# Studies on the neurotrophic factor Manf and the pleiotropic factor Lin-28 during *Drosophila* development

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ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki in **Auditorium 2**, **Korona Information Center**, Viikinkaari 11, Helsinki, on **December 05th 2014**, at 12 o'clock.

Helsinki 2014

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Cover layout by Anita Tienhaara
Cover image: Coronal section of *Drosophila* adult brain. Green: Manf, Red: Glia.

ISBN 978- 951-51-0386-4 (paperback)
ISBN 978-951-51-0387-1 (PDF; http://ethesis.helsinki.fi)
ISSN 2342-3161 (Print)
ISSN 2342-317X (Online)
Press: Hansaprint, Vantaa 2014

To my family

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# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications. The articles are reproduced with the permission from the copyright owners.

I Kallijärvi J, **Stratoulias V,** Virtanen K, Hietakangas V, Heino TI and Saarma M (2012). Characterization of *Drosophila* GDNF receptor-like and evidence for its evolutionarily conserved interaction with neural cell adhesion molecule (NCAM)/FasII. *PloS One* **7:** e51997.

II **Stratoulias V,** Heino TI and Michon F (2014). Lin-28 regulates oogenesis and muscle formation in *Drosophila melanogaster*. *PloS One* **9:** e101141.

III **Stratoulias V** and Heino TI. MANF silencing, immunity induction or autophagy can independently trigger macrophage-like cells in metamorphosing *Drosophila* brain. Submitted.

IV **Stratoulias V** and Heino TI Analysis of the conserved neurotrophic factor MANF in the *Drosophila* adult brain. Submitted.

This thesis is based on original publications, which in the text are referred to by their Roman numerals.

# ABBREVIATIONS

| | |
|---|---|
| ARMET | Arginine rich mutated in early stage tumors |
| APF | After puparium formation |
| ARP | Arginine rich protein |
| BDNF | Brain-derived neurotrophic factor |
| BrdU | 5-bromo-2'-deoxyuridine |
| CCHC | Cysteine-Cysteine-Histidine-Cysteine |
| CDNF | Cerebral dopamine neurotrophic factor |
| CNS | Central nervous system |
| CNTF | Ciliary neurotrophic factor |
| CSD | Cold shock domain |
| DAMP | N-{3-[(2,4-Dinitrophenyl)amino]propyl}-N-(3-aminopropyl)methylamine, dihydrochloride |
| DIOM | Dorsal oblique muscle |
| Dlg | Discs large |
| Dm | *Drosophila melanogaster* |
| DNP | Dinitrophenol |
| DNT | *Drosophila* neurotrophin |
| EcR | Ecdysone receptor |
| ER | Endoplasmic reticulum |
| ESC | Embryonic stem cell |
| FSC | Follicular stem cell |
| gcm | Glial cells missing |
| GDNF | Glial cell-derived neurotrophic factor |
| GFP | Green fluorescent protein |
| GFR | GDNF receptor |
| GPI | Glycosyl phosphatidylinositol |
| GSC | Germline stem cell |
| Imd | Immune deficiency |
| JAK / STAT | Janus kinase protein and the signal transducer and activator of transcription |
| LIF | Leukemia inhibitory factor |
| LSD | Lysosomal storage disorder |
| MANF | Mesencephalic Astrocyte-derived Neurotrophic Factor |
| MAPK | Mitogen-activated protein kinase |
| MARCM | Mosaic analysis with a repressible cell marker |
| MiC | Manf immunofluorescent Cell |
| mRNA | Messenger RNA |
| miRNA | MicroRNA |
| NF-κb | Nuclear factor k |
| NGF | Nerve growth factor |

| | |
|---|---|
| PCD | Programmed cell death |
| PGRP | Peptidoglycan recognition proteins |
| PGN | Peptidoglycan |
| PNS | Peripheral nervous system |
| PRR | Pattern recognition receptor |
| Repo | Reverse polarity |
| RNAi | RNA interference |
| TGF | Transforming growth factor |
| TH | Tyrosine hydroxylase |
| TNF | Tumor necrosis factor |
| TLR | Toll-like receptor |
| Trk | Tyrosine kinase |
| VNC | Ventral nerve cord |
| UAS | Upstream activating sequence |

# ABSTRACT

Developmental biology studies how a single cell will give rise to a fully developed, highly ordered and reproducible living organism. In order for this to occur, the fertilized egg needs to grow, divide and differentiate to produce different cell types. Subsequently, these different cell types need to organize into predefined arrays of committed cell groups to produce specialized tissues and organs, which constitute the living multicellular organism. Although the final product seems enormously complex, the multicellular organism is composed of cells that they all have the same set of genes. Cells differentiate by switching on and off the expression of different molecules and they produce and respond to signals which result in cells growing, dividing and dying in a strict spatiotemporal manner. In this work I aimed to address questions on the molecular mechanisms during development by using the fruit fly *Drosophila melanogaster* as a model system.

The first aim of my studies was to characterize and investigate the role of Manf during *Drosophila* pupation and adulthood. MANF is an evolutionarily conserved neurotrophic factor, which has previously been reported to protect and restore dopaminergic neurons in mammals. In *Drosophila* embryos DmMANF has been shown to be specifically expressed in glial populations, while DmMANF null mutants die early during development while they exhibit specific and significant reduction of dopaminergic neurites. Our data reveal that in pupae and adults, DmMANF has a much wider expression pattern and it is localized both in glia and neurons. This analysis led to the identification of an unusual phenotype in the *Drosophila* pupal brain. We showed when Manf is silenced, or either autophagy or immunity is induced in glia, macrophage-like cells appear in areas of the pupal brain that are normally devoid of cell bodies. We identified molecular markers and pathways that are activated in these cells, as well as some of the unique subcellular features they possess. This study brings new and elegant data in several aspects of glial biology with exciting perspectives for studying brain plasticity and repair.

The second project was to characterize the expression of Gfrl receptor, the *Drosophila* homolog of the mammalian GFRα receptor in the *Drosophila* adult brain. The results showed that although the Gfrl receptor is widely expressed in the fly adult brain, this expression is in surprising contrast to the missing expression of dRet, the *Drosophila* Ret homolog. This data provide novel insights into further elucidation of the Ret-dependent and Ret-independent -GFRα signaling complexes, as well as to further understand the several aspects of invertebrate brain development and function.

The third aim of this study was to investigate the role of the conserved pleiotropic factor Lin28 during *Drosophila* development. Previous studies have shown that Lin28 is one of the factors sufficient to reprogram human somatic cells into induced pluripotent stem cells. By constructing Lin-28 null mutants, our data reveal that Lin-28 mutant files were viable but sub-fertile, exhibiting oogenesis defects. These results will help us improve our understanding of Lin28 role in stem cell maintenance and differentiation.

# REVIEW OF THE LITERATURE

## *Drosophila* as a model system

As Krogh stated back in 1929 "… for a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied" (Krogh, 1929). This concept is central to disciplines related to biology, pharmacology and disease treatment, as scientists have to work with organisms, different from the ones they wish to apply their findings to. This is especially valid in biomedical research and its downstream applications that focus on the human health.

*Drosophila melanogaster*, commonly referred to as fruit fly, is one of the model organism cornerstones of biology research. It has been used continuously for over a century. Attributes such as cost effectiveness, short life span, fewer ethical and legal constrains (compared e.g. to mouse) has rendered the fly a very convenient model organism for generations of scientists. This has in turn led to the generation of a broad arsenal of sophisticated genetic tools and web-based databases. All of the above, along with the extensive functional conservation to human have rendered *Drosophila* the workhorse of modern genetics. Forward genetic approaches have resulted in the identification of a plethora of novel genes that control complex phenomena such as cell division, pattern formation and programmed cell death.

## Neurobiology: the study of the nervous system

The nervous system is a complex network of cells. It transmits signals to and from the brain and the spinal cord from various parts of the body. At the cellular level, the nervous system contains two main types of cells: (i) neurons that are long fibrous cells that transduce information in the form of electric potential and (ii) glia cells that are considered to perform mainly supportive role to neurons. In addition, a third cell type, called microglia, exists in the brain of vertebrates. At the anatomical level, the nervous system consists of two main parts: the central nervous system (CNS) and the peripheral nervous system (PNS) (Price et al., 2011).

In vertebrates, the CNS is composed of the brain and the spinal cord. Invertebrate animals, like *Drosophila*, do not have a spinal cord; instead, they have an analogous structure that is called the ventral nerve cord (VNC). The PNS and the VNC mainly consist of long fibers (neurons) that connect the CNS to all parts of the body. The nervous system is susceptible to malfunction in a wide variety of ways, as a result of genetic defects, physical damage due to trauma or poison, infection or simply aging (Price et al., 2011).

## Neurotrophic factors in vertebrates and their classification

Neurotrophic factors are a unique family of small, secreted proteins that provide trophic and survival-promoting support to neurons. The first neurotrophic factor to be identified was the nerve neurotrophic factor (NGF) (Levi-Montalcini and Hamburger, 1951; Cohen et al., 1954), a finding that was awarded with the 1986 Nobel Prize for Physiology or Medicine. The classical neurotrophic factor theory is largely based on studies on NGF (Levi-Montalcini, 1987). The theory states that during development an excess of neurons is produced. These neurons are programmed to undergo apoptosis, unless they receive sufficient trophic support. This trophic support is provided by tissues that surround the neurons (glia) or the tissues that the neurons innervate (e.g. muscles) in the form of small secreted proteins (i.e. neurotrophic factors). Neurotrophic factors, which are usually secreted in limited amounts, bind to receptors on neurons and they initiate a receptor-mediated intracellular signaling cascade resulting in halted apoptosis. Hence, only neurons that receive neurotrophic factors in sufficient quantities survive and maintain synaptic contacts, while the rest die through apoptosis. This process guarantees the correct matching of the number and properties of neurons to the demands of the innervated tissue (Levi-Montalcini, 1987). Interestingly, although mammalian neurotrophic factors have been studied for over 60 years and neurotrophin receptor homologs have been identified in organisms such as snails, mollusks and flies, no neurotrophic factors were identified in flies until very recently.

In vertebrates, neurotrophic factors are classified in five main families, based on their amino acid sequence and structure: Glial cell-derived neurotrophic factor (GDNF) family ligands [reviewed in (Airaksinen and Saarma, 2002)], neurotrophins [reviewed in (Bartkowska et al., 2010)] and neuropoietic cytokines [reviewed in (Bauer et al., 2007) ]. More recent families are the MANF / Cerebral dopamine neurotrophic factor (CDNF) [reviewed in (Lindholm and Saarma, 2010)] and the Meteorin/Cometin family of neurotrophic factors (Nishino et al., 2004; Jørgensen et al., 2009; Jørgensen et al., 2012). Neurotrophic factors signal through transmembrane receptor tyrosine kinases (Trks) or via kinases that interact with their receptors [reviewed in (Bespalov and Saarma, 2007)]. Each family acts through its distinct receptors and activate its distinct signaling cascade; however, the cellular responses they elicit are often redundant. Up to date, no receptors have been identified for the two more recently discovered families of neurotrophic factors, namely the MANF/CDNF and the Meteorin/Cometin families.

### *GDNF family ligands and their receptors*

The GDNF family members belong to the Transforming growth factor-β (TGF-β) superfamily, containing seven cysteine residues that are conserved in their primary structure and determine their protein fold (Airaksinen and Saarma, 2002). Its members are involved in the development, differentiation and maintenance of many neuronal types [reviewed in (Airaksinen and Saarma, 2002)]. Its founding member GDNF was identified

as a neurotrophic factor that promotes the survival and differentiation of dopaminergic neurons *in vitro* (Lin et al., 1993). GDNF has regenerative properties and along with BDNF (Brain-derived neurotrophic factor) and neurturin, they are the only neurotrophic factors used in clinical trials as a potential treatment for Parkinson's disease [reviewed in (Vastag, 2010)]. Other members of this family include neurturin (Kotzbauer et al., 1996), artemin (Baloh et al., 1998) and persephin (Milbrandt et al., 1998). The GDNF family ligands are secreted as pre-pro-proteins, which need to be cleaved and homodimerize in order to be functional. They signal through a heteromeric signaling complex composed of the transmembrane Trk receptor Ret and a class of co-receptor subunits [GDNF receptor (GFR) α subunits 1 to 4], which are linked to the plasma membrane by a glycosyl phosphatidylinositol (GPI) anchor (Treanor et al., 1996) (**Figure 1A**). The GFRα subunits confer the ligand specificity: GDNF binds to GFRα1, neurturin to GFRα2, artemin to GFRα3 and persephin to GFRα4, although weak crosstalk has also been documented (Airaksinen and Saarma, 2002).

*MANF / CDNF family*

The Mesencephalic astrocyte-derived neurotrophic factor (MANF) is the founding member of this neurotrophic factor family, which consists of two members. MANF was initially discovered as a gene mutated in renal cell carcinomas and named ARMET (Argenine Rich Mutated in Early Stage Tumours) or ARP (Arginine Rich Protein) (Shridhar et al., 1996). However, later studies showed that the polymorphisms attributed to ARP's tumorigenic function were also seen in normal tissue (Evron et al., 1997; Tanaka et al., 2000; Piepoli et al., 2006). Later ARP was identified as a novel factor that supports dopaminergic neurons *in vitro* and was renamed MANF (Petrova et al., 2003). Subsequent bioinformatic and biochemical studies identified a paralog to MANF called CDNF (Lindholm et al., 2007). MANF and CDNF comprise a novel family that is structurally unrelated to classical neurotrophic factors and growth factors (Lindholm and Saarma,

**Figure 1. Neurotrophic factors in vertebrates and their *Drosophila* homologs.** (**A**) In vertebrates, the dimer of GDNF ligand binds to the co-receptor GFRα and the GDNF-GFRα complex will activate the transmembrane receptor Ret. In flies, a Ret receptor (Hahn and Bishop, 2001) and a GFRα (Kallijärvi et al., 2012) receptor homolog have been identified. The ligand for either Gfrl or dRet has not been identified in flies. (**B**) Schematic presentation of the primary structures of *Drosophila* Manf (DmMANF) and the human MANF and CDNF proteins. In all MANF family proteins, the spacing of the 8 cysteines (as indicated by dotted lines) is strictly conserved. (**C**) Schematic presentation of the primary structure of *Drosophila* DNT1 and human members of the neurotrophin family proteins. Although the six-cysteine residues are conserved, the spacing (as indicated by dotted lines) differs even between neurotrophin members that derive from the same species. (**D** and **E**) No *Drosophila* homologs of the neurokine and the Meteorin/Cometin families have been identified.

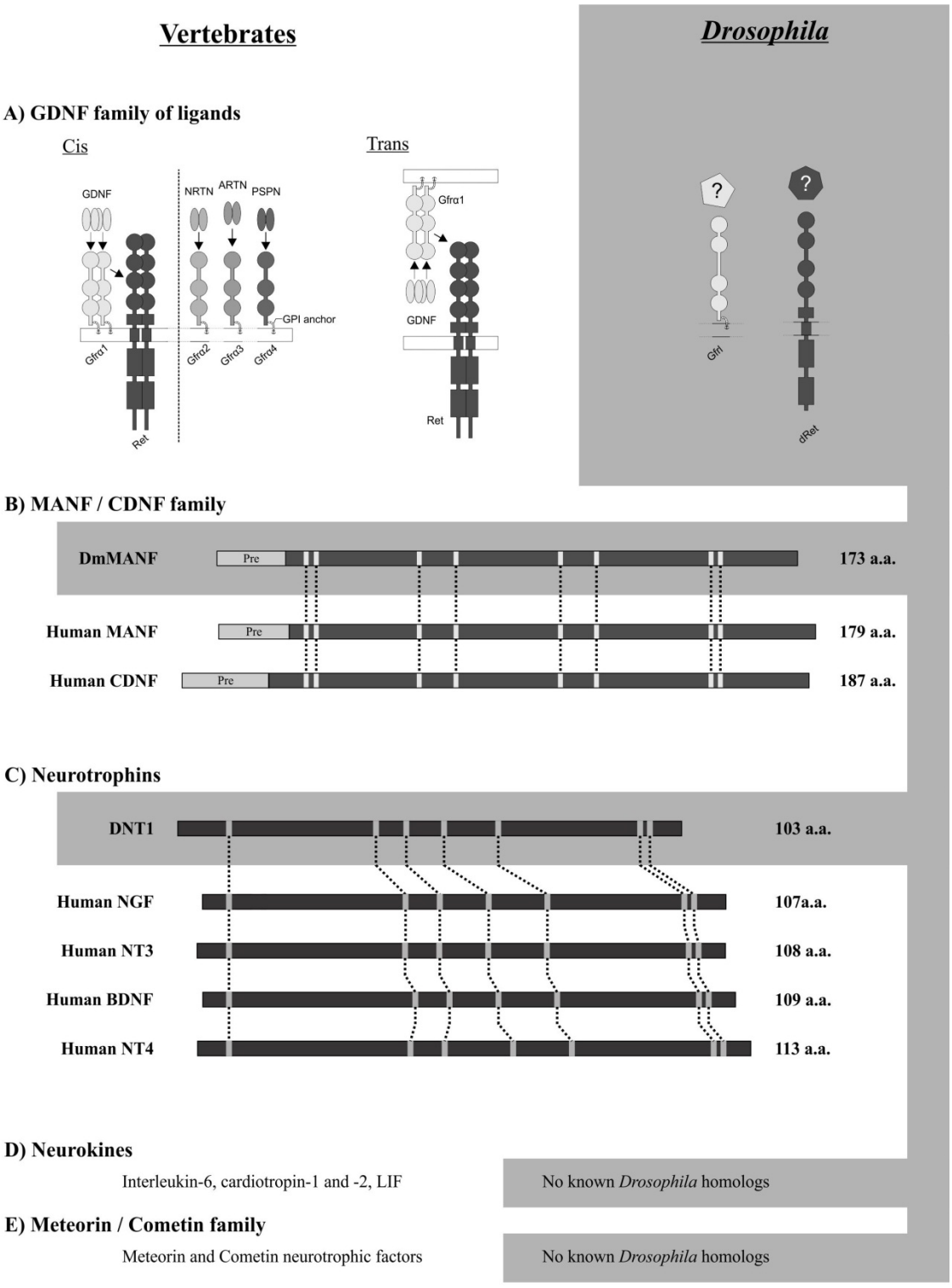
Vertebrates
Drosophila
A) GDNF family of ligands
Cis
Trans
GDNF
NRTN
ARTN
PSPN
GPI anchor
Gfrα1
Gfrα2
Gfrα3
Gfrα4
Ret
Gfrα1
GDNF
Ret
?
?
Gfrl
dRet
B) MANF / CDNF family
DmMANF
Pre
173 a.a.
Human MANF
Pre
179 a.a.
Human CDNF
Pre
187 a.a.
C) Neurotrophins
DNT1
103 a.a.
Human NGF
107a.a.
Human NT3
108 a.a.
Human BDNF
109 a.a.
Human NT4
113 a.a.
D) Neurokines
Interleukin-6, cardiotropin-1 and -2, LIF
No known Drosophila homologs
E) Meteorin / Cometin family
Meteorin and Cometin neurotrophic factors
No known Drosophila homologs

**Figure 1**

2010). They contain a secretion signal, but no pro-sequence, suggesting that they do not need to be enzymatically processed in order to become active. In addition, MANF and CDNF contain eight cysteine residues that are conserved in their primary structure (Shridhar et al., 1996; Petrova et al., 2003; Voutilainen et al., 2009) (**Figure 1B**).

Both MANF and CDNF were shown to specifically protect and restore dopaminergic neurons in rat (Lindholm et al., 2007; Voutilainen et al., 2009) and mouse (Airavaara et al., 2012) models of Parkinson's disease. Because the MANF/CDNF class of neurotrophic factors is structurally unrelated to previously identified neurotrophic factors, it is anticipated that it also activates distinct signaling mechanisms, compared to other neurotrophic factors (Lindholm and Saarma, 2010). This renders MANF protein an interesting molecule that can potentially lead to novel treatments of neurodegenerative diseases, and most importantly, Parkinson's disease.

According to crystal structure studies, the mature human MANF and CDNF consist of two domains: (i) a saposin-like N-terminal domain which may bind to lipids or membranes and (ii) a C-terminal domain, similar to that of thiol/disulphide oxidoreductases and isomerases which suggests that MANF and CDNF may be involved in protein folding in the endoplasmic reticulum (ER) (Parkash et al., 2009). On the molecular level, no upstream or downstream targets for MANF have been identified up to date. Recently however, it has been shown that MANF binds intracellularly to the KDEL receptor in the ER (Glembotski et al., 2012) and several lines of evidence point towards MANF having both extracellular and intracellular functions. From these, the best studied is its role as an ER stress-responsible protein (Apostolou et al., 2008; Palgi et al., 2012).

Both MANF and CDNF are expressed during all developmental stages in a variety of neuronal and non-neuronal tissues (Lindholm et al., 2007; Lindholm et al., 2008), a common feature for all neurotrophic factors. This expression in non-neuronal tissues suggests that they have a role outside the nervous system. In fact, it has recently been shown and that MANF protects cardiac myocytes in myocardial infraction (Glembotski et al., 2012) and that MANF deficiency results in pancreatic β cell depletion and diabetes in mice (Lindahl et al., 2014). In the brain, both CDNF and MANF are relatively widely expressed and are localized in neuronal populations such as cerebellar Purkinje cells (Lindholm et al., 2008). MANF but not CDNF has also been shown to be expressed in tyrosine hydroxylase (TH) positive neurons (Lindholm et al., 2008), while neither CDNF nor Manf is expressed in glial cells in mice (Lindholm et al., 2008). In addition, MANF is strongly expressed in embryonic salivary glands, pancreas, and in adult liver, heart, and testis and more specifically in the early spermatocytes (Lindholm et al., 2008). On the other hand, CDNF is also expressed in the heart, testis, but unlike MANF, it is strongly expressed in skeletal muscle of adult mice (Lindholm et al., 2007).

*Neurotrophins*

The term neurotrophin is often used as a synonym for neurotrophic factor. However, it is generally accepted that neurotrophin specifically refers to four structurally related factors: NGF (Levi-Montalcini and Hamburger, 1951), BDNF (Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3 (Hohn et al., 1990) and neurotrophin-4/5 (Berkemeier et al., 1991; Hallböök et al., 1991) [reviewed in (Bartkowska et al., 2010)]. Neurotrophins are synthesized as large precursors that are cleaved to release the mature and active form. The mature neurotrophins are 12 to 14 kDa in size and bear six cysteine residues, allowing them to form three disulphide bridges, which collectively form a protein structural motif known as cysteine knot (**Figure 1C**). One end of the molecule contains a cysteine-knot, which stabilizes the fold and locks the molecule to its specific conformation (Wiesmann et al., 1999; Wiesmann and De Vos, 2001), while the other end has three hairpin loops that are responsible for the functional differences between the proteins of the neurotrophin families (McDonald et al., 1991). Neurotrophins form homodimers and mediate their action through two classes of receptors, high- and low- affinity receptors. The low affinity receptor ($p75^{NTR}$) enhances the binding of neurotrophins to the high affinity receptors. The high affinity receptors are members of the Trk superfamily.

*Neurokines*

Neurokines, also known as Ciliary neurotrophic factor (CNTF) family after its most prominent member, also include interleukin-6, cardiotropin -1 and -2 and leukemia inhibitory factor (LIF). Members belonging to this category of neurotrophic factors are structurally similar to cytokines. They signal through cytokine receptors and activate the JAK-STAT and MAPK signal transduction pathways. Neurokines are mostly known for their role in the control of neuronal, glial and immune responses to injury and disease, as well as for their role in regulation of neurogenesis [reviewed in (Bauer et al., 2007)].

*Meteorin / Cometin family of neurotrophic factors*

Meteorin (Nishino et al., 2004; Jørgensen et al., 2009) and Cometin (Jørgensen et al., 2012) constitute the most recently described family of neurotrophic factors. Although little is known about their mechanism of action, data indicate that signal through the JAK/STAT and MAPK pathways (Jørgensen et al., 2009; Jørgensen et al., 2012). Interestingly, Cometin is only expressed during development and not in adult animals.

## Neurotrophic factors in invertebrates

Direct neuron-glia interactions and secretion factors that control proper nervous system development and function are well established in *Drosophila* [for review see (Silies and Klämbt, 2011)]. In addition, molecules secreted from axons have been shown to be required for glial survival. Such molecules include the *Drosophila* Neuregulin homolog Vein (Hidalgo et al., 2001) and the TGF-like ligand spitz (Hidalgo et al., 2001; Bergmann et al., 2002). However, no neurotrophic factors in invertebrates i.e. secreted factors that control the number, the survival and they provide trophic support to neurons, were identified until very recently.

Several lines of evidence support the existence of neurotrophic factors in *Drosophila*. Fly neurons are produced in excess and surplus is removed by programmed cell death (PCD) during development (White et al., 1994). In addition, it has been shown that in the *Drosophila* visual system, local absence of glia is accompanied by extensive apoptosis of neurons (Dearborn and Kunes, 2004); however, these signals are still unknown.

The first direct indication for the existence of neurotrophic factors in invertebrates came from the *in silico* identification (Sugaya et al., 1994) and subsequent cloning (Hahn and Bishop, 2001) of the *Drosophila* Ret (dRet), the receptor of the GDNF family of neurotrophic factors. Interestingly, dRet is expressed in the nervous and digestive system, expression pattern that it is in accordance to the mammalian Ret expression (Hahn and Bishop, 2001). Furthermore, chimeric receptors composed from the human extracellular Ret domain and the *Drosophila* intracellular dRet domain, respond to human GDNF (Abrescia et al., 2005), therefore suggesting a degree of functional conservation. However, up to date, no ligands have been identified for dRet and therefore its possible neurotrophic role has not been demonstrated (Sugaya et al., 1994; Hahn and Bishop, 2001). In addition, no homologs of the GDNF family ligands, the cytokine family ligands or the Meteorin/Cometin family appear to be encoded in the *Drosophila* genome or in other invertebrates (Airaksinen et al., 2006), possibly due to low amino acid sequence conservation.

Recently however, and greatly due to the number of animal whole sequencing data and the better performance of search algorithms in identifying sequence and structural homologies, molecules with neurotrophic factor properties in *Drosophila* have started to emerge. To date, two *Drosophila* neurotrophic factor families have been discovered, both of which share homologies to mammalian neurotrophic factors.

### *Drosophila* Neurotrophins (DNTs)

Spätzle and its receptor Toll were originally identified in screens detecting genes required for the dorsoventral axis formation of *Drosophila* embryos (Anderson et al., 1985). Spätzle, like neurotrophins, was shown to be secreted in an inactive form that upon cleavage and homodimerisation can exert its function (DeLotto and DeLotto, 1998;

Ligoxygakis et al., 2002; Weber et al., 2003; Hu et al., 2004). In addition, structural and biophysical data indicate that Spätzle has a cysteine knot domain that is very similar to the cystein knot of neurotrophins (DeLotto and DeLotto, 1998; Mizuguchi et al., 1998; Hoffmann et al., 2008). However, the overall sequence homology between Spätzle and neurotrophins is very low and hence Spätzle has not been considered as relevant to the neurotrophin superfamily. It was not until very recently that it was shown conclusively that Spätzle, along with two paralog proteins, namely DNT1 and DNT2, have neurotrophic properties in flies and belong to the neurotrophin family of neurotrophic factors that is evolutionarily conserved among protostomes and deuterostomes (Zhu et al., 2008) (**Figure 1C**). Interestingly, there are no canonical homologs of the mammalian neurotrophin receptors in flies. However, it was recently demonstrated that Toll receptors are mechanistically similar to vertebrate NTRs and DNT1 and DNT2 bind to Toll-7 and Toll-6 respectively, to exert their neurotrophic functions (McIlroy et al., 2013). In addition, crystallographic data show that the molecular structure of Spätzle/Toll complex is strikingly similar to NGF in complex to $p75^{NTR}$ (neurotrophin receptor) (Lewis et al., 2013).

### *Drosophila* MANF (DmMANF)

Sequence analysis among species also revealed a second neurotrophic factor, DmMANF, as homolog of the mammalian MANF/CDNF family of neurotrophic factors (Palgi et al., 2009). DmMANF shares a striking 50% and 47% amino acid sequence identity with human MANF and CDNF, respectively (Palgi et al., 2009; Lindholm and Saarma, 2010). In addition, the spacing between the characteristic eight-cysteine residues is strictly conserved (**Figure 1B**).

DmMANF, as its mammalian homologs, is also secreted (Palgi et al., 2009). It is maternally contributed to embryos and it is expressed widely in many tissues during embryogenesis and larval stages. In the embryonic and larval CNS, DmMANF is expressed specifically in the cell body glia that surround the TH-positive midline neurons. This subpopulation of glia also express the transcription factor *eagle* and it is identified as a glial subpopulation that is related to the mammalian astrocytes (Ito et al., 1995; Palgi et al., 2009). In addition, DmMANF is also found in longitudinal glia that express the transcription factor *prospero* (Palgi et al., 2009). In embryos, DmMANF does not co-localize with neuronal markers (Palgi et al., 2009).

DmMANF is also highly expressed in many non-neuronal tissues that have a high secretory activity. In addition to being expressed in the CNS during embryogenesis, DmMANF is predominantly expressed in garland cells (Palgi et al., 2012), which are highly endo- exo-cytotic cells showing structural similarities to mammalian kidney podocytes. The role of garland cells is to form a barrier and filtrate the hemolymph (Weavers et al., 2008). Other secretory tissues where DmMANF is expressed during embryogenesis and larval stages are salivary glands, Malpighian tubules, fat body, trachea (http://flyatlas.gla.ac.uk) and ovarian follicle cells (Palgi et al., 2009; Palgi et al., 2012).

*DmManf* null mutants die as early second instar larvae and exhibit specific and significant reduction of dopaminergic neurites, while other neuronal cell populations seem unaffected (Palgi et al., 2009). Interestingly, the fly null phenotype can be rescued by the human MANF (Palgi et al., 2009) and CDNF (Lindström et al., 2013), suggesting that the human and the fly MANF proteins are functionally orthologous and therefore evolutionarily conserved.

## Glia

Glia are abundant cells in the brain of animals including flies and humans. For long, they were thought to have mainly supportive roles. However, this notion is changing as new findings demonstrate that glia cells play an irreplaceable role in all aspects of brain development and function [e.g. insight issue in Nature edited by (Chouard and Gray, 2010)]. In the fly, glia, as well as neurons, derive from neuroblasts. Early glia cells express transiently the transcription factor *glial cells missing* (*gcm*), which turns on the homeodomain gene *reverse polarity* (*Repo*). *Repo* is required for glia differentiation and it is expressed in all glia during both development and adulthood. The only exception is a group of glia cells, namely midline glia, that do not express Repo, but express *gcm*. Interestingly, midline glia are eliminated during pupal stage and do not exist in adult flies (Rusconi et al., 2000). Elimination of midline glia occurs through apoptosis and is the result of elevated ecdysone levels during pupation (Rusconi et al., 2000). In addition to regulating the expression of glial genes, Gcm also regulates the expression of genes such as *draper* (Freeman et al., 2003), which are expressed in macrophages.

Different classes of *Drosophila* glia have been characterized, which share many morphological, functional and molecular similarities with their mammalian counterparts (Barres, 2008; Klämbt, 2009; Hartenstein, 2011). Glia in flies perform versatile functions, including pathfinding and enseathing of axons, they form the blood brain barrier [reviewed in (Edwards and Meinertzhagen, 2010)] and provide trophic support for neurons (Booth et al., 2000). Interestingly, flies do not have a “professional” resident macrophage glial cell type, similar to mammalian microglia in the vertebrate nervous system.

## Microglia –The macrophages of the vertebrate nervous system

Macrophages are highly specialized cells that constitute the cellular immunity of organisms including flies and humans (see later). In mammals, resident macrophages exist in all tissues of the body and are the first line of defense against injury and infection, responding rapidly to disturbances in tissue homeostasis. The resident macrophages of the mammalian nervous system are called microglia. Microglia belong to the glial system of non-neuronal cells and they are broadly distributed throughout the brain and spinal cord (Lawson et al., 1990). They constitute about 10% of the total CNS cell population (Hugh Perry, 1998; Mittelbronn et al., 2001).

Unlike macrophages, which are short-lived and regularly regenerated throughout life from cells in the bone marrow, microglia are long-lived cells – essentially the whole lifespan of the organism. They derive from primitive myeloid progenitors that originate from the extra-embryonic yolk sac (Ginhoux et al., 2010). They enter the nervous system where they proliferated and undergo a profound structural and physiological remodeling to differentiate into microglia cells (Ginhoux et al., 2010). In essence, microglia are the first glial cell population (4.5 weeks gestation for humans) and enter the nervous system before the formation of the blood-brain barrier, which prevents cells (including immune cells) to enter the nervous system. Therefore the initial microglial population that is formed during embryogenesis is the same found in adult animals. Local cell division that maintains the microglial number has been observed in rodents, but it is at a very low rate (Lawson et al., 1992).

Microglia have two main functional aspects: CNS maintenance and immune defense. Under physiological conditions, microglia are found in a “resting” state; they have a small cell body and multiple thin processes that extend in all directions and which continuously scan the environment for changes in brain homeostasis. Upon a brain insult, microglia are “activated”: they change their morphology to an amoeboid form, retract their processes and start upregulating a variety of cell-surface and cytoplasmic molecules, including neurotrophic factors, tumor necrosis factors (TNFs), Toll-like receptors (TLRs), pro-inflammatory mediators and immune response molecules (Rivest, 2009; Perry et al., 2010; Kettenmann et al., 2013). They also become motile and move towards the injury. Under special conditions (e.g. cell death in the CNS), microglia transform further and become phagocytotic. Thus, microglia can produce their effect either through phagocytosis or through secreting various factors with trophic, neuroprotective and transmitter properties (Perry et al., 2010). The nature of the signal that triggers the process of microglia activation is not fully understood, but it can be both due to endogenous and exogenous stimuli [reviewed in (Kierdorf and Prinz, 2013)].

Microglia are activated when the brain homeostasis is disturbed. Virtually, all mammalian brain pathologies are accompanied by activation of microglia, including infections and inflammation. In addition, in CNS tissues from patients with chronic neurodegenerative diseases like Parkinson's disease and prion disease, active microglia are always present in large numbers (Perry et al., 2010). Microglia also have an active role during development and adult circuit plasticity and function [reviewed in (Tremblay et al., 2011)].

## Immunity in *Drosophila*

Immunity is the ability of an organism to resist infection and disease through its immune system. The immune system has two main functions: to recognize invading pathogens and then trigger pathways that will destroy them. Immunity is divided into innate and adaptive immunity based on the specificity of the response towards the pathogen. Innate immunity

is the first and most ancient line of defense against pathogens. It provides resistance to a wide range of pathogens through means of non-specific response such as phagocytosis (cellular immunity) and secretion of antimicrobial peptides, through activation of NF-κb pathways (humoral immunity). Antimicrobial peptides are an evolutionarily conserved component of the innate immune response found in all animals and plants and represent the main form of invertebrate systemic immunity. On the other hand, in adaptive immunity, the response is "custom made" to the pathogen through specific antigen – antibody recognition.

Although it is commonly accepted that *Drosophila* has only innate immunity, this notion is under challenge as new studies show that *Drosophila* has a primitive form of adaptive immunity (Watson et al., 2005; Dong et al., 2006). In addition, *Drosophila* has retained RNA interference (RNAi) as a potent antiviral pathway, in contrast to mammals that have probably lost this ability (Sabin et al., 2010).

## Cellular immune response

As in humans, the *Drosophila* cellular immune response is elicited by leukocyte-like cells, which are known as hemocytes [reviewed in (Wood and Jacinto, 2007; Ulvila et al., 2011)]. Hemocytes are found in the hemolymph (blood) of the fly where they either circulate freely, or they are associated with various tissues and organs. Apart from constituting the cellular immune response during pathological conditions, hemocytes are also responsible for phagocytosis of apoptotic cells during embryogenesis as well as during organ remodeling during metamorphosis (Abrams et al., 1993; Tepass et al., 1994; Franc et al., 1996). The *Drosophila* hemocyte population is divided into three classes: plasmatocytes, crystal cells and lamellocytes, which appear in two different hematopoietic waves during *Drosophila* development (**Figure 2**). From these three hemocyte populations, plasmatocytes are the most abundant, constituting 95% of the embryonic hemocyte population. Interestingly, hemocytes of embryonic origin persist to adulthood (**Figure 2**).

Of special importance, is that after the sealing of ventral nerve cord at 20 hours of development (Schwabe et al., 2005) and the formation of the blood brain barrier, hemocytes surround the ventral nerve cord without entering it (Kurant et al., 2008). Hemocytes have not been identified in the nervous system. Instead glia assume a semi-professional phagocytotic role, a function that is strongly reflected on the molecular level by a strong differential expression of phagocytosis genes in glia (Kurant et al., 2008).

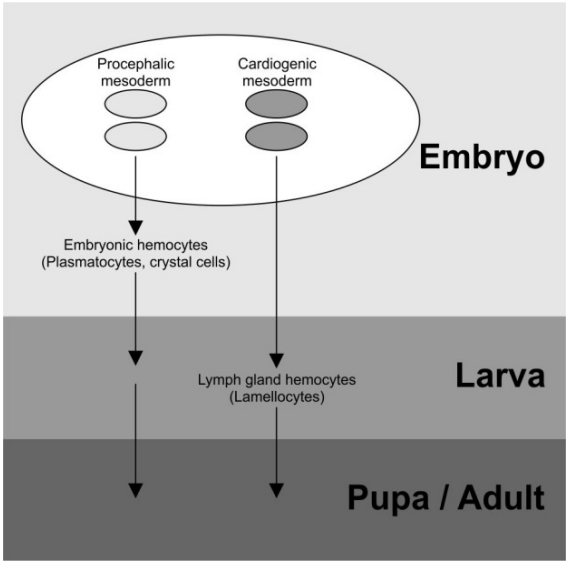


**Figure 2. Hemocyte specification in *Drosophila*.** Embryonic hemocytes arise in the procephalic mesoderm in early embryogenesis, while lymph gland hemocytes derive later in development from cardiogenic mesoderm. Both embryonic and lymph gland hemocytes contribute to the hemocyte population of the pupae and adults. Image modified from (Wood and Jacinto 2007).

## Humoral immune response

NF-κb family members are an evolutionarily conserved class of transcription factors. They regulate numerous fundamental biological processes, among which is the control of the expression of genes encoding anti-microbial peptides. NF-κb proteins are found inactive in the cytoplasm. Upon stimulation, they are activated either by proteolytic cleavage or by degradation of their corresponding inhibitory protein and subsequent release. Consequently, they translocate to the nucleus to control the transcription of immune responsive genes (Hetru and Hoffmann, 2009; Sabin et al., 2010; Ganesan et al., 2011; Kounatidis and Ligoxygakis, 2012).

In flies, three NF-κb proteins, namely Dif, Dorsal and Relish, control almost half of the immune responsive genes (Ganesan et al., 2011). Two well-characterized NF-κb pathways control antimicrobial peptide gene expression in *Drosophila*: the Toll and the Immune deficiency (Imd) pathways. In both cases, peptidoglycans (PGN) on the pathogens cell walls are recognized by pattern recognition receptors (PRRs). The latter are activated and consequently activate NF-κb transcription factors, which in turn translocate to the nucleus to induce an anti-microbial program (**Figure 3**). The *Drosophila* Toll pathway shares similarities with the invertebrate Interleukin-1 and the Toll-like receptor pathways, while the Imd pathway is homologous to the TNF pathway (Hetru and Hoffmann, 2009; Sabin et al., 2010; Ganesan et al., 2011; Kounatidis and Ligoxygakis, 2012).

### *Toll pathway*

The Toll pathway is activated by Gram-positive bacteria and fungi, which are initially, recognized by peptidoglycan recognition proteins (PGRPs) and glucan-binding proteins

(Hetru and Hoffmann, 2009). The latter initiate a cascade that results in the activation of the cytokine Spätzle, which in turn activates the transmembrane receptor Toll (Hetru and Hoffmann, 2009; Kounatidis and Ligoxygakis, 2012). The activation of Toll results in the phosphorylation of Cactus, which is targeted for degradation. Upon Cactus degradation, the NF-κb members Dif and Dorsal are translocated to the cell nucleus, where they act as transcription factors controling the expression of genes encoding immune-responsive peptides (Hetru and Hoffmann, 2009; Kounatidis and Ligoxygakis, 2012).

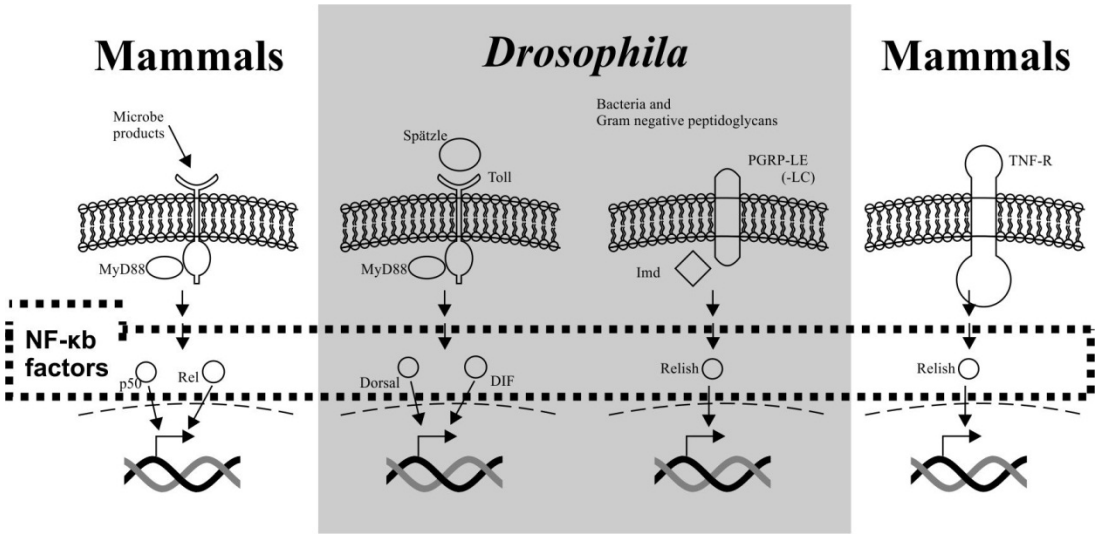


**Figure 3. Comparison of the mammalian and the fly humoral immunity.** In both flies and mammals, a receptor is activated and transduces its signal to the effector molecule that is an NF-κb molecule. The NF-κb molecule will be translocated into the nucleus where it will activate immune responsive genes. In flies, the Toll pathway will activate dorsal, while the Imd pathway will activate the transcription factor Relish.

*Imd pathway*

The Imd pathway is activated by diaminopimelic acid containing PGNs, commonly found on the cell walls of Gram-negative bacteria. These molecules bind to two PGN family members, PGRP-LC and PGRP-LE, activation of which leads to nuclear translocation of the NF-κB factor Relish (Hetru and Hoffmann, 2009; Kounatidis and Ligoxygakis, 2012). Upon immune challenge, Relish is cleaved so its N-terminal fragment to translocate to the nucleus and it acts as a transcription factor to activate immune-responsive peptides, while its C-terminal fragment remains in the cytoplasm (Stöven et al., 2000). Cleavage *per se* however, is not sufficient for Relish translocation (Kleino et al., 2005). Recently, it has been reported nuclear localization of the C-terminal fragment of Relish (Tapadia and Verma, 2012).

### Immunity in the *Drosophila* brain

In comparison to vertebrates brains where microglia are the dedicated immune system, in the fly, no microglia or microglia-like cells have been identified. During both development and adulthood, sessile glia assume a semi-professional immune role which is critical for the elimination of superfluous or damaged cells through PCD and against brain homeostasis disturbances. Glia have been shown to be the main phagocytes in fly embryos (Freeman et al., 2003; Kurant et al., 2008) and their role has been studied in apoptotic clearance and axon pruning during metamorphosis (Awasaki and Ito, 2004; Dekkers et al., 2013) and after neural injury in adult flies (MacDonald et al., 2006).

Microglia-like cells have been identified in mollusks (Stefano et al., 1996), annelids (Elliot and Muller, 1981) and have been shown to appear under *in vitro* conditions in cockroaches (Sonetti et al., 1994). *Drosophila* has macrophages (hemocytes) that display phagocytotic and scavenger properties and perform apoptotic clearance during embryogenesis (Sonnenfeld and Jacobs, 1995). However, once the nerve cord is enseathed and the blood-brain barrier is formed, hemocytes no longer have access to the nervous system (Kurant et al., 2008) and no other professional immune cell type has been identified in *Drosophila* brain (Edwards and Meinertzhagen, 2010).

## Autophagy

Autophagy is a secondary ancient and evolutionarily conserved defense mechanism that organisms use to eliminate pathogens (Yano et al., 2008; Levine et al., 2011). It occurs independently of the Toll and Imd innate signaling pathways. Autophagy is the catabolic mechanism that involves the degradation of a cell's own components via the lysosomal machinery. Eukaryotic cells have evolved this self-cannibalization process as a means to maintain cellular homeostasis during unfavorable conditions. Autophagy is induced under stress conditions, such as starvation, hypoxia, heat and drug treatment. It is well characterized and requires more than 20 autophagy (atg) genes (Xie and Klionsky, 2007).

Autophagy has been implicated as a means of PCD, called autophagic cell death or type II PCD, in contrast to apoptosis (type I PCD) (Schweichel and Merker, 1973). Although this notion is under great debate, recent studies, argue that at least in *Drosophila*, autophagy acts as a mean of PCD, both in stress situations (Berry and Baehrecke, 2007) as well as during normal development (McPhee et al., 2010). Notably, in *Drosophila* the engulfment receptor Draper has been demonstrated to be autonomously required for autophagy associated with cell death, while it is not required for autophagy associated with cell survival (McPhee et al., 2010).

## Oogenesis

The egg is the largest cell in all animals, conversely with the other germ cell, the sperm, which is usually the smallest cell (Alberts et al., 1994). The human egg is visible by the naked eye and it is 1000 times larger than a typical somatic cell. The enormous size of the egg is due to the fact that the egg needs to store large amounts of messenger RNA (mRNA) and proteins that are needed for oogenesis, fertilization and development (Alberts et al., 1994). This is especially true for animals like *Drosophila*, which do not have a placenta and their oocyte is transcriptionally inactive (Alberts et al., 1994).

In *Drosophila*, eggs are continuously produced from stem cell populations, resulting in an independent array of egg chambers, known as ovariole [oogenesis reviewed in (Spradling, 1993)]. Each ovary is composed of several ovarioles, which all have the same anterior-posterior direction (**Figure 4A**). The anterior-most egg chamber, the germarium, houses two stem cell populations: the germline stem cells (GSCs) and the follicular stem cells (FSCs). In each germarium there are two or three GSCs (**Figure 4A and 4B**). The GSC divides asymmetrically to produce another stem cell and a cystoblast. Cystoblasts undergo four mitotic divisions with incomplete cytokinesis to form a cluster of 16 cells (cystocytes), which are interconnected with cytoplasmic bridges called ring canals (**Figure 4C**). Of the 16 cystocytes, only one will become the oocyte, while the other 15 will

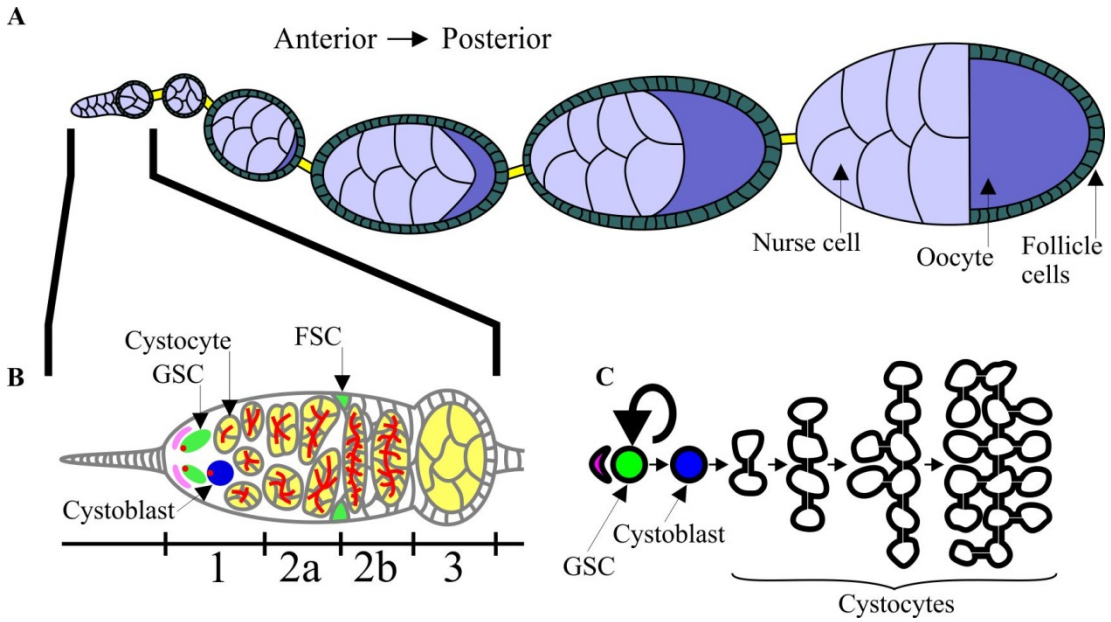


**Figure 4. *Drosophila* oogenesis.** (**A**) Schematic drawing of an ovariole with germarium at the anterior tip and egg chambers of increasing age. The egg chamber consists of three main cell types. The germ line derived oocyte, its endopolyploid sister cells called nurse cells and the follicle cells, which are of somatic origin. (**B**) Magnified view of germarium. Cap cells in pink and spectrosome/fusome in red (**C**) GSCs divide asymmetrically to give rise to a cystoblast and a GSC. The cystoblast will divide four times with incomplete cytokinesis to produce 16 cystocytes.

become the nurse cells. The oocyte is always the posterior-most cell in the egg chamber (**Figure 4A**) and retains a proteinaceous organelle, the fusome, which was present in the original cystoblast. Germ cells and cystoblasts contain a spherical cytoplasmic structure called the spectrosome, which develops into a branched fusome that transverses the intercellular ring canals, linking the cytoplasm of individual cystocytes. The fusome will disappear after the last mitotic division (**Figure 4B**).

Oogenesis in *Drosophila* has been divided into 14 stages. During stage 9, the follicle cells migrate towards the oocyte, leaving only very few cells at the anterior of the egg chamber. At stage 10, a group of anterior follicle cells called border cells, will start to migrate towards the anterior of the oocyte through the nurse cells. This migration is crucial for the formation of the micropyle, which is needed for the egg fertilization.

During oogenesis, the nurse cells are transcriptionally very active. This is pronounced by the large and highly polyploid nurse cell nuclei, compared to the nucleus of the transcriptionally inactive oocyte nucleus that is called the karyosome. Nurse cells produce large amounts of RNA and proteins that are consequently transported to the oocyte through the ring canals, while the follicle cells secrete the vitelline membrane and the chorion.

## Lin-28 and *let-7*

Lin-28 is an evolutionarily conserved RNA-binding molecule that gained ample attention after it was identified to be one of the reprogramming factors used to induce pluripotency in adult human fibroblast cells (Yu et al., 2007). Lin-28 is unique among the reprogramming factors as it is the only RNA binding protein, rather than a transcription factor. Initially, *lin-28* was discovered in a *C. elegans* screen for heterochronic genes that control developmental timing (Ambros and Horvitz, 1984). In *C. elegans* Lin-28 is expressed broadly during the first larval stage and subsequently its levels are reduced during the second and is greatly diminished by the third and fourth larval stages (Moss et al., 1997). Lin-28 downregulation is mediated by two miRNAs, namely *let-7* and *lin-4* (Moss et al., 1997). *lin-28* homologs exist in all bilaterian animals ranging from flies to humans, indicating an ancient origin of this gene (Moss and Tang, 2003). All homologs are composed of four domains, a positively charged linker that binds three RNA-biding motifs: two Cys-Cys-His-Cys (CCHC)-type zinc-binding motifs and a cold shock domain (CSD) (Moss and Tang, 2003). Interestingly, all homologs have conserved *let-7* binding domains, indicating that *lin-28* is regulated by *let-7* (Moss and Tang, 2003). Subsequently, *lin-28* mRNA was shown to be a conserved target of the *let-7* miRNA family both in *C. elegans* and in higher vertebrates (Reinhart et al., 2000; Yang and Moss, 2003). On the other hand, Lin-28 was found to inhibit *let-7* processing (Viswanathan et al., 2008), by physically interacting with the *let-7* precursor, and therefore preventing further processing towards the mature *let-7* form (Newman et al., 2008; Loughlin et al., 2011). Further

studies heavily concentrated on the double-negative feedback loop between these two pivotal molecules, Lin-28 and *let-7*.

*let-7* was also identified in a screen for heterochronic genes that control developmental timing in *C. elegans* (Reinhart et al., 2000). It was one of the first two known miRNAs (Ambros, 2001) and the first miRNA to be discovered in humans (Pasquinelli et al., 2000). *let-7* miRNA is also evolutionarily conserved in all bilaterians and from flies to humans in both sequence and function and is expressed in a similar temporal pattern in diverse species (Pasquinelli et al., 2000). Targets of *let-7*, apart from Lin-28, are the oncogenes RAS (Johnson et al., 2005; Kumar et al., 2008), KRAS (Chin et al., 2008), HMGA2 (Mayr et al., 2007) and c-myc (Sampson et al., 2007). Interestingly, *let-7* was found to have an expression that is reciprocal to Lin-28 expression in various contexts. In mice and humans, Lin-28 is highly expressed in embryonic stem cells (ESCs), but its downregulated in most differentiated adult tissues; on the other hand, *let-7* levels are not detectable in ESC, but its levels increase upon differentiation and are maintained in adult tissues [(Sempere et al., 2004; Thomson et al., 2004; Schulman et al., 2005; Wulczyn et al., 2007; Van Wynsberghe et al., 2011) and reviewed in (Viswanathan and Daley, 2010)].

Subsequently, the role and relationship between Lin-28 and *let-7* has been extensively studied in relevance to cancer in a broad range of cancer types, including ovarian, prostate, colon, lung, epithelial and breast cancers among others [see reviews (Boyerinas et al., 2010; Huang, 2012; Zhou et al., 2013)]. Their relationship during tumorigenesis reflects their reverse relationship during embryogenesis. Lin-28 is largely seen as a powerful regulator of stem cell and germ cell self-renewal, while *let-7* is associated with progression of differentiation (Oishi and Wang, 2011) (**Figure 5**). As a result, both molecules are explored as potential biomarkers (prognostic markers) and theurapeutic targets in cancer research [see reviews (Boyerinas et al., 2010; Huang, 2012; Zhou et al., 2013)].

Recent data indicate that Lin-28 has also a *let-7* independent mechanism of action and plays an important role in translational regulation. A recent genome-wide association study revealed that in human ES and somatic cells Lin-28 target more than 6,000 genes, through a GGAGA binding motif (Wilbert et al., 2012), indicating that Lin-28 plays a prominent role in post-transcriptional regulation. In addition, other studies have demonstrated that Lin-28 directly interacts with and regulates the production of histone H2a (Xu and Huang, 2009), Cyclin A, Cyclin B and Cyclin-dependent kinase 4 (Xu et al., 2009) and IGF-2 (Polesskaya et al., 2007). Interestingly, Lin-28 has also been shown to bind and regulate *Oct4* mRNA (but not *NANOG* or *SOX2*) (Qiu et al., 2010).

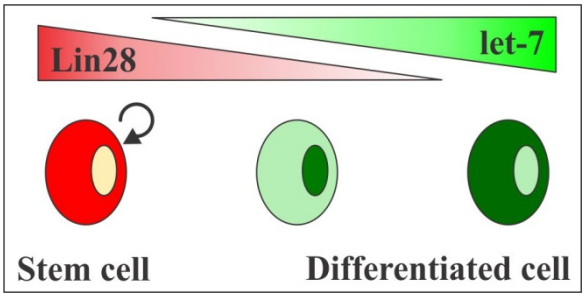


**Figure 5. Lin-28 is highly expressed in stem cells, while *let-7* in differentiated cells.** Lin-28 blocks *let-7* miRNA maturation. As stem cells differentiate, Lin-28 expression decreases, which allows *let-7* processing and increased production of mature *let-7*. Image modified from (Oishi and Wang 2011).

In *Drosophila*, two independent *let-7* null mutants have been created (Caygill and Johnston, 2008; Sokol et al., 2008). Surprisingly, *let-7* null mutant animals are viable and fertile. However, they exhibit multiple defects, including defects in maturation of the neuromuscular junctions (Caygill and Johnston, 2008; Sokol et al., 2008), and late-neurons (Kucherenko et al., 2012). In addition, they exert reduced fecundity (Caygill and Johnston, 2008; Sokol et al., 2008) and defects in the stem cell maintenance in testis (Toledano et al., 2012). On the molecular level, *let-7* is activated by the steroid hormone ecdysone (Chawla and Sokol, 2012), while it regulates the transcription factors abrupt (Caygill and Johnston, 2008) and chimno (Wu et al., 2012), the adhesion molecule Fasciclin 2 (Kucherenko et al., 2012) and the RNA binding protein Imp (Toledano et al., 2012).

# AIMS OF THE STUDY

In this thesis I addressed questions from a wide spectrum of *Drosophila* development. The general aims of this study were:

- To study the expression pattern of the GDNF receptor alpha homolog (GFRl) in the *Drosophila* adult brain

- To analyze the phenotype of the pleiotropic factor *lin-28* null mutant during *Drosophila* development

- To determine whether DmMANF is required for the maintenance of the dopaminergic system in the fly pupal and adult brain

- To characterize and investigate the mode of appearance of an unusual cell type in the *Drosophila* brain

# MATERIALS AND METHODS

Materials and methods used in this work are described in detail in the original articles and manuscripts.

**Table 1. Methods**

| Method | Article |
|---|---|
| **Fly genetics** | |
| Crosses | I, II, III, IV |
| RNAi | III, IV |
| **Tissues of interest** | |
| Pupal / Adult brain | I, III, IV |
| Ovary | II, III |
| Muscle | II, III |
| Nephrocyte | III |
| Testis | III |
| **DNA and RNA methods** | |
| PCR | II |
| mRNA *In situ* hybridization | I |
| **Immunological methods** | |
| Western blotting | III |
| Immunohistochemistry | II, III, IV |
| Lysotracker staining | III |
| **Microscopy** | |
| Bright Field Microscopy | I, II, III, IV |
| Fluorescent Microscopy | II, III, IV |
| Confocal Microscopy | II, III, IV |
| Transmission Electron Microscopy | III |
| **Behavioral studies** | |
| Negative Geotaxis | II |
| Spontaneous Locomotion Activity Assay | II |
| Induced Locomotion Activity Assay | II |
| Oviposition Assay | II |
| **Other studies** | |
| BrdU feeding | III |
| Trauma Induction | III |

**Table 2. Fly strains**

| Fly strain | Code | Source | Article |
|---|---|---|---|
| *alrm-Gal* | | (Doherty et al., 2009) | III, IV |
| *ATM*$^{8}$ | #8624 | BDSC | III |
| *D/Cyo;Dr/Tm6TbSbYFP* | | Gift from I. Salecker | III, IV |
| *delDmGfrl* | | Generated by J. Kallijärvi for I | |
| *da-Gal4* | | (Wodarz et al., 1995) | II, III, IV |
| *Df(3L)Exel6106* | #7585 | BDSC | II |
| *DF(3L)ZN47* | #3096 | BDSC | II |
| *elav-Gal4* | #458 | BDSC | III, IV |
| *elav-Gal4* | #8760 | BDSC | III, IV |
| *FRT82B; tubulin-Gal80* | #5135 | BDSC | IV |
| *Gad1-Gal4* | | (Ng et al., 2002) | IV |
| *gcm-Gal4* | | Gift from A.Giangrande | III |
| *He-Gal4, UAS-GFP* | #8700 | BDSC | III |
| *Hml-Gal4, UAS-GFP* | #30140 | BDSC | III |
| *hop*$^{2}$ | #6032 | BDSC | III |
| *hop*$^{Tum}$ | #8492 | BDSC | III |
| *hsFLP* | #8862 | BDSC | IV |
| *Kr/CyO; D/Tb Sb* | #7199 | BDSC | III, IV |
| *lama-Gal4* | | (Chotard et al., 2005) | IV |
| *lin-28*$^{dF30}$, *lin-28*$^{dF101}$ | | Generated by F. Michon and T. I. Heino for II | |
| *Nazgul-Gal4* | | Gift from B. Altenhein | III |
| NP1243 | v12835 | DGRC | III, IV |
| NP2222 | v112830 | DGRC | III, IV |
| NP2276 | v112853 | DGRC | III, IV |
| NP3233 | v113173 | DGRC | III, IV |
| NP6293 | v105188 | DGRC | III, IV |
| NP6520 | v105240 | DGRC | III, IV |
| *P{EP}lin-28*$^{EP915}$ | #17298 | BDSC | II |
| *P{Δ2-3}* | #3629 | BDSC | II |
| *prospero-Gal4* | | Gift from B. Denholm | III |
| *repo-Gal4* | | (Sepp et al., 2001) | III, IV |
| *Sb*/TM3 *ActGFP Ser* | #4534 | BDSC | II |
| *Stat92E*$^{F}$ | #24757 | BDSC | III |
| *sws*$^{4}$ | #28121 | BDSC | III |
| *sws*$^{olfE-x26}$ | #1015 | BDSC | III |
| *tubulin-Gal4* | | (O'Donnell et al., 1994) | IV |
| *UAS-Atg1*$^{[6A]}$ | | (Scott et al., 2007) | III |
| *UAS-Atg1*$^{[6B]}$ | | (Scott et al., 2007) | III |
| *UAS-Atg1*$^{[GS10797]}$ | | (Scott et al., 2007) | III |
| *UAS-Atg1*$^{K38Q[5B]}$ | | (Scott et al., 2007) | III |

*Table 2 continued…*

| Fly strain | Code | Source | Article |
|---|---|---|---|
| *UAS-DIAP1* | #6657 | BDSC | III |
| *UAS-Dicer-2* | | (Baumgardt et al., 2007) | III, IV |
| *UAS-DmMANF$^{RNAi}$* | v12834 | VDRC | III |
| *UAS-DmMANF$^{RNAi}$* | v12835 | VDRC | III, IV |
| *UAS-grim*, | | (Zhou et al., 1997) | III |
| *UAS-hid, -rpr* (,*-grim*) | | (Zhou et al., 1997) | III |
| *UAS-Hsap\SNCA.A30P* | #8147 | BDSC | III |
| *UAS-lacZ* | | (Brand and Perrimon, 1993) | III |
| *UAS-let-7* | #41171 | BDSC | II |
| *UAS-Manf$^{135}$* | | (Palgi et al., 2009) | III, IV |
| *UAS-mCD8::GFP* | #5137 | BDSC | III, IV |
| *UAS-Neuroglian$^{RNAi}$* | v107911 | VDRC | III |
| *UAS-nGFP* | #4775 | BDSC | II, III, IV |
| *UAS-p35* | #5072 | BDSC | III |
| *UAS-p35* | #5073 | BDSC | III |
| *UAS-PGRC-LC* | #33917 | BDSC | III |
| *UAS-PGRC-LE* | #33054 | BDSC | III |
| *UAS-RelD* | | Gift from S. Cherry | III |
| *UAS-STAT$^{RNAi}$* | v100519 | VDRC | III |
| *UAS-Tefu$^{RNAi}$* | v100008 | VDRC | III |
| *UAS-Toll* | #30900 | BDSC | III |
| *UAS-Toll* | #30901 | BDSC | III |
| *UAS-Tor$^{TED}$* | #7013 | BDSC | III |
| *UAS-zfh1$^{RNAi}$* | v109931 | VDRC | III |
| *w$^{1118}$* | | Umea Stock Center | I, II, III, IV |

**Table 3. Antibodies**

| Antigen | Name | Source/Reference | Article |
|---|---|---|---|
| BAM | | DSHB | II |
| BrdU | | GE Healthcare | III |
| Bruchpilot | nc82 | DSHB | III, IV |
| Caspase-3 | | Cell Signaling Technology | II, III |
| chaoptin | 24B10 | DSHB | IV |
| discs large | 4F3 | DSHB | III, IV |
| DmMANF | | (Palgi et al., 2009) | III, IV |
| DNP | | ICN ImmunoBiologicals | III |
| Draper (mouse) | | (Freeman et al., 2003) | III |
| Draper (rat) | | (Manaka et al., 2004) | III |
| dSTAT | | (Flaherty et al., 2010) | III |
| Ecdysone Receptor | DDA2.7 | DSHB | III |
| E-Cadherin | DCAD2 | DSHB | II |
| elav | | DSHB | III, IV |
| engrailed/invected | | DSHB | III |
| Fasciclin 2 | 1D4 | DSHB | III, IV |
| Fasciclin 3 | 7G10 | DSHB | III, IV |
| Hts-RC | | DSHB | II |
| Lysotracker | | Molecular Probes | III |
| Neuroglian | BP104 | DSHB | III |
| orb | 4H8 | DSHB | II |
| Phalloidin | | Sigma | II, III |
| phospho-Histone 3 | | Upstate Cell Signalling Solutions | II, III |
| Relish-C | | DSHB | III |
| Relish-N | | Gift from S. Stöven | III |
| repo | | DSHB | III, IV |
| Spectrin | | DSHB | II |
| Single-minded | | DSHB | III |
| TH | | DiaSorin | III, IV |
| Twinfilin | | (Wahlström et al., 2001) | III |
| Zfh-1 | | (Postigo et al., 1999) | III |

# RESULTS AND DISCUSSION

## DmMANF expression in the adult CNS (III-IV)

To address how DmMANF is expressed in the adult brain, we performed co-localization studies. We found that DmMANF is more widely expressed in the adult brain than in embryonic and larval CNS. In the adult brain, DmMANF, apart from co-localizing with glial cell bodies, it is also localized with glial processes (IV) (**Table 4**). We further analyzed DmMANF localization and we found that DmMANF is localized in cell bodies and processes of all glial subtypes (IV) [as classified by (Awasaki et al., 2008; Doherty et al., 2009)]. In addition and contrary to the embryonic expression, DmMANF is also localized in neurons, as indicated by co-localization studies when using the pan-neuronal driver *elav-Gal4* (IV). Interestingly, in adults DmMANF is also localized in the cell somas of dopaminergic neurons, but not in their processes, even when DmMANF was overexpessed using a dopaminergic driver *TH-Gal4* (IV).

Because DmMANF is a secreted protein, we wanted to investigate whether DmMANF labeling in glia and dopaminergic neurons is due to DmMANF being expressed cell-autonomously by these cells or not. First, we knocked down the *DmMANF mRNA* by employing the RNAi technique coupled with the upstream activating sequence (UAS)/Gal4 system (Dietzl et al., 2007). We tested the RNAi construct by expressing it ubiquitously and found that the *da-Gal4; UAS-DmMANF*$^{RNAi}$ *UAS-Dicer-2* larvae died as young larvae, phenocopying the DmMANF null mutant phenotype (III). In addition, we verified the specificity of the RNAi construct with Western blot (III).

Next, we knocked down DmMANF specifically either in glia or in neurons by using RNAi. In this case, DmMANF was not detected in the cells that the *UAS-DmMANF*$^{RNAi}$ construct was expressed (IV). Secondly, to conditionally knock-out the DmMANF gene in the brain we created homozygous *DmMANF* deficient somatic clones in the adult brain of an otherwise wild type animal. In addition, we used the MARCM technique (Wu and Luo, 2007), which allows the visualization of single cells by generating a labeled homozygous mutant clone. In both cases, we never detected DmMANF in DmMANF$^{-/-}$ cells (IV). Based on these results, we conclude that DmMANF is actually expressed in both glia and in neurons during pupal and adult stages.

### DmMANF expression in the adult non-neuronal tissues (III)

Immunohistochemical analysis showed that in adult flies, DmMANF is strongly expressed in abdominal nephrocytes, in follicle cells and border cells of the ovary and in investment cone bundles of testis. Furthermore, DmMANF is not expressed in muscles.

**Table 4. Driver lines with which DmMANF co-localizes.**

| Expression | | Stock name | DmMANF expression |
|---|---|---|---|
| **Glial** | Pan-glial | *repo-Gal4* | Yes |
| | Astrocyte-like | NP1243 * | Yes |
| | Astrocyte-like | NP3233 | Yes |
| | Astrocyte-like | *alrm-Gal4* | Yes |
| | Cortex | NP2222 | Yes |
| | Enseathing | NP6520 ** | Yes |
| | Subperineurial | NP2276 | Yes |
| | Perineurial | NP6293 | Yes |
| **Neuronal** | Pan-neuronal | *elav-Gal4* | Yes *** |
| | Dopaminergic | *TH-Gal4* | Yes *** |
| | Neurites | BP104 & Fasciclin 2 antibodies | No |

* Has secondary expression in Ensheathing and Cortex glia
** Has secondary expression in Cortex glia
*** Only at cell bodies

## DmMANF *in vivo* functional studies with focus to the dopaminergic system (IV)

Initially, we wanted to investigate what is the function of DmMANF in the pupal and adult brain, and more specifically to examine whether DmMANF is required for the maintenance of the dopaminergic system during these developmental stages. In order to study the function of DmMANF in later developmental stages, we employed techniques that enabled us to bypass the larval lethality of the DmMANF null mutant. These techniques included clonal analysis, RNA interference (RNAi) and overexpression studies.

## DmMANF overexpression does not affect the dopaminergic system (IV)

First, we assessed whether overexpression of DmMANF affects the overall structure of the dopaminergic network. We expressed a *UAS-DmMANF* construct using a variety of ubiquitous, neuronal and glial drivers (**Table 5**). In all cases, the animals appeared to develop normally and they eclosed to adulthood. In addition, based on immonhistochemistry, their dopaminergic system seemed unaffected.

**Table 5. Driver lines used in DmMANF overexpression studies to test whether the DA system is affected.**

| **Expression** | | **Is DA system affected?** |
|---|---|---|
| Ubiquitous | *daughterless-Gal4* | No |
| | *tubulin-Gal4* | No |
| Neuronal | *elav-Gal4* | No |
| | *Gad1-Gal4* | No |
| | *TH-Gal4* | No |
| Glial | *repo-Gal4* | No |

## DmMANF is not required in a cell autonomous fashion for the survival and differentiation of the main cell types of the brain (IV)

We created MARCM clones of the cell types that DmMANF is expressed, namely glia and dopaminergic neurons, by using the pan-glial driver *repo-Gal4* and the dopaminergic specific driver *TH-Gal4* lines respectively. In this case as well, although DmMANF was knocked out, both glia and dopaminergic neurons developed in the adult brain. In addition, both glia and dopaminergic neurons appeared to have a normal morphology and acquired their typical positions in the *Drosophila* brain. Based on the above results, we concluded that DmMANF is not required in a cell-autonomous fashion for the survival and differentiation of either glia or dopaminergic neurons in the *Drosophila* brain.

## DmMANF requirement for the development of the dopaminergic system after larval stages remains inconclusive (IV)

According to our co-localization studies, DmMANF is expressed in glial cells and in dopaminergic neurons. Consequently, we wanted to see if the dopaminergic system was affected when DmMANF is silenced in those cell populations where DmMANF is expressed in the adult brain. Flies from three time points [late pupation (dark pupae) and 1, 10 and 30 days after eclosion] were subjected to confocal microscopy to assess their dopaminergic network.

First, we concentrated on the effect of DmMANF knockdown in neurons and in dopaminergic neurons. We used the pan-neuronal driver *elav-Gal4* as well as the dopaminergic specific driver *TH-Gal4* to express the *UAS-DmMANF*$^{RNAi}$ transgene. In both cases, the animals eclosed to adulthood, lived for at least 30 days and we did not observe any dramatic locomotion difference when compared to control flies. In addition, staining with TH antibody showed that their dopaminergic system was not affected. Next, we knocked down DmMANF specifically in glial cells. In this case as well, the animals developed normally and did not show any dramatic locomotion defects. In addition, based on TH stainings, their dopaminergic system looked unaffected. This result is surprising

because in *DmMANF* null mutants the embryonic and early larval dopaminergic neurites are seriously affected (Palgi et al., 2009). We reasoned that this discrepancy could be due to RNAi being less effective compared to the null mutant situation. Therefore, we tried to enhance the RNAi effect, by concurrent Dicer-2 overexpression. Dicer-2 is the limiting factor of the RNAi machinery, and concurrent expression of Dicer-2 along with the expression of an RNAi construct is an approach commonly used in *Drosophila* for enhancement of the RNAi efficiency (Dietzl et al., 2007). Our results indicated that the dopaminergic system was not affected when *elav-Gal4* was used. However, when we expressed the *UAS-DmMANF$^{RNAi}$ UAS-Dicer-2* transgene using the *TH-Gal4* construct, we observed a significant loss of the dopaminergic system. Interestingly, we observed the same phenotype when overexpressing Dicer-2 alone using the *TH-Gal4* promoter. It has recently been reported by White *et al* (White et al., 2010), that Dicer-2 expression by the TH promoter alone is sufficient to produce a reduction in TH staining. Therefore, our results regarding the requirement of DmMANF for the development of dopaminergic neurons after larval stages are still inconclusive and require more studies.

### Expression of the *UAS-DmMANF$^{RNAi}$ UAS-Dicer-2* transgene under the pan-glial driver repo produces a novel phenotype (III)

Next, we overexpressed the *UAS-Dicer-2* construct, while knocking down DmMANF with RNAi in glial cells. In this case however, the animals died at different developmental stages, depending on the level of the temperature sensitive UAS/Gal4 system expression (Duffy, 2002). At temperatures that the UAS/Gal4 system is highly expressed, namely at 29$^{o}$C and 25$^{o}$C, the animals died as third instar larvae. Remarkably, at 29$^{o}$C, animals stayed as 3$^{rd}$ instar larvae an extra 6 days compared to controls and wild type. In addition, they exert the same phenotype as described in Iyengar *et al.* (Iyengar et al., 2011): reduced peristaltic contraction frequency and circular path trajectories likely due to loss of normal postural control. This phenotype was not characterized further.

## MiCs – Novel cell type (III)

When we simultaneously knocked down DmMANF and overexpressed Dicer-2 at 18$^{o}$C, animals pupated normally. However, they died as pharate animals and only 3% of them eclosed to adulthood. Interestingly, the dopaminergic system of these pharate animals seemed unaffected. Strikingly, in the brains of the live pharate animals, an excessive number of cells strongly positive for DmMANF appeared in the neuropil areas of the brain (**Figure 6A and B)**. Based on this initial observation, we called this cell type Manf immunofluorescent Cell (MiC). Neuropils are synapse-dense areas filled with axons, dendrites and glial processes and the cell bodies (somas) of neither neurons nor glia are known to exist there. This phenotype was fully penetrant since all pupae examined

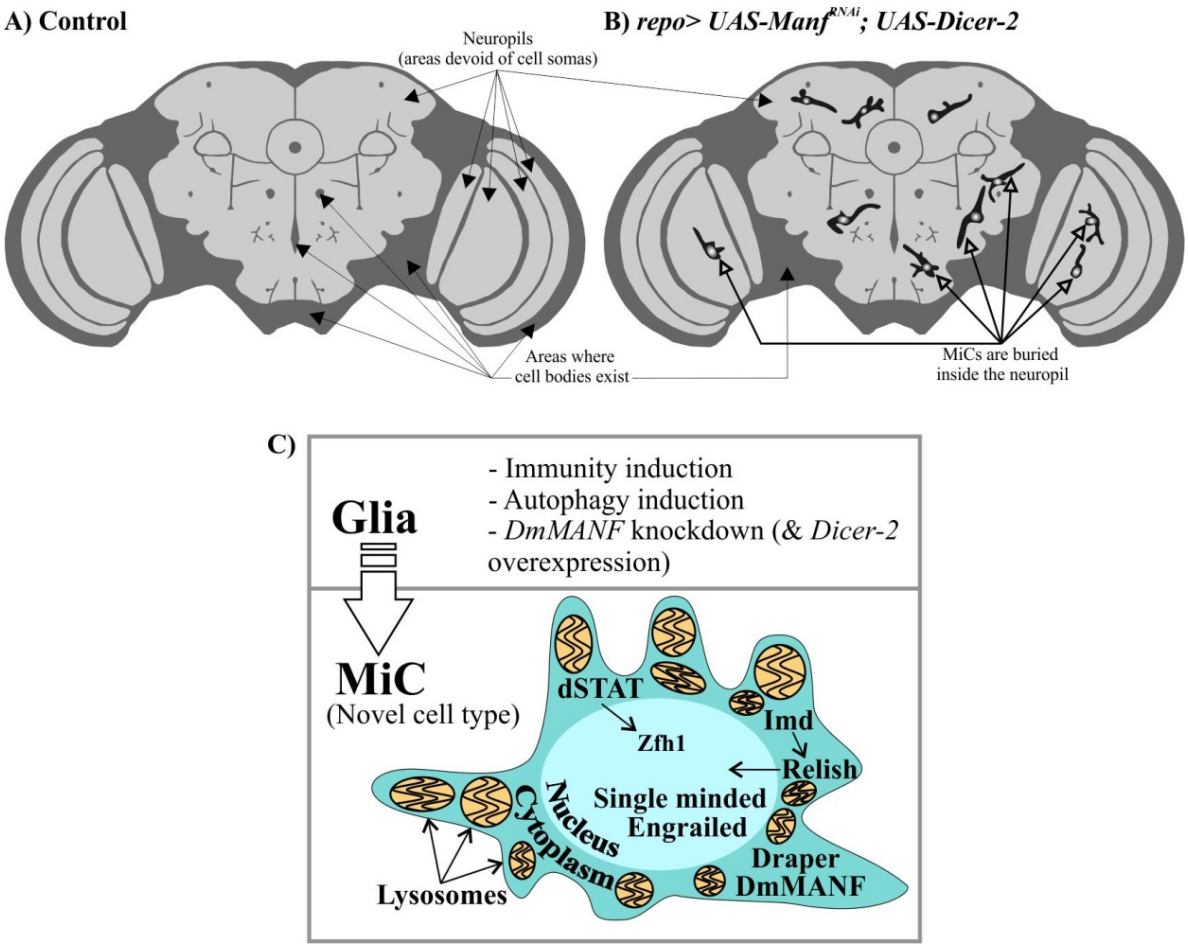


**Figure 6. MiCs appear under three different genetic manipulations (see main text).** (**A**) In insect brains, the cell somas (both glial and neuronal) are predominantly present at the circumference of the brain (dark grey). The central part of the brain is synapse-dense and filled with axons, dendrites and glial processes. These areas are devoid of cell somas and are collectively called neuropils (pale grey). (**B**) The neuropil organization in *repo> UAS-Manf*$^{RNAi}$ *UAS-Dicer-2* brains is not disturbed. However, a previously unidentified cell appears, we call MiC (white arrows). MiCs are found inside the neuropils. In addition, these brains show signs of neurodegeneration (vacuoles) in the first optic chiasm (white speckles indicated by a triple arrow). (**C**) Summary of the main findings: MiCs apperar in the pupal brain, when specifically in glial cells either (i) immunity or (ii) autophagy or (iii) concurrent DmMANF knockdown and Dicer-2 overexpression are induced. MiCs express the transcription factor Zfh1 and have nuclearly accumulated dSTAT and Relish. In addition, they express the transcription factors Engrailed and Single Minded, the conserved neurotrophic factor DmMANF and the engulfment receptor Draper. MiCs are loaded with lysosomes with multilamellar structures as seen in human lysosomal storage disorders.

manifested this phenotype (n>300). Staining with the synaptic markers nc82 and Discs large (Dlg) revealed no staining at the sites where MiCs were located, therefore indicating that MiCs reside in a distinct area in the neuropil. MiCs had elongated arms, indicating that they are migratory and they were found in great numbers both besides the central brain also in the ventral nerve cord. Interestingly, we did not find MiCs in non-neuronal tissues such as muscles (>2 hour old animals), testes and ovaries (>3 day old animals).

MiCs appeared only when DmMANF expression was downregulated concurrently with Dicer-2 overexpression and under the pan-glial promoter *repo-Gal4*. MiCs did not appear when we expressed either *UAS-MANF*$^{RNAi}$ or *UAS-Dicer-2* independently in glial cells. This difference can be due to either the lower efficiency of DmMANF alone, or the synergistic effect of Dicer-2 upregulation and DmMANF downregulation. In addition, MiCs did not appear in the brain when *UAS-MANF*$^{RNAi}$ and *UAS-Dicer-2* constructs were expressed either individually or concurrently in neuronal cells under the pan-neuronal promoter Elav or the TH specific driver (see above), or other drivers, such as subglial specific drivers. Furthermore, MiCs did not appear when we overexpressed *UAS-Dicer-2* along with other RNAi constructs, such as *UAS-Zfh1*$^{RNAi}$ or *UAS-tefu1*$^{RNAi}$ under the glial promoter *repo-Gal4.* These data collectively point towards the MiC phenotype being a result of synergistic effect of Dicer-2 upregulation and DmMANF downregulation in glia cells.

**MiCs do not express the glial marker repo or the neuronal marker elav (III)**

Cell bodies in neuropil areas of the *Drosophila* brain have not been described in bibliography before; therefore, we decided to further investigate the mode of appearance as well as the function and origin of MiCs. The *Drosophila* adult and late pupal brain are anatomically similar and they are composed of two main cell types, neurons and glia, which are detected by the pan-neuronal marker Elav and the pan-glial marker Repo, respectively. Surprisingly, MiCs do not express either of these markers. In order to investigate the possibility that MiCs are transdifferentiated glia, we expressed *UAS-MANF*$^{RNAi}$ *UAS- Dicer-2 UAS-nGreen Fluorescent Protein (GFP)* (or *UAS-mCD8::GFP*) using *repo-Gal4*. This construct enabled us to label with GFP the cells that either transiently or constitutively express Repo. MiCs appeared in the induced brains but were not positive for GFP, while glia expressed GFP as expected. This indicates that MiCs do not express Repo at any point during their differentiation. In spite of this evidence, we were not able to conclusively exclude the cell autonomous model for MiC induction, as the half-life of GPF has not been studied, and it may degrade during a long time period.

**Do Mics derive from midline glia? (III)**

In the developing *Drosophila*, there is a single class of glia that does not express Repo. This glial class is called midline glia and originates from the mesoectoderm, in contrast to

all other glial subtypes that originate from the neuroectoderm. However, midline glia are not known to exist in mid- and late pupae. During normal development they are eliminated by apoptosis in two temporally distinct waves during embryogenesis and mid-pupation, by a process that is regulated by the ecdysone (Rusconi et al., 2000). Interestingly, MiCs express the midline specific transcription factor Single-minded (Crews et al., 1988). This result raises the possibility that MiCs are midline glia that do not undergo apoptosis, but instead survive and migrate to the neuropils. In order to test this hypothesis, we expressed either of the two the anti-apoptotic genes *p35* or *DIAP1* under the midline glia driver *Single-minded-Gal4*. However, in either case MiCs did not appear, indicating that inhibition of apoptosis of midline glia is not sufficient for the appearance of MiCs and also that glial contribution is necessary for the manifestation of the phenotype. In addition, MiCs do not express a second midline specific marker Slit (Rothberg et al., 1990), while they express the transcription factor Engrailed, which is known not to be expressed in midline glia (Kearney et al., 2004). Together, these data point towards MiCs not deriving from midline glia.

**Evidence that MiCs are motile (III)**

The appearance of MiCs in neuropil areas of the brain that are void of cell somas is a strong indication that they are motile. In addition, morphological features, such as elongated cytoplasmic arms also point towards this direction. Unfortunately, it is not technically possible to perform live imaging in *Drosophila* pupal brain in order to confirm their motility. The transcription factor dSTAT is known to specify and maintain cell motility in various models of cell migration in *Drosophila*, including border cell migration (Silver and Montell, 2001), germ cell migration (Xi et al., 2003) and migration of embryonic tracheal cells (Li et al., 2003). We found that dSTAT is expressed in MiCs and is localized in the nuclei, which is a hallmark of the JAK/STAT pathway activation (Agaisse et al., 2003). MICs also express the transcription factor Zfh1 which is a known downstream target of the JAK/STAT pathway (Leatherman and DiNardo, 2008).

**Induction of immunity in glia also results in the appearance of MiCs (III)**

We further investigated whether the appearance of MiCs is exclusively a DmMANF/Dicer-2 related phenotype or does it also appear under other genetic backgrounds. It is well documented that the JAK/STAT signaling pathway is involved in hematopoiesis and immune response both in *Drosophila* and in mammals [reviewed in (Agaisse and Perrimon, 2004)]. Consequently, in *Drosophila* Zfh1 has been implicated in the Imd pathway activation (Kleino et al., 2005; Myllymäki and Rämet, 2012), in hemocyte development in embryos (Frandsen et al., 2008) as well as to inhibit apoptosis in a subtype of embryonic subperineurial glia (Ohayon et al., 2009). Therefore, we investigated whether the artificial induction of the immune response in glia results in the

appearance of MiCs. We activated the two pathways that are responsible for innate immunity in flies, namely the Toll and the Imd pathway, by overexpressing either the Toll receptor, or the PGRP-LE (-LC) receptor, respectively in glia. We found that MiCs appeared when activating the Imd pathway, but not when activating the Toll pathway. In addition, when we overexpressed either receptor in neurons, no MiCs appeared, further indicating that the MiC phenotype is associated with glial behavior.

Consistent with the activation of the Imd pathway, in the *repo-Gal4; UAS-DmMANF$^{RNAi}$ UAS-Dicer-2* pupae we found that the NF-κb transcription factor Relish is expressed in MiCs and it is accumulated in their nuclei. Relish is the key factor in the induction of the humoral immune response in *Drosophila* and is downstream of the Imd pathway, therefore implying that the immune response is activated in MiCs. Based on our confocal studies, we detect anti-Relish staining in the nuclei of MiCs with antibodies that recognize either the N- or the C- terminus of Relish protein. This result challenges previous observations that after cytoplasmic cleavage, it is the N-terminal part of the protein that translocates into the nucleus to act as transcription factor, while the C-terminal part stays in the cytoplasm (Stöven et al., 2000). However, in these studies, the Imd response was triggered by bacterial infections, while in our study, the humoral response is triggered by genetic means. In addition and in agreement with our data, a recent study where the Imd pathway was triggered by genetic means, an antibody that detects the C-terminus of Relish was also detected in the nuclei of cells in Malpighian tubules (Tapadia and Verma, 2012). However, the authors do not comment on this result in their article.

**MiCs express the engulfment receptor Draper (III)**

Various cues, and most importantly the unexpected appearance of MiCs in the brain when brain homeostasis is disturbed, suggest that MiCs resemble mammalian microglia cells. Microglia are the dedicated macrophages of the mammalian brain that upon trauma infliction, infection or neurodegeneration invoke immune response by secreting antimicrobial peptides and by phagocytosis. However, in *Drosophila*, microglial or microglia-like cell population has not been identified. Instead, a number of studies have shown that during both development and adulthood, the resident sessile glial population assumes a semi-professional macrophagocytotic role (Kurant et al., 2008; Freeman et al., 2003; Awasaki and Ito, 2004; Tasdemir-Yilmaz and Freeman, 2014; MacDonald et al., 2006). During this process glia start to express the engulfment receptor Draper (Logan et al., 2012; Freeman et al., 2003; Kurant et al., 2008; MacDonald et al., 2006; Tasdemir-Yilmaz and Freeman, 2014; Manaka et al., 2004). Draper is homologous to the *C. elegans* cell corpse engulfment receptor *ced-1*, as well as to the mammalian *Jedi-1* (Carninci et al., 1996) and *MEGF10* genes (Singh et al., 2010). Interestingly, both *Jedi-1* and *MEGF10* are expressed in glia and play a conserved role in their phagocytic activity (Singh et al., 2010; Wu et al., 2009; Cahoy et al., 2008). Immunohistochemistry staining revealed that MiCs also express Draper, therefore they are potentially phagocytotic.

**Induction of autophagy in glia also results in the appearance of MiCs (III)**

An exciting feature of the engulfment receptor Draper is its association with autophagy. Autophagy is a conserved catabolic process. During autophagy the cell degrades its cellular components using the lysosomal machinery. In *Drosophila*, it has been shown that autophagy has two roles in respect to cell integrity (McPhee et al., 2010). In one case, autophagy can be related to survival of the cells. In *Drosophila* larvae that have been starved, autophagy is induced in the fat body as a mechanism for catabolic degradation of cellular components. In a second case, during pupal histolysis, autophagy is used as a mean of PCD for the degradation of the salivary glands. Interestingly, Draper is cell-autonomously required for autophagy in dying cells but not in autophagy related to cell survival (McPhee et al., 2010). These studies support the earlier studies that have proposed the autophagy as a PCD mechanism that is independent of apoptosis and has been also named as PCD II (Tsujimoto and Shimizu, 2005; Schwartz et al., 1993; Datan et al., 2014). However, the role of autophagy as a means of PCD is still under debate.

To test if there is a link between MiCs and autophagy, we expressed Atg1 (Scott et al., 2007) or the dominant-negative form of Target of rapamycin ($Tor^{TED}$) (Scott et al., 2004) in glia. In both cases MiCs appeared, therefore recapitulating the phenotype with a third evolutionarily conserved genetic approach. In addition, as with the *UAS-DmMANF*$^{RNAi}$ *UAS-Dicer-2* construct and the immune induction, induction of autophagy in neurons did not produce MiCs. Interestingly, the Imd pattern recognition receptor PGRP-LE has been shown to trigger autophagic response (Yano et al., 2008), while upregulation of autophagy has been observed in a number of lysosomal storage disorders (LSDs) (Settembre et al., 2008bb; Settembre et al., 2008aa).

In our perception, the fact that inducing either autophagy, or immunity or downregulating the neurotrophic factor DmMANF specifically in glia cells results in a common phenotype is intriguing. Interestingly though, recent studies indicate that immunity, autophagy and neurodegeneration share common pivotal genes (Levine et al., 2011; Levine and Kroemer, 2008; Chang and Neufeld, 2010). These observations come mainly from the results of genome-wide association studies (Palgi et al., 2012; Petersen et al., 2012; Yano et al., 2008). This phenotype could very well be a striking example of crosstalk among these three evolutionarily conserved mechanisms.

**MiCs are rich in lysosomes (III)**

The fact that MiCS express the phagocytotic marker Draper raises the exciting possibility that MiCs perform phagocytosis. In order to investigate this hypothesis, we performed electron microscopy to gain insights about the ultrastructure of MiCs.

Toluidine blue is a dye with high affinity for acidic molecules and is the standard dye used in transmission electron microscopy to visualize cells in semithin sections before the actual thin-sectioning. In these sections scattered cells that were stained strongly purple were located in neuropil areas. This pattern was highly reminiscent of the distribution of MiCs in confocal sections. Such cells were not seen at all in section of the

control brains. In parallel we exposed *repo-Gal4; UAS-DmMANF$^{RNAi}$ UAS-Dicer-2* brains to DAMP [N-{3-[(2,4-Dinitrophenyl)amino]propyl}-N-(3-aminopropyl)methylamine, dihydrochloride], which is used to detect acidic organelles, such as lysosomes (Anderson et al., 1984). DAMP can be recognized with anti-Dinitrophenol (DNP). These brains unlike control brains had numerous cells in the brain neuropil areas positive for DNP and they co-localized with MiC markers. Staining with the lysosomal marker Lysotracker, revealed that brains from *repo-Gal4; UAS-DmMANF$^{RNAi}$ UAS-Dicer-2* animals were positive also for this lysosomal marker, again the control brains that were negative. Based on these results, it can be concluded that the cytoplasm of MiCs is highly acidic and that they are rich in lysosomes.

Transmission electron microscopy identified cells in the neuropil areas of the brain with dramatic morphology. These cells had intact nuclei, indicating that they were not undergoing apoptosis, while their cytoplasm was filled with lysosomes. The lysosomes were exceptionally large with highly electron dense, transversely stacked membranes. These lysosomes strongly resemble the typical morphology of lysosomes as seen in human LSDs (Burton et al., 2012). Further investigation revealed that in flies, there are several models of LSDs and one them is caused by Saposin (dSap-r) deficiency (Hindle et al., 2011). Interestingly, according to crystallography studies, the N-terminal domains of both Manf and CDNF have saposin-like lipid-binding domains (Parkash et al., 2009). However, contrary to our initial hypothesis, we never showed signs of endocytosis in MiCs, such as membrane internalizations or cellular debris.

### MiCs induced by any of the three genetic manipulations express the same markers (III)

As shown above MiCs appear in the neuropil areas of the pupal brain, as a result of three different genetic manipulations: (i) by the concurrent downregulation of the neurotrophic factor DmMANF, and by the induction of either (ii) immunity or (iii) autophagy in glia. Based on our results, the cells appearing under all three different genetic manipulations are the same cell type. In all three cases, the induced cells express the conserved neurotrophic factor DmMANF, the engulfment receptor Draper, and possess nuclear localization of transcription factors Zfh1, dSTAT and Relish. In addition, in all three cases, MiCs do not express the neuronal marker Elav or the glial marker Repo. Furthermore, in all cases, these brains, unlike the control brains, are positive for the lysosomal marker Lysotracker. In conclusion, the cells that appear in the neuropil areas of the brain under any of the three genetic manipulations are of the same cell type (**Figure 6C**).

### MiCs appear during early pupation and do not divide (III)

A key question in understanding the origin of MiCs is at what developmental stage they appear. We never saw DmMANF+/Zfh1+ cells in the neuropils of larvae. We were able to

first identify DmMANF+/Zfh1+ in the neuropils of 32.5 hours after puparium formation (APF) pupae. The number of MiCs increased greatly at around 80 hours APF, which coincides with the increase in the volume of neuropils. The greatest number of MiCs is found during late pupation (~99 hours APF) or just before the animals die. All late pupae tested at 99 hours APF were alive, as they were moving their legs and proboscis upon their removal from the pupal case. Surprisingly, the ~3% of animals that survived to adults, lived to at least 15 days and they appeared to have very little or no MiCs. Interestingly, MiCs were not positive for cleaved Caspase-3, indicating that MiCs do not undergo caspase-dependent apoptosis during late pupal stages.

Consequently, we wanted to investigate whether MiCs divide during pupation or they originate from a still unidentified location from which they migrate. First, we stained with the mitotic marker phosphorylated Histone 3 (PH3) the brains of 80 hours APF *repo>UAS-Manf$^{RNAi}$ UAS-Dicer-2*. PH3 is an M phase marker of the cell cycle. We never saw MiCs being PH3 positive. Next, we used the synthetic thymine analogue BrdU (5-bromo-2'-deoxyuridine) to perform a BrdU pulse/chase experiment (Boone and Doe, 2008). Late 2$^{nd}$ / early 3$^{rd}$ instar larvae were fed with BrdU for 3 hours and then they were allowed to develop without BrdU until they reached late pupation (more than 100 hours after the feeding). Interestingly, at 99 hours APF all MiCs were positive for BrdU. This result suggests that MiCs divide during late 2$^{nd}$ / early 3$^{rd}$ instar larvae as they incorporated the BrdU. However, after this initial division(s), MiCs do not divide, or they divide at low rates resulting in the BrdU not to be diluted out, but to be maintained in all MiCs found in the neuropil. The PH3 and the BrdU pulse/chase experiments collectively suggest that MiCs do not divide (or divide at a very low rate) during pupation.

**The critical developmental time for MiC induction is first and second instars (III)**

A second key question in understanding the origin of MiCs is at what time during development do glia cells need to be manipulated in order to produce MiCs in the pupal brains. In order to answer this question, we took advantage of the temperature sensitivity of the UAS/Gal4 system (Duffy, 2002) and the higher permissive temperature of the *repo>UAS-TOR$^{TED}$* animals. We shifted animals between a temperature that the UAS-Gal4 system activity is significantly decreased (19$^{o}$C) and a temperature that the UAS-Gal4 has high activity (26$^{o}$C). We found that raising *repo>UAS-TOR$^{TED}$* animals at 26$^{o}$C during either second or third instar larval stage was sufficient for MiCs to appear, irrespective of what temperature the rest of their development occurred. Therefore, we can conclude that the critical time for glia manipulation in order to produce MiCs in the pupal brain is 2$^{nd}$ and third instar larvae. Combining this result with the BrdU pulse/chase and PH3 results, we propose that MiCs originate from cell precursors that divide during 2$^{nd}$ / 3$^{rd}$ instar larva, which they subsequently stop dividing and they are invading the neuropil areas during pupation. Interestingly, these non-dividing cells are long living cells, as they are not caspase-3 positive during late pupation (live more than 100 hours long).

**MiCs do not appear under other conditions tested (III)**

We further explored if there would be other contexts where MiCs could appear. These are summarized in the Supplementary Table S1 of article III. One hypothesis we investigated was whether MiCs appear in fly neurodegeneration models. In tissues from patients with chronic neurodegeneration diseases, active microglia exists in large numbers (Perry et al., 2010). To test this hypothesis we used the alpha-synuclein model of *Drosophila* Parkinson's disease (Feany and Bender, 2000), the *swiss cheese* (Kretzschmar et al., 1997) and the *ATM*$^{8}$ (Petersen et al., 2012) neurodegeneration models. Due to the onset of neurodegeneration in these models [alpha-synuclein model, adult (Feany and Bender, 2000); *ATM*$^{8}$ model, day 7 post eclosion (Petersen et al., 2012)], we tested for MiCs at late pupal stage, as well as in 10-day-old flies. However, in none of the cases we observed MiCs. This result can be explained due to the late onset of the disease in these models which show no signs of neurodegeneration during pupation and this is the reason that no MiCs appear.

In mammals, microglia activation is also induced by trauma induction. We induced traumatic brain injury in the brain of 4-day-old *Drosophila* by using a model developed by Leyssen (Leyssen et al., 2005). Briefly, heads from newly eclosed flies were pierced by inserting a thin needle below the left antenna, through the left optic lobe. Flies were then left to recover for 4 days, prior to dissection. However, in this instance as well, MiCs did not appear. Again, the reason is possibly due to the use of adult animals. Unfortunately, inducing brain trauma in *Drosophila* during pupation is not feasible with current tools.

**MiCs are macrophage-like cells (III)**

Although microglia-like elements have been identified in the ganglia of other invertebrates, microglia or microglia-like cells have never been reported in *Drosophila*. Instead, it has been shown that sessile glia assume the role of the immune-associated cell type in the fly brain during development (Tasdemir-Yilmaz and Freeman, 2014; Kurant et al., 2008; Awasaki and Ito, 2004; Freeman et al., 2003) and during adulthood (MacDonald et al., 2006).

Our data suggests that MiCs are macrophage-like cells and they share similarities to mammalian microglia. First, as during microglia activation, MiCs arise when brain homeostasis is disturbed. In addition, MiCs are likely to be motile and they are only found in the CNS and ventral nerve cord, and not in any other tissues examined. MiCs express the pro-inflammatory mediator Relish, the transcription factor dSTAT and the engulfment receptor Draper. They also express neurotrophic factors, such as DmMANF. Interestingly, recent studies have shown that MANF is strongly upregulated in activated rat brain microglia (Shen et al., 2012).

An unexpected finding was that MiCs have an extremely high lysosomal content. Theoretically, one would expect that a cell type like microglia that is highly phagocytotic would have increased capacity to degrade subcellular material through the lysosomal

pathway. This could be achieved either by having high lysosomal content, or their increase activity or both. Surprisingly, such a relationship between lysosomes and microglia has not been documented. In addition, no connection between microglia and autophagy has been established. Interestingly, LSDs have been connected with upregulation of autophagy (Hindle et al., 2011) and upregulation of innate immunity genes (Alam et al., 2012).

### Are MiCs of hemocyte origin? (III)

Apart from sessile glia, hemocytes are a second cell type that performs phagocytosis in *Drosophila* (Tepass et al., 1994). However, hemocytes have not been shown to enter the brain due to the blood brain barrier. *Drosophila* hemocytes have been extensively studied during embryogenesis and larval stages. Interestingly, they express several markers expressed by MiCs, including Zfh1 (Frandsen et al., 2008), Draper (Manaka et al., 2004), and they have activated the JAK/STAT pathway (Agaisse and Perrimon, 2004). In addition, in *Drosophila*, autophagy is induced in hemocytes in response to bacterial infections (Yano et al., 2008). Unlike in earlier developmental stages, little is known about hemocytes during pupation and adulthood.

We identified two subpopulations that express either the hemolectin or the hemese hemocyte-specific markers in the periphery, but not in neuropils, of late pupal brain with non-overlapping expression pattern. Unexpectedly, both subpopulations expressed the glial marker Repo. This result is rather confusing as it indicates that both of these cell populations are glia rather than hemocytes in the pupal brain. Expression of the *UAS-DmMANF$^{RNAi}$ UAS-Dicer-2* construct by using promoters of either hemolectin-Gal4 or hemese-Gal4 did not trigger the appearance of MiCs. These results suggest that MiCs are not of the same lineage as hemocytes. Furthermore, MiCs were not induced when *gcm-Gal4* was used. *gcm* has a role in the differentiation of both glial (Freeman et al., 2003) and hemocyte (Vivancos and Giangrande, 1997) lineages.

## Expression of the *Drosophila* GDNF receptor-like transcript in the adult brain (I)

Using bioinformatic and biochemical analyses a *Drosophila* gene that encodes a protein with four cysteine-rich GFRα domains and a GPI anchoring site was identified. This gene was named this gene *Drosophila melanogaster Gfr-like* (*DmGfrl*). Further biochemical analysis showed that Gfrl is glycosylated, secreted and GPI anchored on the cell surface, similarly to mammalian GFRα proteins. Expression studies in the embryos revealed that *DmGfrl* expression starts in the *Drosophila* nervous system at stage 13, time that coincides with the start of neuronal differentiation. Further analysis showed that, at least at the embryonic stage, Gfrl positive cells co-localize with neurons and not glia. Also the mRNA expression pattern of *DmGfrl* in relation to *DmRet* was investigated. We found that during embryogenesis, expression of both transcripts coincides temporally. However, based on

the *in situ* analysis, *DmGfrl* and *DmRet* expressions do not overlap either during embryogenesis or during larval stage. I specifically studied the mRNA expression of *DmGfrl* and *DmRet* in the adult *Drosophila* brain. DmGfrl mRNA was abundantly expressed in the cell somas of the central brain. Interestingly, this expression pattern resembles the $GABA_B$ receptor-2 expression in the GABAergic interneurons (Okada et al., 2009). Surprisingly, no *DmRet mRNA* expression was detected in the adult brain. Based on these findings we hypothesize that unlike in mammals, in the fly DmGfrl and DmRet do not interact *in cis*.

## Isolation of a *lin-28* mutant (II)

*Drosophila* has a single gene that is homologous to *lin-28*. It encodes an 195 amino acid protein whose amino acid sequence is 57% identical to *C. elegans* Lin-28 and 47% to human Lin-28A proteins. The Berkeley *Drosophila* Genome Project identified a P element insertion that mapped to *lin-28*'s third intron. This strain, *EP(3)0915*, is homozygous viable, fertile and shows no obvious phenotype. In order to investigate whether this P element insertion causes a mutation to the *lin-28* gene, we examined whether the *let-7* mRNA levels are affected in this strain. It is well established that *let-7* is a highly conserved target of Lin-28 (Viswanathan et al., 2008; Piskounova et al., 2011; Zhong et al., 2010; Loughlin et al., 2011). Lin-28 protein physically binds to the *let-7* precursor, resulting in inhibition of *let-7* maturation. qPCR showed that in the EP(3)0915 line, *let-7* levels are identical to the levels of wild type, indicating that in the EP(3)0915, *let-7* is not affected.

Imprecise P-element excision was used to generate deletions of the *lin-28* gene, from the *EP(3)0915* line. Two hundred lines with potential excision were created, all of which were viable and fertile as homozygotes. One line was selected for further studies, hereafter referred as $lin\text{-}28^{dF30}$. Sequence analysis showed that the P-element excision in $lin\text{-}28^{dF30}$ allele caused a deletion of 1 007 base pairs upstream of the original P-element position. This predicted that the mutant $Lin\text{-}28^{dF30}$ protein lacks the CSD, the linker and the first CCHC domain, while the second CCHC domain would remain unaffected. Combining our sequencing results and findings by Nam *et al* (Nam et al., 2011) that physical interaction between Lin-28 and *let-7* requires both the CSD and the linker domain, we concluded that the $lin\text{-}28^{dF30}$ mutant is a null mutant, at least in respect to its ability to regulate *let-7* mRNA.

### *let-7* levels are down regulated in $lin\text{-}28^{dF30}$ mutants (II)

The mode of action of Lin-28 as inhibitor of microRNA *let-7* is evolutionarily conserved (Viswanathan et al., 2008; Piskounova et al., 2011; Zhong et al., 2010; Loughlin et al., 2011). In *Drosophila*, *let-7* is expressed in ovaries (Sempere et al., 2002). On the other hand, Lin-28 has been shown to be expressed in mouse (West et al., 2009) and in human

(Childs et al., 2012) GSCs [also see review (Spradling et al., 2011)]. We noticed a dramatic increase of *let-7* levels in *lin-28*$^{dF30}$ mutant ovaries. This result shows that the *lin-28* function as a regulator of *let-7* is also conserved in *Drosophila*.

### *lin-28*$^{dF30}$ mutant flies have muscular defects (II)

Previously it has been shown that *let-7* is involved in the muscle formation and remodeling in *Drosophila* (Caygill and Johnston, 2008; Sokol et al., 2008). In *let-7* mutants the abdominal dorsal oblique muscles (DIOMs) were retained after eclosion and during adulthood (Sokol et al., 2008). Under normal conditions, DIOMs are required for eclosion and they are lost 12 hours post eclosion. Therefore, we tested if deletion of *lin-28* and subsequent upregulation of *let-7* would result into a phenotype in DIOMs. In *lin-28*$^{dF30}$ mutant animals the DIOMs were missing in pharate animals. This muscular phenotype was also demonstrated by behavioral studies that showed that 50% of *lin-28*$^{dF30}$ mutant flies either failed to exit the pupal case or died shortly after eclosion. Both *lin-28* and *let-7* are heterochronic genes known to have key roles during cell differentiation, tissue growth and organ formation (Moss, 2007). These roles, along with their reciprocal inhibitory effect they produce, could explain why the phenotypes observed in the *lin-28* and *let-7* mutants have opposite phenotypes, i.e. abnormal number of DIOMs.

### *lin-28*$^{dF30}$ mutant ovaries have supernumerary nurse cells (II)

Lin-28 has been shown to be an important factor in GSC maintenance in mouse (West et al., 2009) and human (Childs et al., 2012) ovaries. In addition, the *Drosophila let-7* mutants show decreased egg laying (Sokol et al., 2008), a phenotype that has not been further studied. Therefore, we investigated whether *lin-28* deletion has also an effect on female fertility. We found that *lin-28*$^{dF30}$ mutant flies laid 5 times less eggs compared to control flies. Next, we examined whether the reduced fertility is due to abnormalities in the oogenesis of *lin-28*$^{dF30}$ mutants. We observed that in *lin-28*$^{dF30}$ mutant ovaries, in 40% of the ovarioles, there was at least one egg chamber that had supernumerary nurse cells. We concentrated on later stages of oogenesis, where this phenotype is easier to score due to egg chamber size and availability of molecular markers. The number of nurse cells in these egg chambers varied from 22 to 47 nurse cells (**Figure 7**). Such a supernumerary phenotype has been described in bibliography before [e.g. *maelstrom* mutant (Sato et al., 2011), maelstrom is involved in the *Drosophila* oocyte axis symmetry].

The increased number of nurse cells can be the result of three possibilities. First, the neighboring egg chambers may fuse. In this case we would expect the new egg chamber to have a number of nurse cells that is multiplication of the number 15 (30, 45 etc.). In addition, we would expect that the number of ring canals would equal the number of nurse cells. Furthermore, we would expect to see nurse cells grouped together into distinct

groups of cells with different dimensions, corresponding to individual cysts within the same egg chamber.

A second possibility is that the increased nurse cell number arises by abnormal control of GSC proliferation. In this case, the number of nurse cells in an egg chamber will not be a direct derivative of number 15. In addition, the number or ring canals would not equal the number of nurse cells (**Figure 4C**). The third possibility is that the supernumerary phenotype is a combined result of the two previous cases.

Thorough investigation of abnormal stage 9 and 10 egg chambers showed that the number of nurse cells is always equal to the number of ring canals. In addition, we observed ectopic oocytes in all cases, grouped together with nurse cells of similar size. Therefore, we concluded that the supernumerary nurse cell phenotype in *lin-28*$^{dF30}$ mutant egg chambers is a compound phenotype of egg chamber fusion and mitotic defects (**Figure 4D**), as reported for the *maelstrom* mutant line (Sato et al., 2011).

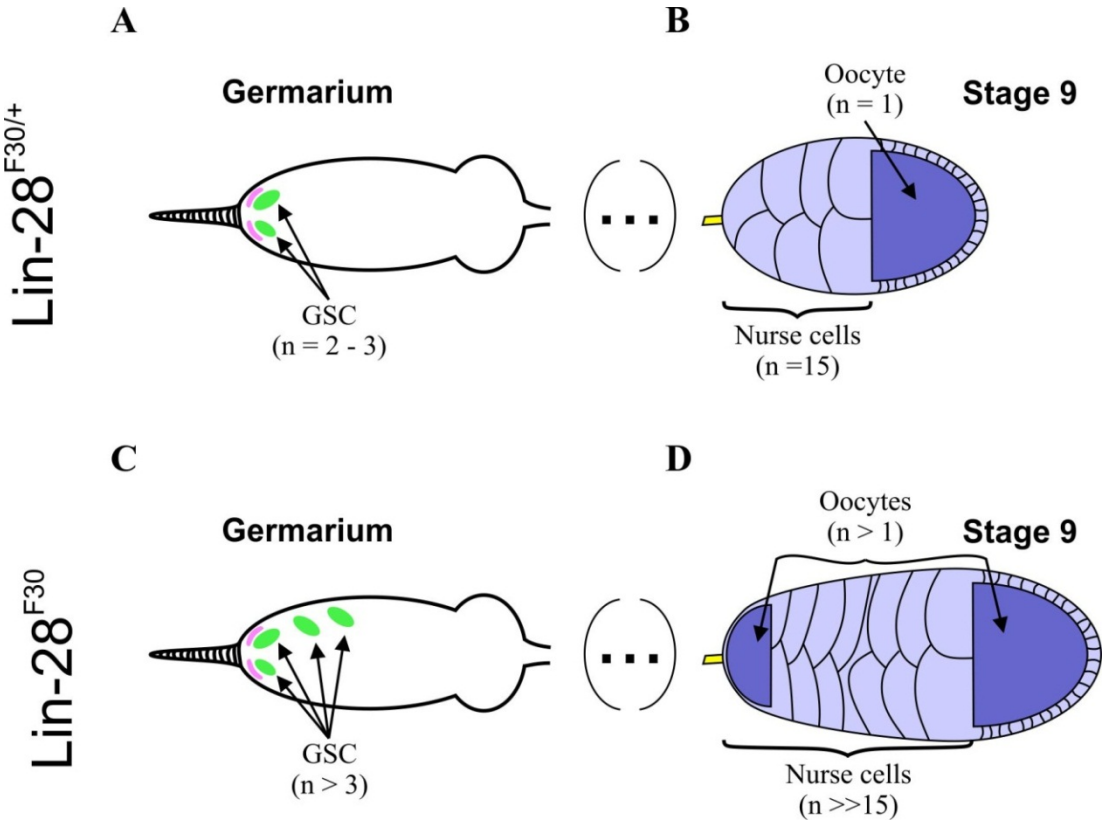


**Figure 7. Drosophila oogenesis in Lin-28$^{F30}$ mutants** (**A**) Control germaria have 2 to 3 GSCs (green). (**B**) Control stage 9 egg chambers have 15 nurse cells (pastel blue) and one oocyte (purple). (**C**) Lin-28F30 germaria exhibit increased number of GSCs, compared to controls (A). (**D**) Lin-28F30 stage 9 egg chambers have multiple oocytes (purple), while the number of nurse cells is greatly increased compared to controls (B).

### *let-7* overexpression recapitulates the supernumerary egg chamber phenotype (II)

Lin-28 has been shown to exert large part of its function through inhibition of *let-7*. The increased *let-7* levels in *lin-28*$^{dF30}$ mutant fly ovaries suggest an interaction of Lin-28 with *let-7* in oogenesis. In order to test this hypothesis, we overexpressed *let-7* specifically in ovarian follicle cells, therefore mimicking the Lin-28 depletion in these cells. Interestingly, in this case as well, we observed supernumerary nurse cells. This result indicates that the supernumerary egg chamber phenotype is, at least partly, the result of *let-7* overexpression. However, in this case all supernumerary egg chambers had 30 nurse cells. This results points towards *let-7* overexpression in follicle cells and can only account for the egg chamber fusion phenotype, but not for the mitosis defect.

### Supernumerary egg chambers have abnormal EcR and Fasciclin 2 expression patterns (II)

Ecdysone is the most important steroid hormone in flies that regulates various developmental decisions. Interestingly, it has been shown that the ecdysone signaling regulates *let-7* during development (Sempere et al., 2002). In addition, the *let-7* target *Abrupt* (Caygill and Johnston, 2008; König et al., 2011) interacts with Ecdysone signaling by a negative feedback loop (Jang et al., 2009). Abrupt also represses the expression of the cell adhesion molecule Fasciclin 2 in the developing *Drosophila* brain (Kucherenko et al., 2012). Therefore, *let-7*, Ecdysone signaling, Abrupt and Fasciclin 2 constitute a complex network that regulates critical developmental decisions, such as border cell migration and GCS differentiation (**Figure 8A**).

Border cell migration in *Drosophila* ovaries initiates at stage 9 and it is critical for both fertilization of the egg as well as for the dorsal ventral patterning of the anterior-posterior patterning of the egg chamber (Montell, 2003). Under normal conditions the Ecdysone receptor (EcR) is expressed specifically in the anterior follicle cells of stage 9 egg chambers (Jang et al., 2009). In concert with the Ecdysone receptor (EcR) expression timing, ecdysone signaling increases during stage 9 to peak during stage 10 (Schwartz et al., 1989). We found that in stage 9 supernumerary *lin-28*$^{dF30}$ egg chambers, the EcR pattern is not restricted in the anterior follicle cells, but it is uniformly expressed in all follicle cells surrounding the egg chamber. Although the precise Ecdysone expression pattern is not known, we suggest that the uniform EcR expression pattern in the follicle cells of supernumerary *lin-28*$^{dF30}$ egg chambers breaks the Ecdysone signaling asymmetry that is required for proper oogenesis and initiation of border cell migration.

Fasciclin 2 is also required for initiation of border cell migration (Szafranski and Goode, 2004). It is expressed ubiquitously in follicle cells through stage 7. At stage 8 and when border cell start to differentiate, Fasciclin 2 expression is confined in a small cluster of cells in the anterior of the egg chamber, known as polar cells. This change in expression is critical for the timing of border cell delamination from the epithelium (Szafranski and Goode, 2004). Again, in stage 8 supernumerary *lin-28*$^{dF30}$ egg chambers we found that the

Fasciclin 2 expression pattern symmetry is disturbed. In these egg chambers, Fasciclin 2 expression is not confined only to 2-3 cells at the anterior of the egg chamber. In contrast, Fasciclin 2 is expressed broadly at the anterior of the affected egg chamber.

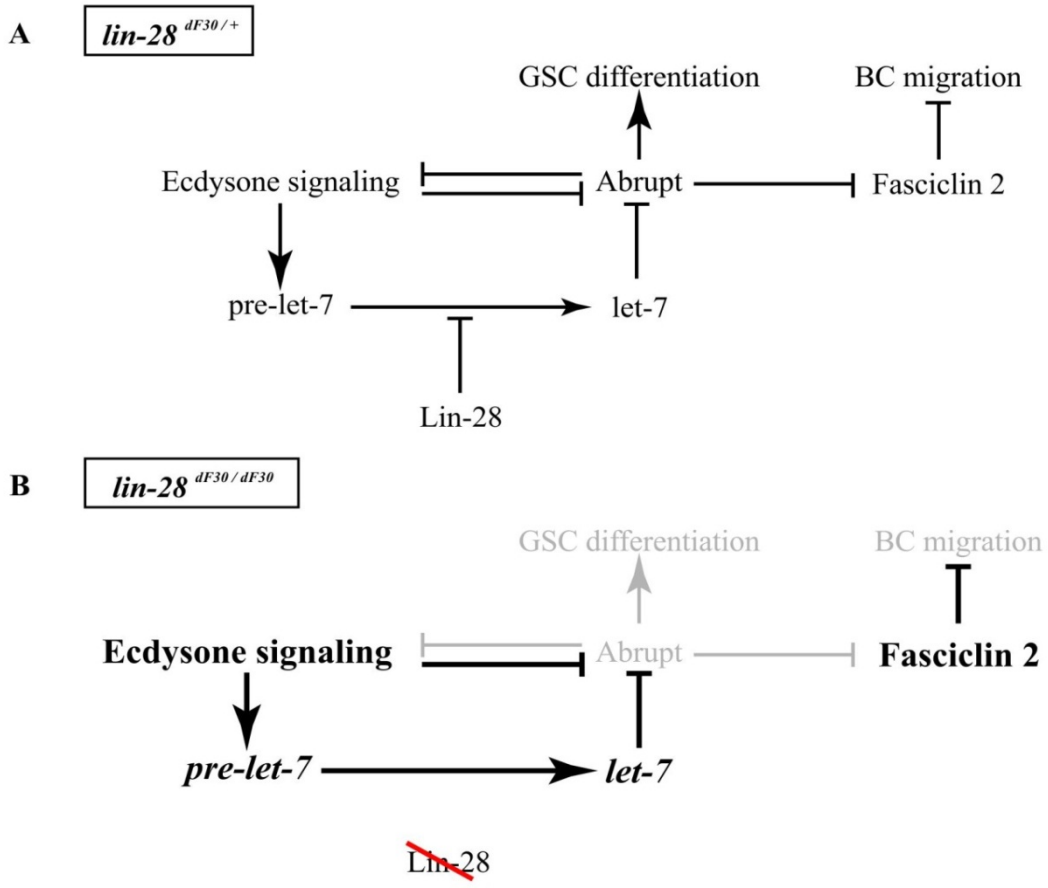


**Figure 8. Network of key factors involved in GSC differentiation and border cell migration in *Drosophila* (based on bibliography).** (**A**) Under normal conditions, *let-7* miRNA maturation is under strict control of Lin-28. (**B**) In *lin-28* null mutants, this *let-7* miRNA maturation control is impaired, leading to GSC and border cell migration defects.

Both the ecdysone-signaling pathway and Fasciclin 2 have important roles in various developmental decisions during *Drosophila* oogenesis. In supernumerary stage 8 and stage 9 *lin-28*$^{dF30}$ egg chambers the tight expression patterns of both the EcR and Fasciclin 2 are greatly impaired. In line with observations, we never observed supernumerary egg chambers of developmental stage older than stage 10, indicating that the supernumerary egg chambers undergo apoptosis during the mid and late oogenesis checkpoints (McCall, 2004). In addition, in supernumerary stage 9 and stage 10 *lin-28*$^{dF30}$ egg chambers we never observed border cells.

### *lin-28*$^{dF30}$ germaria display abnormal GSC differentiation (II)

In order to investigate the mechanism behind the supernumerary nurse cell phenotype observed in *lin-28*$^{dF30}$ egg chambers, we focused on GSCs differentiation in germaria. Spectrin is a marker for cyst identification (de Cuevas et al., 1996) (**Figure 4B**). GSCs are characterized by a spherical proteinous organelle, called the fusome. The fusome is recognized by Spectrin and appears as a round Spectrin accumulation. As GSC divides towards cystocytes with incomplete cytokinesis, the cystocytes are interconnected by the fusome. The fusome, which derives from the spectrosome is also recognized by Spectrin. Therefore, the more differentiated the cystocyte is, the more fusome material will be present and the more branched morphology the Spectrin will reveal (de Cuevas et al., 1996).

In all control germaria, two rounded fusomes next to the cap cells exist, located at the anterior of each germarium, corresponding to the two GSCs. Posteriorly, fusome acquired a branched morphology, indicating the cystocyte formation and subsequent differentiation. In *lin-28*$^{dF30}$ germaria we identified multiple rounded fusomes, corresponding to multiple GSCs. Some of the rounded spectrosomes are localized next to the cap cells; however, other rounded spectrosomes were located more posterior, to areas of the germarium that no GSCs should exist. This result shows that in *lin-28*$^{dF30}$ germaria, there is an increase of undifferentiated GSCs (**Figure 7C**).

To further investigate the GSC differentiation defect of *lin-28*$^{dF30}$ germaria, we visualized the expression of Bag of Marbles (Bam). Molecularly, Bam is under the strict control of Decapentapligic (Dpp) (Chen and McKearin, 2003). Bam is strongly expressed in cystoblasts and to a lesser degree to mitotically active cystocytes, while it is not expressed in GSCs (Chen and McKearin, 2003). Bam is a direct regulator of the switch from stem cell to cystoblast both in *Drosophila* ovaries and testes. Ectopic expression of Bam is sufficient to extinguish stem cell division in the germarium (Ohlstein and McKearin, 1997). On the other hand, in Bam mutant ovaries, the proliferating germ cells appear to behave as stem cells (Ohlstein and McKearin, 1997), highlighting the importance of Bam in the initiation of germ cell differentiation. Interestingly, in *lin-28*$^{dF30}$ germaria we observed that Bam expression is drastically reduced and we did not observe any distinctive strong expression at the cystoblasts. This expression pattern shows that in *lin-28*$^{dF30}$ germaria the Bam-driven initiation of germ differentiation is impaired, result that reiterates the increase of undifferentiated GSCs as shown with the Spectrin staining. These results combined, strongly indicate that in *lin-28*$^{dF30}$ germaria, differentiation of GSCs to cystocytes is impaired, resulting to germ cells that are possibly amplifying. It is a distinct possibility that the amplifying germ cells actually undergo incomplete cytokinesis and progress through the cell cycle within as cyst, as it has been shown to be the case in *bam* mutant testes (Gonczy et al., 1997).

# CONCLUSIONS

Our findings provided valuable information on the role of neurotrophic factors in *Drosophila* (I, III, IV). This is particularly interesting when taking into account that neurotrophic factors in *Drosophila* were an elusive topic until very recently. In addition, article II describes for the first time the characterization of the reprogramming factor Lin-28 in *Drosophila*.

Article IV describes basic research concerning the characterization and functional studies of the novel conserved neurotrophic factor DmMANF. Given that MANF and its mammalian ortholog CDNF have both shown to specifically affect dopaminergic neurons in vertebrates and in *Drosophila* embryos, and because human MANF has an orthologous function in the fly, the focus of this study was to investigate the role of DmMANF in adult flies (IV). To our surprise, these functional analyses did not reveal any obvious effect on the dopaminergic system (article IV). Instead we observed the appearance of numerous obscure cells in the pupal brain, in areas where no cell bodies are known to exist (III). Since this observation was very striking and to our knowledge such a phenotype has never been described before, we decided to concentrate exclusively on the analysis of this novel cell type (III). The results from the Manf study (article IV) show that DmMANF has a wider expression during pupal and adult developmental stages, when compared to embryonic expression. However, article IV does not conclusively resolve whether DmMANF is required for the support of dopaminergic neurons during adult stages, hypothesis that will need further studies to be resolved.

Article III reports the finding and identification of this novel cell type in the *Drosophila* pupal brain that has not been described previously. We named this cell MiC (MANF immunoreactive Cell), based on our initial observation that these cells were strongly DmMANF positive. In addition, these results (III) show that this dramatic phenotype is manifested by employing three different genetic mechanisms *i.e.* not only by silencing *DmMANF* but also when either immunity or autophagy is induced. We identified molecular markers, pathways activated as well as subcellular features of these cells. Importantly, MiCs strikingly resemble mammalian microglia, a cell type that has not been described in *Drosophila* before (III). The results from this study brings new and exciting data on glial and brain biology, as well as on the cross talk of three main evolutionarily conserved mechanisms, namely autophagy, immunity and neurotrophic factors. Further studies could concentrate on the origin of MiCs, their function as well as the molecular mechanisms that drive the appearance of these enigmatic cells.

Article I describes the characterization of the *Drosophila* Gfrα homolog, casting light on the evolutionary aspects of a second family of neurotrophic factors, namely the GDNF family ligands.

In contrast to the studies on the *Drosophila* CNS and neurotrophic factors, article II deals with adult stem cells and the evolutionarily conserved pleiotropic factor *lin-28*. This article shows that the *Drosophila lin-28*, like its mammalian homolog, is involved in the GSC differentiation, therefore we provide valuable insights about the mode of action of

both Lin-28 and its partner *let-7* miRNA. The results of this study contribute in improving our understanding of the pivotal role Lin-28 has in stem cell maintenance and differentiation.

# ACKNOWLEDGEMENTS

This work was carried out at the Institute of Biotechnology and the Division of Genetics, Department of Biosciences, University of Helsinki, with generous financial support from the EU Marie Curie Early Stage Training Action, the Finnish Cultural Foundation, the Ella and Georg Ehrnrooth Foundation and the Helsinki University Funds.

I would like to warmly thank the former director of the Institute of Biotechnology Professor Mart Saarma as well as the Head of the Genetics Department Professor Tapio Palva for providing excellent research infrastructure. In addition, I would like to thank the director of the Developmental Biology Program Academician Irma Thesleff for providing a stimulating scientific environment and numerous pleasant social events.

I am sincerely grateful to my supervisor Docent Tapio Heino for giving me the opportunity for this unique adventure. Thanks for your support, your good advices, your everlasting patience and above all for your trust in me during the years. I feel privileged to have enjoyed freedom to push myself as a researcher, initiate projects and collaborations, explore remote fields of research and visit various laboratories and conferences.

I am honored and delighted to have Dr Iris Salecker as my opponent and Professor Juha Partanen as my custos. I would like to warmly thank Docent Osamu Shimmi for his intellectual support, for being part of my advisory committee and for reviewing my thesis. I am grateful to Dr Igor Adameyko for his support and for his insightful feedback regarding the review of my thesis. I also thank Docent Pekka Heino for being part of my advisory committee, as well as for providing help on student related matters.

During the years I met a number of people that contributed to this moment. In particular I would like to thank the Marie Curie Fellows: Maarja, Sylvie, Roxana, Jens and Sarah for the unforgettable times we had. I also wish to thank all the members of the Developmental Biology Program and most specifically Elina J, Maria[n], Laura L, Shinya and Satu K. I also want to thank Jackie, Richard, Heini, Jukka K and Jaakko M for support and friendship during the years.

I am grateful to Professor Mart Saarma, Professor Juha Partanen and Docent Ville Hietakangas for their scientific contribution and their help when they were asked. In addition I would like to thank Professor Prinz and Dr Pyrowolakis for enlightening scientific discussions.

I would also like to thank Mari and Riitta for sharing valuable time with me. I also want to thank Arja for technical support and being valuable from every respect. Furthermore, I would like to thank the Light Microscopy and the Electron microscopy Units for providing excellent infrastructure, advice and collaboration during the years.

I am more than grateful to have met Frederic Michon. Your friendship, collaboration and currently giving me the chance to acquaint with the “vertebrate” world are simply irreplaceable. Without your contribution, guidance and encouragement this thesis would have been very different.

I also want to thank my friends and family for supporting the progress of this thesis work. I want to thank George, Vassilis, Panos, Theodoros, Manos, Thanasis, Katerina, Camilla and Juha, Risto, Marja and Elina for always being there for me and for their invaluable friendship during the years.

Above all, I want to thank my family for supporting me and encouraging me to pursue my dreams during the years: my father Thanos for teaching me to never be afraid, my mother Aleka for never giving up and my wonderful siblings Dimitris and Tatiana for just being who they are. Most of all, I am grateful to my dear fiancée Minttu for being by my side through thick and thin and offering me her irreplaceable smile that makes all of it worth it.

Helsinki, 3rd November 2014
Vassilis Stratoulias

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