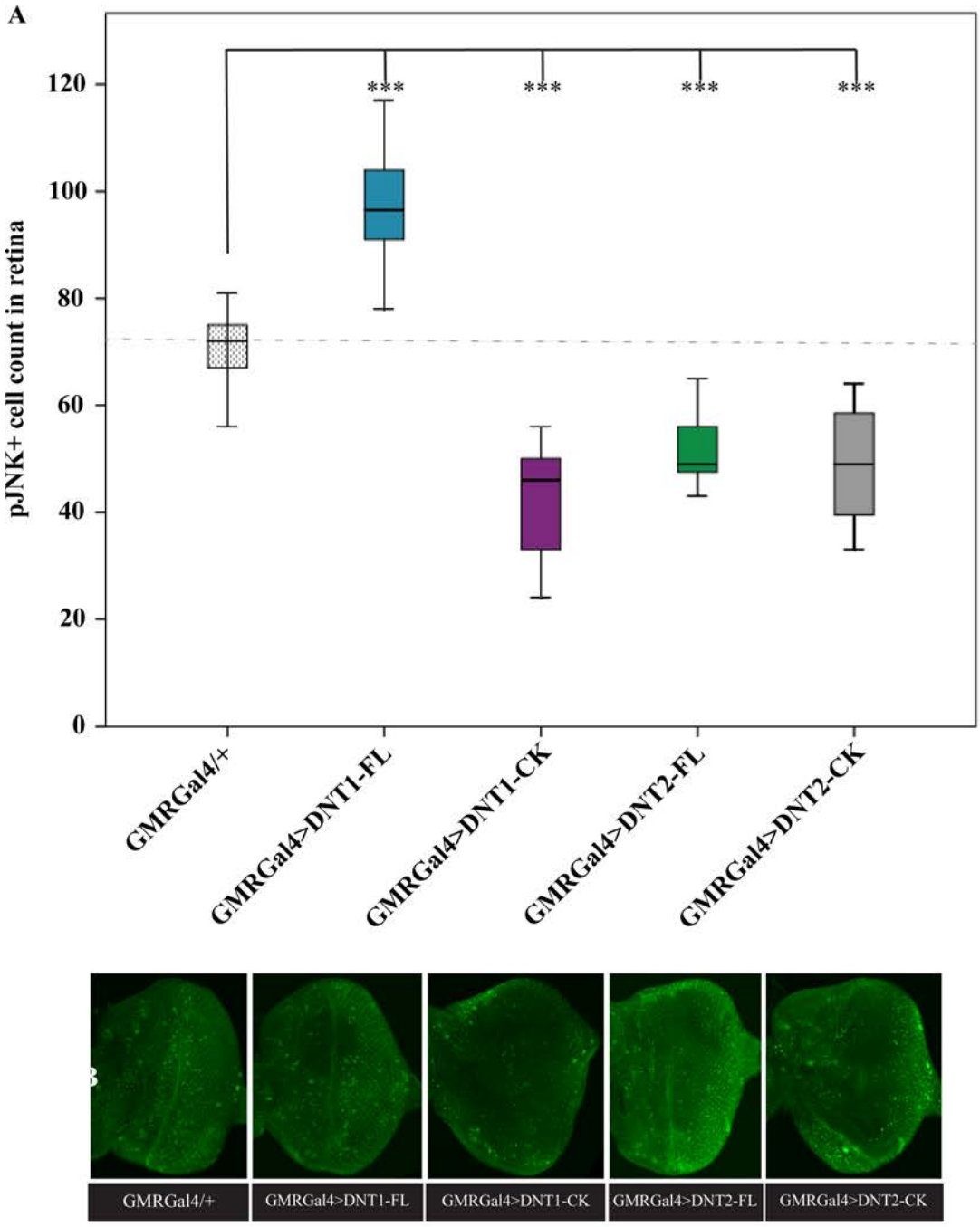
6.2.1 DNTs in the regulation of cell death via the pJNK pathway

As the Tolls appear to be able to influence different outcomes, namely death and survival depending on where they were being expressed and we know that the ligands for Toll-6 and Toll-7 can be promiscuous (McIlroy *et al* 2013) I wondered if the ligand-receptor binding partnership influenced cell fate. And also whether or not the different forms of the ligand determined specific outcomes or if they themselves have distinct functions similar to the Tolls?

In Zhu *et al* 2008, the mature cleaved form of DNT1 (DNT1-CK) was able to cell autonomously regulate neuronal survival in the embryonic CNS, however; both the full length (DNT1-FL) and DNT1 pro domain proteins were unable to reduce neuronal apoptosis in a similar fashion to DNT1-CK. In mammals, apoptosis is achieved via the JNK signalling pathway thus to determine if differentially processed DNTs are able to activate JNK signalling in flies, I used anti-phospho JNK antibodies to stain phospo-JNK+ cells of retina discs of wandering larvae.

Using GMRGal4 to over-express the different forms of DNT1 and DNT2, DNT1-CK, DNT2-CK and DNT2-FL led to a reduction in the number of pJNK+ cells in comparison to the control (Figure 6.1). Therefore it appears that these forms of the DNTs can promote the rescue of cell death in the retina. However; over-expression of DNT1-FL resulted in an

Figure 6.1: DNT1-FL activates the pro-apoptotic pJNK pathway.



DNT1-FL activates the pro-apoptotic pJNK signalling pathway. The graph in panel A shows the over-expression of DNT1-FL results in an increase in the number of pJNK+ cells.Conversley; both forms of DNT2 as well as DNT1-CK lead to a reduction in the number of pJNK+ cells. One Way Anova (*F(4,54)=63.291, p = <0.0001*). The images in panel B are representative of the over-expression of the different forms of the DNTs in retinal discs stained with anti-pJNK.

increase in the number of pJNK+ cells, and it is therefore likely that DNT1-FL is able to activate in wandering larval retina discs the pro-apoptotic JNK signalling pathway.

Consistent with the findings in the embryonic CNS (Zhu *et al* 2008) DNT1-CK, DNT2-FL and DNT2-CK are pro-survival, whereas the full-length form of DNT1 works to activate the pro-apoptotic JNK pathway in larval retina discs.

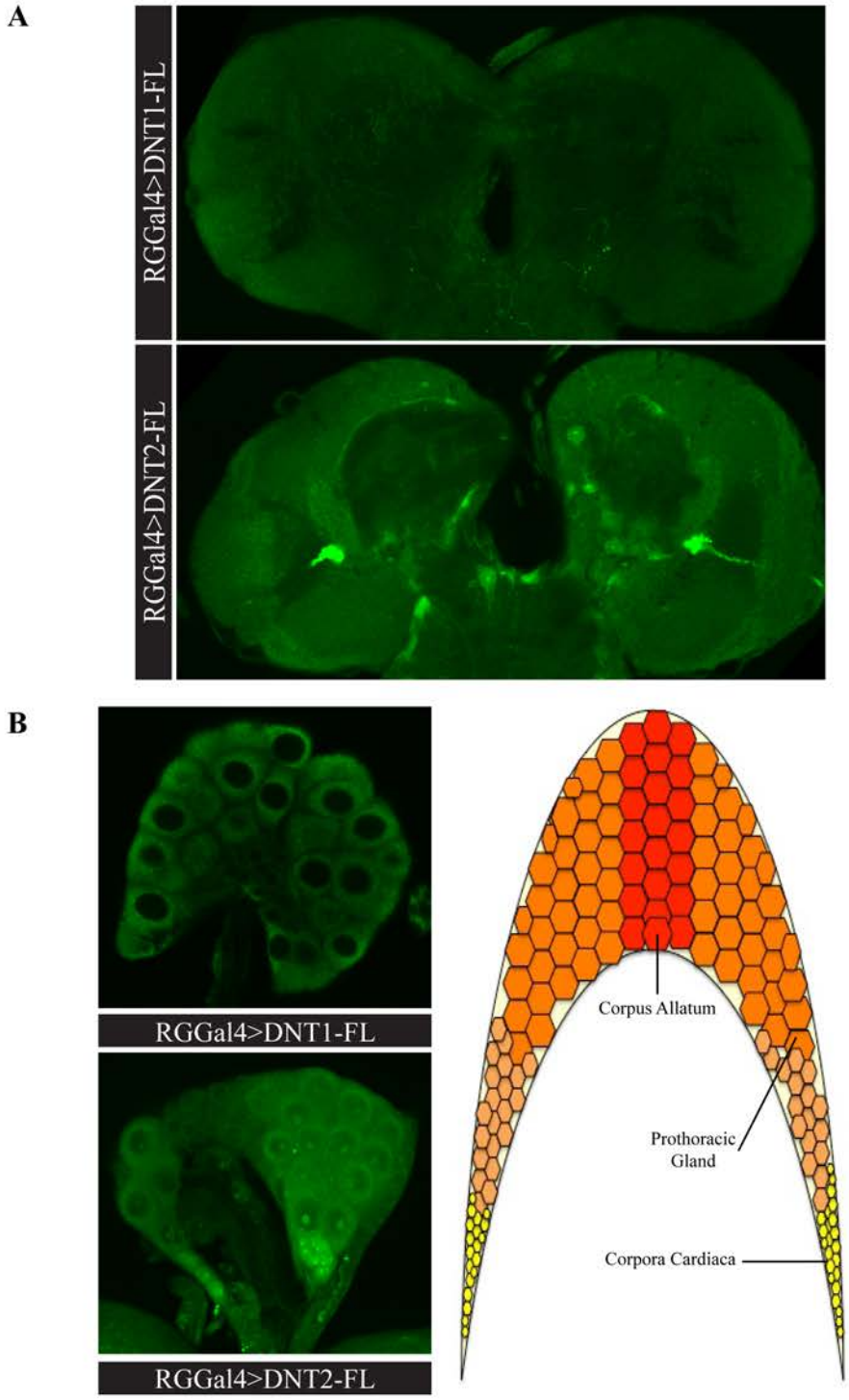
6.2.2 DNTs in the regulation of cell survival via the pERK pathway

In order to investigate which forms of the DNTs, if any, regulate cell survival via the pERK pathway I tested if the *in vivo* over-expression of both DNT1-FL and DNT2-FL (Figure 6.2) in neurons of the larval optic lobes and the ring gland with RG-Gal4. The over-expression of DNT2-FL led to the activation of ERK in a collection of neurons within the optic lobe as well as within the corpora cardiaca of the ring gland. In contrast the over-expression of DNT1-FL did not lead to the activation of pERK in either of these tissues.

6.2.3 MyD88 is expressed throughout the CNS in both larval and pupal developmental stages

Canonical Toll-1 signalling is via the downstream adaptor MyD88. The death domain of MyD88 forms a signalling complex with both Tube and Pelle in order to recruit MyD88 to the membrane (Tauszig-Delamasure *et al.,* 2002; Gay and Gangloff, 2007). Therefore to establish if MyD88 functions as a downstream adaptor of Toll-6, I first determined its expression pattern. I used MyD88^{NP6394}Gal4 in order to drive the expression of membrane tethered

Figure 6.2: Over-expression of DNT2-FL activates ERK signalling



Over-expression of DNT2-FL but not DNT1-FL activates ERK signalling. The images in panel A show that the over-expression of DNT2-FL in the optic lobes results in activation of ERK signalling. The images in panel B show that the over-expression of DNT2-FL in the corpa cardiaca region of the ring gland of the third stage larvae also activate ERK signalling. A schematic diagram of the regions of the ring gland shows the division of cells in this specialised tissue.

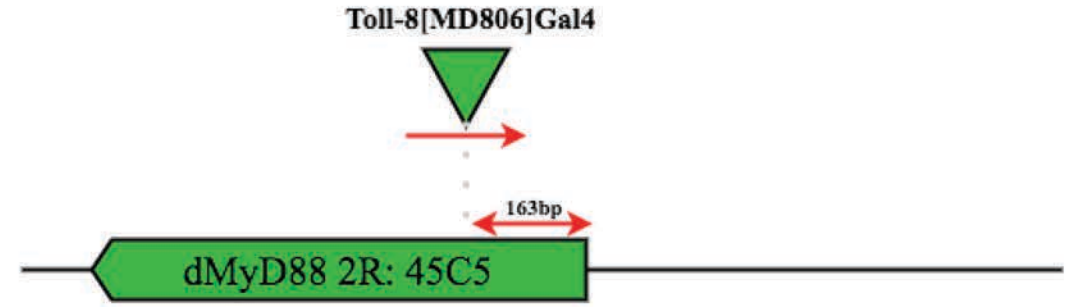
10xUAS-myr-td-Tomato, and anti-DsRed antibodies. In larvae, MyD88^{NP6394} is expressed throughout the CNS in both the VNC and optic lobes (Figure 6.3). The expression of MyD88^{NP6394} is restricted mainly to the thoracic region of the VNC in thoracic interneurons, dorsal projections into and out of the CNS, with some expression in abdominal neurons and central brain region. In pupae MyD88^{NP6394} is expressed at higher levels than were seen in the larval stage (Figure 6.3). Particularly in the VNC were there is an increase in the expression throughout the neuropile and innervations into/out of the neuropile. A lot of the regions where MyD88^{NP6394} is expressed are regions involved in motor circuitry.

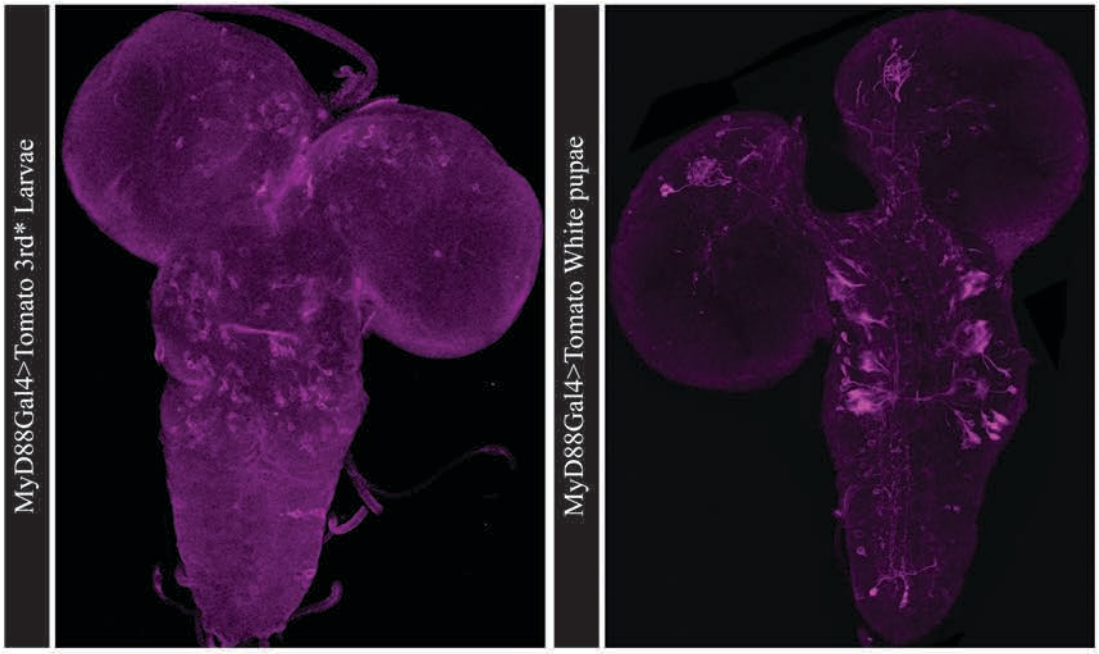
6.2.4 Levels of MyD88 control Eve+ neuron number

As previously mentioned canonical Toll-1 signalling functions via MyD88 (Tauszig-Delamasure *et al.,* 2002). In mammals, mature NTs bind p75NTR in order to promote cell survival via NF-κB activation (Carter *et al.,* 1996; Foehr *et al.,* 2000; Roux and barker 2002; Gutierrez and Davies, 2011), and MyD88 is required for NF-κB activation (Muzio *et al.,* 1997; Wesche *et al.,* 1997). In order to determine if Toll-6 can function via a pro-survival MyD88-NF-κB signalling mechanism I first established if MyD88 is able to regulate neuronal number.

I over-expressed MyD88 in neurons, and tested how this affected Eve+ neuron number in both larval and pupal stages. Over-expression of MyD88 in neurons resulted in an increase in Eve^{+} neurons in larvae (Figure 6.4; ElavGal4>MyD88) in comparison to the control (ElavGal4/+) and there is a further increase in pupae (Figure 6.5; ElavGal4>MyD88), whereas loss of MyD88 function (MyD88CR2.8/DFBSC279) in pupae resulted in a reduction

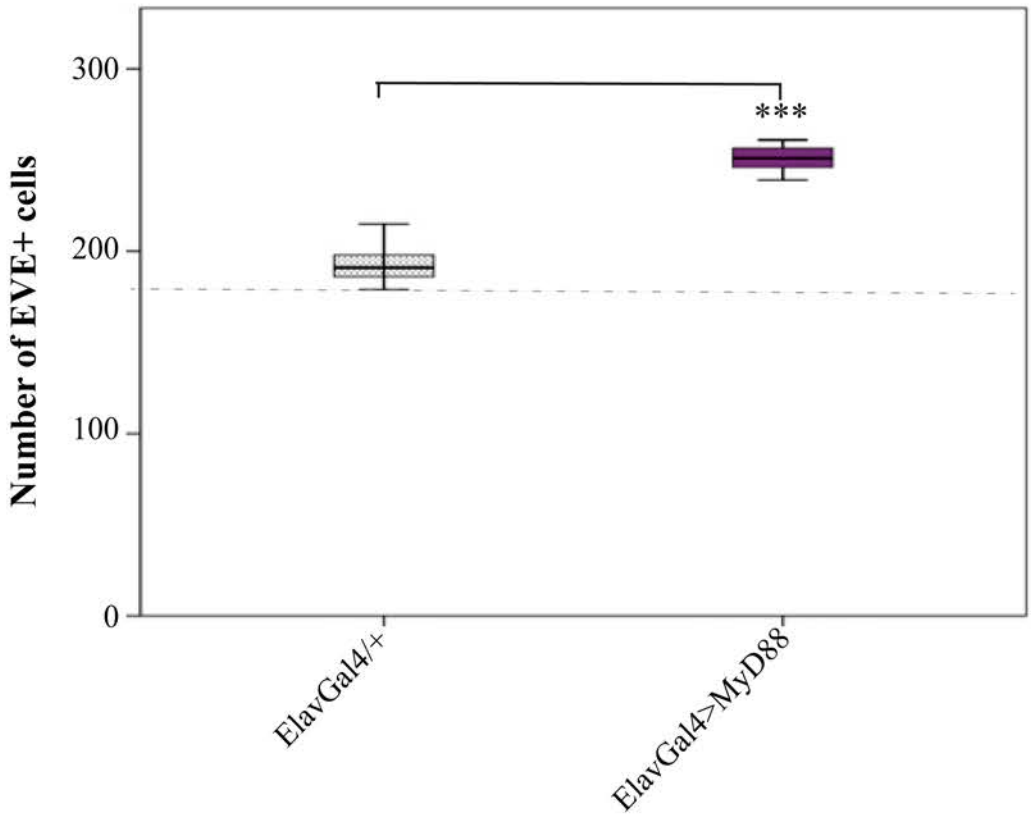
Figure 6.3: MyD88^{NP6394}Gal4 is expressed at higher levels in Pupae

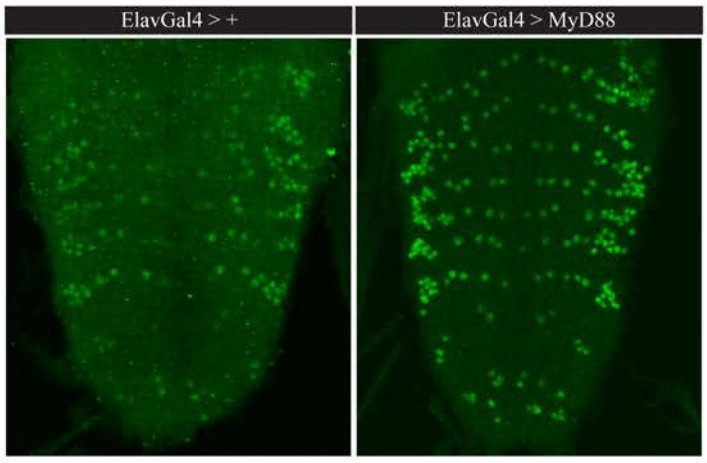




The expression of MyD88 was visualised with MyD88^{NP6394}Gal4>20xUAS-myr-td-Tomato and anti-DsRed. It is expressed widely throughout the CNS in the optic lobes and particularly in the thoracic region of the VNC in thoracic interneurons, dorsal projections into and out of the CNS, with some expression in abdominal neurons and central brain region. The expression of MyD88^{NP6394}Gal4 is upregulated during the pupal stage.

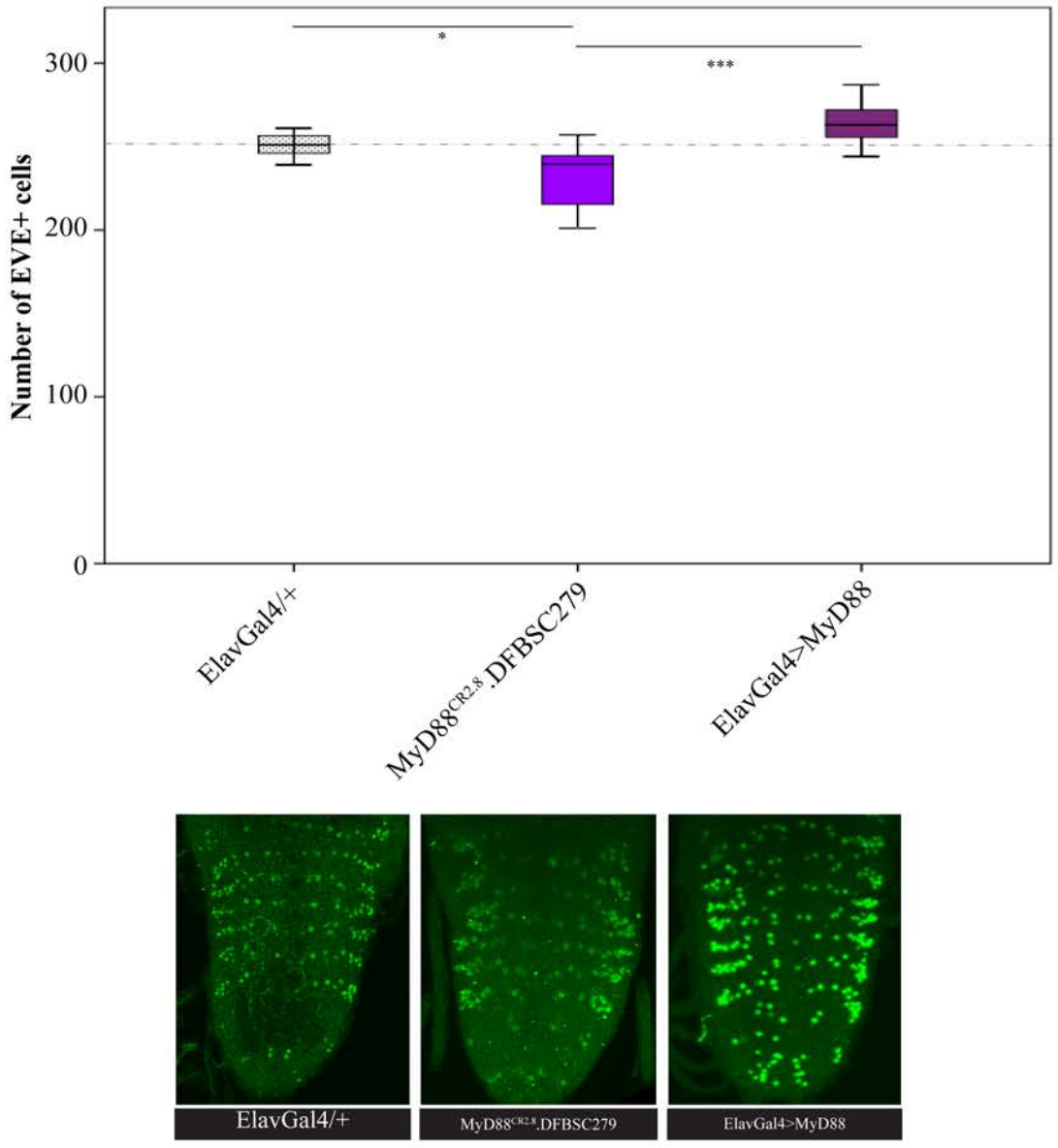
Figure 6.4: Over-expression of MyD88 results in an increase of Eve+ neurons





Over-expression of MyD88 results in an increase in the number of Eve+ neurons. The graph in panel A shows that there is an increase of Eve+ neurons when MyD88 is over-expressed in the VNC indicating that MyD88 is required for neuronal survival. (Students T-Test t= 15.853 , df = 20 , p = 0.000). The images in panel B are representative of the genotypes tested with Eve+ cells stained with anti-Eve and counted automatically using DeadEasy software.

Figure 6.5: Levels of MyD88 control Eve+ neuron number in the pupal CNS.



The level of MyD88 controls Eve+ neuron number. The graph in panel A shows that the overexpression of MyD88 in all neurons (ElavGal4>MyD88) leads to an increase in the number of EVE+ neurons in comparison to the control (ElavGal4/+). Furthermore; MyD88 loss of function ($MyD88^{CR2.8}$.DFBSC279) mutants results in a decrease in the number of Eve+ neurons. Welch One Way ANOVA *($F(2,28)=13.007$, $p = 0.000$).* The images in panel B are representative of the genotypes tested with Eve+ cells stained with anti-Eve and counted automatically using DeadEasy software.

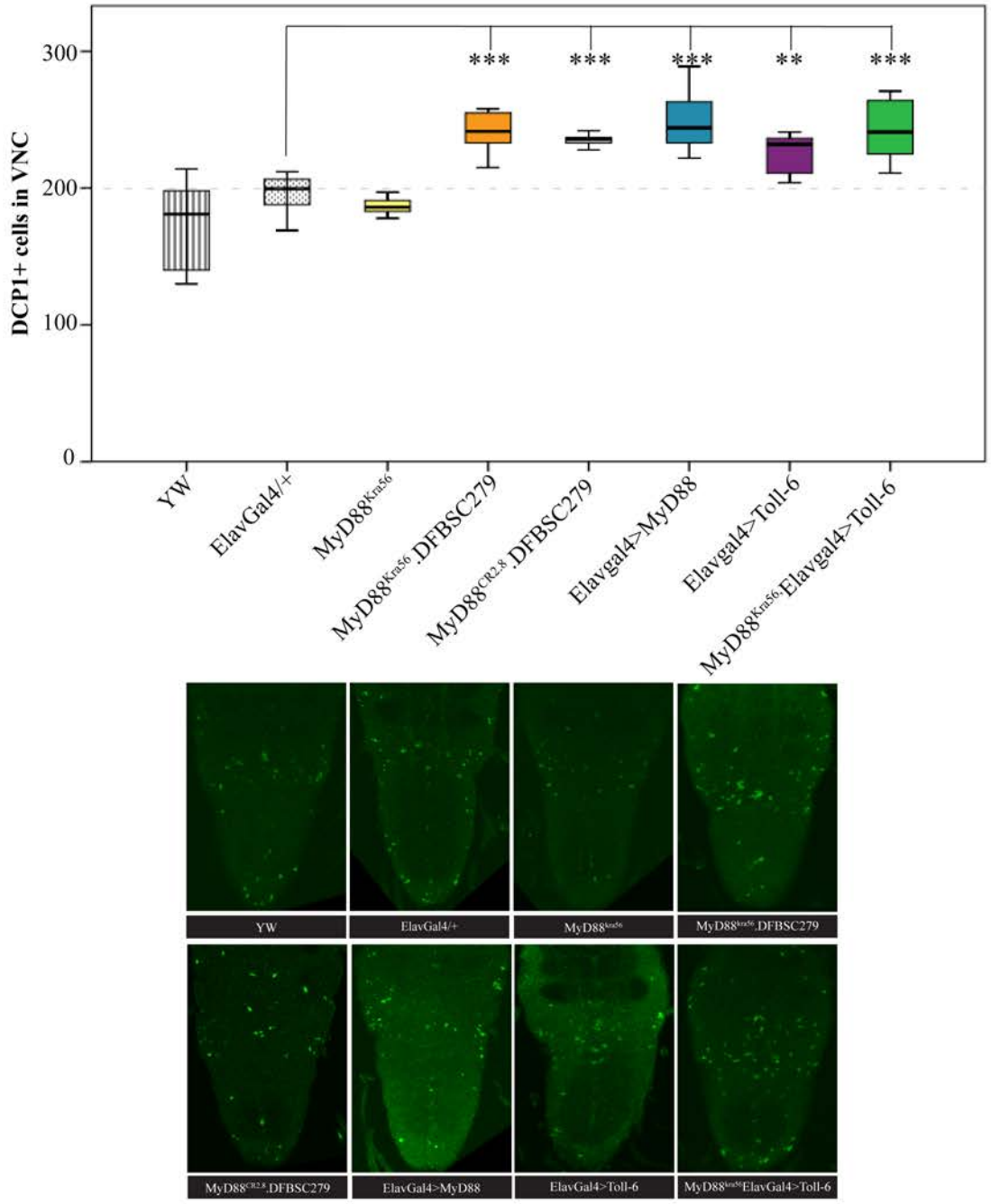
of Eve+ neurons in comparison to the control (ElavGal4/+). This implies that MyD88 promotes neuronal survival at both larval and pupal stages and this effect is particularly important during the pupal stage of development, as there is a further increase in neuronal survival. In order to substantiate the requirement of MyD88 as a pro-survival effector, and its interaction with Toll-6, it was necessary to determine their role in the regulation of a pro-death situation, namely apoptosis.

6.2.5 Apoptosis increases in a MyD88 mutant background

To test whether MyD88 was required downstream of Toll-6 to regulate neuronal survival, it was necessary to determine if it had a role in the regulation of apoptosis. McIlroy *et al.,* 2013 has previously shown that Toll-6 and Toll-7 in the embryonic CNS are able to promote neuronal survival. And I have now shown that Toll-6 is required for cell survival in the larvae. We now know that there is also a period of cell death at the pupal stage of development and needed to determine if MyD88 is required to maintain neuronal survival during this time. Furthermore; $MyD88^{kra56}$ flies are semi-lethal when homozygous and they die at late pupation, another indication of the requirement of MyD88 proper function during this period. To further test if MyD88 was required for survival I tested whether the levels of MyD88 influence the incidence of apoptosis. To test this I used Cleaved *Drosophila* DCP-1 antibodies as a marker of apoptosis and automatic counting of apoptotic cells via Deadeasy software (Forero *et al.,* 2009; Kato *et al.,* 2011; Forero *et al.,* 2012).

Homozygous $MyD88^{kra56}$ apoptosis levels in 10 minute pupae remained the same as in both of the controls (Figure 6.6: YW and ElavGal4/+) but there was a significant increase in

Figure 6.6: Apoptosis increases in a MyD88 mutant background as well as when there is overexpression of MyD88.



Apoptosis increases in a MyD88 mutant background. The graph in panel A shows that apoptotic levels did not change in homozygous $MyD88^{Kra56}$ mutants, but there was a significant increase when put in trans over its deficiency ($MyD88^{Kra56}$.DFBSC279), to the same level as the null CRISPR mutant ($MyD88^{CR2.8}$.DFBSC279). Furthermore there was an increase in apoptosis when MyD88 was over-expressed (ElaGal4> MyD88), when Toll-6 was over-expressed (ElavGal4>Toll-6) and in a $MyD88^{Kra56}$ mutant background ($MyD88^{Kra56}$.Elavgal4>Toll-6). Welch Anova ($F(7,77)$=39.184, p=<0.0001). The images in panel B are representative of the genotypes tested with Dcp1+ cells stained with anti-Dcp1 and counted automatically using DeadEasy software.

apoptosis when in trans over its deficiency (MyD88^{kra56}.DFBSC279) and to the same level as the new CRISPR null mutant generated by the post-doc in our lab Dr Neale Harrison (MyD88$^{CR2.8}$.DFBSC279). These data show that MyD88 is required for cell survival, and Toll-6 may require this signalling pathway for neuronal survival in the CNS. Interestingly when MyD88 is over-expressed (ElavGal4>UASMyD88) there is also an increase in the levels of apoptosis.

However, over-expression of Toll-6 (ElavGal4>Toll-6) in neurons also resulted in an increase in apoptosis, and this increase was furthered in a MyD88 mutant background (MyD88^{kra56};ElavGal4>Toll-6) which is interesting as it implies that Toll-6 has pro-apoptotic functions in the pupal CNS. This effect may be independent of MyD88 function and therefore require a different signalling effector.

6.2.6 dSarm is expressed throughout the CNS of embryos, larvae and pupae

In mammals, SARM (Sterile alpha and armadillo motif-containing protein) binds to TRIF (a TLR adaptor) in order to inhibit signalling (Carty *et al.,* 2006) and can suppress MyD88 mediated signalling (Carlsson *et al.,* 2016). Furthermore, SARM is able to activate neuronal apoptosis (Oneill and Bowie., 2007). In *Drosophila* the homologue of SARM, dSarm, was shown to facilitate axonal destruction following axonal severing (Osterloch *et al.,* 2012) and mediates a signalling pathway involving DNT1 and Toll-8 during immune responses within the trachea (Akhouayri *et al.,* 2011). Therefore I tested if dSarm was involved in the pro-apoptitic signalling that Toll-6 is involved in.

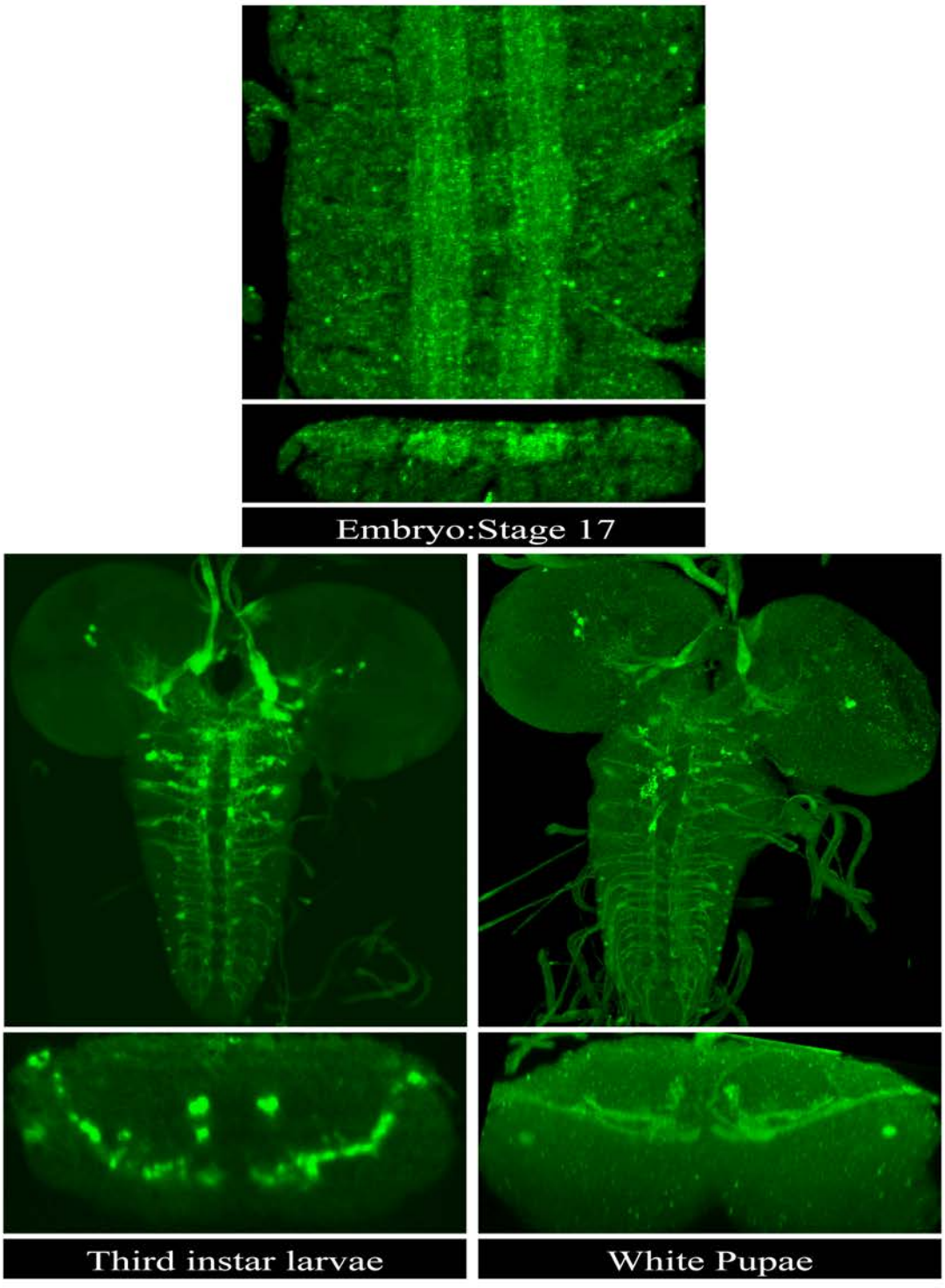
First I wanted to determine how the expression of dSarm relates to that of MyD88 (Figure 6.3) I used immune-histochemistry in the embryonic, larval and pupal stages. dSarm expression was visualized with Ect4^{MIO8854}GFP (Figure 6.7; Ect4 is a synonym of dSarm) which contains a MIMIC-GFP insertion into the dSarm locus and anti-GFP antibodies. In stage 17 embryos, Ect4^{MIO8854}GFP was expressed throughout the CNS neuropile. In both third instar larvae and white pupal stages Ect4^{MIO8854}GFP was dispersed throughout the VNC, particularly within segmentally repeating projections into and out of the neuropile as well as with some projections into the central brain region. The larval patterning was similar MyD88^{NP6394}, however by the pupal stage there are significant differences between the two. Whereas MyD88^{NP6394} is expressed in motor circuits, Ect4^{MIO8854}GFP is localised to regions pertaining to sensory circuits. This indicated that there might be some requirement during the pupal stage for MyD88 and dSarm to have differing functions.

6.2.7 dSarm leads to an increase in apoptosis and antagonises MyD88 function

In order to determine if dSarm facilitates the Toll-6 pro-apoptotic function, I over-expressed EP3610 (EP3610 drives multiple isoforms of Ect4) in neurons in the VNC of 10’ pupae. This resulted in a significant increase in the number of apoptotic cells. Furthermore, when EP3610 was over-expressed in a MyD88 mutant background (MyD88^{kra56};ElavGal4>EP3610) the rate of apoptosis increased further indicating that not only can dSarm induce apoptosis, but it also antagonises Myd88 function in order to do so (Figure 6.8).

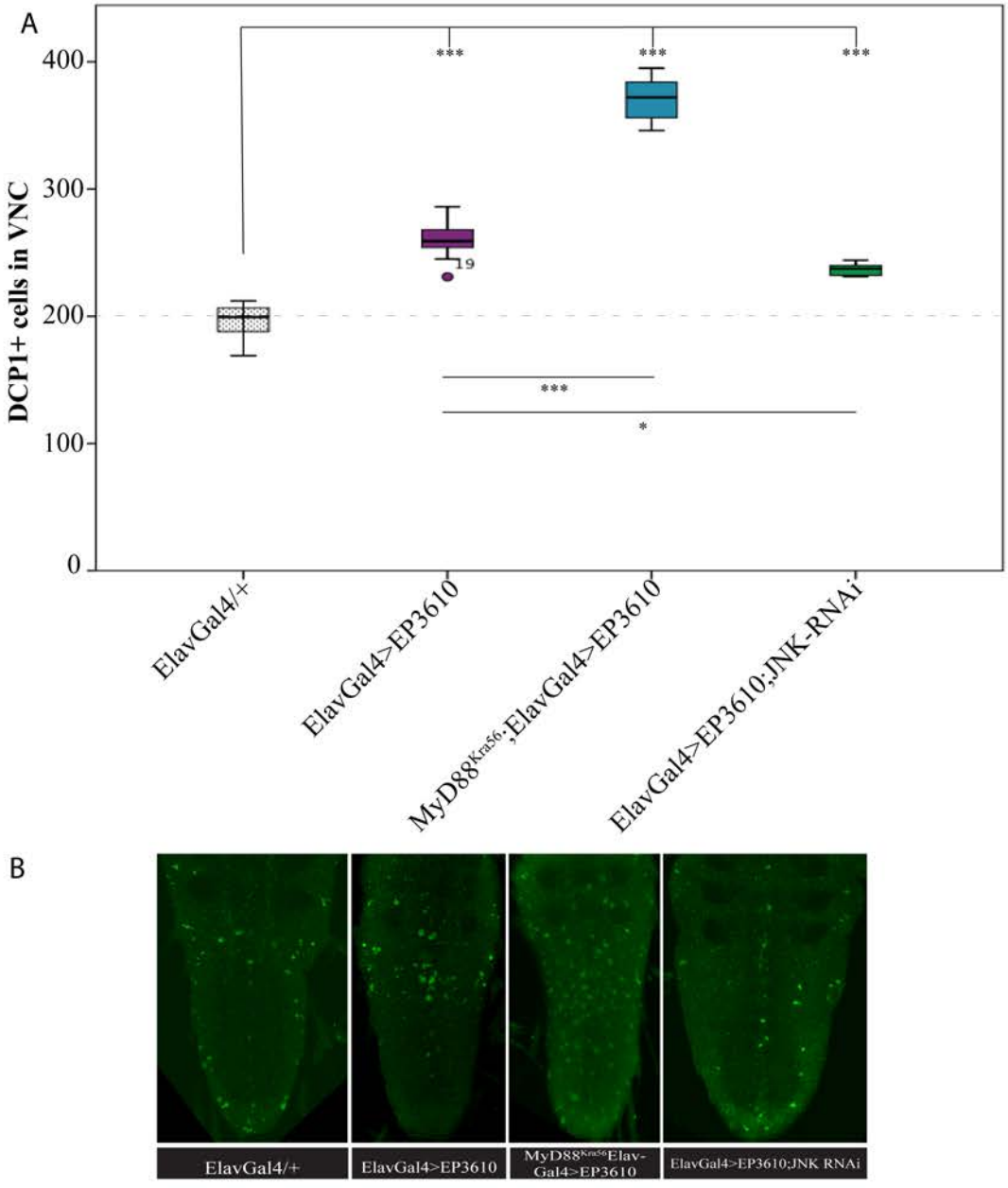
In order to test if dSarm functions via JNK, I tested if knocking down JNK signalling (ElavGal4>EP3610;JNK-RNAi) was able to rescue the increased apoptotic level caused by

Figure 6.7: dSarm is expressed throughout the embryonic, larval and pupal CNS



dSarm was visualised using Ect4^{MIO8854}GFP and anti-GFP antibodies. In the Embryo dSarm was expressed throughout the neuropile, in the larvae and pupae dSarm was expressed throughout many segmentally repeating subsets of CNS neurons as well as projections into and out of the VNC.

Figure 6.8: dSarm leads to an increase in apoptosis, and antagonises MyD88 function



dSarm leads to an increase in apoptosis and is able to antagonise the function of MyD88. The graph in panel A shows that apoptosis is significantly increase when dSarm is over-expressed (ElavGal4>EP3610), and this increase is furthered in a MyD88 mutant background (MyD88^{Kra56};ElavGal4>EP3610) indicated that dSarm may be antagoniseing the function of MyD88. Furthermore; Apoptosis is reduced when over-expressing dSarm when JNK signaling is inhibited via RNAi (ElavGal4>EP3610;JNK-RNAi) indicating that the ability of dSarm to induce apoptosis is reliant upon the JNK signaling pathway. One Way Anova ($F(3,42)$=355.12, p=<0.0001). The images in panel B are representative of the genotypes tested with Dcp1+ cells stained with anti-Dcp1 and counted automatically using DeadEasy software.

the over-expression of dSarm (ElavGal4>EP3610) (Figure 6.8). JNK knockdown resulted in a significant reduction in the level of apoptosis, indicating that dSarm induces apoptosis and via the JNK signalling pathway.

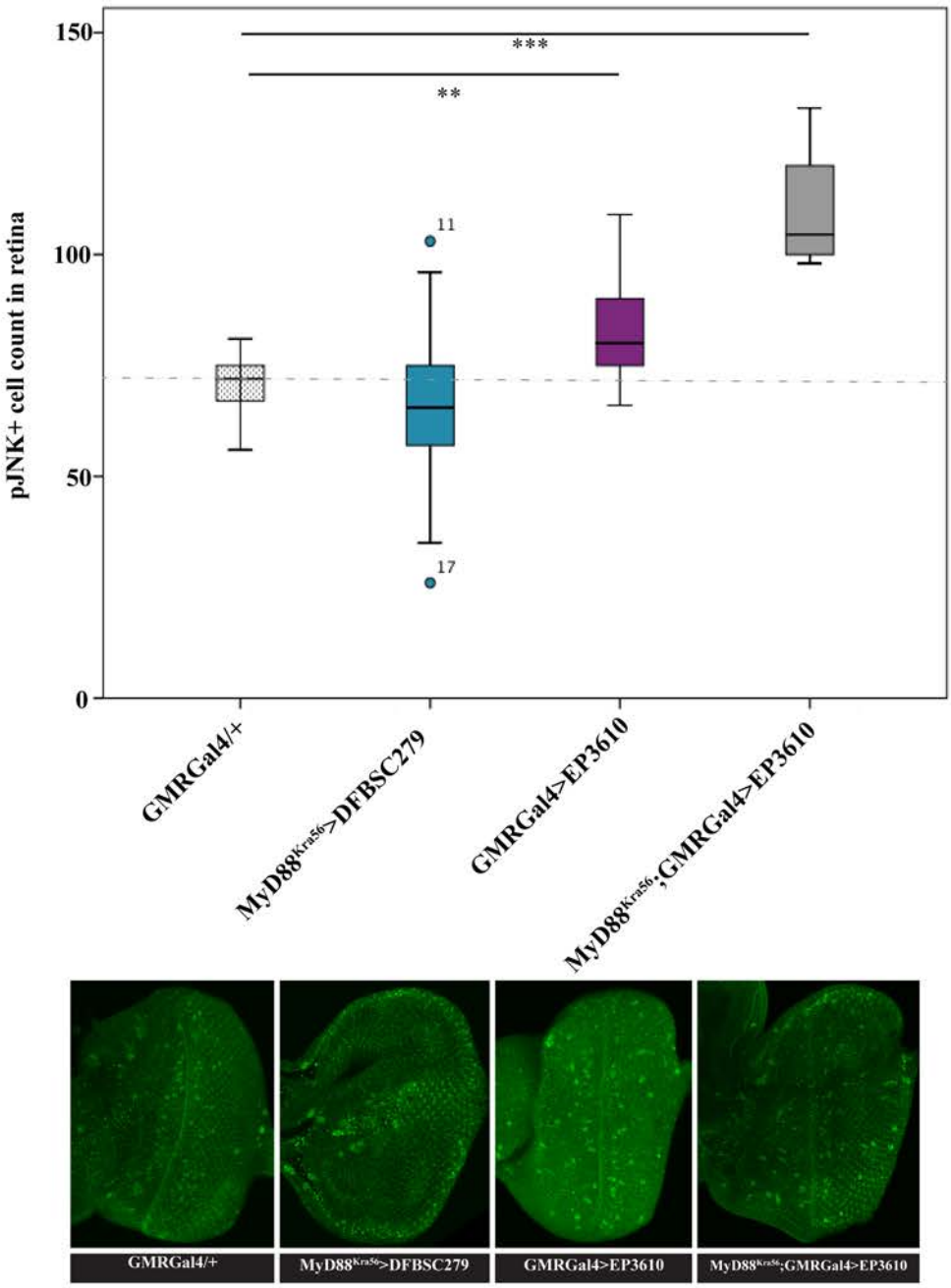
6.2.8 dSarm activates JNK signalling in vivo and antagonises MyD88 function

In order to confirm that dSarm is able to activate JNK signalling in vivo and to determine if MyD88 had any influence, I stained third instar larvae retinal discs (using GMRGal4) with pJNK and counted the number of pJNK+ cells. I used retinal discs as phospho-JNK antibodies stain axons within the neuropile, preventing nuclear signal being visualised. MyD88 mutants ($MyD88^{kra56}$.DFBSC279) displayed no increase of pJNK+ cells in comparison to the control (GMRGal4/+). Over-expression of dSarm (GMRGal4>EP3610) in retina resulted in an increase in the number of pJNK+ cells and this was further increased in a MyD88 mutant background ($MyD88^{kra56}$GMRGal4>EP3610) (Figure 6.9). Therefore dSarm is able to induce apoptosis via the JNK signalling pathway and is able to antagonise MyD88 function in doing so.

6.2.9 Over-expression of dSarm leads to a decrease in Eve+ neurons

In order to determine if the apoptotic effects of dSarm results in the loss of Toll-6 neurons, I used the Eve antibody (Eve neurons express Toll-6) to stain Eve neurons which where counted with Deadeasy software. Both the over-expression of dSarm (Elavgal4>EP3610) as well as in a MyD88 mutant background ($MyD88^{kra56}$ElavGal4>EP3610) resulted in a reduction in the number of Eve+ neurons in comparison to the control (ElavGal4/+) (Figure

Figure 6.9: dSarm activates JNK signalling and antagonises MyD88 function



dSarm activates JNK signalling and antagonises MyD88 function. The graph in panel A shows that MyD88^{kra56} mutants (MyD88^{kra56}.DFBSC279) do not induce Apoptosis, whereas the over-expression of dSarm (GMRGal4>EP3610) increased pJNK+ cell number and this was further increased in a MyD88 mutant background (MyD88^{kra56}GMRGal4>EP3610) indicating that dSarm activates apoptosis and antagonises MyD88 function. One Way Anova (*F(3,38)=9.315, p = <0.0001.*The images in panel B are representative of the genotypes tested with pJNK+ cells stained with anti-pJNK and counted automatically using DeadEasy software.

6.10). This indicates that not only can dSarm induce apoptosis but it also leads to neuronal loss.

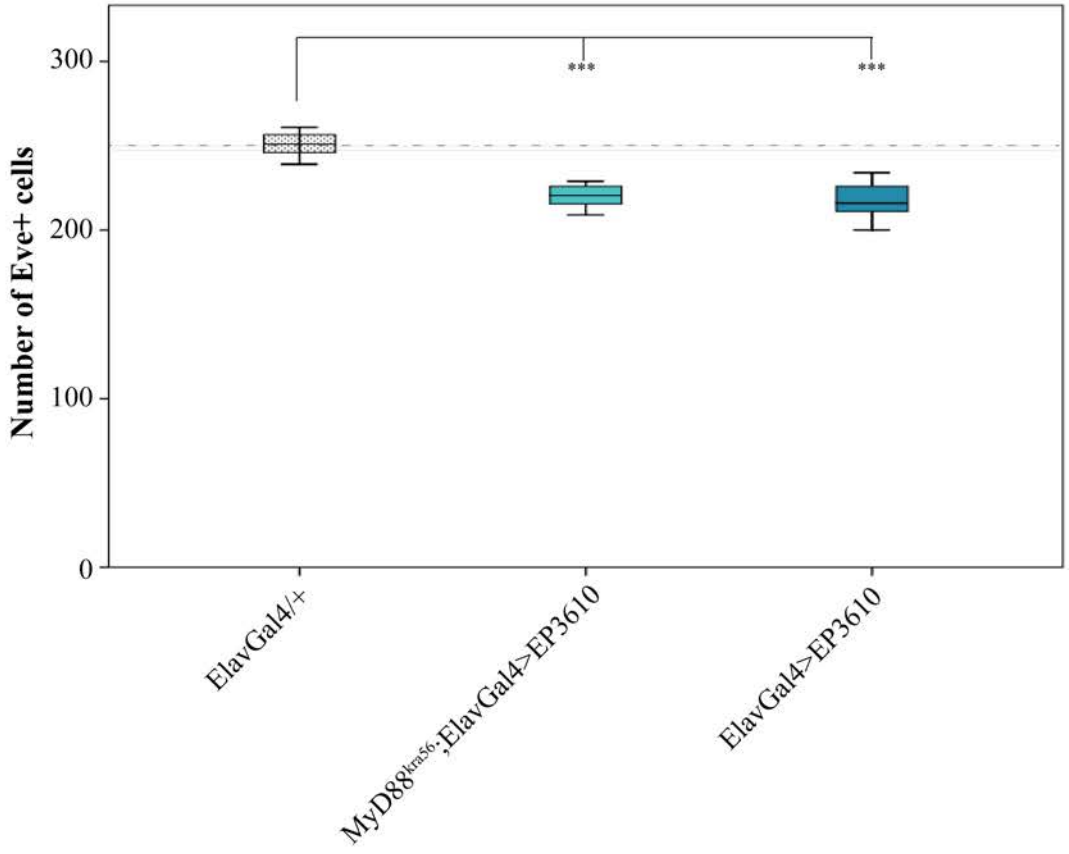
6.2.10 Apoptosis increases in a Wek mutant background

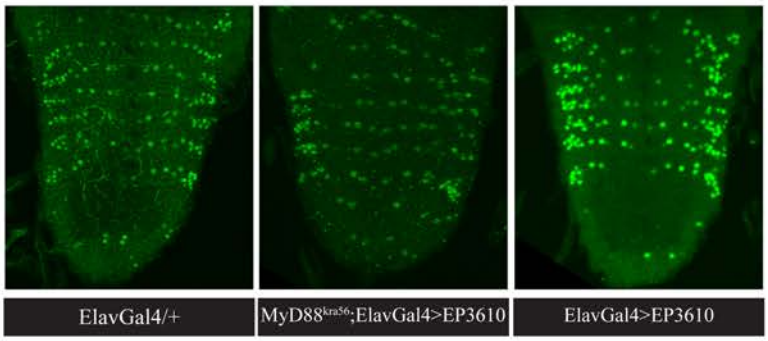
In order for Toll-6 to be pro-apoptotic in the pupal stage of development, there must be another adaptor protein that links both Toll-6 and dSarm in order to regulate their pro-apoptotic functions. Luschnig *et al.,* 2004 identified Weckle (Wek), which encodes a zinc finger transcription factor that controls embryonic dorso-ventral patterning. Furthermore; Chen *et al.,* 2006 identified Wek as an adaptor protein that stabilises the Toll-Wek-MyD88-Tube complex by binding to Toll and then binding to and localising MyD88 to the plasma membrane in embryos. Therefore it was imperative to test the relationship of Wek in the pro-apoptotic functioning of Toll-6 and dSarm.

In order to test this relationship I carried out epistasis experiments using Wek mutant flies to determine the effects of loss and gain of function of Wek on the rates of apoptosis. The over-expression of Wek (Figure 6.11; ElavGal4>Wek) resulted in an increase in apoptosis in comparison to the control (ElavGal4/+), and this was rescued in a Wek mutant background (Wek^{EX14};ElavGal4>Wek). Furthermore; the increase of apoptosis caused by the over-expression of Toll-6 (ElavGal4>Toll-6) is rescued by the loss of Wek function (Wek^{EX14};ElavGal4>Toll-6), indicating that Toll-6 requires Wek in order to induce apoptosis.

The over-expression of Wek in a dSarm loss of function (ElavGal4>dSarmRNAi,Wek) again points towards the requirement of dSarm in inducing apoptosis. The over-expression of

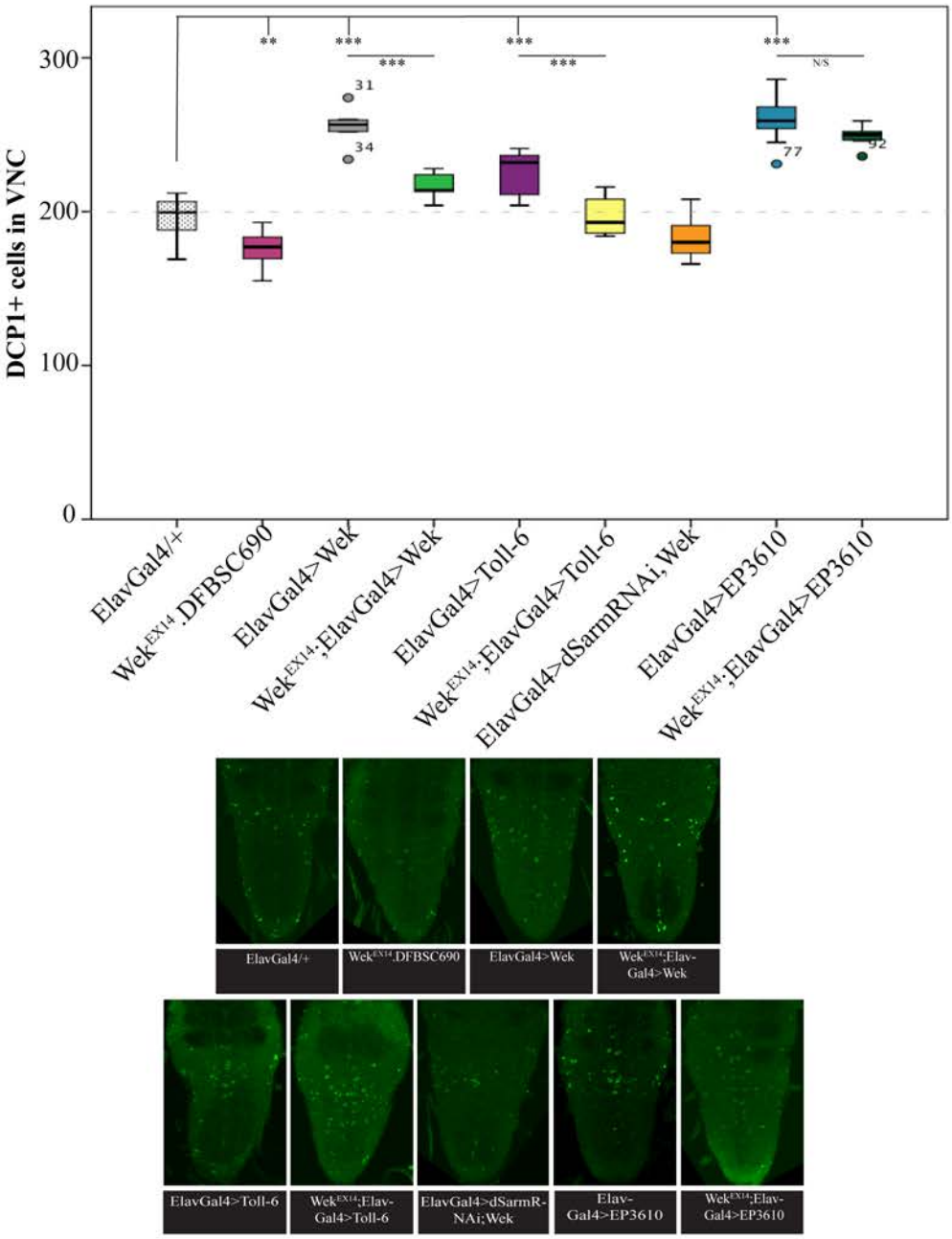
Figure 6.10: Overexpression of dSarm leads to a decrease in Eve+ neurons in the abdominal region of the pupal VNC.





The over-expression of dSarm results in a decrease in Eve+ neurons in the VNC of pupae. The graph in panel A shows that the overexpression of dSarm in neurons (ElavGal4>EP3610) as well as in a MyD88 mutant background (MyD88^{kra56};Elav-Gal4>EP3610) resulted in the reduction of Eve+ neurons. One Way ANOVA *(F(2,30)=59.151, p = 0.000).* The images in panel B are representative of the geno-types tested with Eve+ cells stained with anti-Eve and counted automatically using DeadEasy software.

Figure 6.11: Apoptosis increases in a Wek mutant background



The levels of apoptosis increase in a Weckle mutant background. The graph in panel A shows that apoptosis is reduced in Wek mutants (Wek^{EX14}.DF690) but increased when Wek was over-expressed (ElavGal4>Wek) which was subsequently rescued in a Wek mutant background (Wek^{EX14};ElavGal4>Wek).Toll-6 over-expression (ElavGal4>Toll-6) was also rescued when in a Wek muatnt background (Wek^{EX14};ElavGal4>Toll-6). dSarm over-expression (Elav-Gal4>EP3610) was not rescued in a Wek mutant background (Wek^{EX14};ElavGal4>EP3610) but over-expressing Wek when reducing the levels of dSarm (ElavGal4>dSarmRNAi,Wek) rescues apoptosis caused by Wek suggesting Wek functions upstream of dSarm. One Way Anova ($F(8,83)$=64.148, p=<0.0001). The images in panel B are representative of the genotypes tested with Dcp1+ cells stained with anti-Dcp1 and counted automatically using Dead-Easy software.

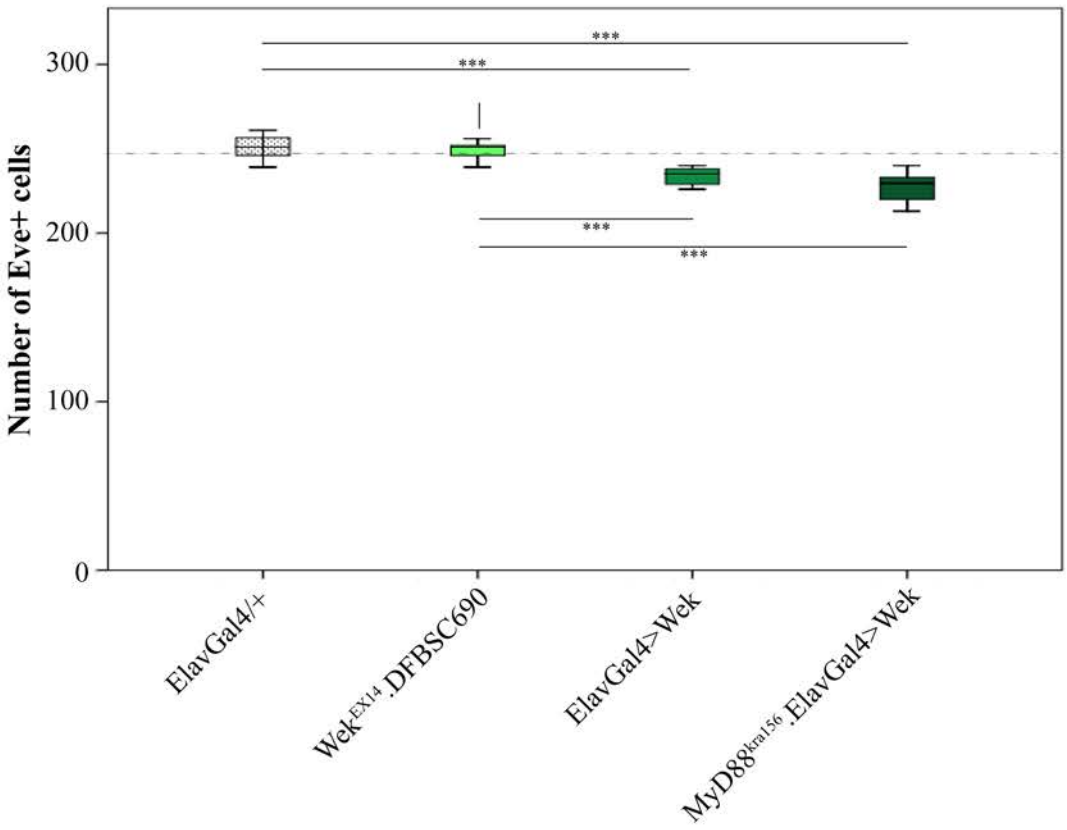
dSarm (ElavGal4>EP3610) results in an increased rate of apoptosis; however; the loss of function of Wek (Wek^{EX14};ElavGal4>EP3610) did not rescue this increased apoptotic rate from the over-expression of dSarm and thus Wek functions upstream of dSarm in order to regulate cell-death (Figure 6.11).

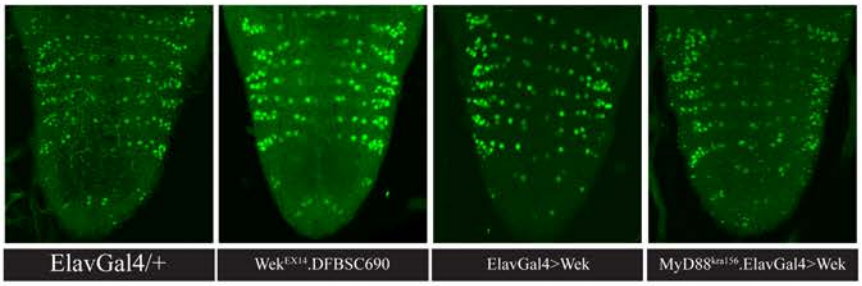
6.2.11 Over-expression of Wek leads to a decrease in Eve+ neurons

As Wek is required for the pro-apoptotic signalling down-stream of Toll-6 and upstream of dSarm, and both Toll-6 and dSarm can lead to apoptosis, it was important to establish the effect of Wek on the regulation of cell number via neuronal loss. In order to confirm if the functioning of Wek results in the loss of Toll-6 neurons I used the Eve antibody to stain Eve+ neurons in white pupae. I counted Eve+ cells in the abdomen (See Figure 2.8 for landmarks) and counted cells using automatic Deadeasy software (Forero *et al.*, 2009; Kato *et al.*, 2011; Forero *et al.*, 2012).

Wek mutants (Figure 6.12: Wek^{EX14}.DFBSC690) had the same number of Eve+ neurons as the control group (ElavGal4/+). However; the over-expression of Wek (ElavGal4>Wek) in neurons as well as in a MyD88 mutant background ($MyD88^{kra56}$.ElavGal4>Wek) resulted in a decrease in the number of Eve+/Toll-6+ cells. This indicates that Wek is not only capable of promoting apoptosis but can also mediate the loss of Toll-6 neurons.

Figure 6.12: Overexpression of Wek leads to a decrease in Eve+ neurons.





The over-expression of Weckle results in an decrease in Eve+ neurons. The graph in panel A shows that the overexpression of Wek in neurons (ElavGal4>Wek) and in a MyD88 mutant background (MyD88^{kra156}.ElavGal4>Wek) leads to a decrease in the number of Eve+ neurons in comparison to the control (ElavGal4/+) and aWek mutant (Wek^{EX14}.DFBSC690). One Way ANOVA *(F(3,36)=27.924, p = 0.000).* The images in panel B are representative of the genotypes tested with Eve+ cells stained with anti-Eve and counted automatically using DeadEasy software.

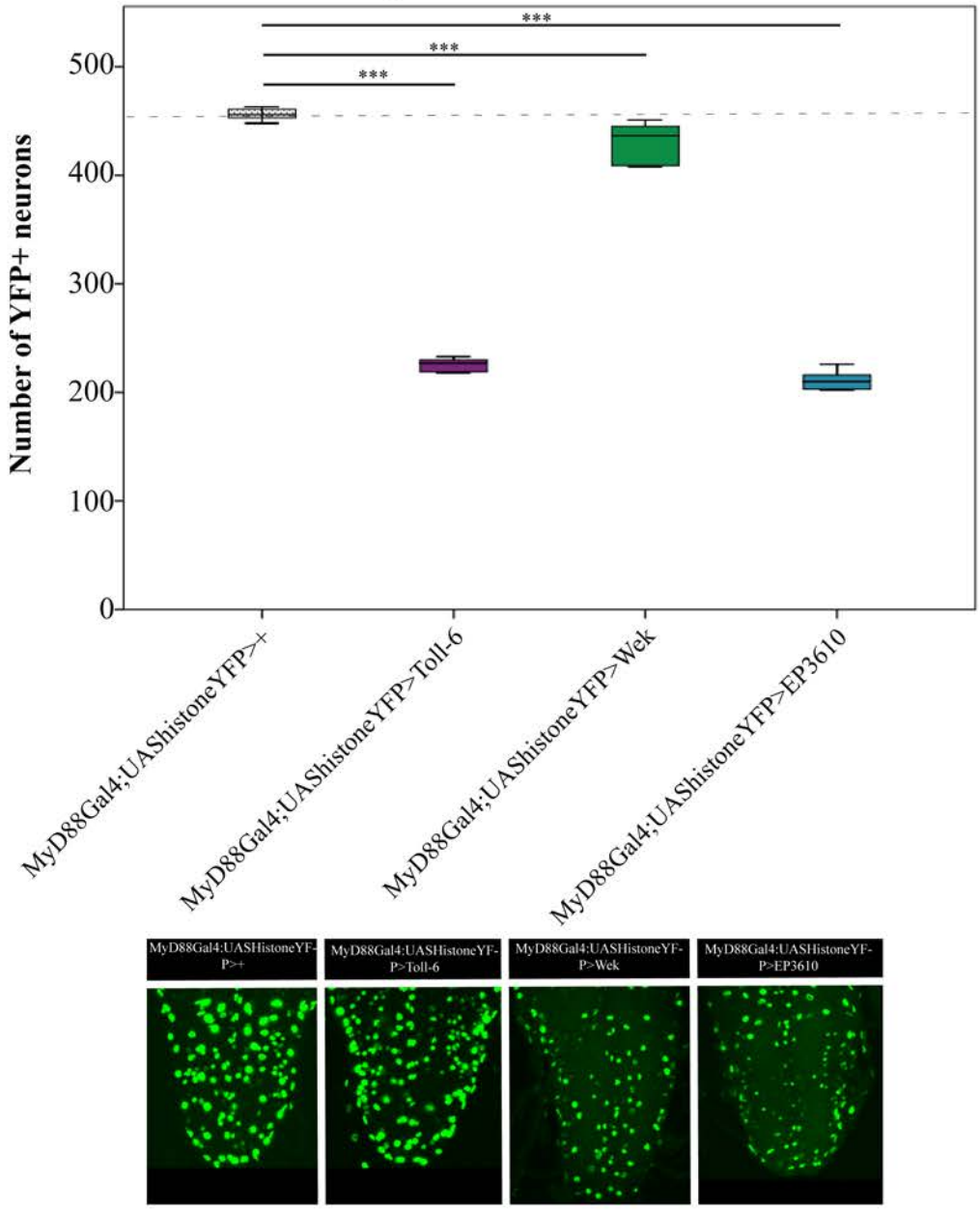
6.2.12 Over-expression of Wek, Toll-6 and dSarm in MyD88 cells leads to a reduction in Histone-YFP+ cell number

It appears that Wek is functioning as the link between pro-survival and pro-death outcomes mediated by the Toll-6 - MyD88 and Toll-6 – dSarm pathways. In order to determine if Wek and dSarm altered the fate of MyD88 neurons, I utilised MyD88Gal4 and drove the expression of Histone YFP, a nuclear marker. The over-expression of Wek (MyD88Gal4;UAShistoneYFP>Wek), Toll-6 (MyD88Gal4;UAShistoneYFP>Toll-6) and dSarm (MyD88Gal4;UAShistoneYFP>Wek), resulted in a reduction of MyD88+ cells (Figure 6.13). It appears as though in pupal VNCs when there is an increase of Toll-6, pro-death outcomes are more likely, most likely facilitated by Wek and dSarm. And similarly the increase of both Wek and dSarm in MyD88 expressing cells has the ability to control cell death downstream of Toll-6.

6.2.13 Over-expression of Wek, Toll-6 and dSarm in MyD88 cells leads to a reduction in Eve+ neurons

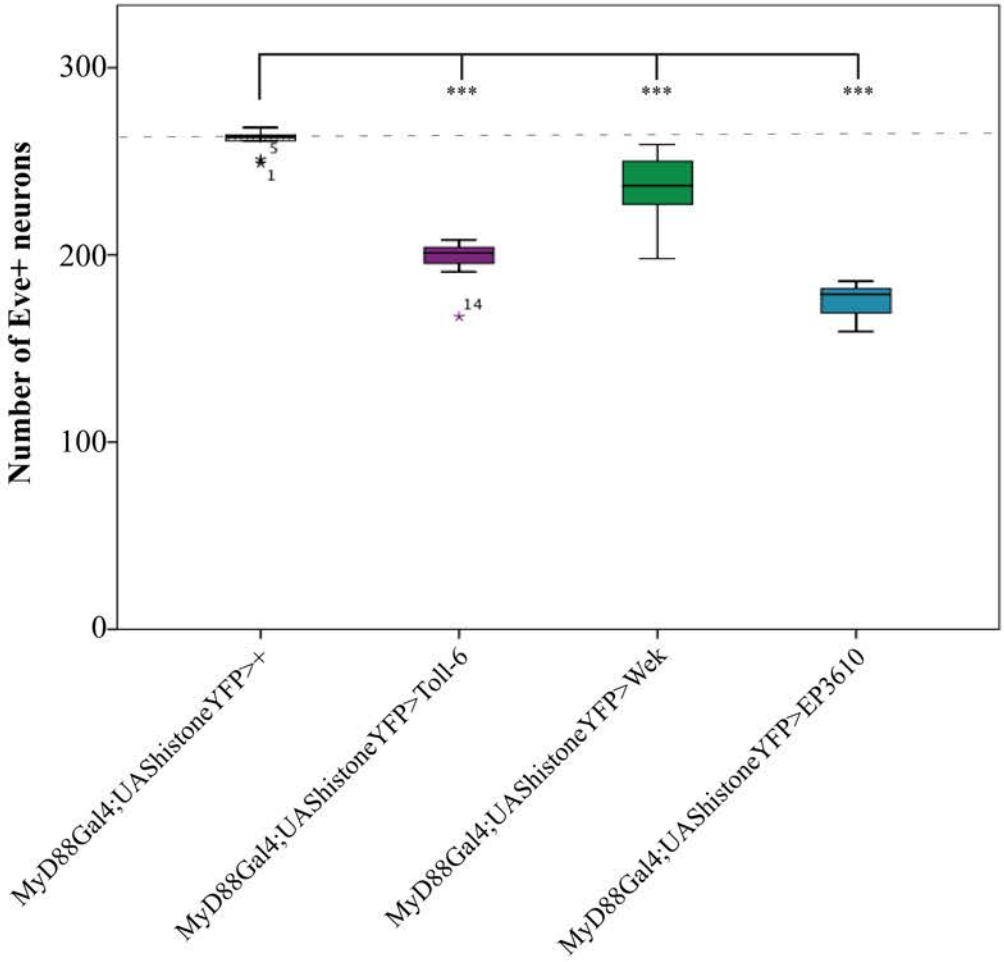
To test this ability to control cell death I looked at the effect the over-expression of Toll-6, Wek and dSarm in MyD88+ cells had on neuronal cell number using Eve antibodies. A similar profile to the over-expression of Wek and dSarm in MyD88Gal4+ expressing cells was seen with Eve+ cell number. Whereby, their over-expression (MyD88Gal4;UAShistoneYFP>Wek, -dSarm) resulted in a decrease in the number of Eve+ neurons in comparison to the controls (Figure 6.14). Thus by increasing the amount of Wek and dSarm in MyD88+ cells there is an increase in the loss of neuronal number, and therefore

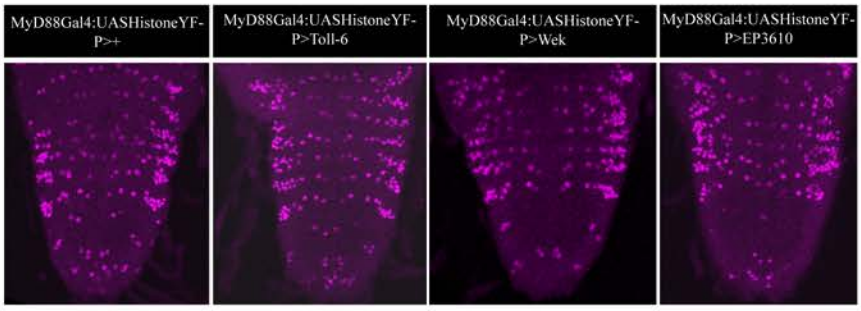
Figure 6.13: Over-expression of Wek, Toll-6 and Sarm in MyD88 cells leads to a reduction in Histone-YFP+ cell number in comparison to controls.



The over-expression of Wek, Toll-6 and dSarm in MyD88 cells results in reduced histone-YFP cell number. The graph in panel A shows that the over-expression of UAS Wek (MyD88Gal4;UASHistoneYFP>Wek) and to a greater extent; UAS Toll-6 (MyD88Gal4;UASHistoneYFP>Toll-6) and UAS Sarm (MyD88Gal4;UASHistoneYF-P>EP3610) in MyD88+ cells driving expression of the nuclear marker Histone-YFP leads to a decrease in the number of YFP+ cells respectivley in comparison to control. One Way Anova ($F(3,23)=996.258$, $p = <0.0001$. The images in panel B are representative of the genotypes tested with Histone-YFP cells counted automatically using Dead-Easy software.

Figure 6.14: Over-expression of Wek, Toll-6 and Sarm in MyD88 cells leads to a reduction in Eve+ neurons in comparison to controls.





The over-expression of Wek, Toll-6 and dSarm in MyD88+ cells results in a decrease in Eve+ neurons. The graph in panel A shows that the over-expression of UAS Wek (MyD88Gal4;UASHistoneYFP>Wek), UAS Toll-6 (MyD88Gal4;UASHistoneYF-P>Toll-6) and UAS dSarm (MyD88Gal4;UASHistoneYFP>EP3610) in MyD88+ cells leads to a decrease in the number of Eve+ neurons respectivly in comparison to controls. One Way Anova ($F(3,24)=59.492, p = <0.0001$). The images in panel B are representative of the genotypes tested with Eve+ stained with anti-Eve and counted automatically using DeadEasy software.

this complex with all three adaptor proteins as well as the receptor, play a crucial role in determining cell outcomes.

6.3 SUMMARY

In this chapter I have shown (Figure 6.15 & Table 6.1):

1) DNT1-FL activates pro-apoptotic JNK signalling
2) DNT2-CK activates pro-survival ERK signalling
3) MyD88 is expressed throughout the CNS motor circuits and is required for neuronal survival
4) In the pupae, Toll-6 is pro-apoptotic, independently of MyD88 function
5) dSarm is expressed in sensory circuits, and expression of dSarm and MyD88 diverge during the pupal stage
6) dSarm promotes apoptosis via JNK signalling pathway and antagonises MyD88 function
7) Overexpression of dSarm results in neuronal loss
8) Wek can promote apoptosis and Toll-6 requires Wek for its pro-apoptotic functions
9) Wek functions upstream of dSarm
10) Wek can mediate the loss of Toll-6 neurons
11) In pupae an increase in Toll-6 results in elevated cell death via dSarm and Wek, and contains positive regulatory feedback mechanisms.

In mammals, the regulation of cell number by NTs is dependent upon the cleavage state of the ligand, the receptor it binds and finally downstream signalling pathways. Full length NTs bind $p75^{NTR}$ and sortilin in order to activate JNK signalling and induce apoptosis. Mature NTs bind $p75^{NTR}$ and Trk receptors in order to activate NF-κB and MAPKinase/ERK signalling pathways (Carter *et al.*, 1996; Foehr *et al.*, 2000; Roux and Barker 2002; Lu *et al.*, 2005; Minichhiello 2009; Gutierrez and Davies. 2011). In this chapter I have shown that only

Figure 6.15 Proposed Model

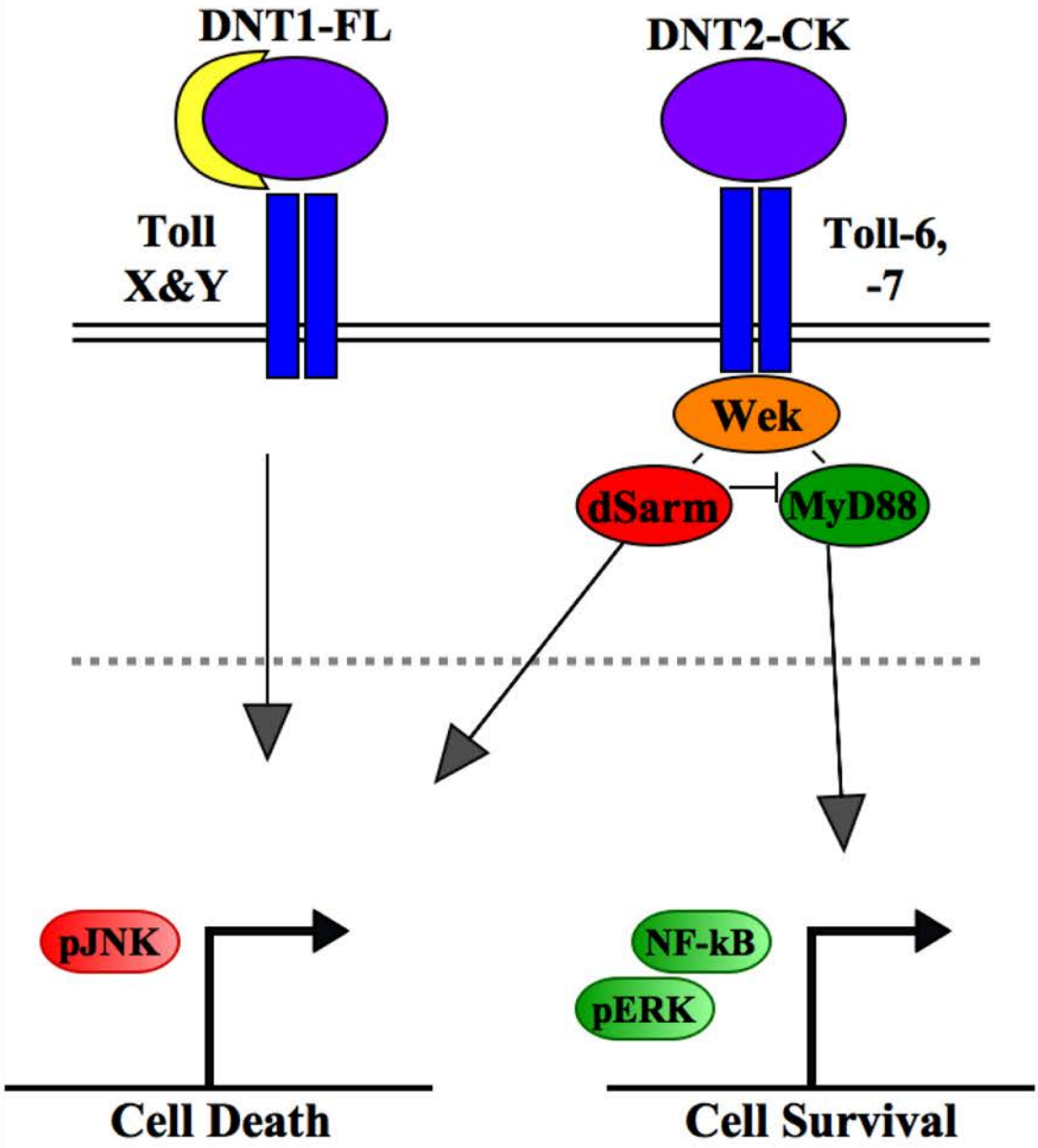


Table 6.1: Table of results from Chapter 6

	CHAPTER 6				
	pJNK activation	pERK activation	Expression of signalling adaptors		
	GMRGal4> OE				
	3* Larvae		Embryo	3* Larvae	Pupae
DNT1-FL	Y	N	-	-	-
DNT1-CK	N	Y	-	-	-
DNT2-FL	N	Y	-	-	-
DNT2-CK	N	Y	-	-	-
MyD88Gal4^{NP6394}	-	-	Y	Y	Y
Ect4^{MIO8854}	-	-	Y	Y	Y

OE	Over-expression

	Genotype significiantly different from control in both OE & KD or shows expression
N	Genotype not significiantly different from control
-	Not tested

DNT1-FL activates pro-apoptotic JNK signalling. In contrast the mature forms of DNT-1 and DNT-2 (Foldi, Anthoney *et al.,* 2017) as well as DNT2-FL are able to activate ERK. Furthermore DNT2-CK can also activate NF-κB signalling downstream of Toll-6 and Toll-7, and Toll-6 and Toll-7 can activate ERK (Foldi, Anthoney *et al.,* 2017).

Interestingly from this assay it appears as though the full-length form of DNT-2 activates ERK. However site directed mutagenesis of DNT-1 and DNT-2 proteins show DNT-1 is secreted as a pro-protein, whereas DNT-2 is cleaved and secreted as a mature protein (Foldi, Anthoney *et al.,* 2017). Therefore, DNT2-FL may not be able to activate ERK as described, instead it may be cleaved and the expression is that of the mature form. Therefore these results are highly similar to what occurs in the mammalian system whereby full-length neurotrophins are able to activate the pro-apoptotic JNK pathway, and cleaved neurotrophins are able to activate the pro-survival pathways.

In flies, DNTs bind Toll receptors in order to promote neuronal survival (Weber *et al.,* 2003; Zhu *et al.,* 2008; McIlroy *et al.,* 2013), and I had previously shown that Toll-6 is pro-survival in larvae. Canonical Toll-1 signalling occurs via MyD88 (Tauszig-Delamasure *et al.,* 2002), and MyD88 can form a signalling complex with both Toll-6 and Toll-7 (Foldi, Anthoney *et al.,* 2017). Therefore in order to determine if MyD88 is involved in the promotion of cell survival, I showed that MyD88 is expressed throughout the CNS of both larva and pupae. Furthermore when MyD88 is over-expressed there was an increase in neuronal number, conversely loss of function resulted in a decrease in neuronal number in pupae.

We then asked if MyD88 mediate the pro-survival functions of Toll-6? Except for pCC and RP2 neurons, all other Eve+ neurons also express Toll-6 (McIlroy *et al.,* 2013) and therefore are a good readout to determine the genetic interactions between both MyD88 and Toll-6. In embryos MyD88^{kra56};Toll-6 double mutants had fewer Eve+ neurons in the CNS, indicating that MyD88 promotes cell survival via functioning downstream of Toll-6 (Foldi, Anthoney *et al.,* 2017).

To further quantify that MyD88 is required for cell survival downstream of Toll-6, I investigated the rates of apoptosis when MyD88 is in excess or reduced. Here I found that in the pupae Toll-6 is pro-apoptotic and this is likely to be independent of MyD88 function. This was interesting, as Toll-6 has previously been shown to be more pro-survival in other developmental stages. Another interesting result was that the over-expression of MyD88 also resulted in an increase in apoptosis. A finding which directly contradicted the increase in apoptosis in a MyD88 mutant background. The causative factor for this effect may be taking place downstream of Toll-6 (or any of the Tolls) but upstream of MyD88. This therefore requires to be investigated further.

As Toll-6 was able to induce apoptosis independently of MyD88, it indicated the involvement of another downstream signalling adaptor. Sarm1 is a known inhibitor of MyD88 (O'Neill and Bowie, 2007), and I have shown that Toll-6 functions via dSarm to induce apoptosis. The functions of dSarm are via the JNK pathway, and by inhibition of MyD88 function. Furthermore not only is dSarm capable of inducing apoptosis but an increase in dSarm levels leads to a reduction in overall neuronal number. Interestingly, whilst Co-immunoprecipitations shows that dSarm can physically interact with MyD88, they show that

there is no direct interaction between dSarm and Toll-6 (Foldi, Anthoney *et al.*, 2017). Therefore the pro-apoptotic Toll-6 signalling required another downstream adaptor in order to facilitate the interaction between dSarm and Toll-6.

Through epistasis experiments I found that Wek is that adaptor. Toll-6 requires Wek in order to induce apoptosis, and dSarm functions downstream of Wek. Furthermore Wek can mediate the loss of Toll-6+ neurons presumably by dSarm and with the possibility that there is negative feedback occurring. However, by increasing the levels of Toll-6, Wek and dSarm the result is a reduction in the number of MyD88+ cells, and increasing Wek and dSarm in MyD88+ cells leads to neuronal loss. The interaction between Wek and Toll-6 requires to be confirmed.

In conclusion the downstream adaptors (Wek, dSarm and MyD88) of Toll-6 that are available both spatially and temporally can switch cell fate outcomes between cell survival and cell death during different developmental requirements. I have shown that Wek acts like a link between MyD88 and dSarm to facilitate Toll-6 signalling outcomes whereby Toll-6 – Wek – MyD88 can lead to cell survival outcomes, whereas Toll-6 – Wek – dSarm lead to cell death outcomes.

CHAPTER 7

DISCUSSION

7.1 Summary of findings

The aims of this thesis were to 1) investigate if all Toll receptors are functionally equal 2) establish if all Tolls can promote cell death as well as cell survival 3) determine how Toll-6 regulates cell number plasticity by characterising its downstream signalling mechanism.

7.2 Members of the Toll family are differentially expressed during development

During my PhD I have established that all Toll receptors are not equal either spatially or temporally. During the determination of the levels of the Toll receptors by RT-PCR during different developmental stages I showed Toll-3, Toll-4 and Toll-9 are expressed at reduced levels in comparison to the other Tolls. Toll-3 and Toll-4 are structurally different, as they do not contain a C-terminal extension. Toll-9 is also structurally different from the other receptors containing only one cysteine rich motif (Tauszig *et al.,* 2000; Imler and Zheng, 2003). However these structural differences are unlikely to be the primary cause of being expressed at lower levels. The protein structure of Toll-9 as stated is different to Toll-3 and Toll-4 and furthermore Toll-5 also has a premature stop codon and was expressed at higher levels during RT-PCR analysis. This raises the possibility that these receptors are more transient in nature or are expressed in fewer cells.

When gcm was over-expressed in embryonic neurons both Toll-3 and Toll-4 were expressed at low levels. Interestingly there was an increase of Toll-9 transcripts when gcm is expressed in neurons in comparison to wild-type conditions. However as RT-PCR is qualitative, qRT-PCR and endogenous expression of the Toll-9 protein would be required to quantify this. As gcm is embryonic lethal only this developmental time point could be investigated.

7.2.1 Differential expression of Toll-2, Toll-3 and Toll-8

In embryos Toll-2 is expressed in all developmental stages (RT-PCR and immuno-histochemical visualisation). In embryos Toll-2 was located in the region of haemocyte development, the procephalic mesoderm. Haemocytes are not only involved in innate immune responses but are also crucial during development. During development haemocytes are pivotal in apoptotic processes as they eliminate cells that are no longer required and in doing so help shape various tissues during embryogenesis (Franc *et al.,* 1990). Furthermore, haemocytes are involved in the production and secretion of extracellular matrix (ECM) molecules, which provide structural and biochemical support to neighbouring cells. Some of the ECM molecules known to be secreted by haemocytes include papilin, peroxidasin, glutactin, tiggrin (Fessler and Fessler., 1989; Fogerty *et al.,* 1994; Nelson *et al.,* 1994; Kramerova *et al.,* 2000), basement-membrane-associated dSPARC (Martinek *et al.,* 2002), MDP-1 (Hortsch *et al.,* 1998), laminin A (Kusche-Gullberg *et al.,* 1992), Cg25C and Viking which are collagen IV molecules (Mirre *et al.,* 1988; Knibihler *et al.,* 1987; Le Parco *et al.,* 1989;Yasothornsrikul *et al.,* 1997). This is interesting; as it has previously been identified that Toll-2 may act as a heterophilic cell adhesion molecule (Eldon *et al.,* 2004; Kleve *et al.,*

2006). It would be ideal to explore the expression of Toll-2 within the NMJ, as many cell adhesion molecules localise to synaptic junctions in order to release neurotransmitters.

During larval development Kambris *et al.,* 2002 determined via RT-PCR that Toll-2 was present within anti-microbial peptide (AMP) producing regions including the fat body, lymph gland and haemocytes. I found that within the CNS of both larvae and adults expression is restricted to primarily to optic lobes and central brain. However, expression was high in the corpus allatum (CA) of ring gland which secretes sesquiterpenoid juvinille hormone (JH) and interacts with ecdysone to determine moulting development into larval, pupal or adult forms (Harvie.,1998; Truman., 2007). In adults JH, affects learning and memory, diapause and innate immunity (Denlinger., 1985;Flatt *et al.,* 2008). These data show that Tol1-2 may have a diverse reportoire of functions that need to be explored further.

There was no embryonic expression of Toll-3 within the CNS, Kambris *et al.,* 2002 were also unable to obtain expression via *in situ hybridisation* and northern blot analysis. However RT-PCR results whilst very low showed that there are at least some cells that express Toll-3 during embryonic development. However as results were gathered using whole embryos it is possible that if Toll-3 is expressed only in a limited number of cells they may be located in tissues outside the CNS. In adults expression of Toll-3 is localised to the lamina and medulla regions of the optic lobes indicating a possible role of Toll-3 in the visual system. Furthermore Toll-3 was also located within the mushrooms bodies that are central to olfactory learning and memory (Heisenberg *et al.,* 1985; Zars *et al.,* 2000; Yu *et al.,* 2006; Krashes *et al.,* 2007) as well as visual attention-like behaviours (Xi *et al.,* 2008). Yagi *et al.,* 2010 could not identify any phenotypic manifestations when Toll-3 was over-expressed in different cells

using numerous Gal4 drivers. However this was not an exhaustive list and may have not been the correct assay to define the function of Toll-3. Therefore more specific testing of Toll-3 in both the visual and learning and memory pathways could elucidate its endogenous function. During the larval stage expression was throughout the VNC neuropile and optic lobes, albeit at very low levels.

Toll-8 was identified via RT-PCR as expressed during all stages of development. Previous reports identified that during embryogenesis Toll-8 is located posterior to wingless expression and in a very similar profile, which do not overlap, to Toll-2 (Kambris *et al.,* 2002) and is involved in neural patterning (Seppo *et al.* 2003; Ayyar *et al.* 2007). I have shown that Toll-8 is distributed throughout larval, pupal and adult CNS tissues. In larvae and pupal stages Toll-8 is located throughout VNC interneurons of thoracic and abdominal regions, projections innervating CNS and in optic lobe throughout medulla and central brain. This is in agreement with Seppo *et al.,* 2003, who identified Toll-8 mRNA expression along the VNC at sites of neuronal differentiation. In adults expression is throughout the medulla, mushroom body and central brain regions. Similar to Toll-3 these regions within the adult brain are known to be involved in the regulation of visual, olfactory learning and memory pathways.

The characterisation of these three Toll receptors highlights some similar and different patterns of expression. All three are located in the optic lobes, which are regions of the visual system. In adults Toll-3 and Toll-8 are both expressed in the mushroom bodies, which are regions required for learning and memory. And only Toll-2 is located in the CA of the ring gland, which is pivotal in hormone release during moulting. Both Toll-6 and Toll-7 are expressed in the embryonic, larval and adult CNS. They are distributed throughout the

locomotor circuits, which include interneurons, motorneurons, and within the FSB and EB of the adult brain (McIlroy *et al.*, 2013). Toll-1 is also expressed during all developmental stages. In the embryo Toll-2 is expressed in the midline glia and CNS axons. In larvae and adults stages Toll-1, similar to Toll-6 and Toll-7 located throughout locomotor circuits and FSB and EB (Sutcliffe B PhD thesis). This highlights the possibility that all three may be involved in different processes within the CNS.

7.3 Toll receptors regulate adult locomotion

To test if the nine Tolls had different or equal functions in locomotion, I examined the activity of adult flies that were over-expressing each of the Tolls or had them knocked-down by RNAi, in neurons or glia. The over-expression of Toll-1 and Toll-9 can influence adult locomotion, and the knockdown of Toll-5 is required in glial cells for locomotion. In neurons there appears to be no strong correlation between adult locomotion and their Toll receptor. Toll-6 and Toll-7 are expressed within locomotion centres, and Toll-7;Toll-6 double mutants have been shown to be required for normal larval locomotion and motor axon targeting (McIlroy *et al.*, 2013). Therefore whilst no phenotype with single knock-down flies were observed during this thesis, it would be interesting to test the double mutant to see if this effect is retained in adult flies. Similarly, there may be synergistic effects in double mutants for other Toll combinations as well.

7.4 Toll-3 in neurons is involved in the regulation of CNS size

As another means to ask whether the nine Tolls have equivalent functions in the CNS or not, I tested the effects of over-expressing or knocking down each of the Tolls in larval neurons or glial cells and measured overall larval CNS size. Toll-3 is required in neurons to regulate the maintenance of CNS size. This is independent of cell number as there was no increase in neuronal number in ElavGal4>UASToll-3 flies. The maintenance of CNS size may however be reliant on an increase in cell size, which was not explored. It was undetermined if any of the Toll receptors were important in the regulation of CNS size when expressed in glial cells. It may be possible that the Toll receptors do not influence glial cell number as increasing glial proliferation results in a longer VNC (Kato *et al.,* 2011; Losada-Perez *et al.,* 2016).

7.5 Toll receptors regulate cell number

In larval VNC the over-expression of Toll-2, -4, -6, -7, -8 and -9 in neurons resulted in an increase in Eve+ neurons. This increase in cell number may be due to increased cell survival or proliferation. McIlroy *et al,* showed that there are increased rates of apoptosis in Toll-6 and Toll-7 mutant embryos. This is consistent with both Toll-6 and Toll-7 as being pro-survival factors in the embryonic and larval stages of development.

In larval retinal discs however, the over-expression of Toll-2 and Toll-9 resulted in an increase of Dcp1+ cells and the knockdown of these genes subsequently decreases apoptotic rates. In contrast to the above findings this indicates that these genes are required for cell death functions in larval retina. It is possible that different genetic pathways are functioning in

the VNC compared to retinal discs in order to regulate cell death or cell survival. It is interesting to note that Wu *et al,* demonstrated that Toll-1 signalling induces caspase independent cell death in retinal discs. They state that the activation of cell death is via Spz6>Toll-1>NF-κB signalling pathway, and not via JNK signalling. However I have used Dcp1 to monitor apotosis in the retinal discs, which is a caspase. However Toll-6 knockdown in retina resulted in an increase apoptosis indicating that it functions as a pro-survival receptor in the larvae.

Furthermore the over-expression of Toll-6 results in an increase in the number of Eve+ neurons and can activate pro-survival NF-KB signalling (Foldi, Anthoney *et al,* 2017) again indicating its role in pro-survival signalling. Interestingly the number of Eve+ neurons after over-expressing of Toll-1 and Toll-3 was the same as the control, and together these results indicate that some of the Tolls are likely to be pro-survival whilst others are pro-apoptotic. However it should be noted that the over-expression of Toll-2 and Toll-9 also resulted in increase Eve+ neuron number. Previously I showed evidence that both of these Toll receptors increased cell death in the retina, a result which may imply tissue specificity These data are also consistent with findings that different Toll receptors are involved in regulating different functions in both immunity and in developmental processes (Tauszig *et al*., 2000; Yagi *et al.*, 2010; McIlroy *et al.*, 2013; Meyer *et al.*, 2014; Pare *et al.*, 2014).

The downstream signalling adaptors may change between tissue or cell, and thus influence the outcome upon over-expression or knockdown of certain genes. Furthermore, it is also possible that there is a compensatory mechanism in place whereby an increase in apoptosis leads to an increase in cell number in order to maintain neuronal homeostasis. In order to confirm if it is

gene regulation or compensatory mechanisms providing differing results, it would be beneficial to make comparable measurements of neuronal number and death in the retina or VNC. Thus my data suggest that tolls regulate cell death via a canonical caspase dependent pathway.

7.6 DNTs in the regulation of cell number plasticity

As different Toll receptors are involved in differentially regulating cell survival and/or cell death, I asked if the same was true of the DNT ligands. I provide evidence that similar to the Tolls the DNTs are able to regulate cell number plasticity by promoting both cell survival and cell death in the *Drosopihla* CNS. I have previously mentioned that DNT-1 and DNT-2 bind Toll-7 and Toll-6 receptors (McIlroy *et al.,* 2013), and that this binding may be promiscuous as Toll-7 can also function as the DNT-2 receptor (Foldi, Anthoney *et al*, 2017). Here I have shown that DNT1-FL is able to activate apoptotic JNK signalling, whereas mature DNT1, DNT2 and DNT2-FL activate the pro-survival signalling ERK pathway (This thesis and Foldi, Anthoney *et al.* 2017). DNT2-FL is cleaved intracellularly giving rise to mature DNT2 protein. In comparison to mammalian NTs, there are remarkable similarities. I have found that pro-NTs function via JNK signalling pathways to elicit cell death functions and conversely it is the mature cleaved NTs that via pro-survival signalling pathways NF-KB and ERK, initiate cell survival (Vetter *et al.,* 1991; Aloyz *et al.,* 1998; Riccio *et al.,* 1999; Corbit *et al.,* 1999; Lee *et al.,* 2001, Nykjaer *et al.,* 2004; Beattie *et al.,* 2002; Harrington *et al.,* 2004; Srinivasan *et al.,* 2004). This mechanism of cell survival and death is dependent upon ligand cleavage processes as well as ligand-receptor complex specificity. This suggests that the structure of the DNT, whether it is a pro or mature NT, binds to differing Toll receptors or receptor

heterodimers in order to activate cell death or survival.

7.7 Downstream signalling events of Toll-6

To elucidate downstream signalling adaptors that facilitate Toll and DNT functioning in cell death or survival, I worked in collaboration with Dr Istvan Foldi and Dr Neale Harrison (Postdocs in the Hidalgo lab at UOB) focusing on Toll-6. I have shown that MyD88 was expressed in the CNS of larval and pupal stages. The levels were higher throughout the pupal stage, which also corresponds to the second wave of cell death and significant remodelling of the CNS. MyD88 is able to influence cell survival as over-expression in neurons results in an increase in Eve+ neuronal number. Furthermore both a MyD88 missense allele as well as a MyD88 null allele resulted in an increase in apoptosis.

Whilst in the embryo and larval stage, Toll-6 promotes cell survival (McIlroy *et al.*, 2013 and this thesis); during pupal development Toll-6 induced apoptosis. This indicates that Toll-6 is able to switch between a pro-survival and pro-death state. Furthermore this effect is independent of MyD88. In mammals TLRs can also signal in a MyD88 dependent or independent fashion. dSarm is expressed in the embryonic CNS as well as in the CNS of both larvae and pupae, and levels increase as development progresses. dSarm is able to induce apoptosis and neuronal loss and is able to antagonize MyD88 pro-survival function in doing so. Furthermore, this pro-apoptotic effect of dSarm is via the JNK signalling pathway.

However, dSarm is unable to bind Toll-6 directly (Foldi, Anthoney *et al*, 2017) and requires the interaction of Wek in order to induce apoptosis. Wek acts upstream of dSarm giving rise to the DNT - Toll-6 – Wek – dSarm – JNK mode of cell death. dSarm can also activate cell death via direct inhibition of MyD88 and Wek can act as the link between Toll-6 function and

cell survival outcomes giving rise to Toll-6 – MyD88 – NF-KB/ERK cell survival. Wek therefore functions like a hinge, and is able to facilitate the actions of either MyD88 or dSarm downstream of Toll-6, depending on the cell requirement.

Over-expression of MyD88 and dSarm can influence Wek levels indicating that Wek is regulated by competitive interactions between dSarm and MyD88.It is therefore likely that the different cell survival or cell death outcomes that are Toll-6 mediated are due to the changing levels of different downstream adaptors. In embryos, it is likely that Toll-6 binds MyD88 and activates cell survival signalling pathways in order to actively maintain neuronal survival for neural circuit formation. Beginning in embryogenesis, Toll-6 is therefore promoting neuronal survival (McIlroy *et al.,* 2013), via the Toll-6 - MyD88 – NF-KB/ERK signalling pathway. This signalling pathway can occur due to the low levels of Wek, which prevents the dSarm pro-apoptotic signalling pathway from being activated and also prevents the interaction and direct inhibition of MyD88 from dSarm.

However, as development continues both MyD88 and dSarm activate an increase in Wek, which by the pupal stage facilitates association of either MyD88 or dSarm with Toll-6. This association with Toll-6 can either activate cell survival via MyD88 and NF-KB as in embryos, or activate cell death via dSarm and JNK signalling. During this stage it is also interesting to note that dSarm can directly inhibit the function of MyD88, which continues the apoptotic signalling mechanism but also prevents the canonical pro-survival signalling. Therefore Toll-6 can not only promote cell survival in embryonic stages, but also promote survival and death during pupal stages.

Regulatory feedback mechanisms have been shown in other parts of Toll signalling. For example both Toll-6 and Toll-7, and DNT-1 can up-regulate the NF-KB homologues Dif and

Dorsal (McIlroy *et al.,* 2013). Furthermore, Toll signalling activates Hippo pathway components, and via negative feedback leads to Pelle phosphorylation and Cactus degradation (Liu *et al.,* 2016). It is becoming ever more apparent that signalling pathways can be in control of numerous different signalling outcomes and not simply single linear signalling events.

For another example of positive regulation, Toll signalling can be activated via the generation of reactive oxygen species (ROS) and DAMPs from CIN cells (Liu *et al.,* 2015).. This activation leads to induction of two possible outcomes. The first, JNK and Mmp1 activation followed by JNK dependent apoptosis occurs in neighbouring CIN cells via TNFα secretion. Or the second pathway whereby JNK activation results the production of ligands for the Toll receptor, positively regulating Toll signalling (Liu *et al.,* 2016).

7.8 Final conclusions, implications of research and future directions

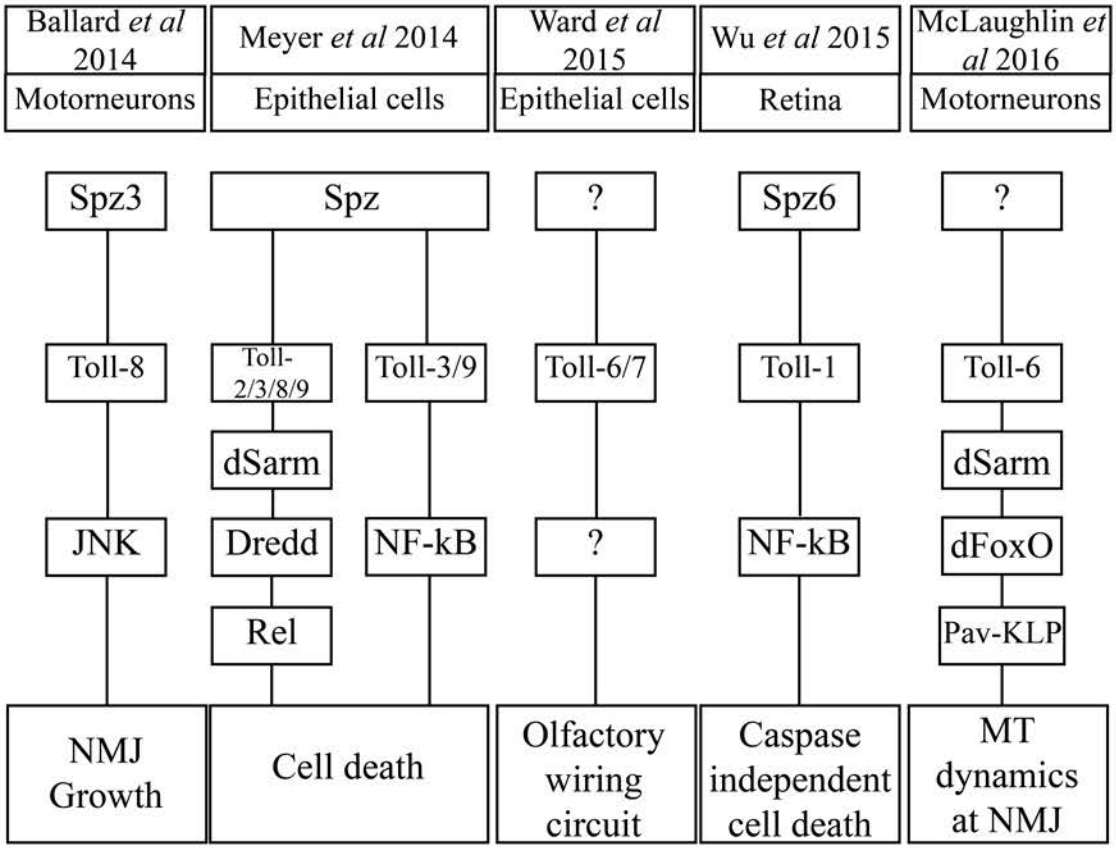
Throughout this thesis I have shown that the Toll receptors are functionally different in their signalling outcomes. Cell survival and death within the *Drosopihla* nervous system is reliant upon the different form (either full length pro-DNT or mature DNT) of DNT that a single (or combination) Toll receptor binds. Then which adaptor proteins are available and functional for downstream signalling pathways. In conjunction with Foldi, Anthoney *et al,* I have shown throughout development different signalling pathways are required to facilitate cell death or survival outcomes. This is reliant upon the changing abundance and availability of the adaptor proteins Wek, MyD88 and dSarm. This system reveals a novel mechanism of cell number control within the *Drosopihla* nervous system. Regulation occurs in a highly plastic manner

via positive and negative feedback mechanisms that drive alternative outcomes. These downstream signalling outcomes are therefore highly context dependent.

Whilst chapter 7 focused solely on Toll-6, I have presented evidence that some of the Toll receptors (in particular Toll-8) are widespread throughout the CNS of *Drosopihla.* Some of the Toll receptors may function in a manner similar to Toll-6 to regulate cell survival via MyD88 - ERK /NF-KB signalling or cell death via Wek - dSarm - JNK signalling. Or they may function via different signalling mechanisms, as throughout this thesis I have shown that context matters. For example time is an important consideration. Toll-6 is initially pro-survival in the embryo and larval stages but is pro-apoptotic from pupal stages. Furthermore, it has become apparent that the specific tissue or cell a Toll receptor is expressed in can have very different signalling outcomes. Toll-2 and Toll-9 regulate neuronal survival in the VNC of larvae, however, they regulate cell death in the retinal discs. Therefore we cannot theoretically extrapolate between different developmental stages, the different tissues or even between the different Toll receptors. There appears to be no general mechanism that governs all of the Toll receptors functionally. There is also the possibility that the Tolls may form heterodimers and certain combination of the Tolls depicts the outcome of a function.

During the course of this PhD, published articles also confirm that the Tolls have very different signalling outcomes through a variety of downstream signalling pathways (Figure 7.1). A recent publication has demonstrated that Toll-6 (and not Toll-7) functions in the promotion of axon transport and structural plasticity of motor neurons through a different complex of signalling mechanisms including dSarm and dFoxO (McLaughlin *et al.,* 2016). Furthermore Ward *et al.,* show independently of downstream signalling both Toll-6 and Toll-

Figure 7.1: Publications from 2014 - 2016 findings discussed in chapter 7



7 are capable of regulating different aspects of olfactory circuit wiring, with Toll-7 primarily involved in ORN axon targeting and Toll-6 in the regulation of PN dendrite targeting. The authors also state that Toll receptor cytoplasmic domains are dispensable for action (Ward *et al.,* 2015). In motor neurons however, McLaughlin *et al.,* demonstrate that Toll-6 requires the cytoplasmic domain to function effectively to promote microtubule stability at the NMJ.

DNT-1, -2 and Spz are required for synaptogenesis, with involvement in synaptic growth and morphology at the neuromuscular junction (NMJ) (Sutcliffe *et al.,* 2013). These neurotrophins regulate growth in a cell specific manner, with Spz regulating growth of NMJ4 and DNT-1 and DNT-2 regulating growth of NMJ 6 and 7 (Sutcliffe *et al.,* 2013). Toll-6 and Toll-7 bind and genetically interact with DNT-2 and DNT-1, most likely in a promiscuous manner (Foldi, Anthoney *et al.,* 2017). These ligand-receptor complexes utilise different signalling mechanisms from vertebrate NTs, which use the canonical $p75^{NTR}$, Trk and sortilin signalling. The DNTs have distinct biochemical properties with DNT2 always being cleaved intracellularly, whereas DNT-1 is secreted in both the pro-DNT protein and mature protein forms. The cleavage proteases involved still remain to be elucidated (Foldi, Anthoney *et al.,* 2017; McIlroy *et al.,* 2013).

Downstream of the Toll receptors McLaughlin *et al.,* 2016 have shown that Toll-6 acts upstream of dFoxO in order to promote growth at NMJ4 and NMJ 6/7. Furthermore they show that Toll-6 regulates microtubule organisation at the NMJ through non-canonical signalling pathways. This pathway does not engage the function of Dorsal, MyD88, Cactus, Pellino, Wek or Relish. Instead works via dSarm and dFoxO and the repression of a mitotic kinesin Pavarotti-KLP (Pav-KLP). Furthermore Ballard *et al,* demonstrate that Toll-8

regulates NMJ growth via a Dorsal - NF-κB and Cactus - IκB independent pathway. Instead, pre-synaptic Toll-8 is activated by Spz3 activating a JNK signalling cascade, resulting in the activation of Jun and Fos and enhancement of NMJ growth (Ballard *et al.,* 2014). McLaughlin *et al,* demonstrate that Toll-6, like Toll-8, activates JNK signalling, but this is not required for NMJ development. And as yet the ligand for Toll-6 and Toll-7 at the NMJ is not known in this Toll-6 – dFoxO – Pav-KLP pathway (McLaughlin *et al.,* 2016).

In the regulation of cell death Meyer *et al,* demonstrates that dSarm promotes apoptosis in epithelial cells during cell competition. The authors propose two different signalling mechanisms dependent upon the cell context. When mutant cells surround a wild type cell, a Spz ligand binds Toll-2, -3, -8 or -9 resulting in the activation of dSarm and downstream apoptotic signalling in order to eliminate it. This is in agreement with my findings whereby Toll-6 works via Wek and dSarm in order to promote cell death. However, the authors also propose that cell death can occur in a dSarm independent manner. In this instance when wild-type cells surround a mutant cell, Spz binds Toll-3 or Toll-9 activating NF-κB transcription factors to promote cell death. Wu *et al,* also propose that within retinal cells Spz6 binds Toll-1 activating NF-κB signalling and this activation results in caspase independent cell death. Neither of these papers investigates the interaction between the Toll receptor, MyD88 and downstream signalling. Interestingly, Adachi *et al,* identified that MyD88 is required for cell proliferation, the induction of NF-KB as well as JNK signalling in mammals (Adachi *et al.,* 1998). Furthermore Tauszig-Delamasure *et al.,* has shown that MyD88 interacts with the apical caspases dFADD and Dredd. These caspases regulate effector caspases such as DCP1 and Drice to drive apoptosis (Tauszig-Delamasure *et al.,* 2002). MyD88 interacts with Toll-1

(Tauszig Delamasure *et al.*, 2002), Toll-6 and Toll-7 (Foldi, Anthoney *et al.*, 2017). However, whether these interactions result in cell death in specific tissues remain to be investigated.

In conclusion, DNTs and Toll receptors and their downstream targets regulate structural plasticity within the CNS of *Drosopihla*. However, there are multiple pathways that govern the signalling outcome, which is completely dependent upon numerous factors. Different neurotrophins bind to a range of Toll receptors to elicit specific cellular outcomes. The outcome is determined by the combination of neurotrophin and Toll, Toll receptor and adaptor protein/s, and downstream signalling mechanisms. I have shown that multiple Toll receptors are involved in a range of functions including locomotion, regulation of CNS size, cell survival and cell death. For Toll-6, we have established that there are two main signalling pathways governing cell death (DNT - Toll-6 – Wek – dSarm – JNK) or cell survival (DNT-Toll-6 - MyD88 – NF-KB/ERK). It will be interesting to determine if other Tolls also regulate cell death and/or survival in the CNS and function via this route. Or if they function in a completely different manner, which present immeasurable possibilities.

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PUBLISHED WORK

Work from this thesis has contributed to a manuscript being accepted by the Journal of Cell Biology. The article is entitled “Three tier regulation of cell number plasticity by neurotrophins and Tolls in *Drosophila*”. A copy of which is attached at the end of this thesis.

Three tier regulation of cell number plasticity by neurotrophins and Tolls in *Drosophila*

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eTOC SUMMARY

A three-tier mechanism involving distinct neurotrophin family ligand forms, different Toll receptors and different adaptors, regulates both cell survival and death. This rich mechanism confers cell number plasticity, could underlie structural plasticity in the nervous system, and structural integrity, homeostasis and regeneration in wider contexts.

ABSTRACT

Cell number plasticity is coupled to circuitry in the nervous system, adjusting cell mass to functional requirements. In mammals, this is achieved by Neurotrophin (NT) ligands, which promote cell survival via their Trk and $p75^{NTR}$ receptors, and cell death via $p75^{NTR}$ and Sortilin. *Drosophila* neurotrophins (DNTs) bind Toll receptors instead to promote neuronal survival, but whether they can also regulate cell death is unknown. Here, we show that DNTs and Tolls can switch from promoting cell survival to death in the central nervous system (CNS), via a three-tier mechanism. First, DNT cleavage patterns result in alternative signaling outcomes. Second, different Tolls can preferentially promote cell survival or death. Third, distinct adaptors downstream of Tolls can drive either apoptosis or cell survival. Toll-6 promotes cell survival via MyD88-NFκB, and cell death via Wek-Sarm-JNK. The distribution of adaptors changes in space and time, and may segregate to distintict neural circuits. This novel mechanism for CNS cell plasticity may operate in wider contexts.

INTRODUCTION

Balancing cell death and cell survival enables structural plasticity and homeostasis, regeneration and repair, and fails in cancer and neurodegeneration. In the nervous system, cell number plasticity is linked to neural circuit formation, adjusting neuronal number to functional requirements (Levi-Montalcini, 1987). In mammals, the neurotrophin (NT) protein family – NGF, BDNF, NT3 and NT4 - regulates neuronal number through two mechanisms. First, full-length pro-NTs - comprised of a disordered pro-domain and a cystine-knot (CK) domain - induce cell death; in contrast, mature NTs formed of CK dimers, promote cell survival (Lu et al., 2005). Second, pro-NTs bind $p75^{NTR}$ and Sortilin receptors inducing apoptosis via JNK signaling, whereas mature NTs bind $p75^{NTR}$ promoting cell survival via NF-κB (Carter et al., 1996), and TrkA,B and C promoting cell survival via PI3K/AKT and MAPKinase/ERK (Lu et al., 2005). As the NTs also regulate connectivity and synaptic transmission, they couple the regulation of cell number to neural circuitry and function, enabling structural brain plasticity(Lu et al., 2005; Minichiello, 2009; Park and Poo, 2013). There is abundant evidence that cell number plasticity occurs in *Drosophila* central nervous system (CNS) development – with neurotrophic factors including neurotrophins and MANF (Palgi et al., 2009; Zhu et al., 2008) -, but fruit-flies lack $p75^{NTR}$ and Trk receptors, raising the question of how this is achieved in the fly. Finding this out is important, as it could lead to novel mechanisms of structural plasticity for both flies and humans.

Drosophila neurotrophins – Spätzle (Spz), *Drosophila* Neurotrophin 1 (DNT1) and DNT2 – share with mammalian NTs the characteristic structure of a pro-domain and a conserved CK of 13-15 kDa, which forms a disulfide-linked dimer (Arnot et al., 2010; Hepburn et al., 2014; Hoffmann et al., 2008a; Hoffmann et al., 2008b; Zhu et al., 2008). Spz resembles NGF biochemically and structurally, and binding its Toll-1 receptor resembles that

of NGF to $p75^{NTR}$ (Arnot et al., 2010; DeLotto and DeLotto, 1998; Hepburn et al., 2014; Lewis et al., 2013; Mizuguchi et al., 1998). *DNT1* (also known as *spz2*) was discovered by homology to *BDNF*, and *DNT2* (also known as *spz5*) as a paralogue of *spz* and *DNT1* (Parker et al., 2001; Zhu et al., 2008). DNT1 and -2 promote neuronal survival, and DNT1, 2, Spz and Spz3, are required for connectivity and synaptogenesis (Ballard et al., 2014; Sutcliffe et al., 2013; Zhu et al., 2008). Spz, DNT1 and DNT2 are ligands for Toll-1, -7 and -6, respectively, which function as neurotrophin receptors and promote neuronal survival, circuit connectivity and structural synaptic plasticity (McIlroy et al., 2013; McLaughlin et al., 2016; Sutcliffe et al., 2013; Ward et al., 2015; Weber et al., 2003; Zhu et al., 2008). Tolls belong to the Toll-receptor super-family, which underlies innate immunity (Imler and Zheng, 2004; Leulier and Lemaitre, 2008). There are nine *Toll* paralogues in flies, of which only Toll-1, -5, -7 and -9 are involved in immunity (Leulier and Lemaitre, 2008; Tauszig et al., 2000). Tolls are also involved in morphogenesis, cell competition and epidermal repair (Ballard et al., 2014; Carvalho et al., 2014; Halfon et al., 1995; McIlroy et al., 2013; Meyer et al., 2014; Pare et al., 2014; Ward et al., 2015; Yagi et al., 2010). Whether DNTs and Tolls can balance cell number plasticity is unknown.

Like the $p75^{NTR}$ receptor, Toll-1 activates NF-κB signaling downstream: a potent neuronal pro-survival factor, with evolutionarily conserved functions also in structural and synaptic plasticity (Gutierrez and Davies, 2011; Hoffmann and Reichhart, 2002; Mattson and Meffert, 2006). Toll-1 signaling involves the downstream adaptor MyD88, which forms a complex with Tube and Pelle (Gay and Gangloff, 2007; Horng and Medzhitov, 2001; Tauszig-Delamasure et al., 2002). Activation of Toll-1 triggers the degradation of the NF-κB inhibitor Cactus, enabling the nuclear translocation of the NF-κB homologues Dorsal and Dorsal-related Immunity Factor (Dif), which function as transcription factors. Other Tolls have also been suggested to activate NF-κB (McIlroy et al., 2013; Meyer et al., 2014).

However, only Toll-1 has been shown to bind MyD88 (Tauszig-Delamasure et al., 2002), raising the question of how the other Tolls signal in flies.

Whether Tolls regulate cell death is also obscure. Toll-1 activates JNK causing apoptosis, but its expression can also be activated by JNK to induce non-apoptotic cell death (Liu et al., 2015; Wu et al., 2015a; Wu et al., 2015b). Toll-2,-3,-8 and -9 can induce apoptosis via NF-κB and dSarm independently of MyD88 and JNK (Meyer et al., 2014). However, in the CNS dSarm induces axonal degeneration, but there is no evidence that it can promote apoptosis in flies (Osterloh et al., 2012). In other animals, Sarm orthologues are inhibitors of Toll signaling and MyD88 (Carty et al., 2006; Yuan et al., 2010), but there is no evidence that dSarm is an inhibitor of MyD88 in *Drosophila*. Thus, whether or how Tolls may regulate apoptosis in flies is unclear.

In the mammalian brain, Toll-Like-Receptors (TLRs) are expressed in neurons where they regulate neurogenesis and apoptosis, neurite growth and collapse - in the absence of any insult (Okun et al., 2011). However, their neuronal functions have been little explored, and their endogenous ligands in neurons remain unknown.

Since Toll-1 and $p75^{NTR}$ share common downstream signaling pathways, and $p75^{NTR}$ can activate NF-κB to promote cell survival and JNK to promote cell death, here we asked whether the DNTs and their Toll receptors could have dual roles controlling cell survival and death in the *Drosophila* CNS.

RESULTS

Different processing for each DNT ligand

Using 3D structural modeling based on the crystal structure of Spz (Lewis et al., 2013), we compared the mature cystine-knot (CK) domains of DNTs with those of mammalian NTs. They all share the structurally conserved CK unique to the NT family, distinct from those of

other growth factors, with the characteristic arrangement of anti-parallel β-sheets and disulfide bridges (Fig. 1A-D). The overhanging wings are out of phase by 90° in the *Drosophila versus* mammalian ligands, possibly reflecting interactions with different receptor types (Fig. B-D). The receptor-binding interface of Spz is not evolutionarily conserved in DNT1 or 2, suggesting distinct receptor affinities (Fig. 1E). Thus, Spz, DNT1, DNT2 are NT ligands with distinctive features.

The pro-domains have distinctive features too. The pro-domains of Spz and DNT2 are disordered coils, whereas that of DNT1 has helices suggesting a globular structure (Fig. 2A and Fig. S1). The DNT1 pro-domain is also twice as long as that of DNT2. The pro-domain of Spz has an α-helix just upstream of the Easter cleavage site, which undergoes a conformational change upon cleavage, essential for the activation of Toll (Arnot et al., 2010). This sequence is not conserved in the pro-domains of the mammalian NTs, nor DNT1 and 2 (Fig. 2A). This suggests that the activation mechanism of Toll by Spz is unique, and distinct from those of Toll-6 and -7 by DNT2 and -1, respectively.

Mammalian pro-NTs are cleaved intracellularly by furin proteases, or extracellularly by Serine-proteases (e.g. BDNF, Fig. 2B). Spz is only secreted full-length, and cleaved extracellularly by the Serine-proteases Easter or SPE (Hoffmann and Reichhart, 2002). Furin sites were absent from the Spz pro-domain, but several highly conserved sites were found in DNT1 and 2 (Fig. 2B). *In vivo* over-expression of mature *Spz-CK, DNT1-CK* and *DNT2-CK* is functional and rescues the respective mutant phenotypes (Hu et al., 2004; Ligoxygakis et al., 2002; Sutcliffe et al., 2013; Zhu et al., 2008). However, S2 cells transfected with DNT1-cystine-knot-C-Terminal-Domain tagged with 3xHA (*DNT1-CK-CTD-HA*) and *DNT2-CK-HA*, did not secrete mature DNTs to S2 cell medium (Fig. 2D lane 3 and lane 8). This either suggests that the pro-domain is required for trafficking in S2 cells, or that S2 cells do not behave like neurons do *in vivo*. S2 cells transfected with wild-type full-length *DNT1-FL-HA*

did not secrete DNT1-FL either, but instead secreted a product truncated at the R283 site (Fig. 2D lane 2), suggesting that cleavage occurs naturally at this site. By contrast, S2 cells expressing *DNT2-FL-HA* invariably secreted the mature CK form of 15KDa (Fig. 2D lane 7). To test if the conserved furin sites were responsible for these cleavage profiles, we carried out site directed mutagenesis of the furin sequences in HA tagged DNT1 and 2 (Fig.2C). DNT1 lacking the furin site at R499 still secreted a product cleaved at R283, but no secreted protein was detected when both R499 and R283 were mutagenized (Fig. 2D, lanes 4 and 5). Thus, DNT1 furin site at R283, which is the most conserved, is functional. Mutagenesis of the DNT2 furin site R284 resulted in the secretion of two products of 30kDa and 18kDa (Fig. 2D lane 10). The 30kDa product corresponds to cleavage at site R214 or R221, implying that cleavage at these sites is unlikely to occur naturally or that cleavage at R284 predominates. The 18kDa product was not detectable in the media expressing wild-type DNT2, suggesting it does not occur naturally and is the result of non-furin cleavage. Mutagenising R214, R221 and R284 sites resulted in the secretion of DNT2-FL-HA from S2 cells, showing that DNT2 can be secreted full-length (Fig. 2D lane 11). These findings showed that the DNT2 furin cleavage site at R284 is functional and it is the predominant cleavage site.

To test whether similar DNT processing occurs *in vivo,* we over-expressed in the retina (with *GMRGAL4*) full length forms tagged at the C-termini with GFP, and visualized the resulting products with anti-GFP in western blots. DNT1was predominantly found in full-length form, and also cleaved at furin sites at 98 (less abundant), 283 (pro-DNT1) and 499 (DNT1-CK-CTD, Fig.2E). DNT2 was found full-length, but predominantly in mature form (DNT2-CK, Fig.2E). These data show that *in vivo*, DNTs are cleaved by furins, and can be found in both pro- and mature forms.

To conclude, each DNT has unique features. DNT1 is more likely found in pro-form than DNT2, and DNT2 is more likely found in mature form. Ultimately, the forms secreted *in*

vivo will depend on the expression profile of proteases, and will be context dependent. The distinct processing mechanisms of Spz, DNT1 and DNT2 suggest functional differences.

Pro-DNT1 activates pro-apoptotic and mature DNTs pro-survival pathways

To ask whether different DNT forms could have distinct functions, we tested whether they could activate pro-apoptotic or pro-survival signaling pathways.

Over-expression of mature DNT1 and -2 promotes cell survival in embryos (Zhu et al., 2008). In mammals, apoptosis is activated by pro-NTs binding $p75^{NTR}$ and activating JNK (Roux and Barker, 2002). Thus, we asked whether the different DNT forms activate JNK signaling, visualised using anti-phospho-JNK antibodies. Over-expression of *DNT1-CKCTD, DNT2-CK* or *DNT2-FL* in the retina reduced the number of pJNK+ cells compared to controls, whereas over-expression of *DNT1-FL* increased pJNK+ cell number (Fig. 3A). Most likely (see Fig.2D,E), DNT2-FL was cleaved intracellularly and secreted as mature CK instead. Thus, pro-DNT1 can activate the JNK pro-apoptotic signaling pathway.

We next tested whether DNTs can activate the pro-survival pathways NF-┤ B and ERK. Stimulating S2 cells with purified mature DNT2-CK induced the phosphorylation of Dorsal (i.e. activation, Fig. 3B). We also transfected S2 cells with *Toll-6* or *-7,* stimulated them with purified mature DNT2-CK, and tested whether it triggered the nuclear translocation of Dorsal or Dif, thus activating NF-┤ B signaling. Subcellular fractionation revealed that DNT2 induced the degradation of the NF-┤ B inhibitor Cactus in the cytoplasm, and the nuclear translocation of both Dorsal and Dif (Fig. 3C and Fig.S2A). These data demonstrate that mature DNT2-CK activates NF-┤ B signaling. Stimulation with DNT2-CK also activated signaling in non-transfected control cells (Fig. 3C). Since S2 cells express multiple Tolls but not Toll-6 (Fig. S2B), this means that DNT2 can also bind other Toll-family receptors. In fact, DNT1 binds Toll-7 and DNT2 binds Toll-6 (McIlroy et al., 2013), but DNT1 could also bind

Toll-6 and DNT2 could also bind Toll-7 (Fig. S3). Thus, binding of DNT1 and 2 to Toll-6 and -7 is promiscuous. Importantly, both Cactus degradation and nuclear translocation of Dorsal and Dif induced by DNT2, were more pronounced in transfected cells than in mock controls (Fig. 3C). This shows that Toll-6 and -7 activate NF-¦ B signaling downstream of DNT2.

To test whether DNTs, Toll-6 and -7 could activate ERK, we over-expressed them and visualized activated anti-phospho-ERK. Over-expression of either *DNT1-FL* or mature *spz-CK* in neurons of the larval brain optic lobe (with *RGGAL4*) did not activate ERK signaling (Fig. 3D). By contrast, over-expression of *DNT1-CKCTD* and *DNT2-CK* did (Fig. 3D). DNT2-FL also activated ERK, but as shown above, DNT2 is readily cleaved prior to secretion. Thus, mature DNT1 and -2 (but not Spz) can activate ERK. Furthermore, over-expression of activated forms of *Toll-6^{CY}* and *Toll-7^{CY}* (McIlroy et al., 2013), in retinae, significantly increased pERK levels (Fig. 3E). Thus, Toll-6 and -7 activate ERK. Together, these data show that DNT1 and 2 can activate the pro-survival signaling pathways NF-¦ B and ERK via Toll-6 and -7, and pro-DNT1 can activate the pro-apoptotic JNK pathway.

To test whether distinct Toll receptors might differentially regulate neuronal number, we asked whether Eve+ neurons were affected by loss or gain of function for Tolls in third instar larvae ventral nerve cords (VNCs). *Toll-7^{P8}/ Toll-7^{P114}; Toll-6^{26}/Toll-6^{31}* double mutant larvae had slightly fewer Eve+ neurons than wild-type, but *Toll-1^{r3}/Toll-1^{r444}* mutants had more (Fig.3F). Conversely, over-expression of constitutively active *Toll-6^{CY}* and *Toll-7^{CY}* in neurons (with *ElavGAL4)* increased Eve+ neuron number (Fig.3F), whereas constitutively active *Toll-1^{10b}* decreased it (Fig.3F). Thus, Toll-6 and -7 promote cell survival, as previously reported (McIlroy et al., 2013), but Toll-1 can be pro-apoptotic. Distinct Toll receptors, and the potential formation of hetero-dimers between different Toll receptors, might switch the response to DNTs from cell survival to cell death (Fig. 3G).

Toll-6 activates pro-survival signaling in the CNS via MyD88

The finding that Toll-6 and -7 could initiate signaling suggested the involvement of the MyD88 adaptor. However, no interactions between Tolls, other than Toll-1, and MyD88 had been previously detected (Tauszig-Delamasure et al., 2002). To test whether Toll-6 or -7 and MyD88 could form a signaling complex, S2 cells were co-transfected with native *Toll-6* or *-7,* or activated *Toll-6*^{CY} or *-7*^{CY} tagged with Flag, and *MyD88* tagged with *V5*. In co-immunoprecipitations, MyD88 co-purified with Toll-6 and -7, and Toll-6^{CY} and Toll-7^{CY} (Fig. 4A). Thus, both Toll-6 and -7 can bind MyD88. This physical interaction could occur *in vivo* as, like Toll-6 and -7 (McIlroy et al., 2013), MyD88 protein was found throughout the embryonic CNS neuropile, and its endogenously tagged downstream targets Dorsal-GFP and Dif-GFP were too (Fig. 4B).

We next asked whether MyD88 is required for the CNS functions of Toll-6 and -7, by testing the effect of mutants on Eve, a reporter for Toll-6 neurons. All Eve+ neurons - except pCC and RP2 - express *Toll-6* (McIlroy et al., 2013). *MyD88*^{kra56} is a hypomorphic allele (Charatsi et al., 2003), ideal to test for phenotypic enhancement or suppression in genetic interactions. *MyD88*^{kra56} mutants had a virtually normal embryonic CNS, but *MyD88*^{kra56} *Toll-6*^{26} double mutants and *MyD88*^{kra56} *Toll-7*^{P8} *Toll-6*^{26} triple mutants had fewer Eve+ neurons (Fig. 4C and Fig. S4). This is consistent with MyD88 functioning downstream of Toll-6 and -7 in the CNS to maintain neuronal survival. In fact, over-expression of *MyD88* in all neurons (with *elavGAL4*) increased Eve+ neurons both in third instar larvae and pupae (Fig. 4D). Conversely, over-expression of *cactus* decreased Eve+ neuron number in larvae (Fig.4D), and loss of *MyD88* function also decreased Eve+ number in pupae (Fig.4D). Together, these data show that MyD88 is required for and can promote neuronal survival.

To verify this, we quantified the effects of altering *MyD88* function in apoptosis. *MyD88*^{kra56} homozygotes are semi-lethal, with a lethality phase at pupariation, indicating this is a critical time for MyD88 function. Using anti-Death Caspase 1 (Dcp1), we counted all dying cells throughout the VNC of white pupae using adapted DeadEasy Caspase software (Forero et al., 2009). In *MyD88*^{kra56} homozygotes apoptosis levels did not differ from controls, but they increased in *MyD88*^{kra56}*/DfBSC279* trans-heterozygotes (Fig. 4E). We generated a *MyD88* null allele using CRISPR/Cas9, *MyD88*$^{cr2.8}$. Trans-heterozygous *MyD88*$^{cr2.8}$*/Df(2R)BSC279* pupae also had increased apoptosis (Fig. 4E). Thus, MyD88 is required for neuronal survival. Together, these data showed that Toll-6 and -7 signal via the canonical MyD88 pathway to promote neuronal survival in the CNS.

However, over-expression of *MyD88* in all neurons also increased apoptosis in pupa (Fig. 4E). This could occur downstream of Tolls, as over-expression of activated *Toll-6*^{CY} or *Toll-1*^{10b} also increased apoptosis in pupae (Fig. 4E). Remarkably, the pro-apoptotic effect of Toll-6 was enhanced when over-expressed in a *MyD88*^{kra56} mutant background (Fig. 4E), suggesting that Toll-6 might induce apoptosis in pupae independently of MyD88.

These data raised two questions: how does MyD88 induce apoptosis? And how can Toll-6 induce apoptosis independently of MyD88?

Toll-6 can induce apoptosis via the MyD88 inhibitor dSarm

In mammals, Sarm1 inhibits MyD88, and can induce neuronal apoptosis (Carlsson et al., 2016; O'Neill and Bowie, 2007). Thus we wondered whether *Drosophila dsarm* might be involved in pro-apoptotic signaling by Toll-6. We over-expressed *dsarm* in all neurons using *EP3610* flies, which drive expression of multiple *Ect4* isoforms (*Ect4* is a synonym of *dsarm*). *Elav>EP3610* increased apoptosis in pupal VNCs (Fig. 5A). Remarkably, over-expression of *dsarm* in a *MyD88*^{kra56} mutant background increased apoptosis further (Fig.

5A). This showed that *dSarm* promotes apoptosis, and antagonises MyD88 function. Apoptosis led to neuronal loss, as over-expression of *dsarm* in normal or *MyD88* mutant pupae, decreased Eve+ neuron number (Fig. 5B). Since *sarm* mutants are embryonic lethal, to further verify this we looked at the embryonic CNS. *dsarm* is expressed throughout the embryonic CNS, as visualised with a $dsarm^{MIMIC\text{-}}GFP$ reporter (Fig.5C). Over-expressing *dsarm*, using either *EP3610* or a single *dsarm* isoform (Osterloh et al., 2012), in all embryonic CNS neurons caused Eve+ neuron loss (Fig. 5D, Fig. S4). Conversely, $dsarm^{4705}/dsarm^{4621}$ mutant embryos had more Eve+ neurons (Fig. 5D and Fig. S4). Together, these data showed that dSarm induces apoptosis and neuronal loss.

JNK is a common pro-apoptotic effector, activated by $p75^{NTR}$ and Sarm1 in mammals, and Tolls in flies (Kim et al., 2007; Roux and Barker, 2002; Wu et al., 2015a). Thus to ask whether dSarm induces apoptosis by activating JNK, we tested whether JNK knock-down could rescue apoptosis caused by *dsarm* over-expression. Indeed, over-expressing *dsarm* in all neurons together with *JNK-RNAi* decreased apoptosis compared to *Elav>EP3610* (Fig. 5A). Thus, dSarm activates apoptosis via JNK. To further verify this, we asked whether MyD88 and dSarm affected activated pJNK+ cells in larval retinae. $MyD88^{kra56}$ */Df(2R)BSC279* mutants had normal pJNK+ cell number, but over-expressing *dsarm* increased pJNK+ cell number (Fig. 5E), and this increased further in a $MyD88^{kra56}$ mutant background (Fig. 5E). This showed that dSarm activates apoptosis via JNK, and antagonises MyD88 function.

To test whether dSarm could inhibit MyD88 through direct physical interaction, we carried out co-immunoprecipitations. S2 cells were co-transfected with *MyD88* tagged with V5 and *dsarm* tagged with HA. Precipitating MyD88 co-purified dSarm, showing that dSarm and MyD88 interact physically (Fig. 5F). Altogether, data showed that Sarm is an inhibitor of MyD88, and it induces apoptosis by antagonising MyD88 and by activating JNK signaling.

But if neuronal apoptosis depends on dSarm, why did MyD88 induce apoptosis in pupa? We had shown that over-expression of *MyD88* increased neuron number, over-expression of *cactus* decreased Eve+ neuron number, and *MyD88* loss of function did not affect pJNK cell number, implying that NF-κB does not directly promote apoptosis. Importantly, apoptosis caused by *MyD88* over-expression in neurons was rescued by *JNK-RNAi* knock-down (Fig.5A), meaning that apoptosis downstream of MyD88 requires JNK. This suggests that MyD88 might induce apoptosis by up-regulating the expression of *JNK, wek or dsarm*.

Our data had shown that Toll-6 can induce apoptosis, that it functions upstream of MyD88 to maintain neuronal survival, but MyD88 is inhibited by Sarm, which also induces apoptosis via JNK. So we asked whether Toll-6 and -7 could activate apoptosis by directly interacting with dSarm, using co-immunoprecipitations. We co-transfected S2 cells with *Toll-6-Flag* or *-7-Flag* and *dsarm-HA*, and found that precipitating Toll-6 or-7 did not co-precipitate dSarm (Fig. 5F). Thus, dSarm does not bind Toll-6 or -7, meaning that dSarm does not directly mediate the pro-apoptotic function of Toll-6.

Thus, data showed that Toll-6 functions upstream of dSarm and MyD88, to regulate neuronal death and survival, respectively (Fig. 5G). But they raised further questions: how can Toll-6 induce apoptosis if it does not bind dSarm? And why does Toll-6 promote cell survival in embryos and apoptosis in pupae?

Pro-apoptotic Toll-6 signaling requires Wek

Our data suggested there might be another adaptor linking Toll-6 to dSarm to enable pro-apoptotic signaling. Weckle (Wek) is an adaptor downstream of Toll-1 that recruits MyD88 to form a signaling complex during embryonic development, but not in innate immunity (Chen et al., 2006). To test whether pro-apoptotic Toll-6 signaling required Wek, we measured

apoptosis in the pupal VNC with anti-Dcp1 in loss and gain of function genotypes. Apoptosis levels decreased in *wek*^{EX14}*/Df(2L)BSC690* mutants compared to controls (Fig. 6A). Conversely, over-expression of *wek* in neurons increased apoptosis (Fig. 6A*).* These phenotypes were rescued by the over-expression of *wek* in neurons in a *wek* mutant background (Fig. 6A). Thus, Wek can promote apoptosis in the CNS.

To test the relationship of Wek with Toll-6 and dSarm, we carried out epistasis analyses. Loss of *wek* function rescued the increased apoptosis caused by the over-expression of *Toll6*^{CY} *(*Fig. 6A), showing that Toll-6 requires Wek to induce apoptosis. Loss of *wek* function did not rescue the apoptosis caused by the over-expression of *dsarm,* meaning that dSarm functions downstream of Wek (Fig. 6A). Furthermore, *dsarm* knock-down rescued the apoptosis caused by the over-expression of *wek,* showing that Wek induces apoptosis upstream of *dsarm* (Fig. 6A). In embryos, over-expression of *wek* caused Eve+ neuron loss (Fig. 6B and Fig. S4). In pupae, the number of Eve+ neurons did not change in *wek*^{EX14}*/Df(2L)BSC690* mutants, but decreased upon *wek* over-expression *(*Fig. 6C). Together, these data showed that Wek can promote apoptosis and neuronal loss downstream of Toll-6 and upstream of dSarm.

To test whether Wek could bind Toll-6 and dSarm, we carried out co-immunoprecipitations. We co-transfected S2 cells with *wek-HA* and *Toll-6-Flag* or *dSarm-Flag*, and found that precipitating Toll-6 or dSarm also brought down Wek (Fig.6D). Thus, Wek can bind both Toll-6 and dSarm.

To conclude, Wek is required downstream of Toll-6 to induce neuronal apoptosis via dSarm (Fig.6E). But a question still remained: why could Toll-6 promote cell survival in embryos, and cell death in pupae?

Adaptor profiles change in space and time

Our data suggested that the relative levels of MyD88, dSarm and Wek could determine neuronal life or death. Thus we used *MyD88GAL4* to ask how increasing the levels of Wek and Sarm relative to normal MyD88 levels would affect neurons. Over-expression of *wek* in MyD88+ cells decreased Eve+ neuron number in pupae compared to controls, and over-expression of *sarm (EP3610)* decreased Eve+ neurons further (Fig. 7A). Using the nuclear reporter *Histone-YFP,* over-expression of *wek* reduced cell number in pupae, and over-expression of *dsarm* reduced cell number even further (Fig. 7B). Remarkably, concomitant neuronal over-expression of *wek* with *MyD88* knock-down resulted in the most severe cell loss in pupal VNCs (Fig.7B). Since over-expression of *wek* alone had only a mild effect, this reveals that normally Wek is in a tug of war between dSarm and MyD88 signalling, that MyD88 and dSarm have antagonistic functions regulating cell number, and that Wek can engage both pathways downstream of Toll-6. Thus, relative levels of Wek, Sarm and MyD88 determine cell survival or death downstream of Tolls.

Toll-6 maintains neuronal survival in embryos and can promote both neuronal survival or death in pupae, suggesting that its signaling adaptors change over time. To test this, we used quantitative real time reverse-transcription PCR (qRT-PCR) and measured *MyD88, dsarm* and *wek* transcript levels in whole stage 17 embryos and in the dissected CNS of second and third instar larvae (L2, L3) and 1 day-old pupae. *MyD88* mRNA levels were high in embryos, decreased in L2 CNS, increasing again between L3 and white pupae (Fig.7C). Relative to MyD88 transcripts, *dsarm* mRNA levels were high in embryos, decreasing thereafter (Fig. 7C) and *wek* mRNA levels were virtually absent in embryos, and increased from L2 on (Fig. 7C). *wek* expression was consistently lower than that of *dsarm* and equal to *MyD88* from L2 onwards (Fig. 7C). The low levels of Wek in embryos suggest that in the embryonic CNS, Toll-6 can bind MyD88 to activate cell survival, but since there is no Wek, it cannot activate the dSarm pro-apoptotic pathway. In the pupa, in the presence of Wek, Toll-6

can activate either cell survival via MyD88 or cell death via dSarm. Thus, the temporal regulation of *wek* expression explains the different outcomes of Toll-6 function over time.

To visualise whether the spatial distribution of MyD88 and dSarm may also change, we used a *dsarm*$^{MIMIC\text{-}GFP}$ insertion, and *MyD88GAL4*^{NP6394} to drive the expression of membrane tethered *10xUAS-myr-td-Tomato* and anti-DsRed antibodies. Both were widely expressed throughout the embryonic CNS neuropile (Fig.4, Fig.5), widespread in larvae (Fig.7D), and more restricted in pupae (Fig.7E). In pupae, *MyD88>myr-td-Tomato* was distributed throughout the VNC, but prominently in thoracic interneurons, potentially linked to the motor circuitry (Fig. 7E). dsarm$^{MIMIC\text{-}}$GFP was distributed throughout the VNC, but prominently in ventral projections, apparently sensory circuits (Fig. 7E). These distinct patterns suggest that following cell number regulation, neural circuits acquire a characteristic composition of Toll-signalling adaptors.

Mammalian NTs can induce signaling from mammalian TLRs

To test whether the link between neurotrophins and Toll receptors might also occur in mammals, we carried out signaling assays with TLR2 an TLR4, which are cell membrane receptors present in the mammalian brain, and TLR5, an intracellular receptor(Gay et al., 2014). HEK293T cells were transfected with TLR2, 4 and 5, and a NF-κB luciferase reporter, and signaling was measured following stimulation with increasing concentrations of mature BDNF or NGF (Fig. S5). Whereas there was no effect upon stimulation of TLR2 or TLR5 with either NGF or BDNF, both ligands induced signaling in cells transfected with TLR4 (Fig. S5). Furthermore, treatment with NGF or BDNF altered the response of TLR2, -4, -5 to stimulation with their canonical innate immunity ligands (Fig.S5). This means that mammalian NTs can influence mammalian TLR signaling.

DISCUSSION

DNTs and Tolls regulate cell number plasticity by promoting both cell survival and death in the *Drosophila* CNS, through a three-tier mechanism.

In the first tier, each DNT has unique features, conducive to distinctive functions (Fig. 8A). Spz, DNT1 and DNT2 share with the mammalian NTs the unequivocal structure of the CK domain unique to this protein family. However, DNT1, -2 and Spz have distinct pro-domain features and are processed differently, leading to distinct cellular outcomes (Fig. 8B). Spz is only secreted full-length, and cleaved by Serine proteases (Hoffmann and Reichhart, 2002). DNT1 and -2 are cleaved intracellularly by conserved furins. In cell culture, DNT1 was predominantly secreted with a truncated pro-domain (pro-DNT1), whereas DNT2 was secreted mature. *In vivo,* both pro- and mature DNTs were produced from neurons. Interestingly, DNT1 also has an isoform lacking the CK domain (Zhu et al., 2008) and Spz has multiple isoforms with truncated pro-domains (DeLotto et al., 2001). Thus, *in vivo,* whether DNT1 and 2 are secreted full-length or cleaved, and whether Spz is activated, will depend on the proteases that each cell type may express. Pro-DNT1 activates apoptotic JNK signaling, whereas mature DNT1 and 2 activate the pro-survival NF-κB (Dorsal and Dif) and ERK signaling pathways. Mature Spz does not activate ERK. This first tier is evolutionarily conserved, as mammalian pro-NTs can promote cell death whereas furin-cleaved mature NTs promote cell survival (Lu et al., 2005). NF-┤ B, JNK and ERK are downstream targets shared with the mammalian NTs, downstream of $p75^{NTR}$ (NF-┤ B, JNK) and Trks (ERK), to regulate neuronal survival and death (Lu et al., 2005; Minichiello, 2009; Roux and Barker, 2002). Thus, whether a cell lives or dies will depend on the available proteases, which ligand type, and ligand-cleavage product it receives (Fig. 8A).

In a second tier, we showed that the specific Toll-family receptor activated by a DNT matters (Fig. 8B). Toll-6 and -7 could maintain neuronal survival, whereas Toll-1 had a predominant pro-apoptotic effect. Since there are nine Tolls in *Drosophila*, some Tolls could have pro-survival, whereas others pro-apoptotic, functions. Different Tolls also lead to different cellular outcomes in immunity and development (McIlroy et al., 2013; Meyer et al., 2014; Pare et al., 2014; Tauszig et al., 2000; Yagi et al., 2010). Thus, the life or death of a neuron will depend on the Toll or combination of Tolls it expresses (Fig. 8B). We also showed that binding of Spz to Toll-1 is most likely unique, but DNT1 and 2 bind Toll-6 and -7 promiscuously, and DNT1 and 2 with Toll-6 and -7 activate NF-┤ B and ERK, whereas pro-DNT1 activates JNK. This suggests that ligand pro-domains might alter the affinity for Toll receptors, and/or facilitate the formation of hetero-dimers between different Tolls, and/or with other co-receptors, to induce cell death. A 'DNT-Toll code' may regulate neuronal number.

In a third tier, available downstream adaptors determine the outcome between cell survival and death (Fig. 8C). Toll-6 and -7 activate cell survival by binding MyD88 and activating NF-┤ B and ERK (whether ERK activation depends on MyD88 is not known), and Toll-6 can activate cell death via Wek, dSarm and JNK signaling. We have shown that Toll-6 binds MyD88 and Wek, which binds dSarm; that dSarm binds MyD88, and promotes apoptosis by inhibiting MyD88 and activating JNK. Wek also binds MyD88 and Toll-1 (Chen et al., 2006). So, evidence suggests that Wek recruits MyD88 and dSarm downstream of Tolls (Fig. 8C). Since Toll-6 binds both MyD88 and Wek, and Wek binds both MyD88 and dSarm, Wek functions like a hinge downstream of Toll-6 to facilitate signaling via MyD88 or dSarm, resulting in alternative outcomes. Remarkably, adaptor expression profiles change over time, switching the response to Toll-6 from cell survival to cell death. In the embryo, when both MyD88 and dSarm are abundant, there is virtually no Wek, and Toll-6 can only bind MyD88 to promote cell survival (Fig.8C). As Wek levels rise, Toll-6 signaling can also induce cell

death. If the Wek-Sarm-JNK route prevails, Toll-6 induces apoptosis; if the Wek-MyD88-NF-κB route prevails, Toll-6 signaling induces cell survival (Fig.8C).

Thus, the cellular outcome downstream of DNTs and Tolls is context and time dependent. Whether a cell survives or dies downstream of DNTs and Tolls will depend on which proteases are expressed nearby, which ligand it receives and in which form, which Toll or combination of Tolls it expresses, and which adaptors are available for signaling (Fig. 8).

How adaptor profiles come about or change is not understood. A neuronal type may be born with a specific adaptor gene expression profile, or Toll-receptor activation may influence their expression. In fact, MyD88 reinforces its own signaling pathway, as Toll-6 and -7 up-regulate Dorsal, Dif and Cactus protein levels (McIlroy et al., 2013), and TLR activation increases Sarm levels (O'Neill and Bowie, 2007). We showed that apoptosis caused by MyD88 excess depends on JNK signaling. Since JNK functions downstream of Wek and dSarm, this suggests that MyD88 – presumably via NF-κB – can activate the expression of *JNK, wek* or *dsarm*. By positively regulating *wek* expression, MyD88 and dSarm could establish positive-feedback loops reinforcing their alternative pathways (Fig.8C, bottom). Since dSarm inhibits MyD88, mutual regulation between them could drive negative feedback. Positive and negative feedback loops underlie pattern formation and structural homeostasis, and could regulate neuronal number in the CNS too. Whether cell autonomous or non-autonomous mechanisms result in the diversification of adaptor profiles, either in time or cell type, remains to be investigated.

Either way, over time the Toll adaptors segregate to distinct neural circuits where they exert further functions in the CNS (Fig.8C). Toll-1, -6 and -8 regulate synaptogenesis and structural synaptic plasticity (Ballard et al., 2014; Halfon et al., 1995; McLaughlin et al., 2016). Sarm regulates neurite degeneration, and in the worm, it functions at the synapse to determine neuronal identity (Chuang and Bargmann, 2005; Osterloh et al., 2012). The

reporters we used revealed a potential segregation of MyD88 to the motor circuit, and dSarm to the sensory circuit, but this is unlikely to reflect the endogenous complexity of Toll-signalling circuitry, as *dsarm*^{MIMIC} has a GFP insertion into one of eight potential isoforms and *dsarm* also functions in the motor system (McLaughlin et al., 2016). Importantly, cell death in the normal CNS occurs mostly in late embryogenesis and in pupa, coinciding with neural circuit formation and remodeling, when neuronal number is actively regulated. Thus, the link by DNTs and Tolls from cell number to circuitry offers a complex matrix of possible ways to regulate structural plasticity in the CNS.

We have uncovered remarkable similarities between *Drosophila* Toll-6 and mammalian TLR signalling involving MyD88 and Sarm. All TLRs except TLR3 signal via MyD88 and activate NF-κB (Gay and Gangloff, 2007; Gay et al., 2014). Neuronal apoptosis downstream of TLRs is independent of NF-κB, and instead depends on TRIF and Sarm1 (Kaiser and Offermann, 2005; Kim et al., 2007; Ma et al., 2006; Mukherjee et al., 2013). Sarm1 is a negative regulator of TLR signaling, an inhibitor of MyD88 and TRIF (Carty et al., 2006). *sarm1* is expressed in neurons, where it activates JNK and promotes apoptosis (Kim et al., 2007; Mukherjee et al., 2013; Osterloh et al., 2012). However, the endogenous ligands for TLRs in the normal, undamaged brain, are not known. Our preliminary analysis has revealed the intriguing possibility that NTs either can bind TLRs, or induce interactions between Trks, p75^{NTR} and TLRs. It is compelling to find out whether TLRs regulate structural plasticity in the mammalian brain in concert with NTs.

To conclude, DNTs with Tolls constitute a novel molecular system for structural plasticity in the *Drosophila* CNS. This could be a general mechanism, to be found also in the mammalian brain, and in other contexts too, such as epithaelial cell competition and regeneration, and altered in cancer and neurodegeneration.

MATERIALS AND METHODS

Genetics

Mutant and reporter stocks

Control stocks were *yw* and/or outcrosses of *yw,* as most transgenic flies were all in a *w-* background. *MyD88^{kra56}* is an EMS induced hypomorphic allele(Charatsi et al., 2003) (gift of B. Moussian, Tübingen, Germany), *wek^{EX14}* is an excision loss of function allele(Chen et al., 2006) (gift of J.L. Imler, CNRS, Strasbourg). *dsarm^{4705}* and *dsarm^{4621}* are loss of function alleles of *dsarm* (gift of Marc Freeman, University of Massachusetts) Deficiencies *Df(2R)BSC279* lacks the *MyD88* locus and *Df(2L)BSC690* the *wek* locus, respectively. *Dorsal-GFP* (*w^{1118};PBac{dl-GFP.FLAG}VK00033/TM3, Sb^{1})* and *Dif-GFP (w^{1118};Pbac{Dif-GFP.FPTB}VK00033)*, are both GFP exon-trap lines. *Ect4^{MI00854}* and *sarm* are synonyms for the same gene, and *Ect4^{MIMICGFP} (yw;MiMicECT4[MI08854])* is a MIMIC insertion bearing GFP into the Ect4 locus. Stocks were balanced using CyOlacZ and TM6BlacZ to identify mutant embryos, or SM6aTM6B balancers carrying Tb- to identify mutant larvae and pupae. Double and triple mutants and other stocks were generated by conventional genetics.

Over-expression in vivo

GAL4 drivers: (1) *w;; elavGAL4* for all neurons; (2) *w; GMRGAL4* for the retina (gift of Matthew Freeman, University of Oxford); (3) *w; RG-GAL4* drives expression in the ring-gland and in a single neuron in the optic lobes (our unpublished data); (4) *w;MyD88GAL4: yw;P{GawB}MyD88^{NP6394}/Cyo, P{UAS-lacZ.UW14}UW14* (Bloomington Stock Centre). These were crossed to: (1) the membrane tethered reporter *w;; 10xUAS-myr-td-Tomato* (gift of B. Pfeiffer); (2) Activated forms of Tolls: *w;;UASToll-6^{CY}* and *w;;UASToll-7^{CY}(McIlroy et al., 2013)* and UASToll-1^{10b} (gift of J.M.Reichhart). (3) *w; UAS-MyD88-full-length* (gift of J.

Kagan); (4) *w; UAS-dsarm* (gift of Marc Freeman) drives expression of the *dsarm* cDNA(Osterloh et al., 2012) and w^{1118};*P{EP}EP3610/TM6B,*Tb^{1} drives expression of all *Ect4 (dsarm)* isoforms (Bloomington) (*Ect4* and *sarm* are synonyms for the same gene); (5) *UAS-wek-HA; UAScactus-HA* (FlyORF). (6) *w[11]; UAS JNK RNAi [P(GD10555*) (VDRC34138, Vienna Drosophila Research Centre); *UAS-dsarmRNAi; UAS-MyD88RNAi* (VDRC32396).

Structural modeling of DNTs and comparison to mammalian NTs

DNT1 and DNT2 were modelled on their closest structural homolog Spz using Modeller software (Webb and Sali, 2014), which builds *ab initio* the loops that were not observed crystallographically in Spz. The same method was used to complete the 3d model of Spz. The structure of the BDNF protomer is known in the context of heterodimerization with either NT3 (Robinson et al., 1995) or NT4 (Robinson et al., 1999). We generated a 3d model of the BDNF homodimer based on these heterodimers by substituting the neurotrophin with BDNF and performing energy minimization in Modeller (Webb and Sali, 2014). Protein sequences were analysed by Clustal Omega (Sievers et al., 2011) and Tcoffee (Notredame et al., 2000). Figures were generated in PyMol (DeLano Scientific, San Carlos, CA) using the lowest energy models with least clashes and best geometry according to Verify3D (Bowie et al., 1991) and MolProbity (Chen et al., 2010), respectively.

Bioinformatics and sequence analysis

Analysis of pro-domain: was carried out using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/), a secondary structure prediction programme.

Identification of conserved furin sites

Potential furin-cleavage sites in DNT1 and DNT2 were identified by PiTou prediction tool (Tian et al., 2012). To test the predicted cleavage sites mutant DNT1 and DNT2 constructs were generated by site-directed mutagenesis (see details below). S2 cells were transfected with full-length, truncated or mutant forms of DNT1 or DNT2 cloned into *pAct5c-3xHA* expression vector (see details below). After transfection, cells were separated from culture media and lysed in NP-40 buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Igepal CA-630). HA-tagged proteins in cell lysates and culture media were detected by anti-HA antibody using standard Western blots.

Primer design

Primers were designed using the public resource Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). For site-directed mutagenesis primers were designed using QuickChange Primer Design on-line tool (http://www.genomics.agilent.com/primerDesignProgram.jsp). For qRT-PCR to detect which Toll receptors are expressed in S2 cells, primer-BLAST was used to design specific primers (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Molecular biology

Generation of fusion constructs

Full-length or truncated cDNAs of DNT1 and DNT2 were cloned into an expression vector using a standard Gateway procedure, inserting them first into *pDONR* and subsequently into *pAct5c-3xHA* to generate the following constructs: *pAct5c-DNT1-FL-3xHA; pAct5c-DNT1 (Sp+CK+CTD)-3xHA; pAct5c-DNT2-FL-3xHA; pAct5c-DNT2 (Sp+CK)-3xHA.* Cloning to generate HA-tagged *dsarm* was also carried out using the Gateway system. *dsarm* cDNA was amplified from a *pUAST-dSarm* plasmid (gift from Marc Freeman) then subcloned first into

pDONR and subsequently into the destination vector resulting in *pAct5c-dsarm-3xHA. UAS DNT1-FL-GFP* and *UAS-DNT2-FL-GFP* were tagged at the C-terminus with GFP by cloning: *DNT1-FL-GFP* was cloned into pUASt using conventional ligation and transgenesis; *DNT2-FL-GFP* was cloned by Gateway cloning into *pUAS-GW-GFP*, followed by conventional transgenesis, using *white* as the selection marker. For all primers, see list in Table S2.

Generation of *MyD88$^{cr2.8}$* mutant allele by CRISPR/Cas9

A *MyD88* CRISPR mutant allele was created by designing a guide RNA targeting exon 1 of MyD88, using http://crispr.mit.edu/, with primers: MyD88BbSI sense gtcgCCGAGGGAGTTATGGACTCC and MyD88 BbSI anti-sense aaacGGAGTCCATAACTCCCTCGG, cloned in to the *BbsI* site of the pCFD3 U6.3 vector and verified by sequencing. Transgenic flies bearing U6.3 MyD88 gRNA were generated by ϕC31 transgenesis (injections by BestGene Inc). Flies bearing the guide RNA (*yscv;;U6.3MyD88gRNA attp2/TM3(sb)*) were crossed to flies carrying Cas9 driven by the nanos promoter (*ym{nosCas9}ZH2A*). Independent balanced stocks were stablished from F1 males (*w;MyD88^{CRISPR}/CYO*), and sequenced. *MyD88$^{cr2.8}$* bears a 7bp deletion that causes a frameshift at amino acid 64, and a premature stop codon at amino acid 94. This corresponds to the start of the Death Domain (DD, amino acids 90-172). This allele lacks the DD and TIR domains and is therefore a null allele. The sequence of the lesion is given below, the guideRNA sequence is given in **bold:**

```
MyD88WT         GTCAGTTATCGGCGTTATCGCACCGCTGGCATGGTGGTG**GCCGAGGGAGTTATGGACTCC**
MyD88CR2.8      GTCAGTTATCGGCGTTATCGCACCGCTGGCATGGTGGTGGCCGAGGGAGTTATG------
                **************************************************

MyD88WT         GGGTCGGGATCGGGCACGGGAACGGGCTTGGGGCACTTCAACGAGACCCCATTATCCGCA
MyD88CR2.8      -GGTCGGGATCGGGCACGGGAACGGGCTTGGGGCACTTCAACGAGACCCCATTATCCGCA
                 ************************************************************
```

and the aminoacid sequence:

```
MyD88WT         MRPRFVCHQQHSVAHSHYQPHSHFHHHTHRHPNPPHHHHIYGATDVSYRRYRTAGMVV**AE**
MyD88CR2.8      MRPRFVCHQQHSVAHSHYQPHSHFHHHTHRHPNPPHHHHIYGATDVSYRRYRTAGMVV**AE**
                ************************************************************
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MyD88WT          GVMDSGSGSGTGTGL------GHFNETPLSALGIETRTQLSRMLNRKKVLRSEEGYQRDW
MyD88CR2.8       GVMGRDRARERAWGTSTRPHYPHWASRPAPSCPACSTOP
```

Reverse transcription-PCR

RT-PCR was performed to see which Toll receptors are expressed in S2 cells. Total RNA was isolated from S2 cells by Trizol (Ambion) reagent following a standard protocol. Reverse transcription was carried out by using GoScript system (Promega). Standard PCR reaction was performed to amplify Toll receptor cDNA fragments using *Taq* DNA polymerase (Invitrogen). For list of primers see Supplemental Table S2.

Site directed mutagenesis

One or more point mutations were generated in *pAct5c-DNT1-FL-3xHA* and *pAct5c-DNT2-FL-3xHA* fusion constructs by site-directed mutagenesis according to (Wang and Malcolm, 1999)). The following mutant expression clones were used for S2 cell transfection: *pAct5c-DNT1-FL-R499G-3xHA; pAct5c-DNT1-FL-R283/499G-3xHA; pAct5c-DNT2-FL-R284G; pAct5c-DNT2-FL-R214/221/284G-3xHA.* For primers, see Table S2.

Quantitative Real Time PCR (Q-RT-PCR)

From 2 hour staged egg collections at 25°C, whole dechorionated embryos were harvested 20 hours after egg laying (AEL), and the CNS was dissected from L2 larvae at 48 hours AEL, L3 at 96 h AEL, and pupa 0-12 hours after puparium formation (APF), samples were placed immediately into TRI reagent (Ambion #AM9738) and frozen at -80°C. Total RNA was extracted from 20 embryos or 20 dissected larval or pupal CNS, using TRl and following manufactures instructions. cDNA was synthesized from 200ng of total RNA using the GOScript reverse transcription system (Promega #A5001) using random primers, diluted

three-fold for qPCR reactions and 2µl used per reaction. 'No-reverse-transcription' controls were run alongside cDNA reactions. Transcript levels were determined in triplicate for each sample using SensiFAST Hi-ROX SYBR GREEN (bioline #BIO-92020) run on an ABI Prism 7000 sequence detection system. The reference gene was *RpL32*, as it remained constant over the course of development. Primers used are given in Supplemental Table S1.

To obtain fold change values using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) for the developmental profiles of *MyD88, dsarm* and *wek,* the Ct value of *Rpl32* was subtracted from the Ct value of each gene and developmental time point to obtain ΔCt. All values were then normalized to the calibrator, which for this set of experiments was *MyD88* mRNA at embryo (ΔΔCt). Three independent biological replicates were carried out per experiment, and the mean ± standard deviation is provided in Fig. 5b.

Cell culture

Cell culture, transfection, stimulation and subcellular fractionation

S2 cells were maintained at 27 °C in InsectXpress medium (Lonza) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin-glutamine (Gibco). TransIT2020 (Mirus) transfection reagent was used to express target proteins in S2 cells. To stimulate S2 cells with mature DNT2-CK, S2 cells were transfected with *pAct5c-Toll-6-3xHA* or *pAct5c-Toll-7-3xHA* and were grown over-night in a 6-well plate (2 x 10^6 cells/well) Cells were serum starved for at least 6 hours and then were treated with purified DNT2-CK (50 nM) for 5-60 minutes.

To separate nuclear and cytoplasmic fractions, cells were pelleted and washed in ice-cold PBS at 500g for 5 min at 4°C. The cells were lysed in ice-cold harvest buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 0.5 M Sucrose, 0.1 mM EDTA, 0.5% TritonX-100, 1 mM DTT supplemented with a protease inhibitor cocktail (Thermo) and 5 mM NaF and 2 mM Na_3VO_4)

for 5 min on ice. Lysate was spun at 800g for 10 min at 4 °C. Supernatant was treated as cytoplasmic/membrane and pellet was treated as nuclear fraction. Cytoplasmic/membrane fraction was transferred in an empty tube and subsequently purified by centrifugation at 14.000g for 10 min at 4°C. Nuclei pellet was re-suspended in buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT supplemented with protease inhibitor cocktail and 5 mM NaF and 2 mM Na_3VO_4) and spun at 800g for 10 min at 4 °C. Supernatant was discarded and the pellet was re-suspended in buffer C (10 mM HEPES pH 7.9, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40, 1mM DTT supplemented with a protease inhibitor cocktail and 5 mM NaF and 2 mM Na_3VO_4) and incubated on ice for 30 min. Nuclear fraction was purified by centrifugation at 14.000 g for 10 min at 4 °C.

To analyse total cell lysate S2 cells were pelleted and washed in ice-cold PBS then lysed in RIPA buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 0.1% SDS, 0.1% Na-deoxycholate supplemented with a protease inhibitor cocktail and 5 mM NaF and 2 mM Na_3VO_4). Total cell lysate or subcellular fractions were analysed by SDS-PAGE followed by Western blot using standard procedures.

Co-Immunoprecipitations from co-transfected S2 cells

Co-immunoprecipitations from S2 cells were carried out as previously(McIlroy et al., 2013). S2 cells were transfected with the following combinations of plasmids: (1) *pAct5c-Toll-6-3xHA* and *pAct5c- Pro-TEV6HisV5-DNT1-CK-CTD; pAct5c-Toll-7-3xHA* and *pAct5c-Pro-TEV6HisV5-DNT2-CK; (2) pAct5c-MyD88-V5* and *pAct5c-Toll-6-3xFLAG* or *pAct5c-Toll-6^{CY}-3xFLAG* or *pAct5c-Toll-7-3xFLAG* or *pAct5c-Toll-7^{CY}-3xFLAG; (3) pAct5c- dsarm-3xHA* and *pAct5c-MyD88-V5; pAct5c- dsarm-3xHA* and *pAct5c-Toll-6^{CY}-3xFLAG* or *pAct5c-Toll-7^{CY}-3xFLAG; ; (4) pAct5c-Wek-3xHA* and *pAct5c-Toll6^{CY}-3xFLAG* or *pAct5c-dsarm-3xFLAG*. *pAct5c-MyD88-V5* plasmid was a gift of S. Wasserman; *.pAct5c-Wek-3xHA* was a

gift from J.L.Imler. Cells were collected 48h after transfection and lysed in NP-40 buffer or in FLAG affinity chromatography (FAC) buffer (50 mM HEPES pH:7.5, 80 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 0.2% TritonX-100) supplemented with protease inhibitor cocktail. Immunoprecipitations from lysates were carried out using mouse anti-V5 antibody in combination with protein-A/G magnetic beads (Thermo) or anti-FLAG antibody-conjugated agarose or magnetic beads (Sigma). Proteins were analysed by SDS-PAGE and western blotting, as described below.

Luciferase Reporter Assay in mammalian cells

HEK293 cells were seeded at 1 x 10^5cells/well in a 96-well plate 36 h prior to transfection with jetPEI (Polyplus). NF-κB-dependent gene expression was determined using a luciferase reporter construct concomitantly with indicated TLR vectors. The Renilla luciferase-thymidine kinase encoding plasmid (pRL-TK) was used to normalize for transfection efficiency, and pcDNA3.1 empty vector was used to maintain constant DNA. Cells were stimulated in a dose-dependent manner using neurotrophic agents: hNGF-β (H9666, Sigma), hBDNF (R&D Systems) or mNGF-7S (N0513, Sigma). Transfected cells were lysed using Passive lysis buffer (Promega) and assayed for luciferase and Renilla activity using luciferase assay reagent (Promega). Luminescence readings were corrected for Renilla activity and expressed as fold increase over unstimulated control values. Data is presented as mean +/- SEM of one of three independent experiments. Statistical analysis was performed using Two-Way ANOVA where we compared TLR signaling upon stimulation with varying concetrnations of NTs, or upon stimulation with both canonical innate immunity ligands and NTs.

Immunostainings

In vivo immunostainings in larval and pupal CNS

Dissections, fixations and immunostainings were carried out following standard procedures, except that for stainings to detect apoptosis in the pupal CNS only pupae within the first 10 minutes of puparium formation were used, to minimize biological variability in apoptosis levels over time. Primary antibodies used were: rabbit anti-GFP (1:500 in larvae and pupae, 1:1000 in embryos, Invitrogen #A11122), rabbit anti-DsRed (1:100, Clontech #632496), rabbit anti-βgal (1:5000, Cappel), mouse anti-Eve (1:5-1:10, DSHB 2B8), Mouse anti-Eve (1:20, DSHB 3C10), mouse anti-pERK (1:500 in retina and 1:100 in optic lobe, Cell Signaling #9106); mouse anti-Repo (1:250, DSHB 8D12); rabbit anti-pJNK (1:200, Promega V7931); rabbit anti-Myd88 (1:250)(gift of Steve Wasserman); rabbit anti-Dcp1 (cleaved *Drosophila* Dcp-1 (Asp216), 1:500 Cell Signaling 9578S). Secondary antibodies were directly conjugated Alexa488, 546 and 647 (1:250, Molecular Probes) or biotinylated mouse or rabbit (1:300) followed by avidin amplification using the Vectastain ABC Elite kit (Vector Labs) or the Tyramide Signal Amplification kit (Life Technologies T20922), using manufacturers instructions. For sample sizes see Supplemental Table S2.

Western blots

Western blotting was carried out following standard procedures. Primary antibodies used were: mouse anti-V5 (1:5000, Invitrogen, #R960-25), rabbit anti-FLAG (1:2000, Sigma, #F7425); mouse anti-Histone-H1 (1:10000,Upstate, #05-629); mouse anti-Tubulin (1:10000, Sigma, #T9026); chicken anti-HA (1:2000 and 1:5000, Aves, #ET-HA100), mouse anti-HA (12CA5)(1:2000, Roche, #11 583 816 001); mouse anti-Dorsal (7A4) (1:500, Hybridoma Bank), mouse anti-Cactus (3H12) (1:500, Hybridoma Bank), rabbit anti-Dif (1:500)(gift from D. Ferrandon). Secondary antibodies used were: anti-mouse HRP (1:5000, Vector Labs, #PI-

2000), anti-rabbit HRP (1:5000, Vector Labs, #PI-1000), anti-chicken HRP (1:10000, Jackson ImmunoResearch, #703-035-155).

Microscopy and imaging

Imaging

For microscopy, samples were mounted either in 70% Glycerol 30% PBTriton (larval and pupal fluorescent CNS, and non-fluorescent embryos) or in Vectashield (H-1000, Vector Labs; fluorescent embryonic CNS), Wide-field microscopy was carried out in a Zeiss Axioplan 2 microscope and 63x objective; images were taken under Nomarski optics with an AxioCam colour camera, and Zen Zeiss software. Fluorescent microscopy was carried out using secondary antibodies directly conjugated to Alexa 488, Alexa 546 and Alexa 647 (Molecular Probes). Laser-scanning confocal microscopy was carried out at room temperature using a Leica SP2-AOBS and a 40x or 63x lens at 512x512 or 1024x1024 pixels resolution, with 0.5 or 1 μm steps, and a Zeiss LSM 710 with 25x (oil) lens at 512x512 pixels resolution, with 1μm steps. Confocal image acquisition was carried out with Leica or Zeiss software, as per system. Each confocal stack comprised 100-300 images, which were processed as follows: (1) for image data, using Image J, to view the entire stacks of images, carry out horizontal and transverse projections, and rotate images; occasionally, a median or 'dust & scratches' filter was applied to a projection image, over the whole image. Adobe Photoshop was used to adjust levels, rotate and crop image and adjust image size to 300d.p.i. Adobe Illustrator was used to compile figure plates. (2) For quantitative data (e.g. number of Dcp1+ or Eve+ or YFP+ cells), we used the image-J plugins DeadEasy Larval Glia (which counts nuclear stains) and DeadEasy Caspase for Larvae (for apoptotic cells), as previously described and validated (Forero et al., 2012; Forero et al., 2009; Kato et al., 2011). DeadEeasy

analyses the entire stacks of images in 3D and identifies cells based on pixel intensity and 3D volume, and counts cells automatically in an entire CNS in 3D in less then a minute.

Quantitative data analysis

Penetrance is the frequency with which a phenotype is manifested within a population; expressivity the severity of the phenotype. Eve+ cells in embryos analysed under wide-field Nomarski optics were counted manually under an Axioplan 2 microscope and a 63x objective. Fluorescent pJNK+ cells in the retina were counted manually within the stacks of confocal sections, using ImageJ and the Cell Counter macro.

Dcp1+ apoptotic apoptotic cells from the entire VNC of the CNS were counted automatically using DeadEasy Caspase for Larvae (see above)(Forero et al., 2009), specific for apoptotic cells and optimized for the larval/pupal CNS (Kato et al., 2011). The entire VNC was counted, using the edges of the optic lobes as anterior boundaries. Eve+ in the larval CNS were counted automatically with DeadEasy Larval Glia software, which counts nuclear stains (see above)(Forero et al., 2012). For Eve+ cell counting, the thoracic (T1-T3) and posterior tip cells were excluded as cells there are too packed together, and only the cells from abdominal segments A1-A6 were counted.

Quantification of pixel intensity was carried out with ImageJ, setting a fixed Regions of Interest over the area posterior to the morphogenetic furrow or over the morphogenetic furrow, and the mean intensity in this area was normalized over the mean intensity of a fixed region of interest over the eye disc, anterior to the morphogenetic furrow.

Statistical analysis

Statistical analyses were carried out using SPSS and GraphPad Prism. Continuous data – e.g. number of Dcp1+, pJNK+ and Eve+ cells – were analysed first for normality, using curve

shape or kurtosis and skewness, and testing the homogeneity of variance with a Levene's test. If the Levene's test was not significant, One Way ANOVA was used, and Welch ANOVA if samples did not pass the Levene's test. Multiple genotypes were compared to a single control with post-hoc Dunnett, or compared to each other using Bonferroni, multiple comparison corrections tests. For TLR signaling, data were analysed using Two-Way ANOVA, and Dunnett post-hoc tests for multiple comparisons to NT=0 controls. For genetic experiments, reproducibility was confirmed by the overall large population sizes and consistent results in multiple repetitions of the experiments; for cell culture data, Q-RT-PCR and co-immunoprecipitations, the experiments were carried out at least three times. All p values, tests and sample sizes are provided in figure legends and further details in Supplemental Table S1.

Supplemental Material includes: five figures (Supplemental Fig.S1-S5), Table S1 with all genotypes, sample sizes and statistical analysis details and Table S2 with a list of primers used.

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FIGURE LEGENDS

Fig. 1 Structural models of DNTs compared to Spz and mammalian NTs. **(A-D)** Crystal structures and homology models of mammalian NTs and DNTs compared in the same

orientation relative to the CK. Representations in: **(A,B,D)** cartoon; **(B', D')** molecular surface color-coded by protomer; **(B'', D'')** surface charge distribution with a gradient from red to white to blue, corresponding to electronegative to neutral to positively charged molecular surfaces. Mammalian NTs **(A, B: BDNF, D: NGF)** and DNTs **(B, D)** have conserved CK but deviating beta-hairpin wings. Compare Spz and NGF (**A**, **D**), and DNT2 (green and grey) with BDNF (magenta and grey) in ribbon representation **(C). (E)** Spz residues mediating Toll-1 binding are not conserved among DNTs. Clustal annotation is * for identity, : for increased similarity). In Red: Spz residues from the proximal Spz chain interfacing with Toll-1; in yellow: from the distal Spz chain; blue triangles: conserved areas. Of 33 Toll-contact residues in Spz, only 11 are conserved in DNT1 and 2, with a single identical residue Tyr^{64} in Spz.

Fig. 2 DNT1 and -2 are cleaved by conserved furin proteases. (A) The pro-domain α–helix of Spz (box) required to activate Toll-1 is not conserved in DNT1 and 2, yellow highlight: corresponding sequences are not conserved. **(B)** The pro-domains of DNT1 and 2 but not Spz have conserved furin sites. **(C)** Site directed mutagenesis of furin sequences. **(D)** Mutant DNT1-FL-HA and DNT2-FL-HA forms, expressed in S2 cells and visualized in western blot with anti-HA from lysate and secreted medium. FL: full length; CK: cystine-knot; CTD: C-terminal domain; black arrowheads: normal forms; red arrows, mutant products. **(E)** Anti-GFP western blot upon over-expression of C-terminus-tagged DNTs in the retina with *GMRGAL4* shows that furin cleavage occurs in vivo.

Fig. 3 DNTs and Tolls activate pro-apoptotic and pro-survival pathways. (A) Over-expression of DNTs in larval retina with *GMRGAL4* altered pJNK activation. Box-plot graph: One-Way Anova $p<0.001$, *Dunnett post-hoc. **(B)** Stimulation of S2 cells with purified

DNT2-CK induced Dorsal phosphorylation. **(C)** Stimulation of S2 cells with DNT2-CK provoked the degradation of the cytoplasmic inhibitor Cactus, and the nuclear translocation of Dif and Dorsal, particularly in *Toll-6* or *-7* transfected cells. **(D)** Over-expression of *DNT1-CK-CTD* and *DNT2-CK*, but not *DNT1-FL* or *spz-CK*, activated ERK in *RGGAL4* neurons of the larval optic lobe, n=5-11. **(E)** Over-expression of activated T*oll-6*^{CY} and *-7*^{CY} in the retina increased pERK, *GMRGAL4>TORDER* is a positive control. Bar chart, error bars: standard devisation (s.d.), One-Way ANOVA p<0.0001, *Dunnett post-hoc, n=8-13. **(F)** Distinct effect of loss and gain of function for Tolls in Eve+ neuron number in larvae. Box-plots: Left: One Way Anova p<0.0001, *post-hoc Dunnett; Right: One Way ANOVA p<0.0001, *post-hoc Dunnett, n=5-22. **(G)** Different ligand forms and Toll receptors can induce either cell survival or death. For genotypes, statistical details and sample sizes, see Table S1. **p<0.01, ***p<0.001. .> means GAL4/UAS. Scale bar: 50µm.

Fig. 4 Toll-6 promotes cell survival via MyD88. (A) Co-immunoprecipitations showing that MyD88-V5 binds Toll-6Flag and Toll-7Flag, and activated Toll-6^{CY}Flag and Toll-7^{CY}Flag. IB: immuno-blot; IP: immuno-precipitation. **(B)** Anti-MyD88, and exon trap reporters Dorsal-GFP and Dif-GFP visualized with anti-GFP, are distributed throughout the embryonic CNS neuropile. Left: Horizontal views; right: transverse sections. **(C)** Loss of Eve+ neurons (arrows) in the CNS in *MyD88*^{kra56}*Toll7*^{P8}*Toll-6*^{26} triple mutant embryos. For quantification see Fig.S4. **(D)** Altering MyD88 signalling affects Eve+ neuron number. Box-plots: Larvae: One Way ANOVA p<0.001; *post-hoc Dunnett; Pupae: Welch ANOVA p<0.01, *post-hoc Dunnett, n=8-12. **(E)** Apoptotic cells visualized with anti-Dcp1 in white-pupal VNCs and counted automatically with DeadEasy software. Box-plot: Welch ANOVA p<0.001, *Dunnett post-hoc. *p<0.05, **p<0.01, ***p<0.001, n=5-16. For statistical details, see Table S1. > means GAL4/UAS. Scale bar: (B,C) 100µm; (D,E) 50µm.

Fig. 5 dSarm antagonizes MyD88 and promotes apoptosis downstream of Toll-6. (A) Apoptotic cells visualized with anti-Dcp1 in pupal VNCs, and quantified with DeadEasy. Box-plots: Left: Welch ANOVA $p<0.0001$; *Bonferroni post-hoc. Right: One Way ANOVA $p<0.0001$, *post-hoc Tukey, n=9-16 **(B)** Eve+ neuron number in the abdominal VNC of L3 larvae is regulated by dSarm. Box-plot: One Way ANOVA $p<0.0001$, *post-hoc Dunnett, n=9-12. **(C)** Anti-GFP in *dsarm*^{MI08854}*GFP* is distributed throughout the embryonic CNS neuropile. **(D)** Loss and gain of *dsarm* function affects Eve+ neuron number in embryos. For quantification see Fig.S4. **(E)** dSarm can activate JNK signaling, seen with anti-pJNK, in the larval retina. Box-plot: One-Way ANOVA $p<0.001$, *Dunnett post-hoc, n=4-18. **(F)** Co-immunoprecipitation from S2 cells showing that dSarm binds MyD88, but does not bind Toll-6 or -7. IB: immuno-blot; IP: immuno-precipitation. **(G)** dSarm inhibits MyD88 and activates JNK, promoting apoptosis. **$p<0.01$, ***$p<0.001$ See Table S1. >: GAL4/UAS. Scale bar: (A, C,E) 50µm; (B) 100µm.

Fig. 6 Wek mediates the pro-apototic function of Toll-6 upstream of dSarm. (A) Apoptotic cells in the white-pupal VNC visualized with anti-Dcp1 and quantified with DeadEasy. Box-plot: Welch ANOVA $p<0.001$, *Bonferroni post-hoc, n=8-16. **(B)** Over-expression of *wek* in all neurons with *elavGAL4* caused loss of Eve+ neurons in embryos (quantification in Fig. S4), and **(C)** in pupae. Box-plot: One Way ANOVA $p<0.001$, *post-hoc Dunnett, n=5-13. **(D)** Co-immunoprecipitation from S2 cells showing that Wek binds Toll-6 and dSarm. IB: immuno-blot; IP: immuno-precipitation. **(E)** Wek recruits dSarm and MyD88. **$p<0.01$, ***$p<0.001$, n.s.= not significant. See Table S1. >: GAL4/UAS. Scale bar: 50µm.

Fig. 7 Adaptors matter and change in space and time. (A) Over-expression of *wek* or *dsarm (EP3610)* with *MyD88GAL4* decreased Eve+ neuron number in pupal VNCs. Box-plot: One Way ANOVA p<0.001, *post-hoc Dunnett, n=6-9. **(B)** *MyD88*-expressing cells visualized with *MyD88GAL4*^{NP6394} and nuclear *Histone-YFP* are lost in pupae by altering levels of adaptors. Box-plot: Welch ANOVA p<0.001, *Bonferroni post-hoc, n=5-9. **(C)** qRT-PCR showing temporal profile of mRNA levels for *MyD88, dsarm* and *wek,* from whole embryos, L2 and L3 larval CNS and pupal CNS, normalized to *MyD88* mRNA in embryos (three biological replicates, mean ± standard deviation). **(D,E)** Expression of *MyD88* visualized with *MyD88*^{NP6394}*GAL4>20xUASmyr-td-tomato* and anti-DsRed, and of *dsarm* visualized with *Ect4*^{MI08854}*GFP* and anti-GFP, in the VNC of L3 larvae and pupae. Horizontal views, transverse views on the right for each. **p<0.01, ***p<0.001. See Table S1. >: GAL4/UAS. Scale bar: 50μm.

Fig. 8 Three-tier regulation of cell number plasticity by DNTs and Tolls. (A) Tier 1: Different ligand forms result from cleavage by furin proteases, and isoforms, and can lead to different cellular outcomes. Pro: pro-domain; CK: Cystine-knot domain; CTD: C-terminal domain. **(B) Tier 2:** Different Tolls can lead to different outcomes. **(C) Tier 3:** different adaptors downstream of Tolls drive alternative cellular outcomes. Adaptor expression changes in time and space. In embryos, Wek levels are low, and dSarm and MyD88 have independent functions. As Wek levels rise, it recruits dSarm and MyD88, and dSarm inhibits MyD88. Toll-6 promotes cell survival via MyD88 in the embryonic CNS, and with Wek it can also induce apoptosis in pupa. Surviving cells segregate into potentially overlapping but distinct neural circuits.

Fig. S1 Structural analysis of the pro-domains of Spz, DNT1 and DNT2 reveals

unique features in each ligand. Pink barrels indicate α-helices, orange arrows β-strands, lines coils, and yellow sequence highlight indicate the putative sequence that might correspond to the Spz α-helix in yellow involved in the activation of Toll-1. This helix is absent in DNT1 and -2 suggesting that their mechanism for receptor activation differs from that of Spz and Toll-1.

Fig. S2 S2 cells express Toll-1, -2, -5, -7 and -8. (A) Western blot showing the purity of subcellular fractionation samples for Fig.3C: anti-tubulin is restricted to the cytoplamisc/membrane (C/M) fraction, and anti-Histone-1 is enriched in the nuclear fraction (N). **(B)** Reverse transcription Ploymerase Chain Reaction **(**RT-PCR) from S2 cells, using primers to each of the Tolls, showing the presence of bands corresponding to expected product sizes, which are indicated on the left. The experiment was repeated in two consecutive passages of S2 cells, shown here as top and bottom gels. GAPDH was used as a house keeping control. Negative control reactions were performed in the absence of reverse transcriptase. S2 cells express Toll-1, Toll-2, Toll-5, Toll-7 and Toll-8, but not Toll-6.

Fig. S3 DNT1 and 2 bind Toll-6 and -7 promiscuously. Co-immunoprecipitations from S2 cells co-transfected with Toll-6HA or Toll-7HA, and DNT1-CK-CTD-V5 or DNT2-CK-V5, respectively. First three panels are controls. On the right: precipitating the ligands with anti-V5 brings down the receptors detected in western blot with anti-HA. Mock: no stransfection; CK: cystine-knot; CTD: caroxy-terminal domain; IP: immunoprecipitation; WB: western blot.

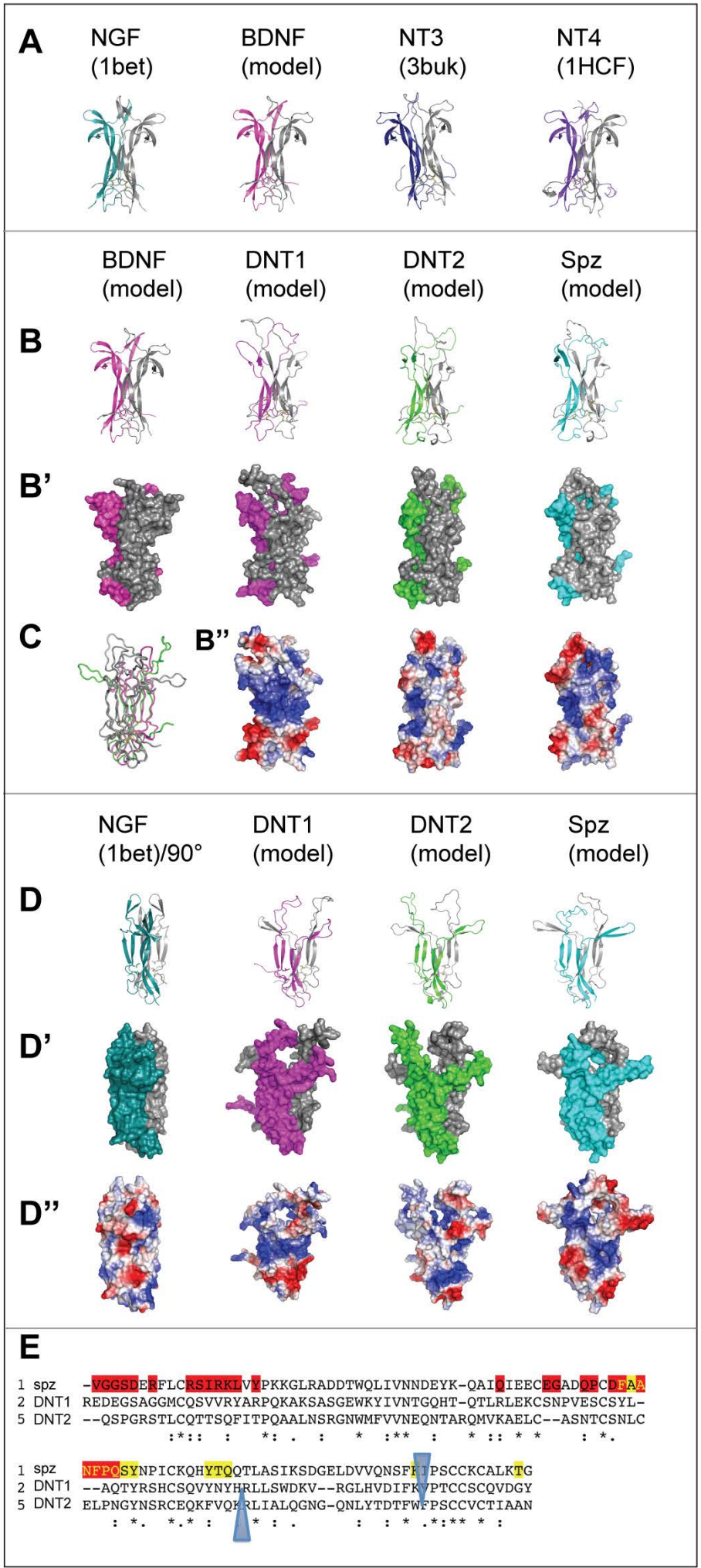
Fig. S4 Penetrance of Eve+ neuron number phenotypes in the embryonic CNS. (A-E) Percentage bar charts showing phenotypic penetrance of decrease in Eve+ neuron

number. **(A)** Dorsal aCC, pCC, RP2 Eve+ neurons are lost most prominently in *MyD88 Toll-7 Toll-6* triple mutants. Chi Square p<0.0001, Bonferroni multiple comparisons correction ***p<0.001, n=52-170. **(B)** Loss of dorsal aCC, pCC, RP2 Eve+ neurons in *dsarm* mutants. Chi Square p=0.0002, Bonferroni multiple comparisons correction ***p<0.001, n=41-170. **(C)** Loss of EL+U/CQ Eve+ neurons in MyD88, Toll-7 and Toll-7 loss of function mutants, double and triple mutants. Chi square all together p=0.0002, Bonferroni multiple comparisons correction ***p<0.001, n=28-119. **(D)** Eve+ ELs and U/CQs are lost in embryos over-expressing *sarm* in all neurons, and in $dsarm^{c705}/dsarm^{4621}$ mutants. Chi square all together: p<0.0001; post-hoc Fisher's Exact test and Bonferroni correction: **p<0.001; ***p<0.001, n=33-106. **(E)** ELs and U/CQs are lost in embryos over-expressing *wek* in all neurons. Fisher's Exact test ***p<0.001, n=106, 269. **(F-G)** Phenotypic penetrance of increase in the number of Eve+ neurons. **(F)** The number of dorsal Eve+ neurons aCC, pCC and RP2 increases in *dsarm* mutant embryos. Chi square *p<0.05, n=41, 170. **(G)** The number of EL+U/CQ Eve+ neurons increases in *dsarm* mutant embryos. Fisher's Exact test **p<0.01, n=16, 91. For statistical details, p values and sample sizes, see Table S1.

Fig. S5 Mammalian NTs elicit signaling from TLR4 and alter the response of several TLRs to their canonical immunity ligands. HEK293T cells transfected with TLR2, 4 and 5, stimulated with varying concentrations of purified NGF and BDNF cysknot dimmers, and their canonical ligands, activated or modulated a luciferase reporter NF-┤ B signaling readout. NGF and BDNF induced signaling from TLR4 but not TLR2 or 5. Two-Way ANOVA: for TLR4 BDNF: p<0.0001; for TLR4 NGF: p<0.0001. Dunnett post-hoc multiple comparison corrections to NT=0 controls, three repeats per experiment. NGF and BDNF altered the response of TLR4, TLR2 and 5 to their canonical ligands involved in innate immunity. This suggests either that BDNF and NGF compete with the canonical ligands to

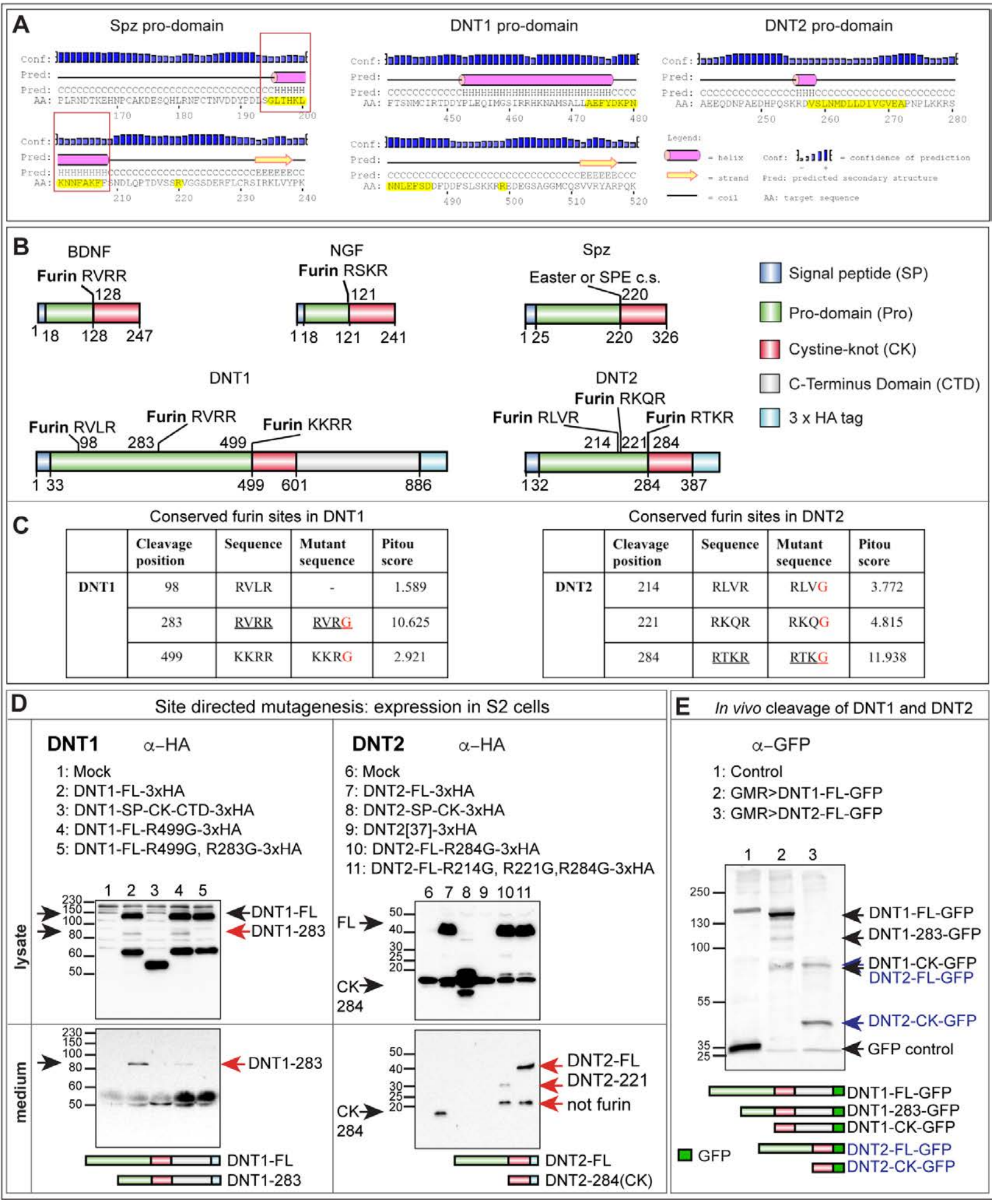
activate TLRs, or they bind other receptors that then modify signaling by TLRs. Two-Way ANOVA $p<0.0001$. Dunnett post-hoc multiple comparison corrections to NT=0 controls, three repeats per experiment. *$p<0.05$; **$p<0.01$; ***$p<0.001$. All data are from three replicates. For statistical details and sample sizes, see Table S1.

Foldi_Figure 1
Structural models of DNTs compared to Spz and mammalian NTs



Foldi_Figure 2

DNT1 and -2 are cleaved by conserved furin proteases.



	Cleavage position	Sequence	Mutant sequence	Pitou score
DNT1	98	RVLR	-	1.589
	283	RVRR	RVRG	10.625
	499	KKRR	KKRG	2.921

	Cleavage position	Sequence	Mutant sequence	Pitou score
DNT2	214	RLVR	RLVG	3.772
	221	RKQR	RKQG	4.815
	284	RTKR	RTKG	11.938

Foldi

Figure 3 DNTs and Tolls activate pro-apoptotic and pro-survival pathways

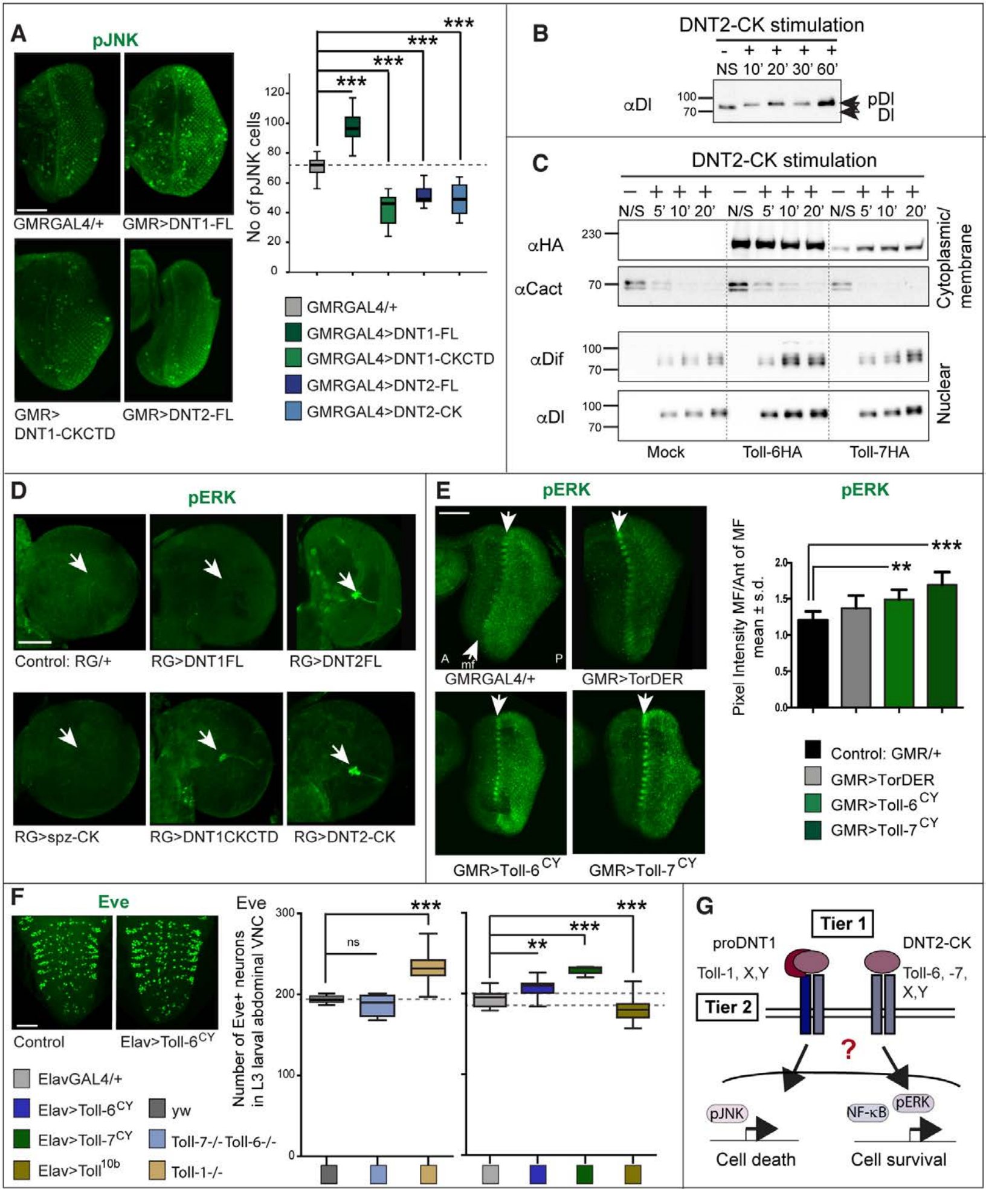
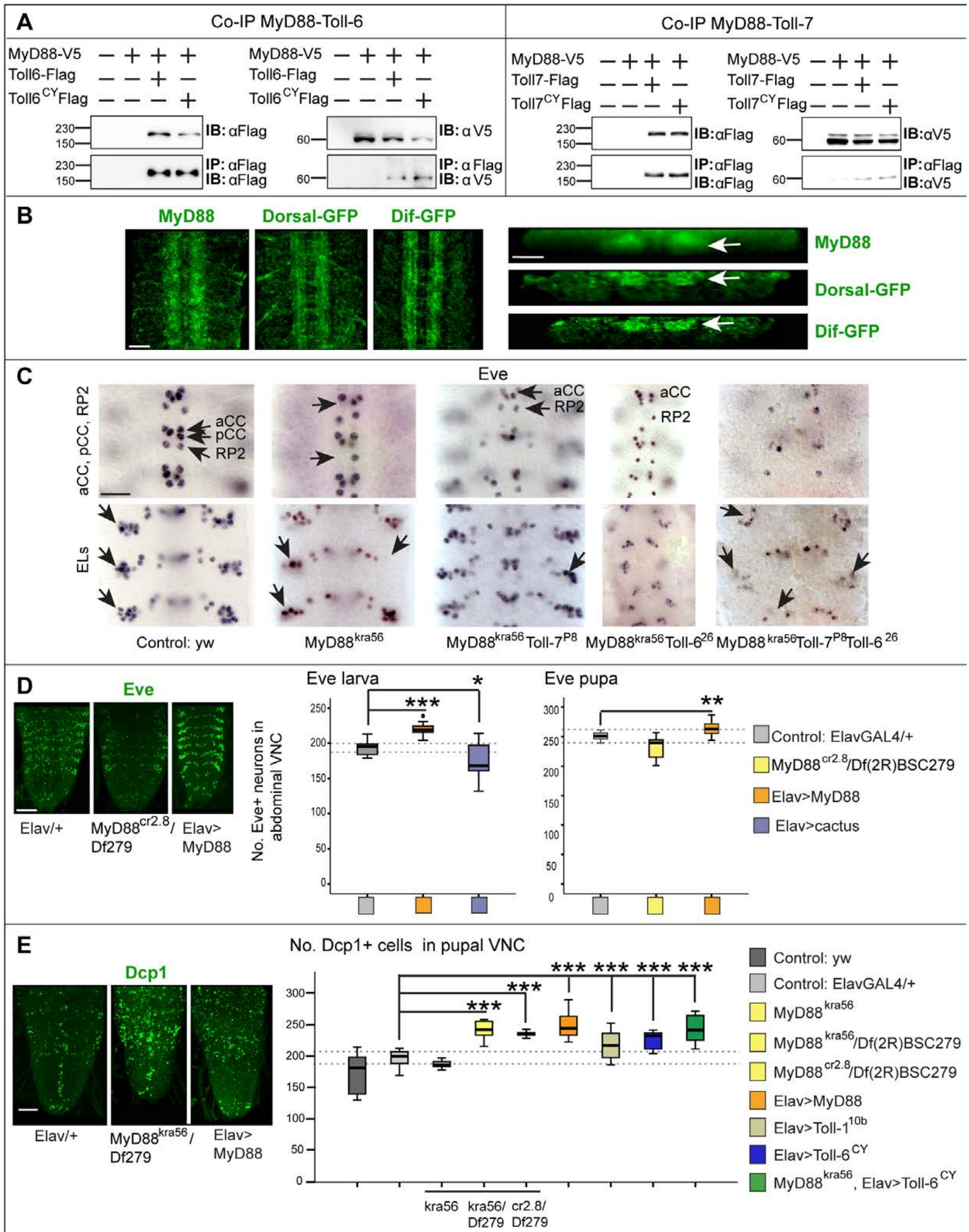
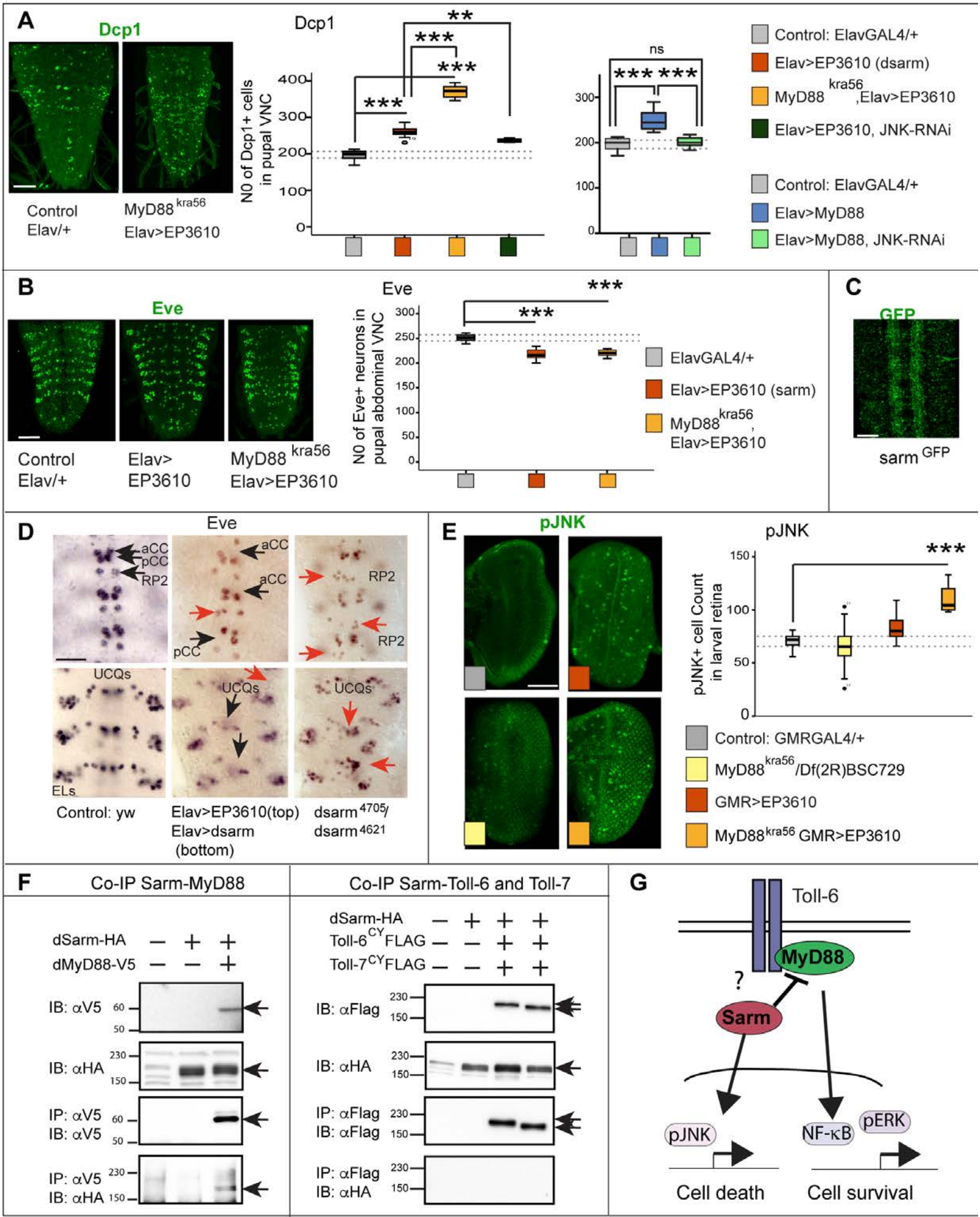


Figure 4 Toll-6 promotes cells survival via MyD88



Foldi_Figure 5
dSarm antagonises MyD88 and promotes apoptosis downstream of Toll-6



Foldi_Figure 6 Wek mediates the pro-apoptotic function of Toll-6 upstream of dSarm

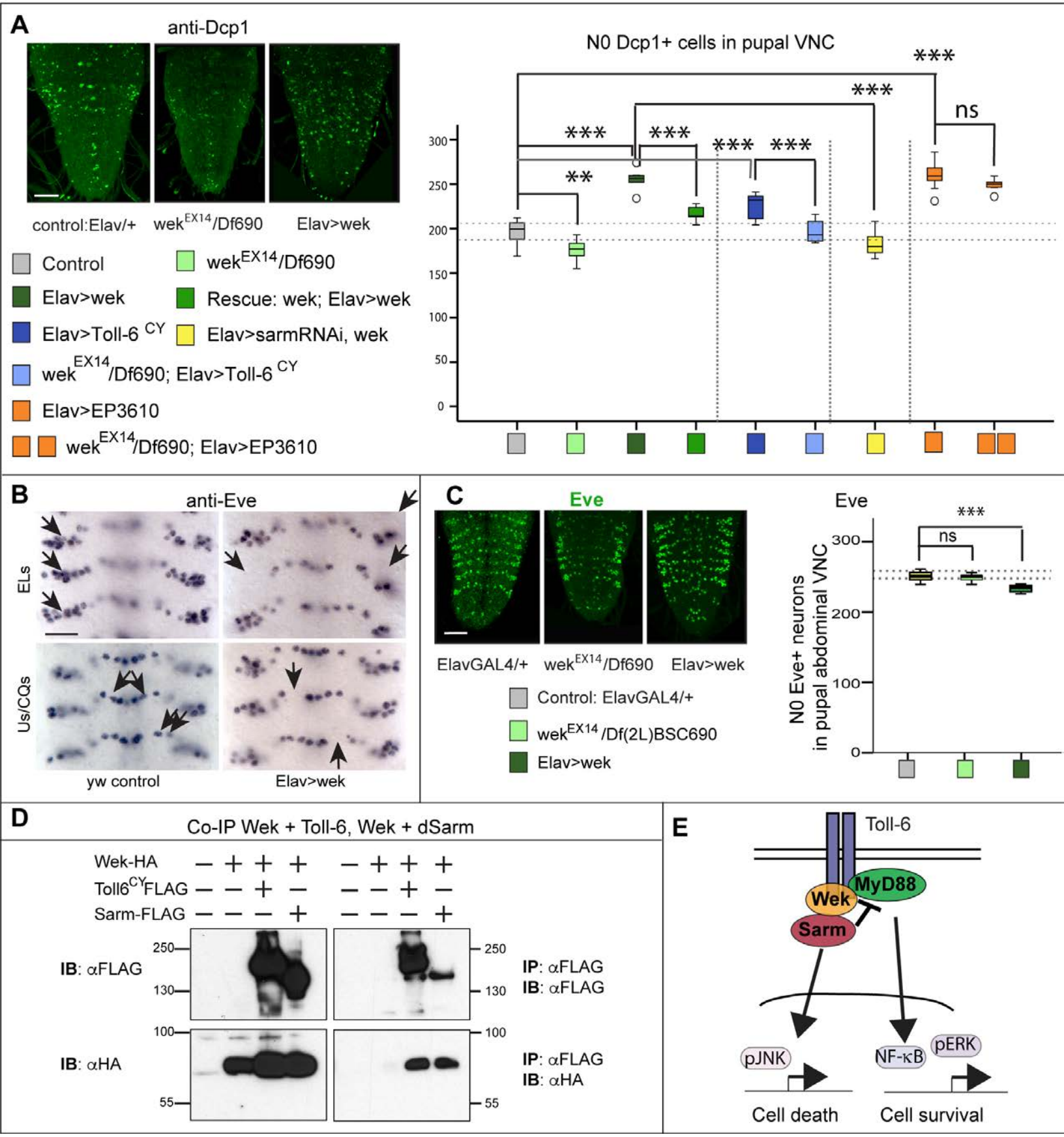
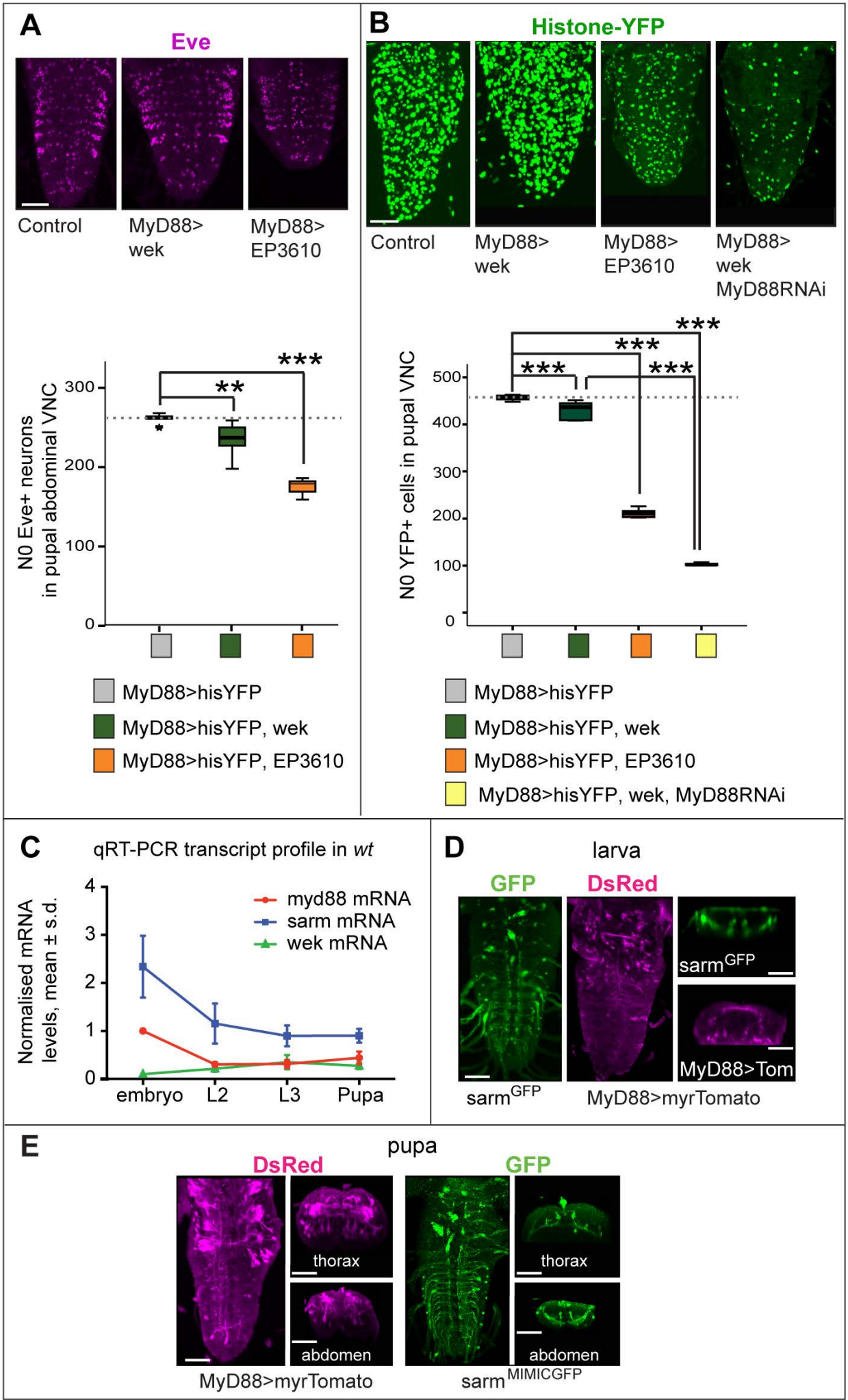


Figure 7 Adaptors matter and change in space and time



Foldi_Figure 8

Three-tier regulation of cell number plasticity by DNTs and Tolls.