Role of Integrins in the *Drosophila* Eye Imaginal Disc Glia Migration

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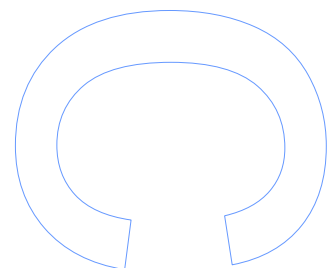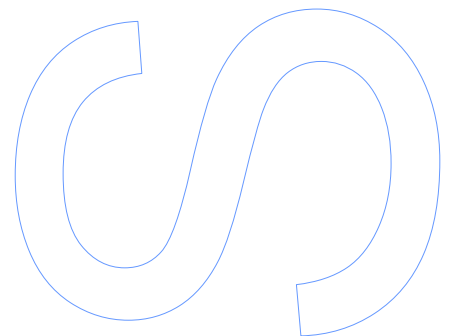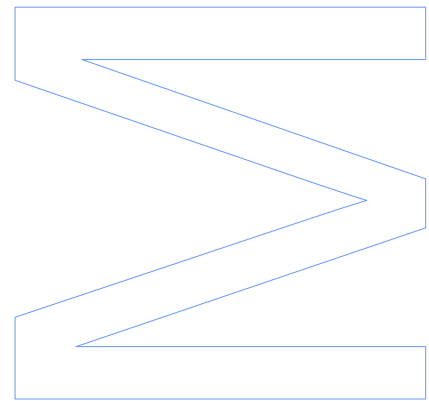
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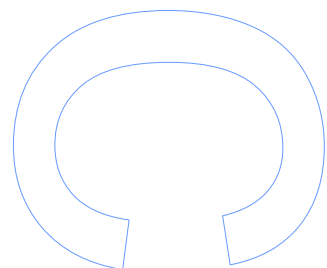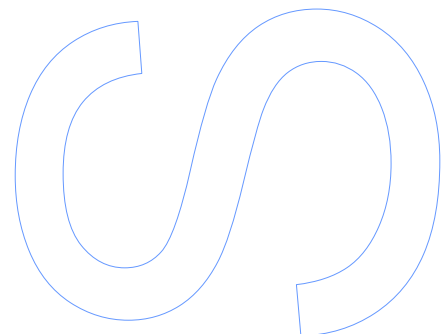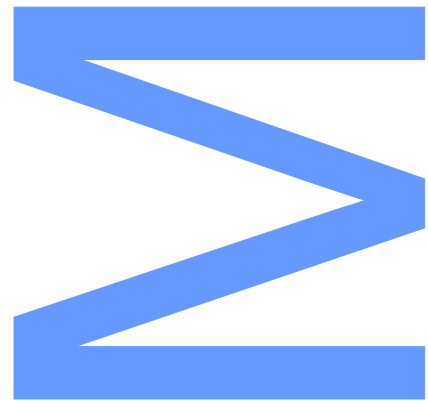Lígia Tavares, PhD, IBMC

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Acknowledges

Abstract

Glial cells are responsible for providing indispensable physical and metabolic support to neurons. For over a century, it was believed that the only function of glia was to support neurons. That idea is now discredited; they are important for proper development of the nervous system. Glial development is modulated by external signals from neurons through both direct cell-cell contact and secreted factors and also signals provided by the extracellular matrix (ECM) and by neighboring cells. Several signals are transduced into glia by specific receptors, such as integrins, an important family of ECM receptors. Previous studies demonstrated that integrins are expressed by all major mammal glial subtypes and play important roles in both neuronal and glial developmental processes in the nervous system. Nevertheless the large number of monomer combinations to form heterodimers of the integrin family and the technical struggles to manipulate genes in mammals limit the understanding of *in vivo* functions of integrins in glia. *Drosophila* has a much modest family of integrin subunits comprehending five alpha and two beta subunits. *Drosophila melanogaster* is an outstanding model for its simplicity, fast life cycle and the wide range of genetic tools available. Additionally the L3 larvae eye imaginal disc where photoreceptor differentiation initiates concomitantly with glia migration is the ideal system to address several questions of glial development, in addition to glia-neuron interactions.

Previous work in the lab has shown that integrin depletion specific in glia cells, by combination of the GAL4-UAS system and RNA interference (RNAi) knockdown, is lethal in late stages of development, suggesting a significant role for integrins on the nervous system development. In L3 larvae, depletion of integrin in glia cells causes migration defects into the eye imaginal disc.

The aim of this study is to understand how the eye imaginal disc is formed and the optic stalk maintained in the absence of glial cells. These questions were addressed by mainly electron microscopy for a better understanding of the ultrastructure of the eye disc and optic stalk in the absence of integrins.

Results obtained indicate a disruption of the eye disc basal lamina – that usually separates the photoreceptors cells from glial cells and axons. Additionally axons loose the circular structure and R8 ommatidia surrounded by glia membranes are no longer seen. There are also an increased number of mitochondria in axons.

In summary specific depletion of integrins in glia cells is essential to the proper nervous system development. Opposite to the current view photoreceptors are able to differentiate and correctly project their axons even in the absence of glia cells in the eye imaginal disc showing they do not work as guideposts for axonal targeting. Nevertheless axons need glia for their correct development as they show signs of the increased stress due to their absence.

Key words: Glia, Photoreceptors, *Drosophila melanogaster*, Imaginal Eye Disc, Migration, Integrins

Resumo

As células da glia são responsáveis por fornecerem aos neurónios indispensável suporte físico e metabólico. Há mais de um século, acreditava-se que a única função da glia era exclusivamente dar suporte aos neurónios. Agora, essa ideia é descreditada. As células da glia são importantes para o correto desenvolvimento do sistema nervoso. O desenvolvimento da glia é modulado por fatores externos provenientes dos neurónios através do contacto direto entre células e de fatores secretados; e também de sinais fornecidos pela matriz extracelular e por células vizinhas. Diversos sinais são traduzidos nas células da glia por recetores, tais como integrinas, uma família de recetores da matriz extracelular. Estudos prévios demonstram que as integrinas são expressas pela maioria dos subtipos de glia mamíferos e têm um papel importante nos processos de desenvolvimento neuronal e glial no sistema nervoso. Contudo, o grande número de combinações de monómeros para formar os heterodímeros de integrinas e os problemas técnicos para manipular genes em mamíferos limitam a compreensão das funções das integrinas *in vivo* nas células da glia. A *Drosophila* tem uma família de integrinas mais pequena, contendo cinco subunidades alfa e duas beta. *Drosophila melanogaster* é um bom modelo devido à sua simplicidade, ciclo de vida rápido e uma variedade de técnicas de manipulação genética disponíveis. Adicionalmente, o disco imaginal do olho na fase larval L3, quando os fotorrecetores iniciam a diferenciação ao mesmo tempo em que as células da glia migram em direção ao olho, são o sistema ideal para responder a várias questões sobre o desenvolvimento da glia e interações glia-neurónio.

Estudos prévios do grupo mostram que a depleção de integrinas especificamente nas células da glia, por combinação do sistema GAL4-UAS e *knockdown* através de RNA de interferência (RNAi), é letal em estágios tardios do desenvolvimento, sugerindo um papel significante das integrinas no desenvolvimento do sistema nervoso. Nas larvas L3, a depleção de integrinas nas células da glia causa defeitos de migração para o disco imaginal do olho.

O objetivo deste estudo é perceber como o disco imaginal do olho é formado e como se dá a manutenção da estrutura em forma de cone que liga o disco imaginal ao lóbulo cerebral ("optic stalk") na ausência de células da glia. Estas questões foram respondidas principalmente com recurso a microscopia eletrónica de transmissão para

obter um melhor entendimento da ultra estrutura do disco imaginal do olho e do "optic stalk" na ausência de integrinas.

Os resultados indicam uma disrupção da lâmina basal do disco do olho, que é a estrutura que separa o corpo celular dos fotorrecetores das células da glia e axónios. Além disso, os axónios perdem a estrutura circular e as omatídias R8 deixam de ser envolvidas por processos membranares de glia. Adicionalmente, há um aumento do número de mitocôndrias nos axónios.

Resumidamente, a depleção específica de integrinas nas células da glia é essencial para o desenvolvimento adequado do sistema nervoso. Contrário à visão previamente estabelecida os fotorrecetores são capazes de se diferenciar e projetar corretamente os seus axónios mesmo na ausência de células da glia no disco do olho pelo que a glia não funciona como postos de sinalização para os axónios. No entanto há a indicação dos axónios precisarem das células da glia para outras funções uma vez que apresentam sinais de stress devido à sua ausência.

Palavras-chave: Glia, Fotorrecetores, *Drosophila melanogaster*, Disco imaginal do olho, Migração, Integrinas

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List of abbreviations

CNS   Central Nervous System

dpp   Decapentaplegic

ECM   Extracellular matrix

FAK   Focal adhesion kinase

FGF   Fibroblast growth factor

gcm   Glial cells missing

GFP   Green fluorescent protein

HRP   Horseradish peroxidise

If   Inflated

ILK   Integrin-linked kinase

mew   Multiple edematous wing

MF   Morphogenetic furrow

MTJ   Myotendinous junction

mys   Myospheroid

OS   Optic stalk

PG   Perineurial glia

PNS   Peripheral nervous system

PS   Position specific

repo   Reversed polarity

RGD   Arginine-Glycine-Aspartic acid

RNA   Ribonucleic acid

RNAi   RNA interference

SJ   Septate junctions

SPG	Subperineurial glia
TEM	Transmission electron microscopy
UAS	Upstream activation sequence
Wb	Wing blister
WG	Wrapping glia
WT	Wild type

1. Introduction

1.1 Glial cell biology in *Drosophila*

The fruit fly *Drosophila* nervous system includes a central nervous system (CNS) and a peripheral nervous system (PNS), both including neurons and glia that arise from multipotencial neuroblasts, similar to vertebrates (Pfrieger and Barres 1995). Throughout evolution the percentage of glia in the nervous system usually increases. For instance, glia corresponds to 25% of cells in the *Drosophila* brain, 65% in the rodent brain and outnumbers neurons in the human brain (Pfrieger and Barres 1995; Azevedo, Carvalho et al. 2009).

Invertebrates have a smaller amount of glial cells and a less complex nervous system. Different glia subtypes are present in the invertebrate nervous systems and have several structural, morphological, molecular and functional resemblances as vertebrate glia (Freeman and Doherty 2006) (Table 1). Thus invertebrate organisms have been considered to address fundamental questions concerning glia-neuron interactions and glia development.

1.1.1 Importance of glia

Fruit fly glia is essential in many aspects of nervous system development. For instance, *Drosophila* glia can conduct and assist axon outgrowth, targeting and termination. In embryos, glia supply particular guidance signals to assist axon cross or circumvent the CNS midline (Harris, Sabatelli et al. 1996; Kolodziej, Timpe et al. 1996; Mitchell, Doyle et al. 1996; Kidd, Bland et al. 1999). Additionally, sensory and motor axons need peripheral glia situated at the PNS/CNS transition zone to guide these axons into appropriate fascicles to enter the CNS (Sepp, Schulte et al. 2000; Sepp, Schulte et al. 2001). Through development, immature glia commonly pre-pattern neuronal migration pathways and supply molecular guidance cues for neuritis (Auld 1999).

In the developing visual system, glial cells function as intermediate targets providing for a subset of photoreceptor axons needed to terminate correctly in the optic lobe (Poeck, Fischer et al. 2001) and form a compartment boundary for diverse visual centres (Tayler, Robichaux et al. 2004). During metamorphosis, glia assists axon pruning independent of axon fragmentation in the developing mushroom body, the memory centre in the *Drosophila* CNS (Watts, Schuldiner et al. 2004; Awasaki, Huang et al. 2011).

Beside their developmental roles, *Drosophila* glia is important for nervous system function. *Drosophila* glia expresses glutamate transporters and glutamine synthetase, which preserve neurotransmitter homeostasis (De Pinto, Caggese et al. 1987; Caizzi, Bozzetti et al. 1990; Caggese, Barsanti et al. 1994; Seal, Daniels et al. 1998; Besson, Soustelle et al. 1999; Kawano, Takuwa et al. 1999; Rival, Soustelle et al. 2004). A subtype of surface glia - subperineurial glia – form extensive pleated septate junctions among themselves and constitute the blood-brain barrier (in CNS) or blood-nerve barrier (in PNS) to protect internal neurons and axons from pathogens and high concentration of potassium ions in the hemolymph which would largely abolish excitability of axons, then ensuring the appropriate ionic and biochemical environment (Baumgartner, Littleton et al. 1996; Auld 1999; Stork, Engelen et al. 2008).

Furthermore, glia can act in response to neuronal injuries in adult flies and clear neuronal debris when axons are severed (Watts, Schuldiner et al. 2004; MacDonald, Beach et al. 2006).

Drosophila have not evolved saltatory conductance, however they need fast electrical conductance as well as vertebrates. Thus fruit flies follow two different and independent strategies to guarantee fast conductance. In some central neuronal networks there developed large calibre axons (Allen, Drummond et al. 1998), while in the PNS axons are insulated by many glial sheaths to guarantee insulation (Stork, Engelen et al. 2008).

1.1.2 Glial cell types in *Drosophila*

By using diverse genetic tools, similar glial subtypes are found both in the CNS and PNS of *Drosophila* and vertebrates (Freeman and Doherty 2006; Hartenstein 2011).

Three main classes of glia have been categorized, based on their morphology and position, in the larval brain and ventral nerve cord: the surface glia, the neuropil glia and the cortex glia (Edenfeld, Stork et al. 2005; Freeman and Doherty 2006).

Surface glia enclose the *Drosophila* CNS with a two layered sheath of flat cells that comprise outer perineural glia (PG) and inner subperineural glia (SPG) (Pereanu, Shy et al. 2005). Cell bodies and nuclei of SPG cells are very large and flattened (Awasaki, Lai et al. 2008; Stork, Engelen et al. 2008). There are very few SPG cells given their dimension (Pereanu, Shy et al. 2005; Stork, Engelen et al. 2008). PG cells are positioned outside the subperineural sheath (Awasaki, Lai et al. 2008; Stork, Engelen et al. 2008), and these cells are more numerous than SPG cells and have an elongated or multilobulated shape (Awasaki, Lai et al. 2008; Stork, Engelen et al. 2008).

Cortex glia span the thickness of the cortex and extend processes profusely around neuronal cell bodies, giving rise to a honey-combed structure of glial processes that occupy the spaces between neuron bodies (Pereanu, Shy et al. 2005). Cortex glia membranes make important physical contact with the blood-brain barrier and oxygen supplying tracheal elements for that they are proposed to provide gas and nutrient to the neurons, like the vertebrate astrocytes (Ito, Urban et al. 1995; Pereanu, Shy et al. 2005). Cortex glial cells have a small and rounded cell body and nucleus (Pereanu, Shy et al. 2005; Awasaki, Lai et al. 2008).

Neuropil glia is restricted to the inner neuropile areas of the brain where they extend sheath-like membrane processes around axonal fascicles, acting in a similar way of the vertebrate oligodendrocyte (Klämbt, Jacobs et al. 1991; Pereanu, Shy et al. 2005).

1.1.3 Glial development

Glia development has been well characterized in *Drosophila.* In *Drosophila,* though glia arise from different sorts of lineages in the CNS and PNS, glial progeny can be identified early on by the expression of the genes *glial cell missing (gmc*, also known as *glide*) and *reversed polarity* (*repo,* also known as *rk2*), which represent the key molecular factors of glial cell fate and differentiation (Campbell, Goring et al. 1994; Hosoya, Takizawa et al. 1995; Jones, Fetter et al. 1995).

In embryos, transiently expression of the transcription factor *gcm* is both required and enough for specifying the glial fate and determination, except for the CNS midline glia

(Hosoya, Takizawa et al. 1995; Jones, Fetter et al. 1995; Vincent, Vonesch et al. 1996; Akiyama-Oda, Hosoya et al. 1998). *gcm* function as a binary genetic switch that decides whether developing neural cells will become neurons or glia.

However, mammalian *gcm* homologs (*gcm1* and *gcm2*) are mainly found in non-neural tissues appearing to have no *in vivo* role in glial specification and it may be controlled by different molecular mechanisms in the *Drosophila* and vertebrate nervous system (Kim, Jones et al. 1998). Even so, later aspects of glia development as proliferation, interaction with neurons, extracellular environment and neuronal ensheathment share a lot of resemblances at the molecular level with vertebrates (Freeman and Doherty 2006).

Tabel 1 Similarities between *Drosophila* and vertebrate glial subtypes (adapted from (Freeman and Doherty 2006)).

Vertebrate glial subtype	Primary function	Distribution	Comparable *Drosophila* glial subtype
Astrocytes	Trophic support of neurons, synapse modulation	Embedded in CNS cell cortex, ensheathing synapses, CNS surface	Cortex glia (and a subset of surface glia)
Oligodendrocytes	Neuronal ensheathment, trophic support of neurons, myelination	Ensheathing axons in CNS	Neuropil glia
Microglia	Immune surveillance, macrophage function	Throughout CNS	Cortex, surface and neuropil glia
Schwann cells	Ensheathment and support of peripheral nerves, myelination	Ensheathing PNS nerves	Peripheral glia

1.2   Eye Imaginal Disc and Optic Stalk

1.2.1 Eye Imaginal disc differentiation

The eye imaginal disc, a small epithelial monolayer sac of larval tissue, gives rise the adult fly compound eye. The larval eye-antennal disc originates to the eye, antenna, ocelli, palpus and the head cuticle (Pappu and Mardon 2004).

The eye imaginal disc offers a system in which neurons and glia extremely coordinate their development. Glia migration has to be accurately synchronized, both temporally and spatially, with neuronal development.

The retina of the *Drosophila* compound eye is organized into roughly 750 ommatidia that are single eye repeating units, arranged in a regular hexagonal array (Wolff 1993). *Drosophila* eye development is a extremely dynamic process and it starts in the late second instar larva with the initiation of the front edge of the wave of retinal patterning, called the morphogenetic furrow (MF) (Ready, Hanson et al. 1976). At the MF neurogenesis is initiated. The MF is marked by a dorso-ventral indentation of the disc epithelium that appears at the posterior margin of the eye disc and progresses anteriorly (Wolff and Ready 1991). This progress of the MF through the eye marks the differentiation of photoreceptors or 'R-cells', the light sensing neurons of the ommatidium (Wolff and Ready 1991).

Every ommatidium encloses eight photoreceptor neurons (R1-8) and eleven supplementary cells, counting with cone, pigment and bristle cells (Wolff 1993; Pappu and Mardon 2004). At the end of larval development 26 rows of ommatidia have been formed and during early pupal development another seven are added (Wolff 1993).

A few rows posterior to the MF the recent photoreceptors project their axons into the basal layer of the disc, where they turn and then advance to the posterior edge of the eye disc all the way through the optic stalk (OS), a thin tubular structure that mediates the connection between the eye disc and the brain (Choi and Benzer 1994). The growth cone of each ommatidium target distinct regions in the optic lobe (Tayler and Garrity 2003). There are three basic classes of R-cells: R1-R6, R7 and R8. R1-6 targets the lamina, while R7 and R8 terminate in the deeper medulla region (Tayler and Garrity 2003).

Glial cells in the eye disc and in the optic stalk express Repo, a homeobox protein that is also expressed by almost every glial cells in the embryo (Halter, Urban et al. 1995; Xiong and Montell 1995). In the visual system, glial cells introduce small processes, called capitate projections, into the photoreceptor termini that have been suggested to be important for normal synaptic function in the lamina (Trujillo-Cenoz 1965; Stark and Carlson 1986; Fabian-Fine, Verstreken et al. 2003; Curtin, Wyman et al. 2007)

1.2.2 Origin of Optic Stalk Glia

The larval optic nerve is called the Bolwig nerve and goes along the peripodial membrane, throughout the apical part of the eye and antennal discs. The Bolwig nerve joins the eye imaginal disc to the brain and therefore foreshadows the upcoming OS through which all axons from photoreceptor neurons are send towards the brain (Silies, Yuva-Aydemir et al. 2010).

The origin of the glia in the optic stalk is unclear. They might be originated in the CNS and migrate along the Bolwig nerve through a process analogous to the migration of the peripheral glia along the segmental nerve at the late embryonic stages (Silies, Yuva-Aydemir et al. 2010).

Though, these glial cells only been observed in the second larval instar when 6-25 glial cells have been visualized in the OS (Rangarajan, Gong et al. 1999). Through early embryonic stages after the Bolwig organs are formed and the anlage of the eye disc is created, a small number of glial cells, possibly derived from the brain lobes, populate the initial section of the Bolwig nerve which afterwards will turn out to be the OS (Silies, Yuva-Aydemir et al. 2010).

OS formation depends on the activity of the focal adhesion kinase (fak) because mutations in fak modify the optic stalk morphology and interrupt axonal conductance, portentous that Integrin based attachment to the ECM is needed for differentiation of glial cells (Murakami, Umetsu et al. 2007; Ueda, Grabbe et al. 2008).

1.2.3 Glial Role in Axonal Outgrowth

The retinal basal glia (RBG) is originated in the OS and migrates into the disc (Choi and Benzer 1994; Rangarajan, Gong et al. 1999) what has been considered crucial for axon outgrowth (Rangarajan, Gong et al. 1999). RBG enter the eye disc at the beginning of retinal patterning at the early third instar larval phase with a posterior-to-anterior wave of morphogenesis occurring. They also migrate from posterior to anterior to occupy the basal layer of the eye epithelium, with the leading edge behind the MF (Choi and Benzer 1994) (Figure1A). This migration is tightly related to photoreceptor differentiation, as the number of glial cells entering the eye disc raises with the number of photoreceptors differentiated (Choi and Benzer 1994).

The leading edge of glial migration is defined approximately by the row of differentiating photoreceptors whose axons have started on turning posteriorly. The trail between MF and the anterior border of glial cells seems to be significant for targeting R-cell axons into the OS (Hummel, Attix et al. 2002). On the basal side of the eye disc, the glial cells are enclosed by a collagen-rich extracellular matrix (ECM).

Both glia migration into the eye disc and axon targeting into the OS are very tightly coordinated processes but so far not completely understood. It has been suggested a role for hedgehog (Hummel, Attix et al. 2002) and Dpp (Rangarajan, Courvoisier et al. 2001) for glia migration but is not clear how they work.

The current view in relation to axonal targeting into the brain is that axons need physical contact with, and support from RGB. When a dominant negative form of Ras is induced in glial cells (with Omb-Gal4 driver), glial cells are inhibited from migrating into the eye imaginal disc making axons to fail to target the optic stalk (Rangarajan, Gong et al. 1999). However, the study of *gcm* mutants, which lack all glia except midline glia, shows that the majority of axon pathways in the embryo, and particularly the longitudinal axon tracts, can grow and develop in the absence of glial cells, suggesting a purely spur role for glia (Hosoya, Takizawa et al. 1995; Jones, Fetter et al. 1995).

1.2.4 Glial cells in the Eye Imaginal Disc and Optic Stalk

The multiplicity of *Drosophila* glial cells was first noticed using electron microscopy studies (Jacobs and Goodman 1989; Klambt and Goodman 1991; Ito, Urban et al. 1995; Sepp, Schulte et al. 2000; Sen, Shetty et al. 2005).

At the early third instar previous to the entrance of the photoreceptor axons, two major classes of glia are observed around the Bolwig nerve in the OS: inner carpet cell (SPG) and the outer perineurial glia (PG). The PG constitutes the outermost layer of glial cells and is located just under a thick neural lamella (Silies, Yuva-Aydemir et al. 2010). Inside the optic stalk glial cells assume a spindle like shape, creating a dense mesh of cells around the photoreceptor axons (Silies, Yuva et al. 2007).

However, the developing eye disc seems to have a larger variety of glial cell types as so far have been described six morphologically different glial cell types (Rangarajan, Courvoisier et al. 2001). The diverse glial cell types identified in the eye disc might be originated from different progenitor pools or derive in a sequential mode from progenitor cells that migrate into the eye disc. There are no less than two layers of glial cells, in a way that a wrapping glial layer is covered up by a surface glia layer (Hummel, Attix et al. 2002).

1.2.4.1 Carpet Cells

As in other parts of the nervous system, the SPG cells are apical to the PG and in the eye disc they also set up the blood brain barrier by using pleated septate junctions that form a thigh epithelium. In the eye disc there are only two giant specialized SPG cells, the carpet cells which have enlarged nuclei, and septate junctions are found between the two of them (Silies, Yuva et al. 2007).

The two carpet cells are continually establish in the OS during larval stages. Thus the carpet cells are probably forming a permeable barrier around the OS. Once an OS is recognized in the second instar larvae, carpet cells can be visualized there (Silies, Yuva et al. 2007). The carpet cells achieve from behind the MF all the way into the lamina (Silies, Yuva et al. 2007). In the eye disc, the carpet cells separate WG from non WG (Silies, Yuva et al. 2007).

Following the photoreceptors send out their axons through the OS, the carpet cells anterior membranes expand out forming a flat mesh-like structure in the eye disc whereas the posterior membranes retain a tubular structure in the OS (Silies, Yuva et al. 2007). The carpet cells are the leading cells for glial migration and they demark the glia limit of migration (Silies, Yuva et al. 2007).

1.2.4.2   Perineurial Glia and Wrapping Glia

The squamous PG cells create a single layer around the outside of the carpet cells. Within the OS, the carpet cell separates the PG cells from the Bolwig axons (Silies, Yuva et al. 2007). Here the PG cells are named for the morphological, molecular and structural resemblances to other PG in the CNS and PNS. In the OS, the PG are variable in motility and differentiation features (Silies, Yuva et al. 2007).

In the eye disc, through larval stages only PG cells can divide (Silies, Yuva et al. 2007). The amount of PG cells is raised as the carpet cells grow making sure there is a stable coverage of PG cells on the external surface of the carpet cell. PG cells get in touch with nascent photoreceptor axons just when they arrive at the edge of the growing eye field (Silies, Yuva et al. 2007) (Figure 1B).

Upon contact with axons, the PG cells are pulled from the carpet cells and are reprogrammed from a migrating cell type to a differentiating WG cell type, leading to a complete wrapping of ommatidial axon fascicles (Figure 1C).

In the posterior domain, glial cells often extend cellular processes that follow the axons through the OS to the brain (Silies, Yuva et al. 2007). The WG cells are the ones that wrap the axons while surface glia surrounds them (Rangarajan, Courvoisier et al. 2001; Hummel, Attix et al. 2002). WG can wrap more than one fascicle (Silies, Yuva et al. 2007).

The WG are easily recognized in the OS and eye disc for their elongated process along photoreceptor axons toward the brain and not so long extensions into the apical region of the eye disc. The eight axons from a single ommatidia are constantly bundled together on their way to their targets in the brain (Franzdottir, Engelen et al. 2009).

The WG membrane extensions are positioned involving these axon bundles and are considered to help to retain this isolation. In the eye disc, simple protrusions are sent out by the WG to wrap a limited number of photoreceptor axonal bundles, different from the WG in the peripheral nerve, which extend several processes to ensheath all axons in the nerve. The formation of sheaths by the WG is regulated by the FGF signalling pathway. Changes of FGF signalling pathway can cause hypo- or hyper-ensheathment in the eye disc and OS, if there is down-regulation or up-regulation of FGF signalling, respectively (Franzdottir, Engelen et al. 2009).

1.2.5 Glial migration

To perform their role in axon guidance or support, glia has to be in the proper position respecting the neurons locations. For that several populations of glial cells have to migrate over many cell diameters within a limited period of time during development, as happens with midline glia in the CNS of *Drosophila* and oligodendrocytes in the vertebrate optic nerve (Small, Riddle et al. 1987; Klämbt, Jacobs et al. 1991).

Also during the development of the imaginal disc glia is required to migrate. As development progresses, glial cells population increases in the eye imaginal disc (from the optic stalk) showing their prominent migratory abilities (Klambt 2009) and at the end of larval development approximately 350 glial cells are found per eye imaginal disc (Silies, Yuva et al. 2007). Glial cell number needs to match the progression of the MF and the wrapping of axons requires to be synchronized with axonal growth (Figure 1).

Once PG cells are pulled from the carpet cells to differentiate into WG; the location at the leading edge of the carpet cell is replaced by the next line of PG cells and a new cycle of migration and differentiation can begin (Silies, Yuva-Aydemir et al. 2010). Therefore migration of glia appears to be regulated by the carpet cells that pack all migratory PG cells, so carpet cell confine the migration of glial cells (Silies, Yuva-Aydemir et al. 2010).

The timing of PG migration and carpet cells distribution is essentially regulated to match photoreceptors differentiation. Thus at first PG migration is repressed by the eye disc epithelium through a Casein Kinase called gilgamesh, the eye specification

transcription factors (*eyeless, eye absent, sine oculis*) and secreted hedgehog protein (Hummel, Attix et al. 2002).

OS glial migration is specially triggered by the differentiation of photoreceptor axons cells (Rangarajan, Gong et al. 1999) through a process that involves the hedgehog, decapentaplegic and fibroblast growth factor (FGF) signalling pathways (Rangarajan, Courvoisier et al. 2001; Hummel, Attix et al. 2002; Franzdottir, Engelen et al. 2009). In addition, these signalling pathways have functions in glial proliferation in the eye disc. During late third larval instar, continuous PG migration and proliferation raise glia number in the disc, which coordinated and matches in ommatidia formation (Choi and Benzer 1994; Silies, Yuva et al. 2007).

FGF signalling controls PG proliferation, migration and differentiation through a switch among two FGF ligands, Pyramus and Thisbe (Franzdottir, Engelen et al. 2009), both of which produced by photoreceptors. In carpet cells Pyramus modulates PG proliferation and motility. The second FGF ligand Thisbe can be detected just by pioneer migrating PG when they migrate near to the MF and past the boundary of the carpet cells. The lack of a carpet cell layer lets the Thisbe to bind the FGF receptor (heartless) on the PG surface and start the fate change from PG to WG (Franzdottir, Engelen et al. 2009).
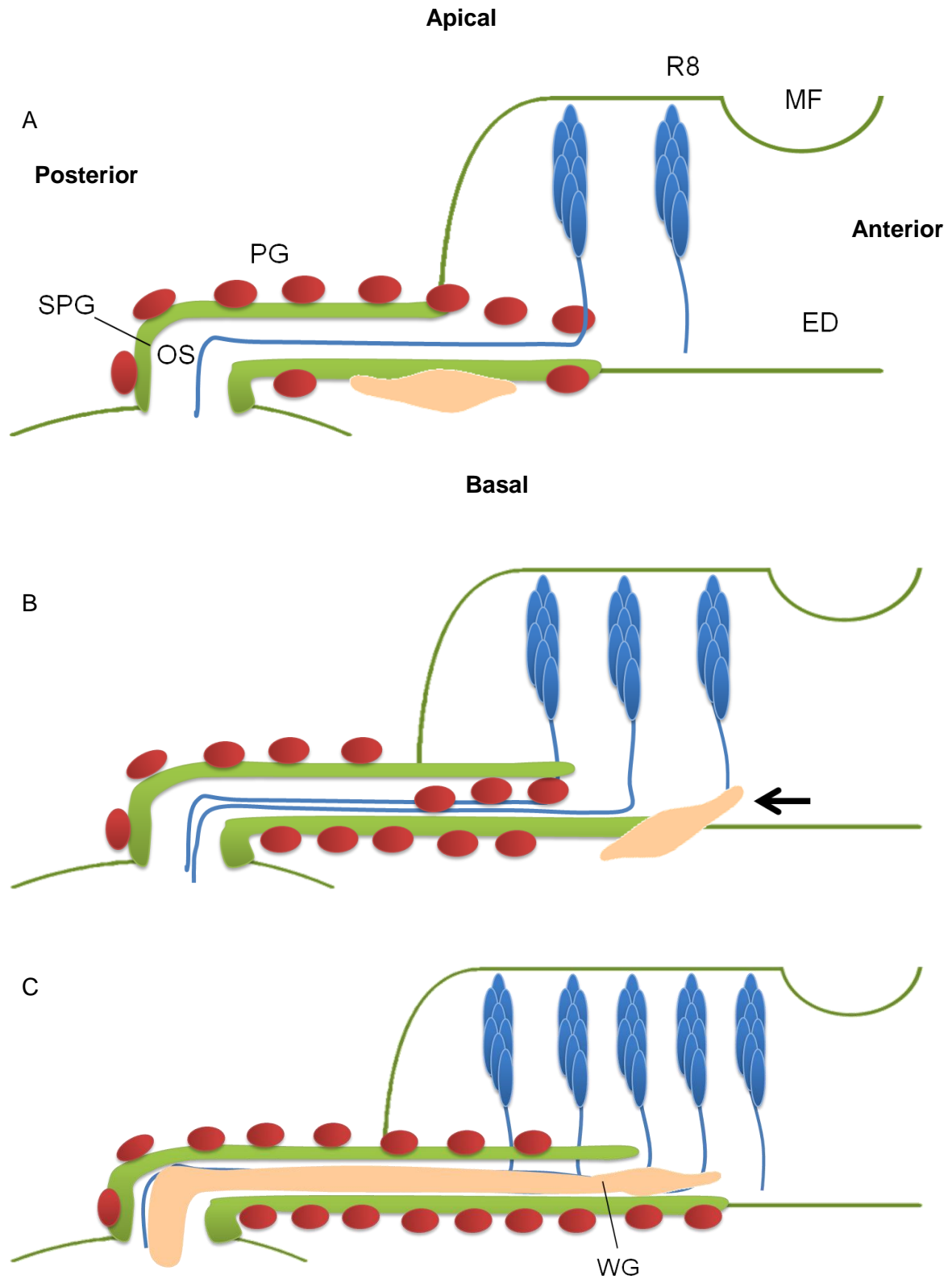
Figure 1 Glia development of glia in the eye disc. As photoreceptors differentiate the number of glia migrating to the eye disc increases. MF, morphogenetic furrow; ED, eye disc; OS, optic stalk; PG, perineurial glia; SPG, subperineurial glia; WG, wrapping glia. **A**. Migration of glia. Photoreceptors start to differentiate and to target their axons basally and then posteriorly towards the OS. **B.** Contact to axons (arrow). **C.** Wrapping.

1.3   Integrins

As several other types of cells in multicellular organisms, also glial cells count on surface cell adhesion molecules (CAMs) to connect to each other and to the extracellular matrix (ECM). CAMs are not simply required to hold cells together; in addition they are implicated in a variety of cell activities, including: survival, cell proliferation, dead, polarization, differentiation, growth, and migration. Diverse kinds of CAMs, counting with immunoglobin superfamily, cadherins and integrins are present in animals from invertebrates to vertebrates in which they are expressed by defined cell types, located to particular subcellular domains and mediating specific cell adhesions mechanisms (Hynes and Zhao 2000). Integrins act as traction receptors that can either send out signals or sense changes in the extracellular matrix (Takada, Ye et al. 2007). Integrins mediate force-resistant adhesion, polarization in response to extracellular cues, and cell migration by integrating the cytoskeleton with points of attachment in the extracellular environment. In addition integrins mediate stable adhesion to basement membranes, the formation of extracellular matrices and migration on such matrices, the formation of platelet aggregates, the establishment of intercellular junctions in the immune system, and bacterial and viral entry during infectious diseases (Arnaout, Mahalingam et al. 2005).

The family of cell adhesion receptors integrins have been demonstrated to play essential roles in mediating both interactions between cells and between cells and ECM. Due to interactions on both sides of the citoplasmatic membrane, integrins are able to mediate cell attachment to explicit substrates and transduce intracellular signals to control particular characteristics of cell activity (Legate, Montanez et al. 2006).

Integrins are transmembrane proteins that form heterodimers containing one alpha and one beta subunit. All subunits are type I transmembrane glycoproteins composed by a larger extracellular portion (alpha subunit has around 700 amino acids and beta has approximately 1000 amino acids) and a shorter intracellular tail (with less than 75 amino acids excluding the vertebrate β4 subunit) (Danen 2006). The extracellular region is composed by several domains which usually fold into an elongate stalk connected to a globular ligand-binding head region (Xiong, Stehle et al. 2001; Shimaoka, Takagi et al. 2002; Xiong, Stehle et al. 2002; Xiao, Takagi et al. 2004; Arnaout, Mahalingam et al. 2005).

The extracellular head region of most integrins binds to ECM glycoproteins as laminins and collagens in basement membranes or connective tissue components as fibronectin. The intracellular tail, even though being short, is constituted by several binding motifs that are able to form complexes with a big number of downstream proteins (Zaidel-Bar, Itzkovitz et al. 2007).

The integrins, as with several other CAMs, are preserved throughout the animal kingdom. At least one alpha and one beta integrin subunit have been cloned from the most ancient animals, the sponges (Brower, Brower et al. 1997; Pancer, Kruse et al. 1997) where integrins have already significant functions in cell-cell adhesion (Wimmer, Perovic et al. 1999). *Drosophila* hosts five alpha and two beta subunits (Brown 2000). The integrin family increases significantly in the vertebrates. In mammals there are 24 integrins identified to date, resultant from different pairings between the 18 α and 8 β subunits (DiPersio, Shao et al. 2000; Hynes and Zhao 2000; Arnaout, Mahalingam et al. 2005).

Whereas the vertebrate integrins develop new extracellular ligand binding and intracellular features and contribute to more complicated biological functions that are not present in lower animals, many still conserve the essential structure, composition and functional characterizations as their ancient homologous (Figure 2). There is no evident homology among the integrin α and β subunits; sequence identity between α subunits is approximately 30% and between β subunits about 45%, pointing that these gene families evolved by gene duplication (Figure 2) (Takada, Ye et al. 2007).

In mammals, the affinity of specific integrin pairs for their extracellular ligands is strongly related by their heterodimeric arrangement and by cytoplasmic signals from within the cell (inside-out signaling). The relationship of the α and β cytoplasmic tails seems to be needed to retain integrins in the inactive state (Ginsberg, Partridge et al. 2005).

It has been suggested that on binding extracellular ligands, mammalian integrins cluster in the interior of the cell near the membrane and transduce signals inside (outside-in signaling) (Takada, Ye et al. 2007).
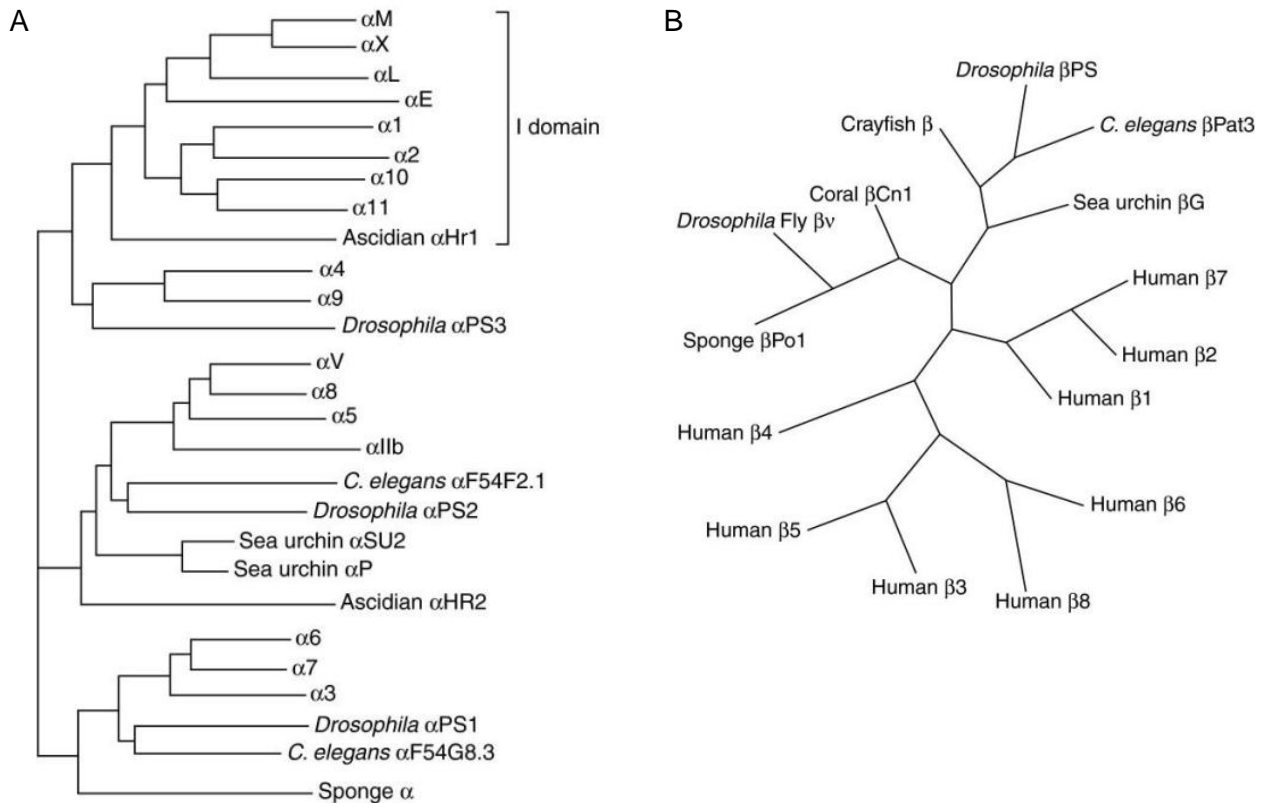
Figure 2 Phylogenetic trees of integrin subunits. Trees for (**A**) integrin α and (**B**) integrin β subunits are adapted from (Miyazawa, Azumi et al. 2001) and (Brower, Brower et al. 1997), respectively.

1.3.1 Outside-in and Inside-out Signalling

Integrin mediated processes count on the binding of integrins to the intracellular cytoskeleton through the commonly short integrin cytoplasmic tails; such binding allows the bi-directional communication of force across the cytoplasmatic membrane (Calderwood, Shattil et al. 2000; Evans and Calderwood 2007). Additionally to the integrin mechanical roles, they send out chemical signals into the cell (outside-in signalling), giving information on its position, environment, conditions of adhesion and surrounding matrix (Hynes 2002; Miranti and Brugge 2002). Besides to outside-in signalling, integrins are able to control their affinity for extracellular ligands. To do this integrins undergo conformational modifications in their extracellular domains that happen in response to signals imposed upon the integrin cytoplasmic tails – inside-out signaling or activation (Calderwood 2004).

The binding of talin to the β subunit cytoplasmic tail has been considered crucial in integrin activation (Tadokoro, Shattil et al. 2003; Calderwood 2004; Ginsberg, Partridge et al. 2005). In *vivo* the essential role of talin in integrin was shown by studies with transgenic mice that demonstrate the significance of talin-integrin interactions for platelet aggregation (Nieswandt, Moser et al. 2007; Petrich, Marchese et al. 2007). Talin also binds to several cytoskeletal and signalling proteins (Gingras, Bate et al. 2008), binding activated integrins directly to cytoskeletal and signalling systems.

One of the first recognized integrin signaling molecules was focal adhesion kinase (FAK), which acts as a scaffold for phosphorylation-regulated signaling and is essential for adhesion mediated by growth-factor signaling, integrins turnover, cell migration and Rho-family GTPase activation (Mitra, Hanson et al. 2005).

Like FAK, integrin-linked kinase (ILK) is an indispensable protein that has a main role as a signalling scaffold in integrin adhesions. ILK takes part in a heterotrimeric complex with the LIM-domain protein PINCH and the actin- and paxillin-binding protein parvin (Legate, Montanez et al. 2006).

The FAK- and ILK-binding protein, paxillin is an additional important signalling scaffold that is early engaged to integrin adhesions (Deakin and Turner 2008). Collectively, they act as intermediate mediators between the binding of regulators and effectors of the Rho family of small GTPases (e.g. the CrkII-DOCK180-ELMO complex and PIX), kinases (e.g. FAK, Src and ILK), actin-binding proteins (e.g. vinculin and the parvins), and phosphatases (e.g. PTP-PEST).

1.4   Integrins in *Drosophila*

The fruit fly has a small integrin family, which comprises five alpha (αPS1-αPS5) and two beta (βPS and βv) subunits, generating five integrin pairs (Takada, Ye et al. 2007). Traditionally, these integrins were called PS proteins for their position specific rather than their expression pattern in specific cell type in the *Drosophila* wing epithelial cells (Brower, Wilcox et al. 1984; Leptin, Aebersold et al. 1987).

Integrin genes get the name according to their mutant phenotypes. The genes *multiple edematous wing* (*mew*), *inflated* (*if*) and *scabs* (*scab*) / *volado* (*vol*) encode for αPS1-3 subunits, respectively, and the βPS subunit is encoded by *myospheroid* (*mys*) (Table 2, Figure 3).

Tabel 2 Integrins, proteins related to focal adhesion complexes, ECM components proteins and genes in *Drosophila* and vertebrate homologous.

Protein	Gene	Vertebrate homolog
Integrin αPS1	*mew*	Integrin α3, α6 and α7
Integrin αPS2	*if*	Integrin αIIB, α5, α8 and αV
Integrin αPS3	*scab* or *vol*	
Integrin αPS4-5	*αPS4-5*	
Integrin βPS	*Myo*	Integrin β1, β2 and β7
Integrin βPv	*βInt-v*	
Talin	*Rhea*	Talin-1 and Talin-2
Laminin α1,2	*Wb*	Laminin α1 and α2
Laminin α3,5	*lanA*	Laminin α3 and α5
Laminin β	*lanB*	Laminin B
Laminin γ	*lanB2*	Laminin γ
Perlecan	*Trol*	Perlecan
Collagen IV	*Vkg*	Collagen IV

The αPS1 subunit is homologous to vertebrate α3, α6 and α7, whereas αPS2 is comparable to vertebrate α5, α8, αv, αIIb (Figure 2, Figure 3, Table 2) (Takada, Ye et al. 2007). Moreover, the sequence likeness extends to their ligand binding profile. Being so, that αPS1βPS (PS1) integrin receptor and αPS2βPS (PS2) are, respectively,

receptors for laminin and arginine-glycine-asparte (RGD) sequence-containing ECM proteins (Bunch and Brower 1992; Zavortink, Bunch et al. 1993; Gotwals, Fessler et al. 1994; Gotwals, Paine-Saunders et al. 1994).

The αPS3-5 integrin subunits are not as conserved in vertebrates (Hynes and Zhao 2000). They are strongly related to each other and seem to be a consequence from recent duplication events. The αPS3βPS integrin receptor has been demonstrated to bind to both forms of laminin even though it does not seems to have an ortholog in vertebrates (Stark, Yee et al. 1997; Schock and Perrimon 2003).

Between the two integrin β subunits present in *Drosophila*, βPS has a sequence homology preserved in more than one vertebrate β subunit (β1, β2 and β7). The other integrin β subunit, the βv, has no evident vertebrate orthologs (Table 2) (Takada, Ye et al. 2007). The βPS has been shown to form heterodimers with αPS1-3 in a wide set of cells and tissue. Its deletion causes embryonic lethality (Leptin, Bogaert et al. 1989). The expression of the integrin βv subunit is mainly constrained to the developing midgut endoderm and their precursors, and βv deletion does not cause lethality (Yee and Hynes 1993).

In *Drosophila,* as in vertebrates, the main ECM receptors are integrins. From embryos to adults and in several tissues and cell types, integrins have significant functions in several processes like cell migration regulation (Martin-Bermudo, Alvarez-Garcia et al. 1999; Bradley, Myat et al. 2003), mediating established cellular adhesion (Brabant, Fristrom et al. 1996; Bradley, Myat et al. 2003), adjusting axonal outgrowth (Hoang and Chiba 1998), preserving stem cells niche (Tanentzapf, Devenport et al. 2007; O'Reilly, Lee et al. 2008) and short term memory (Grotewiel, Beck et al. 1998) just to mention a few.
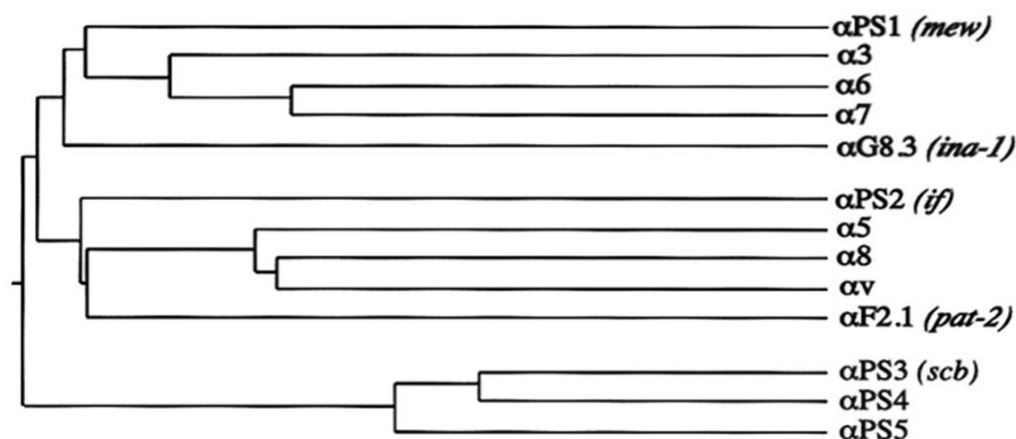

Figure 3 The five integrin α subunits of *D. melanogaster*. Gene names are given in italics. αPS1, which bind laminin, is mainly closely related to a set of vertebrate integrins (α3, α6, α7) that bind laminin. αPS2, which bind to RGB amino acid sequence, is closest related to RGD-binding vertebrate integrins (α5, α8, αv, αIIb) (Adapted from (Hynes and Zhao 2000)).

1.4.1 Integrins Mediated Cell Adhesion

Stabilized cell adhesions are essential for ensuring tissue architectures and are made directly by junctions between cells and not by adhesions of cells to ECM. In the fruit fly, integrins distinguish diverse ECM ligands through the alpha subunits relative specificity, for instance PS1 recognize laminins and PS2 recognize RGD-containing proteins (Bunch and Brower 1992; Fristrom, Wilcox et al. 1993), as triggrin, tenascin-major and laminin α2 (Graner, Bunch et al. 1998).

These two heterodimers are often expressed in juxtaposed tissues with ECM between them and mediate indirect cell to cell adhesions (Fristrom, Wilcox et al. 1993; Brower, Bunch et al. 1995). In the *Drosophila* wing, for instance, the dorsal and ventral epithelium layers are tightly attached to each other through a thin basement membrane present between them (Fristrom, Wilcox et al. 1993). The αPS1 subunit is expressed in the dorsal layer while the αPS2 subunit is present in the ventral layer (Brower, Wilcox et al. 1984; Brower and Jaffe 1989). Mutants of either α or β integrin subunits cause separation of the epithelial layers from each other giving rise to wing blisters (Brower and Jaffe 1989; Wilcox, DiAntonio et al. 1989; Brabant and Brower 1993).

A different example of cell adhesion mediated by integrins is the myotendinous junction. In embryos and larvae, the specific epidermal tendon cells help longitudinal muscles to attach to each other and also to connect to the animal body wall. These epidermal tendon cells constitute the myotendinous junction and transduce muscle contractions to the cuticle (Bate and Rushton 1993). Around muscles and tendon cells is deposited extracellular matrix that mediates through cell surface integrin receptors their connections with each other. The heterodimer PS1 is expressed by tendon cells whereas PS2 is more abundant at the end of every muscle (Bogaert, Brown et al. 1987; Leptin, Bogaert et al. 1989).

In integrin mutant embryos, the somatic muscles contract into spherical structures and are mostly disconnected from the epidermis (Wright 1960; Newman and Wright 1981; Leptin, Bogaert et al. 1989; Brown 1994). These studies in wing and muscle attachment show that different integrins are able to connect two cell layers to opposite areas of the basement membrane.

Other essential feature of integrin-mediate adhesion is that integrin complexes are very dynamic. In embryonic and larval myotendinous junctions, FRAP (fluorescence recovery after photobleaching) experiments demonstrated the turnover of integrin adhesion complexes (Yuan, Fairchild et al. 2010). As the animal grows old and along with the growth of the myotendinous junction, there is a decrease of the amount of integrin complex components that suffer turnover. This implies that during development, in cell to ECM adhesions the turnover of integrins is regulated and is adapted to maintain normal tissue development (Yuan, Fairchild et al. 2010).

In developing and adult muscles, the beginning and maintenance of sarcomere reliability requires integrin mediated cell to ECM adhesion (Sparrow and Schock 2009). For instance, in adult fly muscles, integrin-dependent adhesion down-regulation causes progressive loss of the sarcomere cell architecture and muscle formation (Perkins, Ellis et al. 2010), implying that integrin mediated adhesions are not just passive connections.

1.4.2 Integrins Mediated Cell Migration

Integrin binding to ECM components mediates not only stable cell attachment to the ECM and to other cells; it also uses ECM to regulate cell migration. In cell migration, the contribution of fruit fly integrins was confirmed with studies of integrin mutants and from integrin distribution.

Integrin mediated cell migration has been characterized in several systems as dorsal closure, midgut migration, salivary gland development and tracheal system formation.

The majority of the integrin mediated embryonic migration steps have similar characteristics for both ectodermal and endodermal migrating cells; both cell types migrate along a mesodermal substratum and the ECM is frequently placed between the migrating cells. For instance, the fuit fly midgut is composed by two sets of endodermal primordial cells – the anterior midgut primordium and the posterior midgut primordium – which arise individually from anterior and posterior embryonic regions (Reuter, Grunewald et al. 1993). The two primodial cells use a substratum supplied by visceral mesoderm to migrate (Reuter, Grunewald et al. 1993; Tepass and Hartenstein 1994).

Midgut cells present mainly the integrin βPS subunit (Leptin, Bogaert et al. 1989). After removal of the genes encoding βPS subunit (*mys*) from the embryo midgut primordial cells, from both maternal and zygotic origin, migration is deferred but can ultimately be completed (Roote and Zusman 1995; Martin-Bermudo, Alvarez-Garcia et al. 1999). Moreover, full removal of αPS1 and αPS2 subunits does not create all the same phenotype of the embryos lacking βPS (Roote and Zusman 1995).

During embryonic to pupal stages, the other beta subunit βv is highly specific to the midgut endoderm (Yee and Hynes 1993). Once the two integrin beta subunits (βPS and βv) are removed the midgut primordial migration is entirely blocked (Devenport and Brown 2004). This implies that both integrin beta subunits may work with the same propose to control midgut cell migration.

Another example of the role of integrins in cell migration is the *Drosohpila* larval tracheal system, which is constituted by two main trunks and six primary branches that transport oxygen from the exterior environment to the internal tissues. The trachea is derived from tracheal placodes that are sets of embryonic ectodermal cells, which migrate in different directions to give rise to the tracheal branches (Manning 1993). Migration of the visceral branch, one of the six primary tracheal branches, is particularly impaired in integrin α and β subunit mutants (Boube, Martin-Bermudo et al. 2001); furthermore, the tracheal truck is sporadically disrupted (Stark, Yee et al. 1997).

Another characteristic of integrin subunits in tissues is its specific distribution what may mediate different migratory events. In trachea and midgut, migrating primordial cells express both αPS1 and αPS2; while visceral mesoderm expresses just αPS2 (Martin-Bermudo, Alvarez-Garcia et al. 1999; Boube, Martin-Bermudo et al. 2001). This proposes that distinct integrins dimers may have different roles in leading cell migration and sustaining the substrate.

In the nervous system, glial cells are extremely motile cells; there are in *Drosophila* a great number of examples of that; in the embryonic peripheral nerve glial cells are able to migrate long distances (Sepp, Schulte et al. 2000), to the larval eye imaginal disc (Rangarajan, Gong et al. 1999) and pupal wing (Aigouy, Van de Bor et al. 2004). Furthermore the evidences that PNS glia follow axons, like happens in fly embryo (Sepp, Schulte et al. 2000), zebrafish lateral line (Gilmour, Maischein et al. 2002), chicken Schwann cells (Carpenter and Hollyday 1992); and the fact this cells migrate as chains of cells make a strong suggestion that cell-cell interactions play a role in

migration. Nevertheless, in *Drosophila* it has not been described yet the role of integrins in glial cell migration.

1.5   Glia and Integrins in Disease

Several genes and cellular processes in the nervous system are conserved in flies (Yoshihara, Ensminger et al. 2001). More than 60% of identified human genes causing diseases have a fly orthologue (Rubin, Yandell et al. 2000). As there is significantly less genetic redundancy in *Drosophila* comparative to vertebrate models, characterization of disease-causing gene function is frequently less complicated. Many of the cellular processes important in mammalian neurodegeneration, as the signalling pathways that orchestrate apoptosis, intracellular calcium homeostasis and oxidative stress are well conserved in flies, as are the proteins that control these processes. The *Drosophila* nervous system is formed by 200,000 neurons and supporting glia in comparison to the millions of neurons found in the mammalian brain.

Though simpler, fly neurophysiology is extremely alike to its mammalian counterpart. For example, fly neurons show synaptic plasticity and neurotransmission mediated by a lot of similar neurotransmitters, synaptic proteins, receptors and ion channels as the ones found in the mammalian brain (Yoshihara, Ensminger et al. 2001). In addition as being important in glia and glial diseases, there is a growing pile of implications about the role of glia in diseases – from neurodegenerative disorders like Alzheimer's to neurodevelopmental ones like autism and Rett syndrome (Sloan and Barres 2013). Multiple sclerosis is maybe the prototypic glial disease, where different roles for glia are being shown (Fields 2010).

Integrins are essential to the etiology and pathology of several other diseases. By integrating and transducing information into and out of the cell, integrins control several aspects of cell functioning as cell localization, shape, spreading and motility and therefore are significant determinants of both health and disease. Integrins have been suggested in the pathogenesis of inflammatory disease, platelet aggregation, tumour progression as well as osteoporosis and macular (Millard, Odde et al. 2011).

1.6   Aim

Previous work in the lab has shown that depletion of βPS integrin specifically in glial cells causes migration defects into the eye imaginal disc. Despite the absence of glial cells in the developing retina, photoreceptor axons enter the optic stalk in direction to the brain. This phenotype is very interesting as on one hand an eye imaginal disc is formed without glia and one other hand the photoreceptor axons find their way to the optic stalk. This is an unexpected result as it was shown that axons need glia as guideposts to enter the optic stalk.

So, the aim of this work is to better understand the role of integrins in glia development but also comprehend and how the eye imaginal disc is formed and the optic stalk maintained in the absence of glial cells. For that I will optimize electron microscopy techniques for the eye imaginal disc of Drosophila to compare the ultrastructure of the eye disc of βPS knockdown with that of the wild-type.

2. Material and Methods

2.1 Fly Stocks and Genetics

The Gal4 driver specific for glial cells used in this study was UAS Dicer2:*repo-GAL4/SM6^TM6B*. It was used a GFP protein-trap insertion: Vkg::GFP. The following UAS strains were used in this study: *UAS-LacZ* (II), *UAS-βPS-RNAi* and *UAS-αPS2-RNAi*. Drosophila lines were obtained from the Bloomington distribution centre. Crosses were carried out at 25°C with Repo>Dicer2>LacZ as a control.

2.2 Electron microscopy analysis

The electron microscopy protocols were not established for the eye imaginal disc tissue, so different procedures were used for optimization.

2.2.1 Protocol 1 – Standard Procedure

L3 larvae tissues were fixed in 4 % PFA (A) or 1 % gluaraldehyde (B) in 0.1 M cacodylate buffer overnight (ON) at 4°C. After fixation the samples were washed in cacodylate buffer. Following that, a 1 hour (h) post fixation step was done using 2 % osmium tetroxide in 0.1 M cacodylate buffer. The samples were washed in distilled water followed by another post fixation step with 2 % uranil acetate in water during 30 minutes (min) at room temperature (RT). The samples were dehydrated with ethanol and soaked in propylene oxide for 10 min. After that the propylene oxide was replaced by a mixture (1:1) of propylene oxide and Epon 812 resin and incubated for 30 min. The mixture was then replaced by 100 % Epon resin for 24 h. Finally the Epon was replaced by a fresh one and let to polymerize at 60 ^{o}C for 48 h.

2.2.2 Protocol 2 – Heavy Metal Staining Method

L3 larvae tissues were fixed with 1 % glutaraldehyde, 2 % formaldehyde with 2 mM calcium chloride in 0.15 M cacodylate buffer pH 7.4 at 35 ^{o}C for 5 min. A second fixation step was done for 2 h on ice in the same solution. Samples were washed in cold cacodylate buffer containing 2 mM calcium chloride. Then the tissues were

incubated with 3 % potassium ferrocyanide in 0.3 M cacodylate buffer with 4 mM calcium choride and combined with an equal volume of 4 % aqueous osmium tetroxide.

After washed with distillated water, the samples were incubated with tiocarbohydrazide solution for 20 min, at RT. Samples were washed again and then placed in 2 % osmium tetroxide aqueous for 30 min, at RT. Following that, the samples were washed and incubated in 1 % uranyl acetate aqueous, at 4 $^{\circ}$C ON. Samples were then washed and placed in a lead aspartate solution and incubate at 60 $^{\circ}$C for 30 min. Samples were washed and dehydrated using ice-cold solutions of ethanol and then soaked in propylene oxide. The resin mixture is progressively increased: 25 % Epon:propylene oxide; 50 % Epon:propylene; 75 % Epon:propylene; each for 2 h. At the end samples were placed in 100 % Epon ON and then into fresh 100 % Epon for 2 h. In the final step the Epon was replaced and let to polymerize at 60 $^{\circ}$C for 48 h.

2.2.3   Protocol 3 – *Drosophila* adapted from (Pereanu, Shy et al. 2005)

L3 larvae tissues were fixed in 2 % glutaraldehyde in PBS at 4 $^{\circ}$C for 2 h and post-fixation in 1 % osmium tetroxide in 0.1 5M cacodylate buffer for 30 min (on ice). Specimens were washed several times in PBS and dehydrated in graded ethanol on ice. Then the specimens were soaked in propylene oxide for 10 min. Samples were left overnight in a 1:1 mixture of Epon and propylene oxide and then 5 h in unpolymerized Epon. After that, they were transferred to a mold, oriented and placed at 60 $^{\circ}$C for 48 h to permit polymerization of the Epon 812.

For all the different protocols after Epon polymerization blocks were sectioned (50-70 nm) in a RMC PowerTome PC=XL ultramicrotome. Sections were mounted on net grids and treated with uranyl acetate and lead citrate.

Transmission electron microscopy images were obtained with a Jeol JEM-1400 with an Orius Sc1000 Digital Camera, acquired with GATAN software.

Images were then exported to Photoshop CS2 for compilation and montage. The montage was done manually with panels of at least 6x5 images which had an overlape with eachother of 15%.

2.3   Immunohistochemistry

The following primary antibodies were used: mouse anti-Repo (DSHB) at 1:10 and anti-HRP-Cy5 conjugated at 1:100. Secondary antibody used was Alexa Fluor® 568 goat anti-mouse IgG (H+L) from Molecular Probes at 1:1000.

Dissection and fixation for immunofluorescence was performed according to standard procedures. L3 larvae were dissected and washed in PBS, then fixed in 3.7 % PFA for 20 min at RT followed by 3 washes of 10 min in PBS 0.1 % triton. Samples were incubated with primary antibodies in PBS 0.1 % triton for 2 h at RT. After, samples were washed (3 x 10 min) with PBS 0.1% triton and incubated with secondary antibody together with DAPI (1:1000) for 1:30 h at RT. The samples were washed (3 x 10 min) in PBS 0.1% triton and moved to 50 % glycerol where they were keep at 4°C at least 20 min for an increase in density. Finally, samples were finely dissected and mounted in glycerol.

2.4   Statistics

The number of axons in the optic stalk and the ratio of mitochondria per axon were expressed as mean ± standard deviation (M±SD). The mean and standard deviation were calculated in Excel® and all graphic charts were made with Prism 5® (Graphpad Software). All comparisons were performed with Student T-test. Significant differences were assessed at a 95% confidence interval.

3. Results

3.1   Optimization of Electron Microscopy Protocol

In electron microscopy, the ultrastructure needs to be conserved as close as possible to the *in vivo* state. However the majority of protocols available for electron microscopy regard mammal tissues that have very different physical properties and are generally much more resistant than the ones from *Drosophila* larvae. The principal requirements for a good electron microscopy protocol are quality and reproducibility. Therefore, it was necessary to optimize a protocol that would preserve the entire eye imaginal disc and optic stalk without major artefacts. One of the most critic points is fixation as it crosslinks proteins by chemical fixation, with aldehydes, what helps to preserve the real ultrastructure of the tissue.

I started by using the standard protocol for electron microscopy available in the Histology and Electron Microscopy Service. It was used two different fixations, one with paraformaldehyde (Figure 4A) and another with glutaraldehyde (Figure 4B). Paraformaldehyde, a monoaldehyde conserves ultrastructure well but penetrates faster than the dialdehyde, glutaraldehyde. Post fixation was done by osmium tetroxide which interacts with unsaturated lipids and is electron-dense, therefore staining cell membranes phospholipids. *En bloc* treatment with uranyl acetate helps to stabilize phospholipids. In this protocol dehydration is done through a rising concentration series of ethanol, which is not as strong as an organic solvent as acetone.

In this protocol fixation was very poor, as can be seen by examining the dilated and misshapen mitochondria christae (Figure 4), also the distension between the two nuclear membranes and the presence of extracellular spaces (Figure 4), and the overall lack of contrast.

The second protocol was designed for 3D electron microscopy. In this protocol the last contrast step done in the grid with lead citrate, is absent. Because of that contrast is applied during the preparation of the samples what increases the contrast of the membranes (Figure 5).
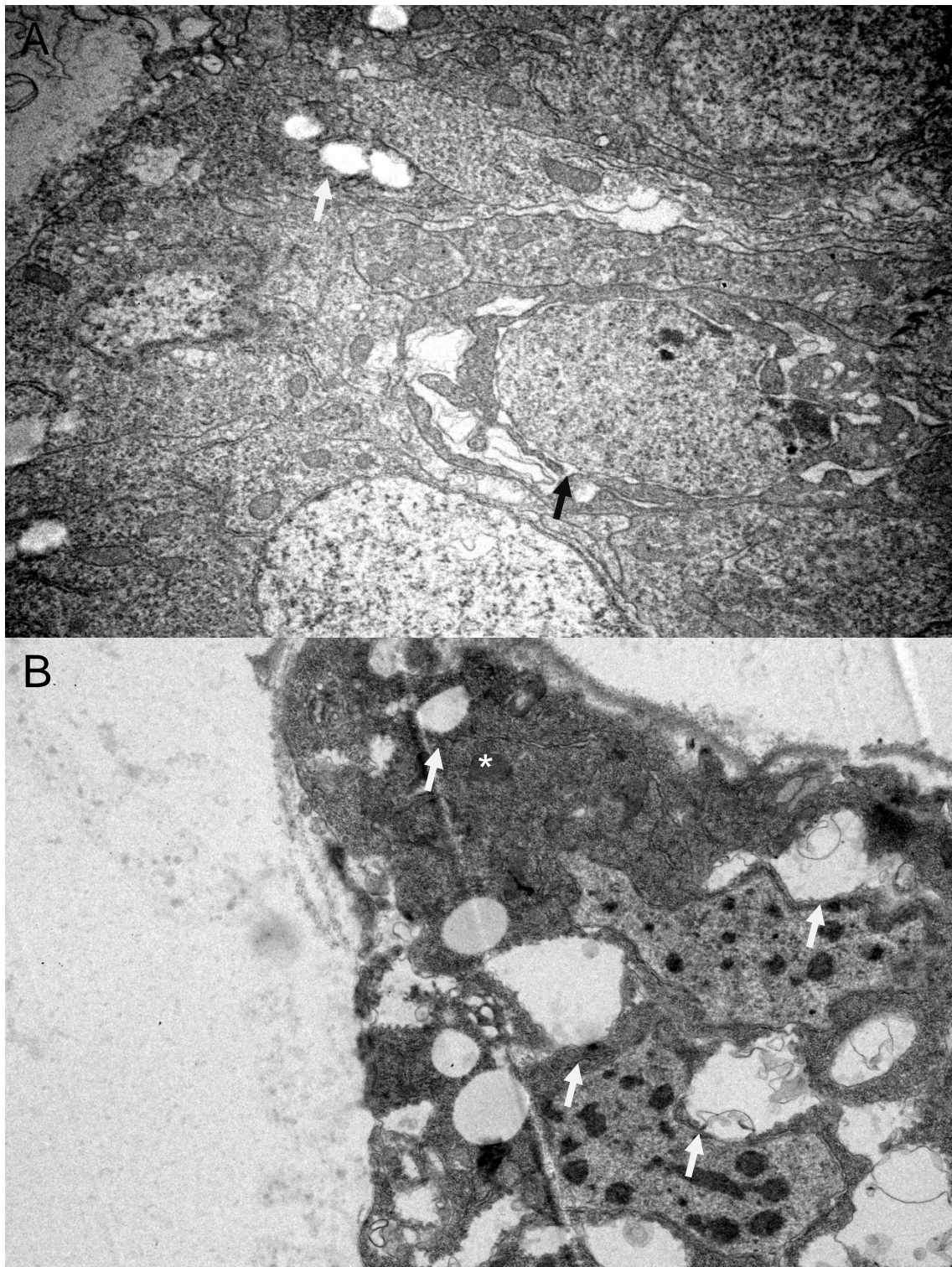
Figure 4 Optimization of electron microscopy techniques, Protocol 1. **A**, Fixation with PFA, the tissue could not hold together during fixation and there are visible hollows (white arrow). Here are also visible spaces between the two nuclear membranes (black arrow). **B**, fixation with glutaraldehyde. Here there also hollows were specially in periphery of the tissue. Here is observed dilated and misshapen mitochondria christae (asterisk).

In this protocol the osmium tetroxide-potassium ferrocyanide method was used to improve membrane contrast in tissues that were poorly preserved by aldehyde fixation (Karnovsky 1971). The mechanisms of staining by osmium-ferricyanide mixtures are not totally understood, however they are certainly complex. Thiocarbohydrazide will then crosslink with osmium what will stain carbohydrates; this is followed by *en bloc* lead aspartate staining. *En bloc* staining with lead aspartate avoids the grid-staining step and consequently samples can be analysed on the microscope and photographed right after thin-sectioning. Dehydration was performed with ethanol and the last step with the organic solvent acetone. Acetone appears to cause less tissue shrinkage than ethanol.

This protocol increases the contrast of samples however what is thought to be artefacts (as they did not appear in other protocols) was present very frequently (Figure 5). DNA seems to be poorly stained as photoreceptor nuclei (asterisk in Figure 5) have a greyish tonality, different from darker heterochromatin stain seen in the standard protocol (Protocol 1). Furthermore, the overall tissue was not recognizable, so montage in the bloc was random, and section between samples could not be compared.
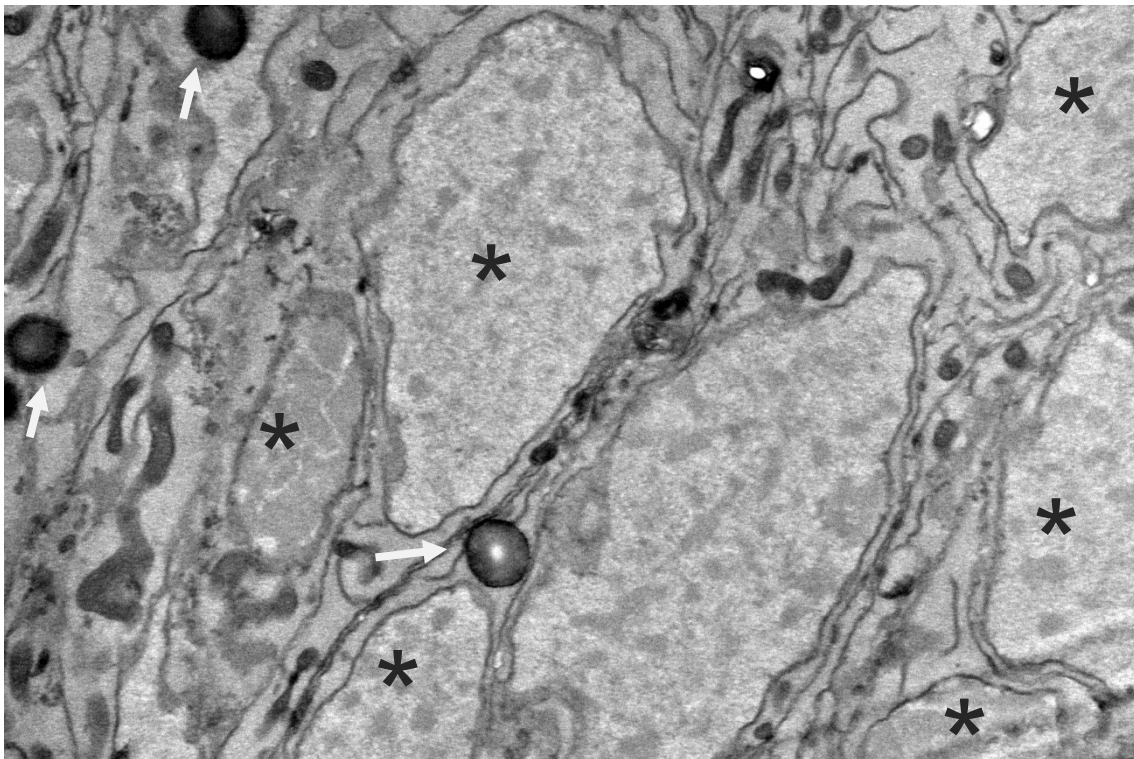

Figure 5 Optimization of electron microscopy techniques, Protocol 2 – Photoreceptors and pigment cells. The nuclei of photoreceptors (asterisk) have a greyish tonality, different from the standard protocol (Protocol 1), which show a darker heterochromatin staining. The presence of artefacts was frequent (white arrow).

The third protocol tested was adapted from one used for *Drosophila* brain (Pereanu, Shy et al. 2005). This one follows generally the same steps of the Protocol 1 with few alterations. For instance, all steps were performed on ice, for the impregnation of solutions to be slower. As Drosophila larvae tissues are smaller and more fragile than those from mammals, a slower procedure has shown to preserve tissues better and to present good details. As shown in Figure 6 photoreceptors nuclei present a dense and dark heterochromatin, very easy to identify and the two faces of the nuclear envelop can be easily detected (Figure 6A). Mitochondria christae are also well preserved and maintain its shape (Figure 6B).

For the reasons explained before it was chose the Protocol 3 as it was less complicated and reproducible, and also it accomplish the requirements of contrast and image quality.
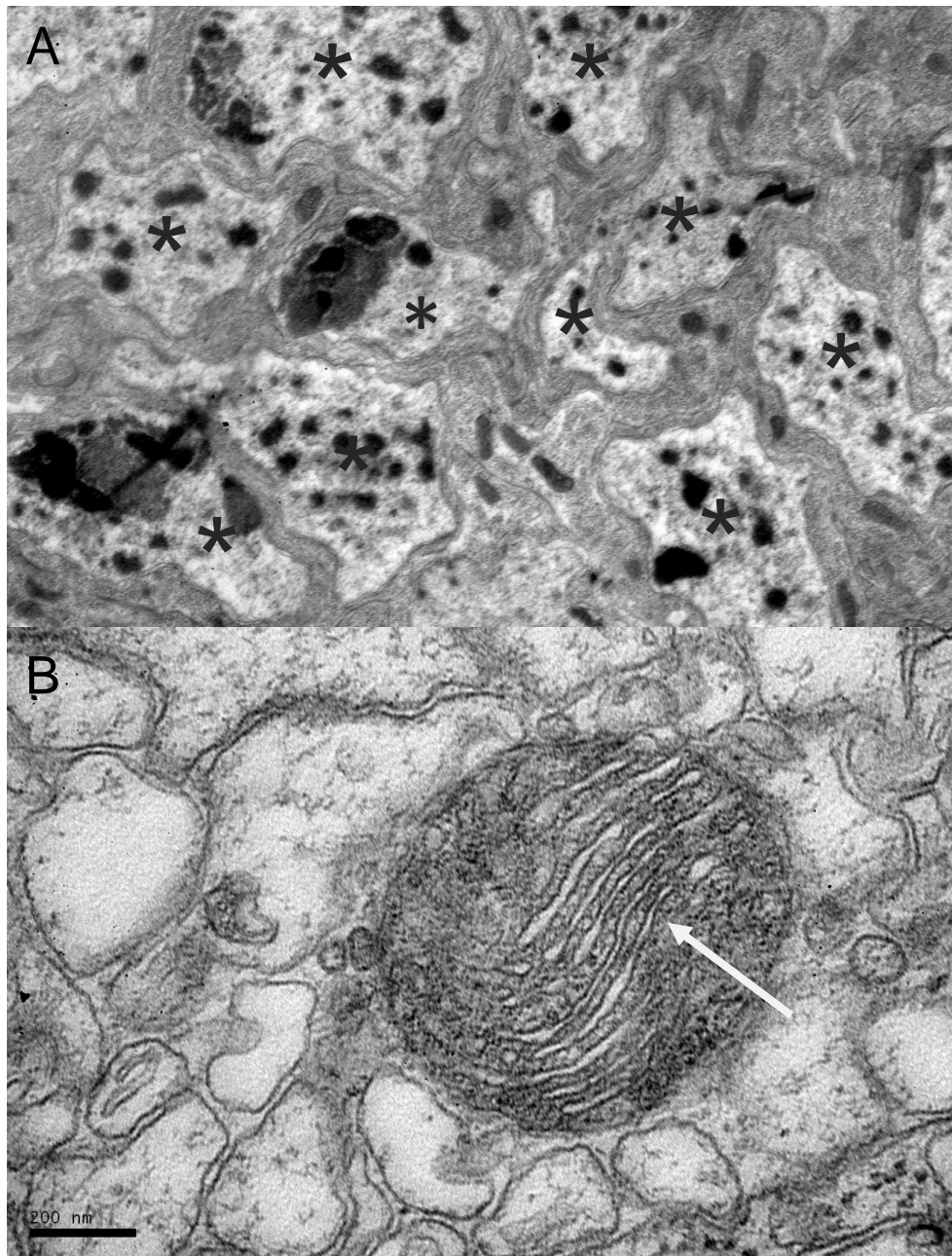
Figure 6 Optimization of electron microscopy techniques, Protocol 3. **A.** Photoreceptors nuclei (asterisk) present a dense and dark heterochromatin and the nuclear envelop presents the two faces. **B.** Mitochondria christae are preserved and well shaped (arrow).

3.2   Eye Imaginal Disc and Optic Stalk Morphology

The most apical region of the eye disc is formed by a layer of squamous epithelium, the peripodial membrane, under which photoreceptors, pigment and accessory cells are found. Glial cells migrate through the basal region of the eye disc where they ensheath the photoreceptor axons (Silies, Yuva et al. 2007). The overall morphology of transversal cuts of the eye disc is represented in Figure 7.
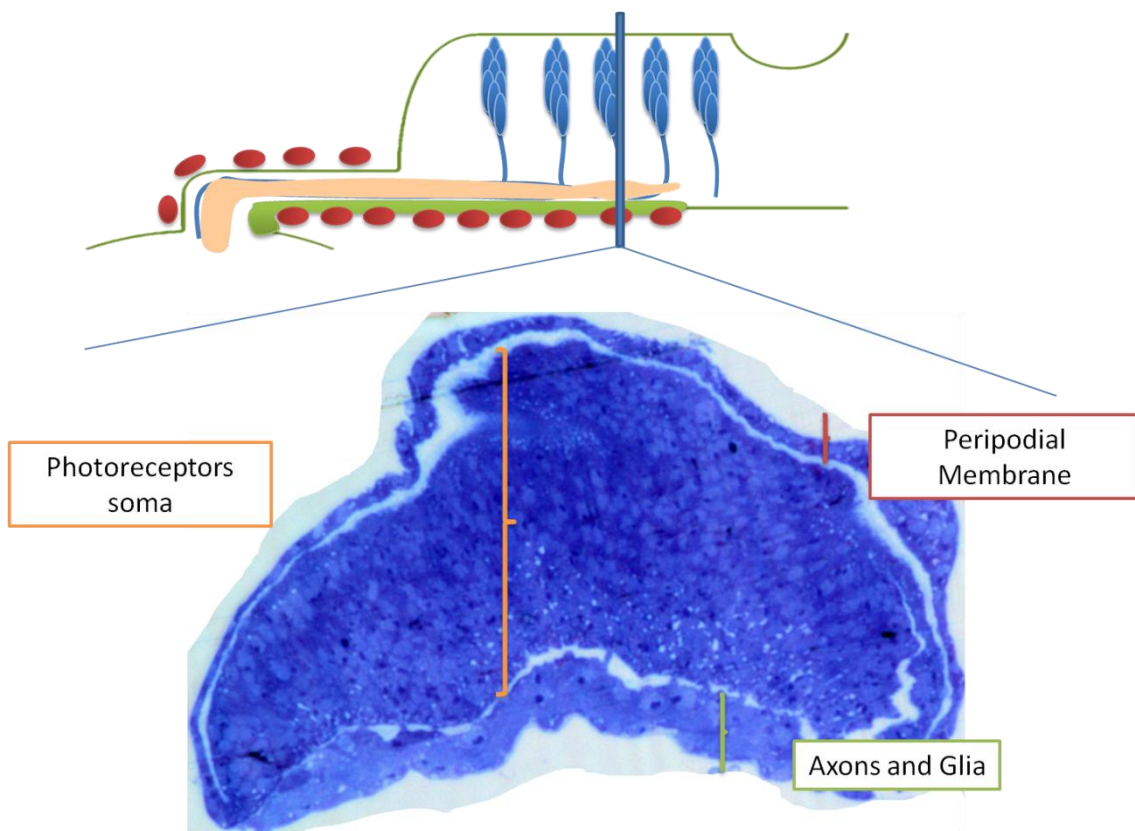

Figure 7 Overall morphology of the eye disc. The discs were cut transversally in order to have transversal R8 axon bundles. The peripodial membrane is the most apical structure, a very thin region of squamous epithelium cells very different from those of the disc. Beneath the peripodial membrane there are the photoreceptors soma that are sending out basally their axons to the most basal region where glial cells are present.

In the late larval stages photoreceptor send out their axons from the eye disc through the optic stalk (OS), a tube-like structure linking the eye disc to the brain, and then into the brain lobe (Tayler and Garrity 2003). At the same time, glial cells migrate from the OS into the eye disc (Rangarajan, Gong et al. 1999). Primarily there are two glial

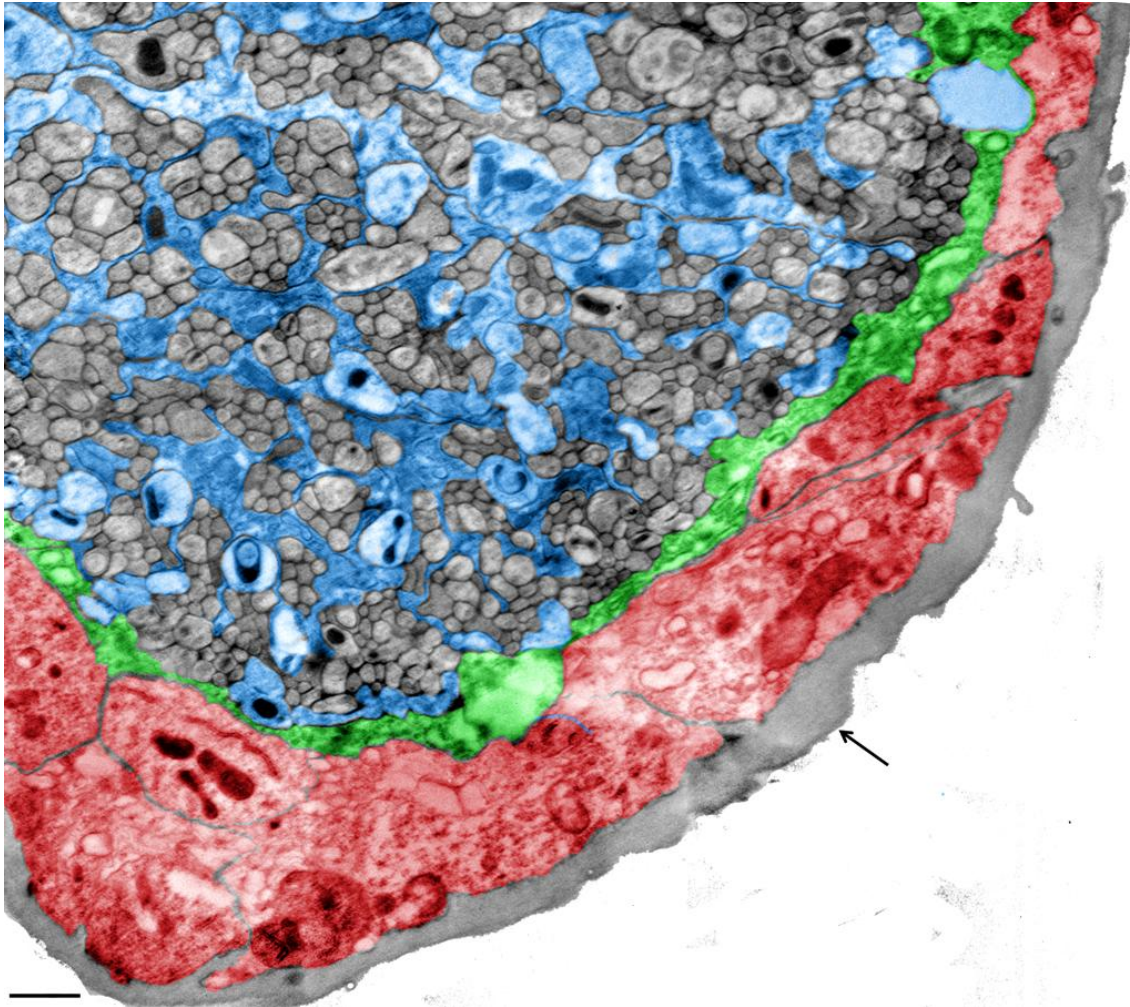subtypes in the OS: two central carpet glia and several adjacent perineurial glia (PG) cells (Figure 8).

Figure 8 Wild-type optic stalk morphology. Repo>Dicer2>LacZ TEM picture of a third instar larvae OS. Around the OS there is a dense neural lamella (arrow). The PG cells surrounding the entire OS are highlighted in red. Underneath PG cells are present the carpet cells, highlighted in green. Carpet cells create a continued narrow ring along the whole OS. Glial processes that ensheath individual R8 bundles are highlighted in blue. It can be seen that not all R8 bundles are surrounded by glial processes, especially the ones in the periphery. [Scale — 1μm]

The two carpet cells are a specific subset of subperineurial glia (SPG) and show a sheet-like structure all around the OS (Figure 8, highlighted in green). In middle to late L3 larvae stages, photoreceptor axons from the developing eye disc migrate through the OS (Silies, Yuva et al. 2007). Adjacent to the carpet glia there is a layer of PG; these cells have a cuboid shape with fine cell protrusions (Figure 8 and 9).
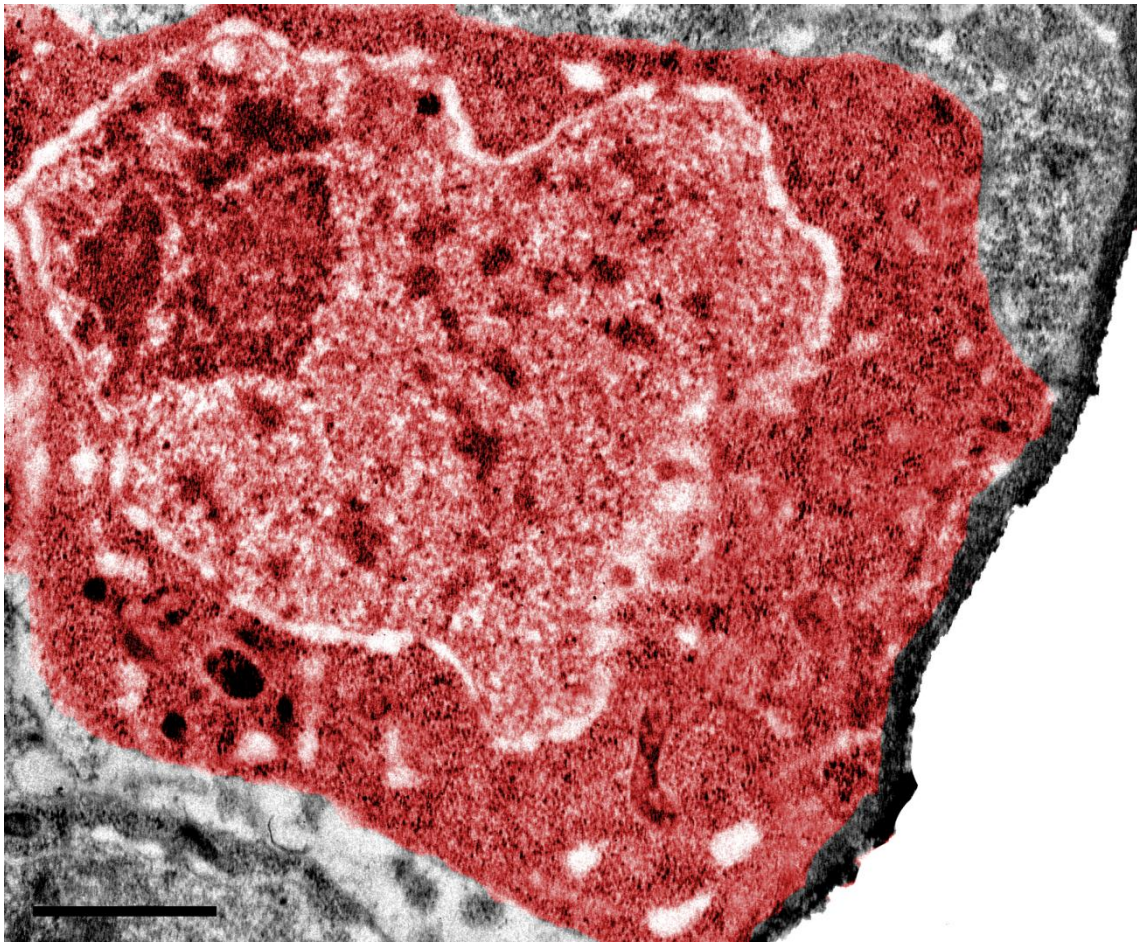
Figure 9 Perineurial glia in the optic stalk of wild-type L3 larvae. PG cells are beneath carpet glia cells and form a single outer layer all around the OS, and exhibit a cuboid shape. [Scale — 1µm]

Differentiated WG send out their processes from the eye disc through the OS while wrapping the axons of photoreceptors (Figure 10A,B). There is still a single layer of PG cells all along the OS axis (Figure 8).

In the eye disc besides the PG cells and carpet cells there are at least two kinds of WG cells, according to their electronic density. In Figure 11 can be seen some lightest blue cells that have more protrusions and wrap more bundles of R8 axons. It can also be detected a greyish blue type with less processes and protrusions and that wrap only a few bundles.
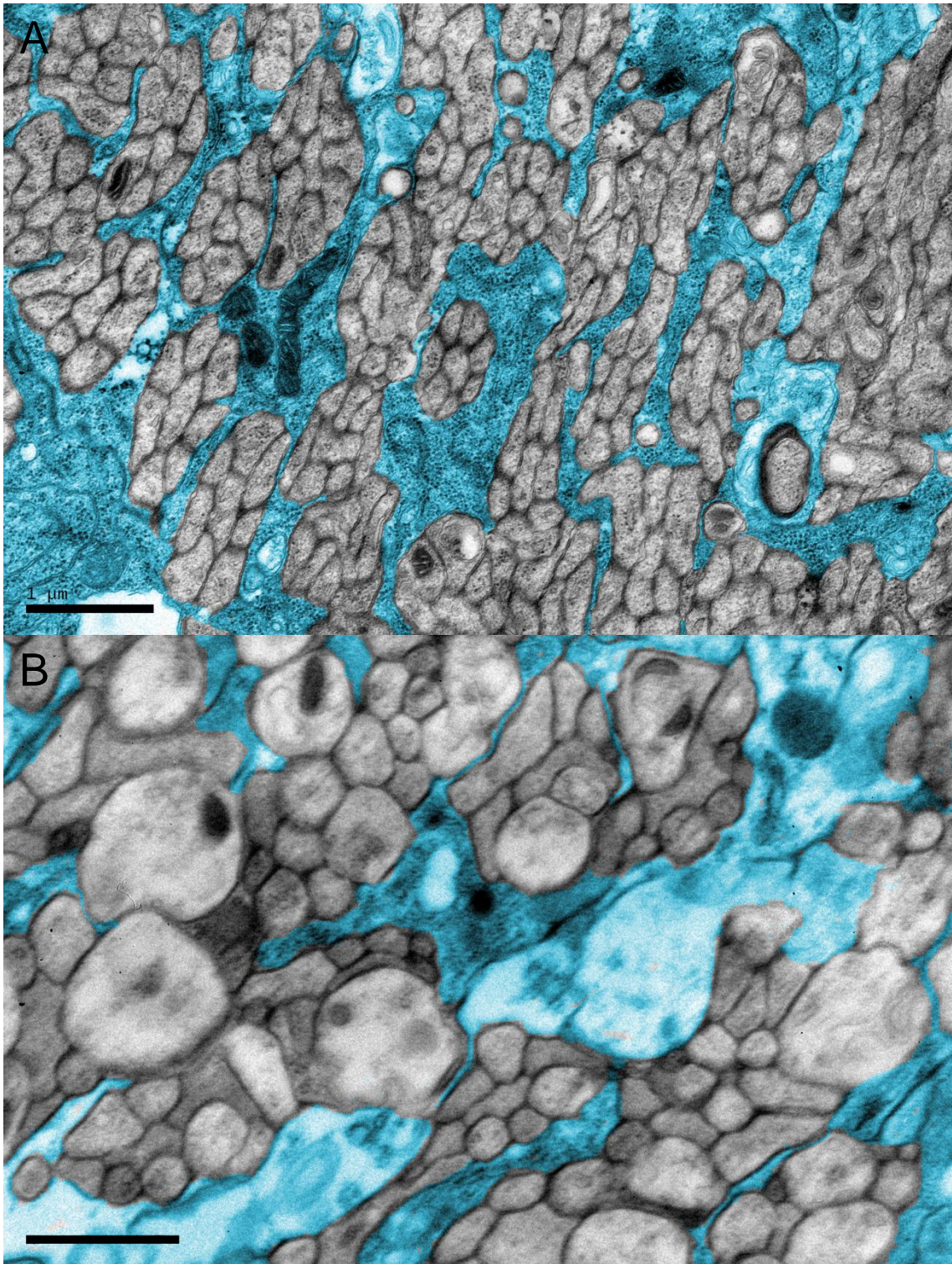
Figure 10 Wrapping glia and photoreceptor axons. **A.** Eye disc WG ensheathing photoreceptor axons in bundles of eight axons (highlighted in blue). **B.** Optic Stalk WG. Axons are not still wrapped in bundles of eight axons. Axons are not as compact as in eye disc. (highlighted in blue). [Scale — 1µm]
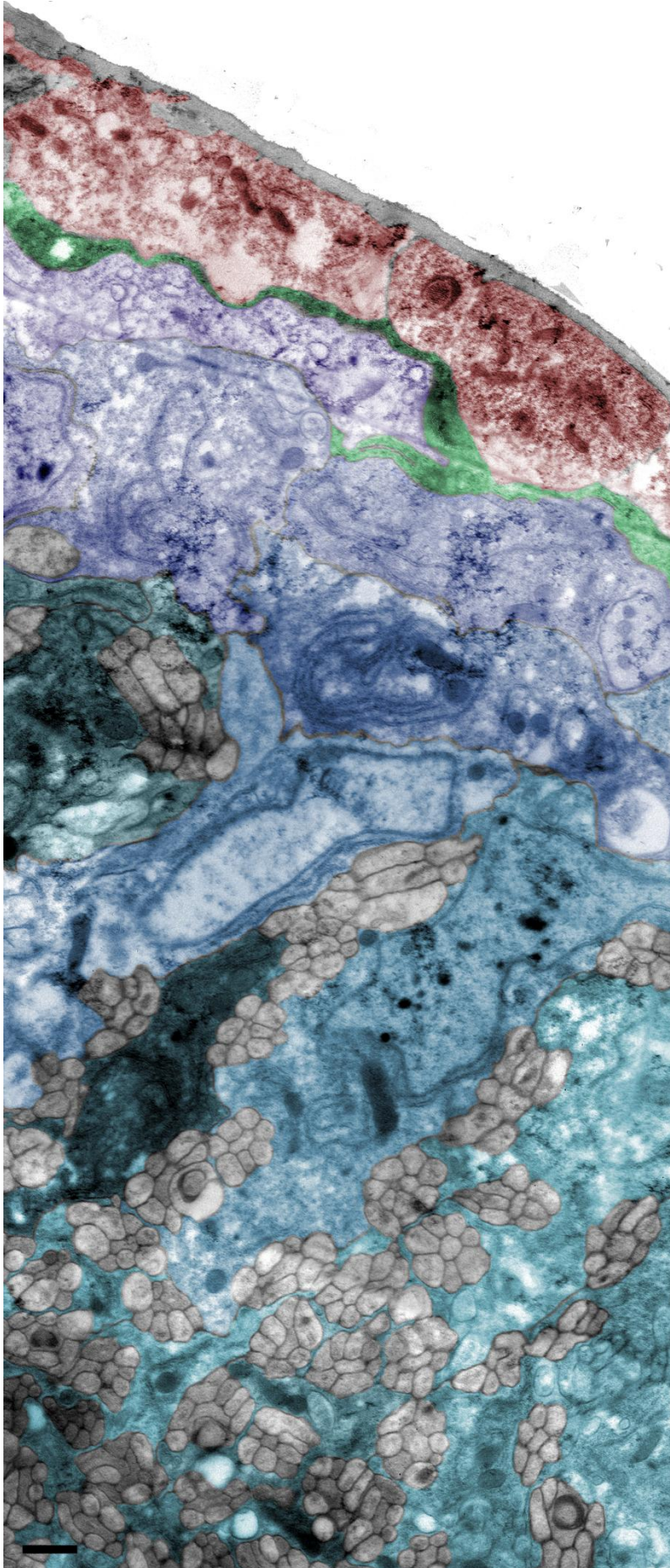
Figure 11 Eye disc glial cells and R8 axons. PG cells are present in the outermost layer (highlighted in red). Carpet cells show thin protrusions as in the OS (highlighted in green). The PG cells that cross carpet cells and contact newly formed axons differentiate in WG (highlighted in blue). There are different types of WG according to the electronic density. The lightest blue ones have more protrusions and wrap more bundles of R8 axons. The greyish blue ones have less processes and protrusions and wrap only a few bundles. [Scale — 2µm]

The Drosophila eye ultrastructure is very complex. There is an additional region, between the photoreceptor soma and the region where glial cells migrate (basal region), that is called basal lamina (Figure 12, highlighted in orange). However this is not a rigid layer as it is interrupted when differentiating photoreceptors extend their axons that migrate to the basal region and for glial that extend their processes to begin the wrapping process in the photoreceptors region (Figure 13).
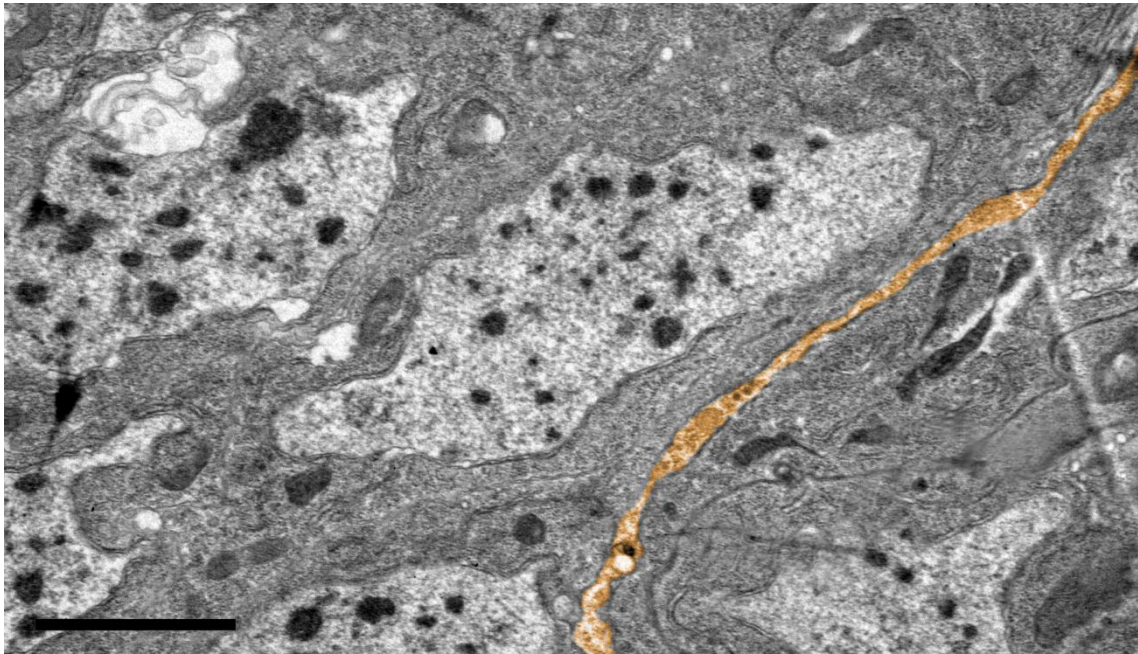

Figure 12 Basal lamina. The basal lamina (highlighted in orange) separates the glia cells region in the left (basal) and the photoreceptor soma in the right (apical). [Scale — 2µm]
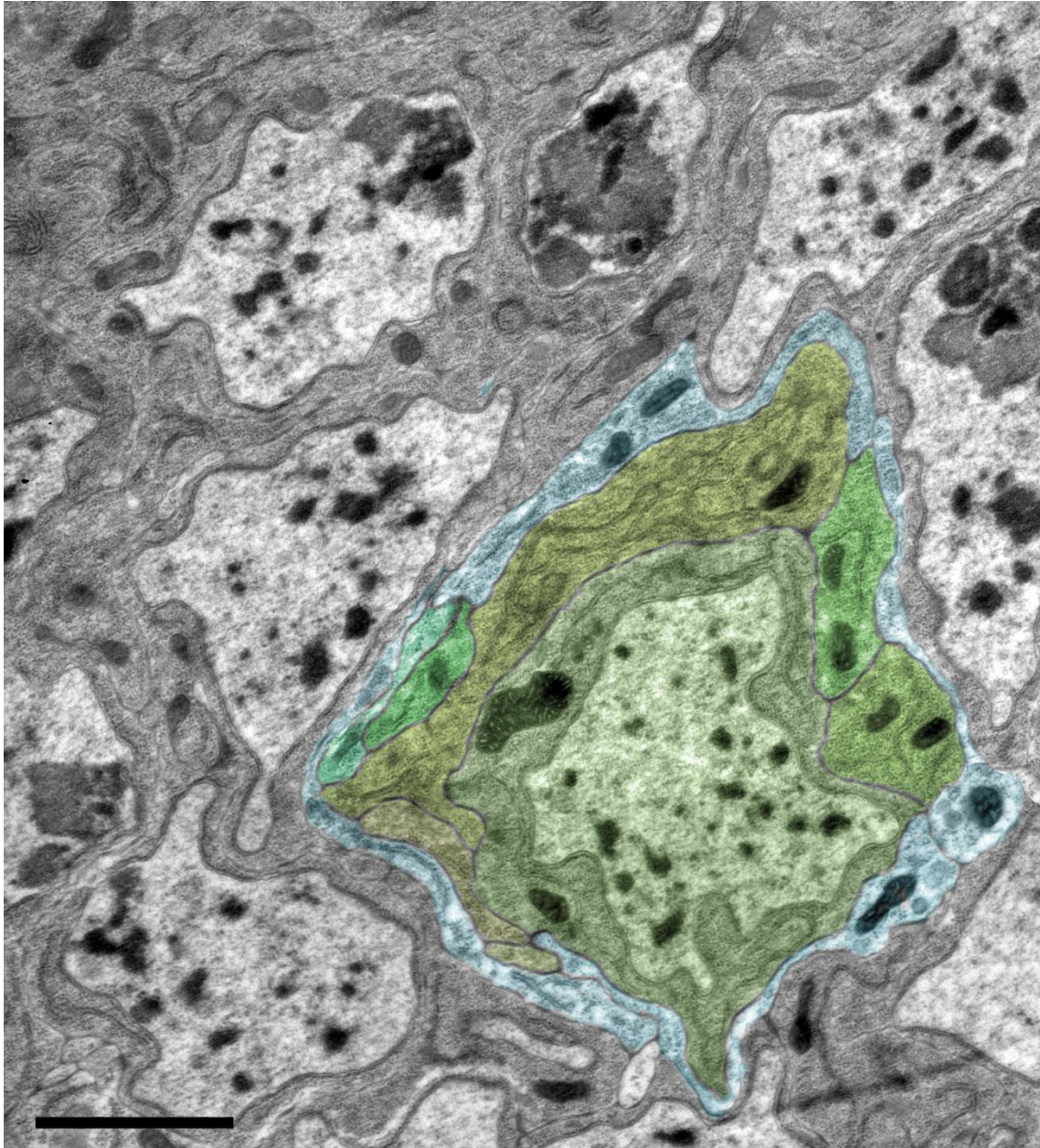
Figure 13 Beginning of the wrapping process. Glial cell processes (highlighted in blue) start the wrapping of the eight photoreceptors (highlighted with different shades of green). [Scale — 2µm]

3.3 Knockdown of βPS integrin in glial cells prevents glial cells migration to the eye disc and causes morphologic changes

It has been demonstrated that the βPS integrin interacts genetically with *Fak56D* and, regulates PG cells distribution. Nevertheless, there is no confirmation of the role of integrins in very important features of glial cells, for instance whether integrins influence migration or differentiation of glial cells. Loss-of-function mutants of *mys* (βPS) are embryonic null; consequently in this study we use RNA interference technique (RNAi) to explore integrin function and development in the eye disc and OS of L3 larvae. It was used *repo* as driver, as it is only expressed in glial cells. So, the yeast transcriptional activator Gal4 is only expressed by glial cells. In the RNAi system, the generic Gal4/UAS system is used to drive the expression of a hairpin RNA that is processed by the RNAse III Dicer into siRNAs. The result is two strands of siRNA: one complementary and other non complementary. The non complementary one is degraded. The other strand is incorporated into a nuclease complex, the RNA-initiated silencing complex (RISC) and functions as a lead RNA to direct RISC-mediated sequence-specific mRNA degradation.

To study the function of integrin in glial cells were performed RNAi experiments using *repo-GAL4* to drive RNAi only in glial cells. For βPS integrin was used βPS RNAi with Dicer2. Dicer2 is an enzyme present in the RNAi pathway and its overexpression will further activate the RNAi pathway increasing the knockdown efficiency.

The βPS RNAi eye disc show dramatic changes when compared to the wild-type counterparts as no glial cells were detected in the eye disc (Figure 14 and 15). In addition to that we also see disruption of the basal lamina (Figure 14). This implies that the eye disc phenotype is due to disruption of integrin based focal adhesion complex in glial cells.

The absence of glial cells in the eye disc is especially noticeable by the nonexistence of bundles of eight axons that are characteristic of a wild-type eye disc (Figure 15). In addition the wild-type eye discs axons generally have a regular calibre, what is not reproduced in the absence of glia cells. In βPS RNAi the eye disc axons have different shapes and calibers (Figure 16). So, glial cells are important to regulate the caliber and shape of axons.
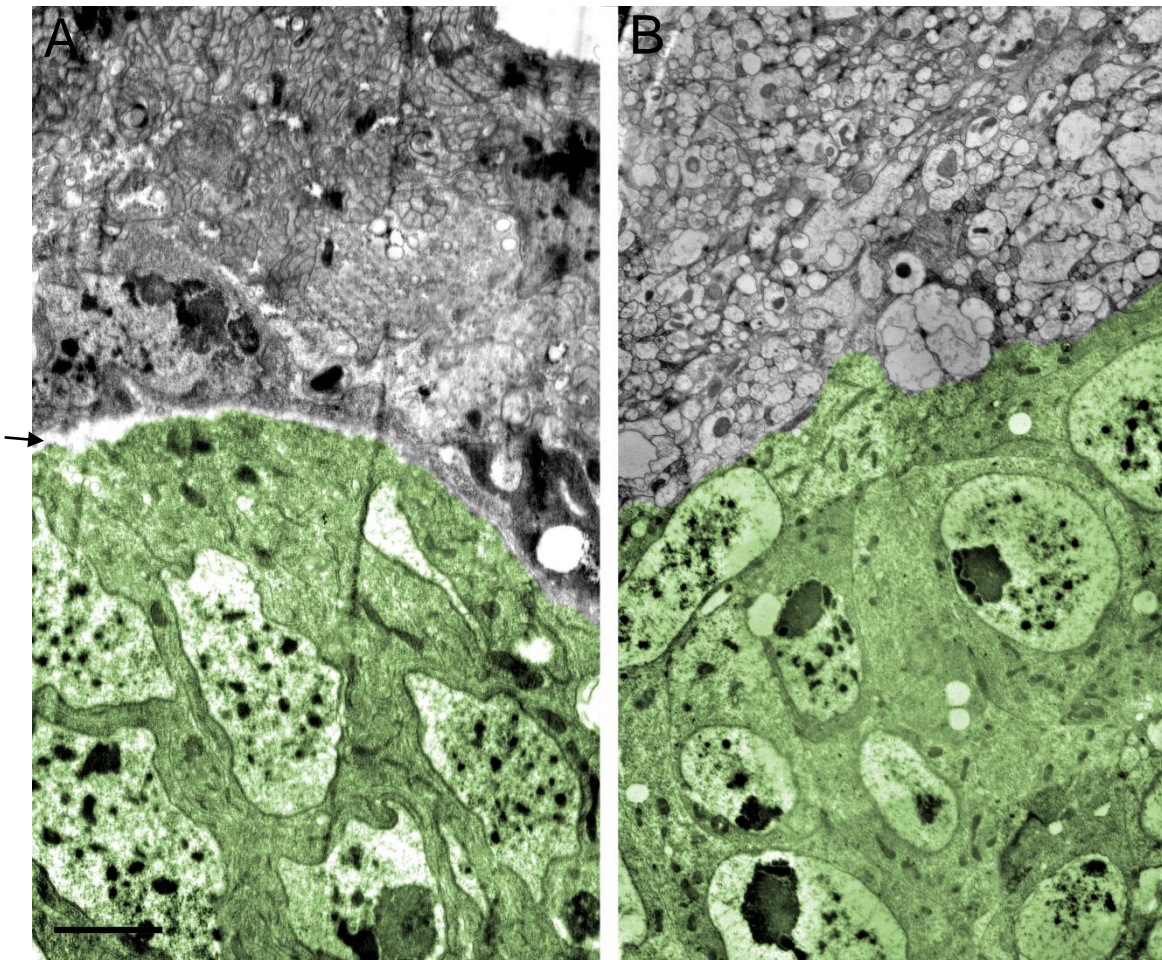
Figure 14 βPS integrin is required for glial migration. (**A**) In the normal eye disc the photoreceptors (highlighted in green) are separated by glial cells by a thin basal lamina (arrow). (**B**) In βPS RNAi eye disc, glial cells are absent of the most basal region. It is not observed any matrix components that separating regions. [Scale — 2μm]

Another consequence of the absence of glial cells is an increase of the mitochondria content in the axons (Figure 17, Figure 18).
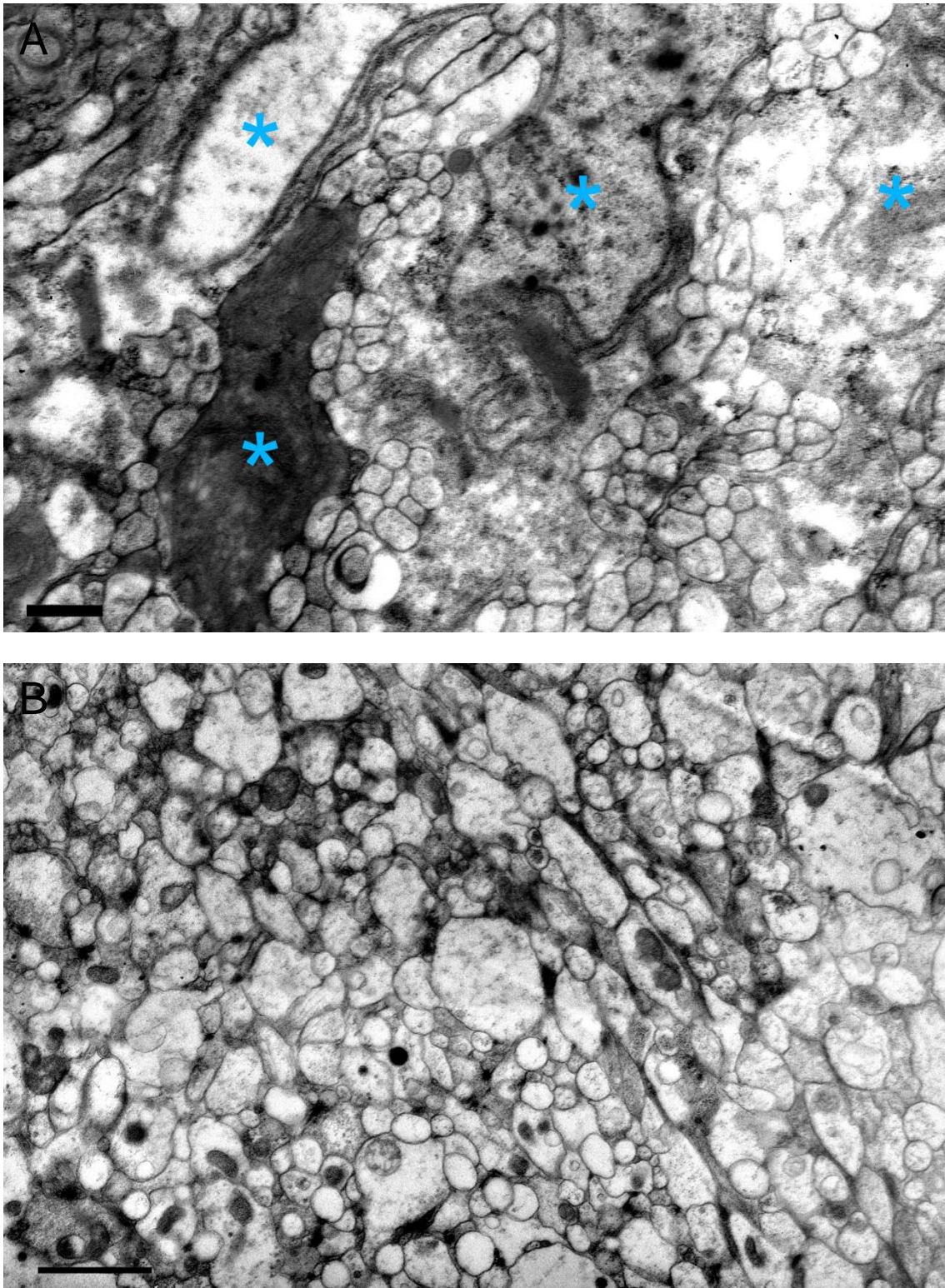
Figure 15 Eye disc basal region. (A) Wild-type glial cells (blue asterisk) extend processes to form the eight axons bundles. (B) In βPS RNAi there not glial cells present neither eight axons bundles [Scale — 2μm]
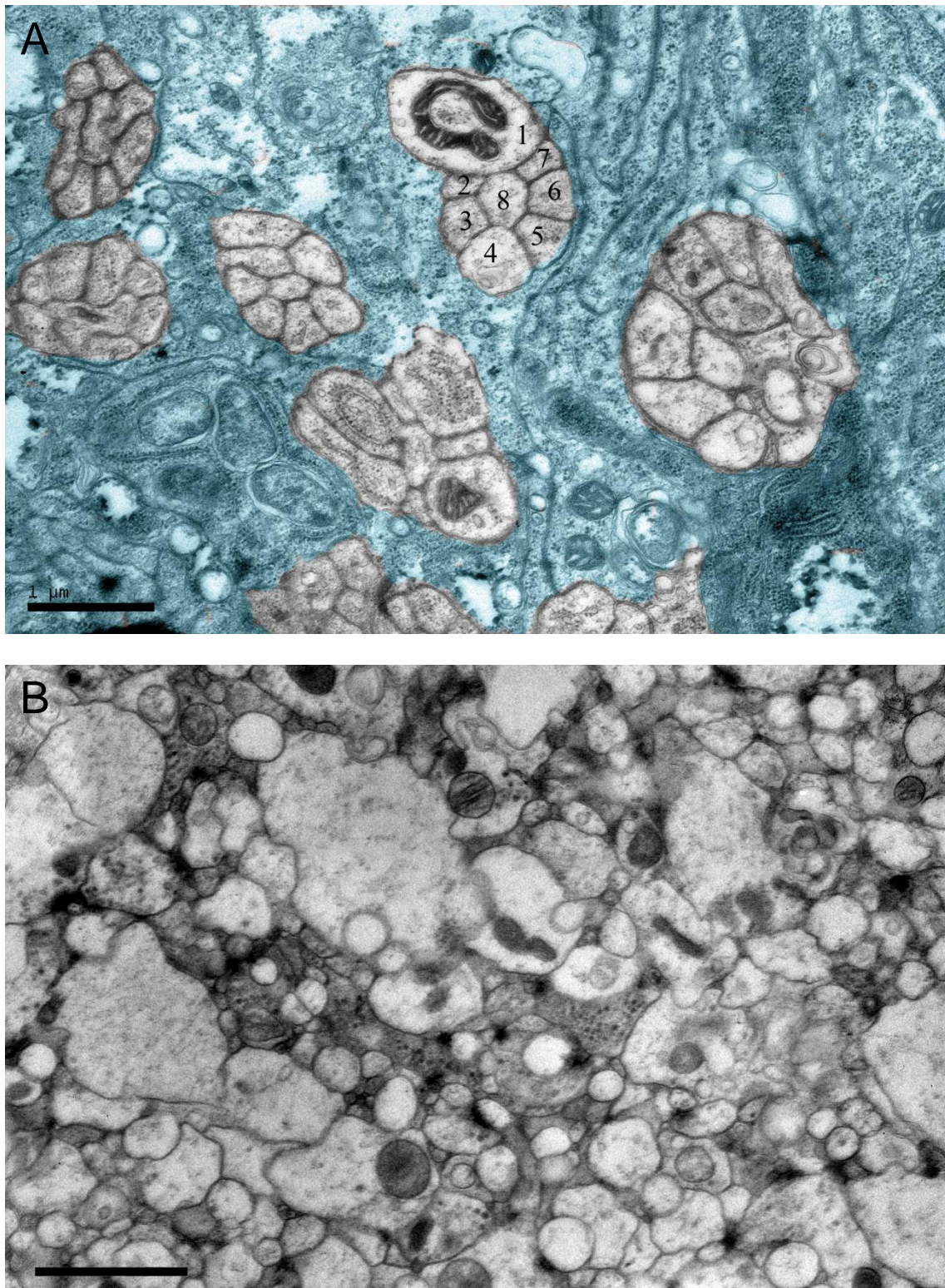
Figure 16 Differences in axon shapes in (A) wild-type and in (B) βPS RNAi eye discs. (A) In the wild-type the axons are in bundles of eight and insulated by glial processes (highlighted in blue) and present a regular caliber with less than 0.5 μm of diameter. (B) In the βPS RNAi eye disc the axons present very different shapes and calibers. [Scale — 1μm]
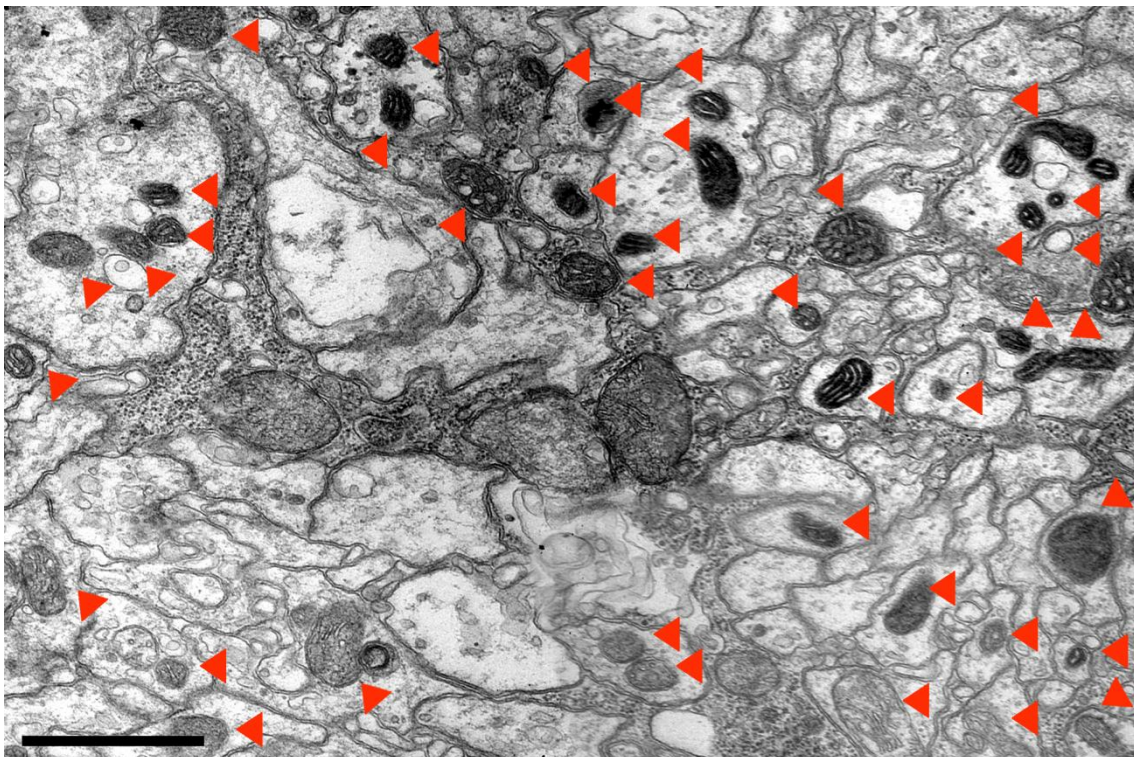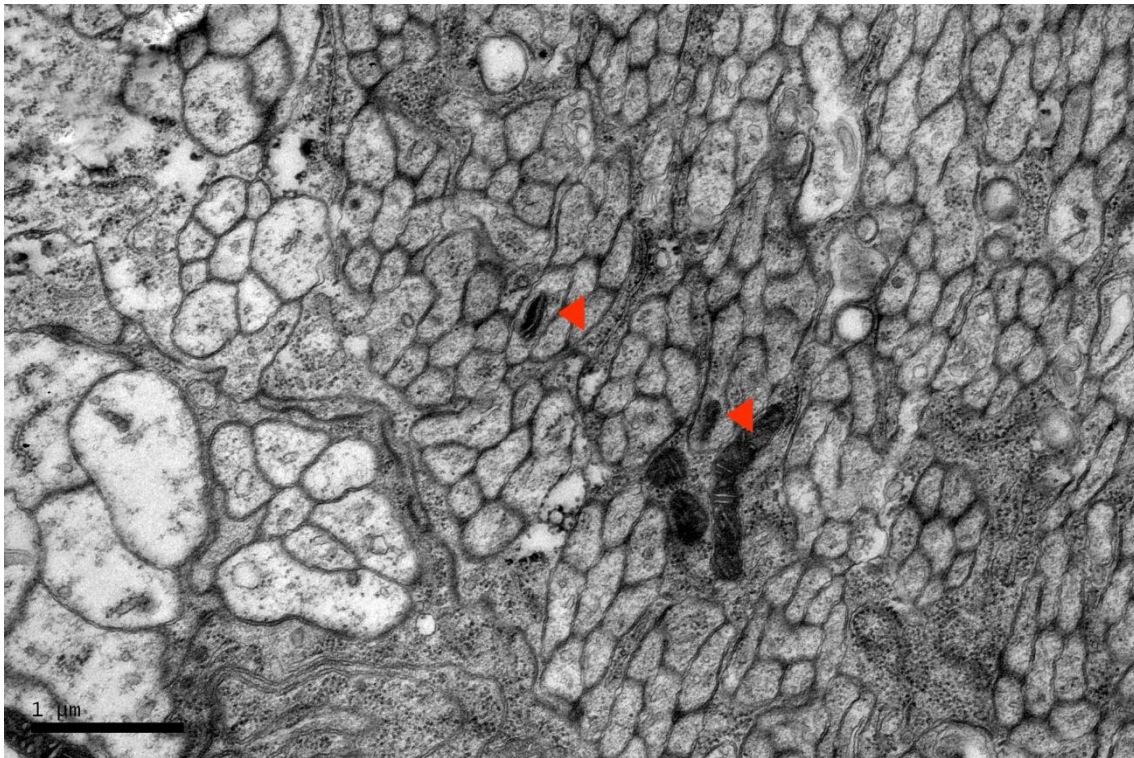
Figure 17 Axonal mitochondria. (A) Wild-type eye disc axons do not exhibit much mitochondrion (orange arrowhead) as (B) βPS RNAi eye discs which present a high density of mitochondria in axons. [Scale — 1µm]
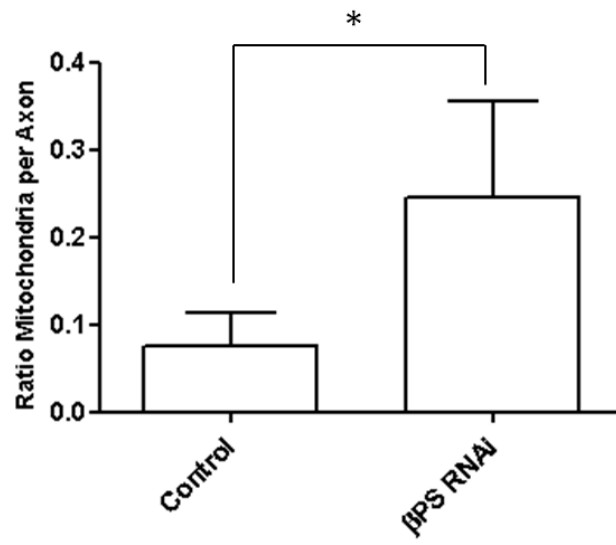
Figure 18 Ratio of mitochondria per axon of control (n=4) and βPS RNA expressing (n=3) eye disc. Bars represent standard deviation. P<0.05

3.4 Glial Cells are not required for Axonal Outgrowing into the Optic Stalk

To further characterise the axonal outgrowing the OS was further analysed. The ultrastructure analysis of the OS was also useful to understand how is the OS formed in the absence of cells.
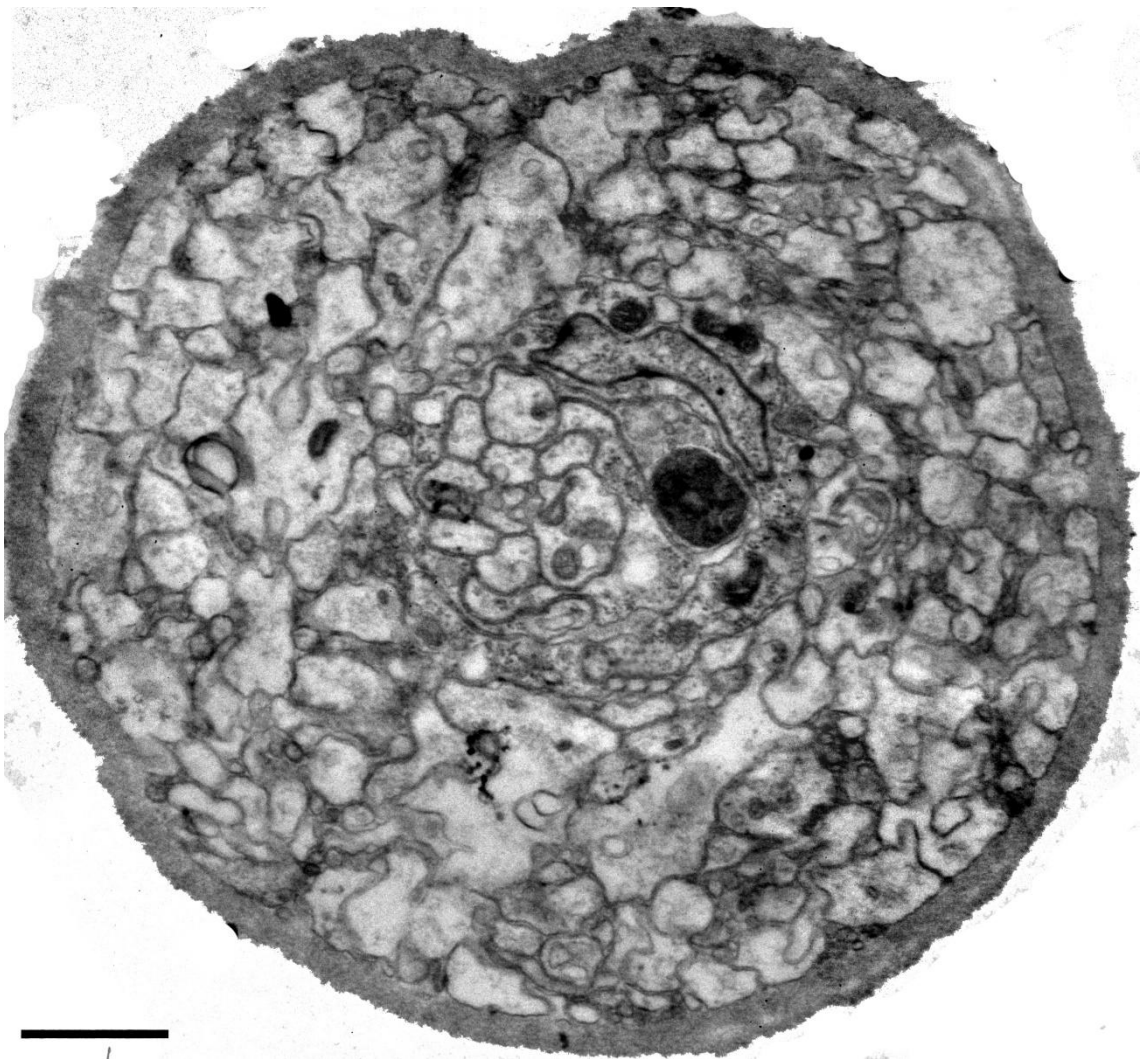
Figure 19 Optic stalk from βPS RNAi L3 larvae. There is no evidence of the presence of cells. Axons are very irregular in size and shape showing various membrane irregularities [Scale — 1μm]
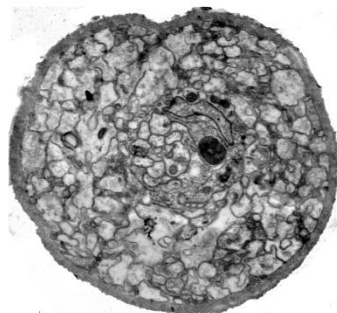
Control

βPS RNAi

Figure 20 To scale optic stalks of control and βPS RNAi. OS area is markedly different as Repo>Dicer2>βPS RNAi is more than 95% smaller than the control (Repo>Dicer2>LacZ). [Scale — 1μm]

βPS RNAi OS presents a fine and slim appearance (Figure 20). On average βPS RNAi OS has a section area of about 95% smaller than the control situation (Figure 20). Analysing the βPS RNAi OS morphology it is evident that it lacks cellular content (Figure 19) confirming immunofluorescence results in confocal microscope (previous results from the lab). So, βPS integrin is responsible for the migration of glial cells, not only in the eye disc, but also to enter the OS.

In addition to the absence of glial cells in the OS, it is evident several axonal defects namely irregular shape and a messy overall morphology of the tissue comparing to the organization of the control OS (Figure 19 and 20). However it was not only the lack of glial cells that diminish the area of the βPS RNAi OS. Comparison with control shows that fewer axons were crossing the RNAi expressing OSs (Figure 21). In average, there is around 80% less axons crossing the OS in βPS RNAi. Nevertheless we cannot conclude that the axons are lost in the eye disc as most likely this decrease in the axon number is due to development defects and a delay in photoreceptors differentiation, as βPS RNAi eye discs are often smaller.

Rangarajan et al. suggested that axons need physical contact with, and support from glial cells for axonal targeting into the brain to occur. However, we show that photoreceptor axons successfully penetrated the OS in the absence of glial cells, suggesting that glia is not necessarily a guidepost in axonal outgrowth through the OS.

This data suggest that βPS integrin plays a role in glial migration into the OS and that the absence of glia is important but not essential for axonal outgrowing into the OS.
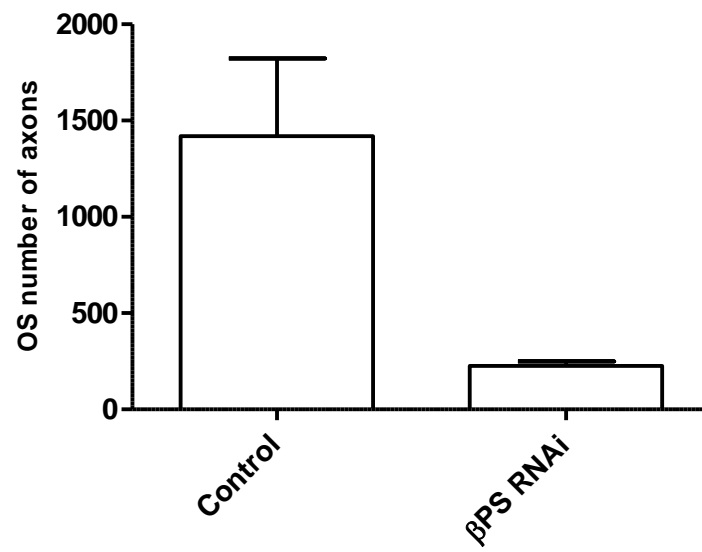
Figure 21 Number of axons in the optic stalk of control (n=2) and βPS RNAi (n=2) situation.

3.5 αPS2 Integrin Subunit is the Partner of βPS in Glial Migration and both coordinate matrix organization

As integrins are obligate heterodimers that contain one alpha and one beta subunit, it was necessary to check which alpha subunit was the partner of βPS in regulating glial migration to the eye disc. Of the two beta subunits (βPS and βν) in *Drosophila*, βPS is extremely important being the only β subunit indispensable for the organisms. RNAi for the other α-subunits (mew, If, Scab) were tested but only in *repo*>Dicer2>If RNAi was observed the same phenotype as in *repo*>Dicer2>βPS (Figure 22). Inflated (If) is the Drosophila αPS2 (Wilcox, DiAntonio et al. 1989).

To further understand how is the OS maintained in the absence of glial cells the extracellular matrix was analysed in the presence and absence of glial cells. For that, the gene trap Viking (Vkg)-GFP (Drosophila homologue of mammalian Collagen IV) was used as an ECM marker. For that the area occupied by Vkg in Control and If RNAi was measured and normalised by the OS area (Figure 22).

In Repo>Dicer2>If RNAi Vkg occupies in average 17 % of the total area of the OS while in Repo>Dicer2>LacZ Vkg occupies only approximately 10 % of the OS. It was described that integrins have a role in the organization of the extracellular matrix (Pae, Dokic et al. 2008). So, the width of Vkg can be explained by a disorganization of the ECM in the absence of αPS2.
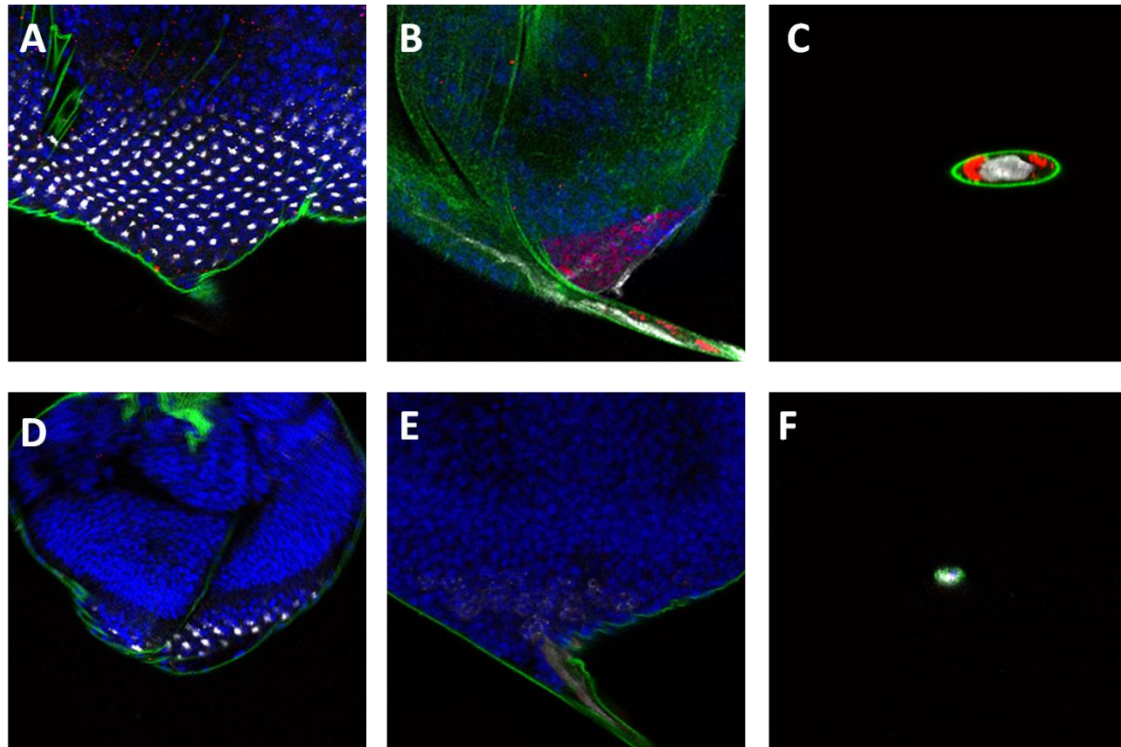
Figure 222 Extracellular matrix collagen-GFP in L3 larvae eye disc and optic stalk. **A**, **B** and **C** Vkg:GFP *repo*>Dicer2>LacZ photoreceptor, glia and OS view, respectively. **D**, **E** and **F** Vkg:GFP *repo*>Dicer2>If RNAi photoreceptor, glia and OS view, respectively. Nuclei are stained by DAPI (blue) and Vkg-GFP is shown in green. Glia cells are marked by Repo (red), and photoreceptor axons by HRP (grey).

4. Discussion and Final Remarks

In vertebrate there are many indications that integrin-mediated ECM interactions have significant roles in regulating glial cells development, though several basic questions are unclear. Studying the role of integrins in glial cells will significantly increase our knowledge of the orchestrated development of glia and neurons. Nevertheless, in vertebrate glia two hindrances considerably limit the progress of integrin function investigation. First it is complex to disrupt vertebrate genes in a tissue specific manner and creating transgenic animals and mutations are expensive and take a long time. Integrin germline mutations cannot be used as are often lethal (Walsh and Brown 1998). Second the family of integrins in vertebrates is large, counting with eighteen α and eight β subunits, which in glia have spatial and temporal expression patterns. The increased number of mammalian subunits may lead to compensatory events, when knocking down a specific subunit, making the examination of an integrin specific function quite difficult. Therefore the use of genetic models is an extreme advantage. An excellent model to study integrin function in glial development is *Drosophila melanogaster*. As referred before, fruit fly glial cells have several functional and structural resemblances with vertebrate glia. Particularly the eye system allows the study of glia-axon, glia-glia and glia-ECM interactions from earliest stages of development. Furthermore, *Drosophila* has a much modest family of integrin subunits that have been studied, in other issues, and demonstrate some conserved functions (Catterson, Heck et al. 2013; Comber, Huelsmann et al. 2013).

4.1 βPS Integrin Subunit Effect in Glial Migration

Drosophila integrins are involved in several aspects of glial development. Some of them, such as proliferation, migration and axonal ensheathment have been shown to be integrin dependent and be regulated by diverse integrin downstream signalling pathways in the vertebrate nervous system (Milner, Edwards et al. 1996; Milner, Huang et al. 1999; Etienne-Manneville and Hall 2001; Garcion, Faissner et al. 2001; Feltri, Graus Porta et al. 2002; Barros, Nguyen et al. 2009; Camara, Wang et al. 2009; Afshari, Kwok et al. 2010; Berti, Bartesaghi et al. 2011). However for the reasons mentioned above studying integrins in vertebrates is rather complicated and results are often not very clear.

This study demonstrates that glia plays a role in eye disc photoreceptors development. Surprisingly it was shown that specific loss of βPS integrin compromises glial migration into the optic stalk and eye disc. This was a very unexpected result as the only study done so far in glia integrins demonstrates that integrin is important for glia process extension and wrapping but that cells were still able to migrate (Xie and Auld 2011). Furthermore, RNAi mediated knockdown of βPS and If integrin subunits in glia determined this integrin complex as essential for glial migration.

In the absence of glial cells in the eye disc it was visible a disruption of the basal lamina what suggest that glial have a role in the formation of basal lamina. Other possibility is that glial cells integrins organize and rearrange the components of the basal lamina, what goes according to other studies that suggest a role of integrins for ECM components rearrangement (Pae, Dokic et al. 2008; Legate, Wickström et al. 2009).

This study shows that the integrin subunit αPS2 (*If*) is the partner of βPS in mediating glial migration. Interestingly previous studies had shown that αPS2 is located in the anterior region of the eye disc (Brower, Piovant et al. 1985) but they were only looking at photoreceptors. Now it would be interesting to analyse the expression profile of this integrin in the retinal glia.

4.2   Importance of Retinal Glial Cells for photoreceptor axons

Glial cells are important to regulate the caliber and shape of axons (Figure 16). Glial cells insulate bundles of axons completely separating different bundles. This means that two different insulated bundles would not be affect by the electric impulse of each other. In βPS RNAi imaginal discs large caliber axons can be seen, what could be a strategy to decrease electric resistance. When axons have larger diameters electrons do not collide as much and consequently create less resistance. This is in accordance to Ohm's law. Resistance is the ratio between the voltage difference across an object and the current that passes through the object due to the existence of voltage differences. As in βPS RNAi imaginal discs axons are not insulated by glial cells, to maintain the current that passes through the axon constant the resistance should be smaller, as they are inversely proportional. Axons with smaller caliber could be the ones that are still growing and so what is visible is the tip of the migrating axon. Alternatively smaller axons could be degenerating axons once carpet cells are not

present in the eye disc to form the blood brain barrier (BBB) to protect internal axons from pathogens and high concentration of potassium ions in the hemolymph, which would largely abolish axonal excitability. The huge increase in the mitochondria number in βPS RNAi eye discs (Figure 17 and 18) could be exactly a sign of oxidative stress in the axons directly due to the absence of glial cells or indirect through BBB disruption.

4.3   Revision of the Model for Glial Migration

In previous studies, Rangarajan and collaborators (Rangarajan, Gong et al. 1999) have shown that axonal targeting into the optic stalk needs physical contact with, and support from glia (Figure 24). However, the study of *gcm* mutants, which lack all glia except midline glia, shows that the majority of axon pathways in the embryo, and particularly the longitudinal axon tracts, can grow and develop in the absence of glial cells, suggesting a purely driving role for glia (Hosoya, Takizawa et al. 1995; Jones, Fetter et al. 1995).

Rangarajan model

A

Optic Stalk

Eye disc

B
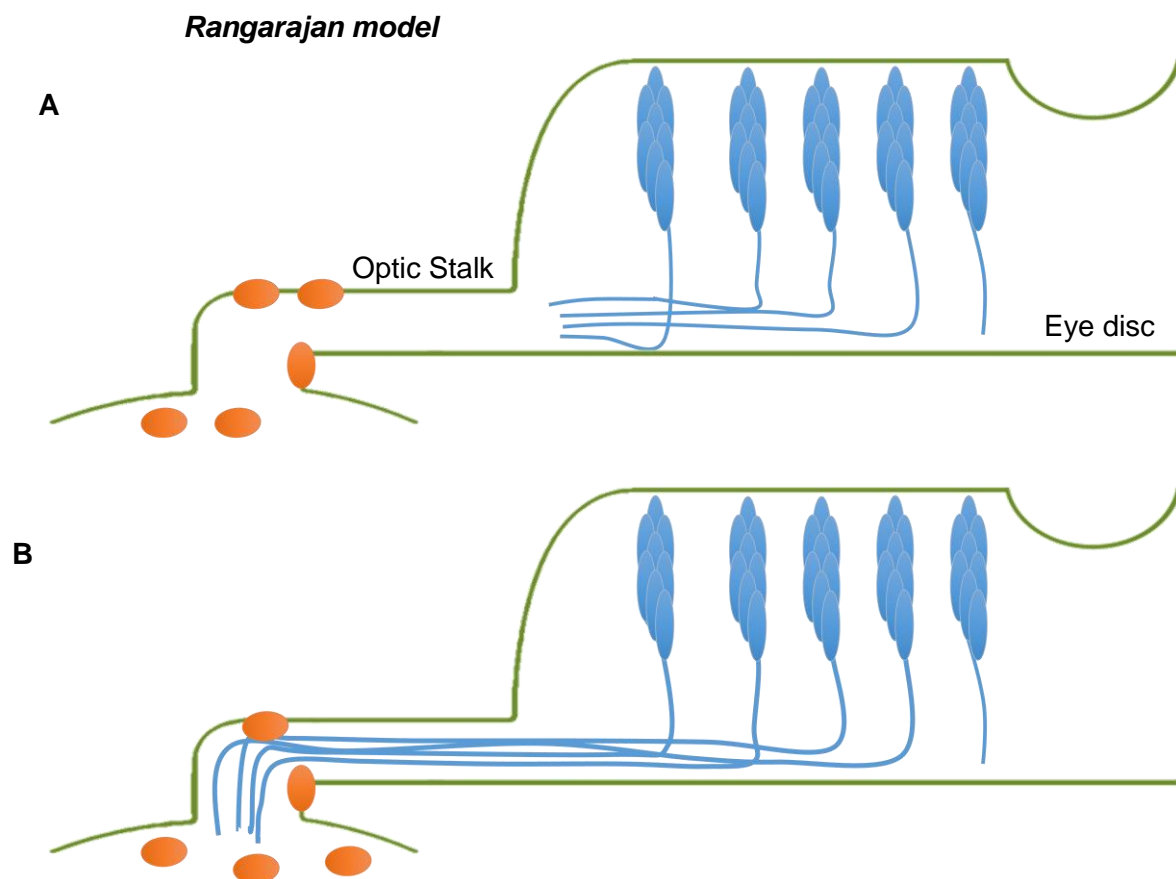
Figure 23 Rangarajan model for glial migration. Rangarajan suggested that for axonal outgrowth (blue) to occur into the optic stalk at least one glial cell (orange) has to be present in the eye disc (**A**). These cells will function as guideposts for axons to enter the optic stalk (**B**).

Also, Choi and Benzer (Choi and Benzer 1994) according with their experiments in mutants with no photoreceptors, suggested that the glia migrate into the eye disc alongside established photoreceptor axons (Figure 25). This goes against the suppose glia role in the guidance of photoreceptor axons.

Choi and Benzer model
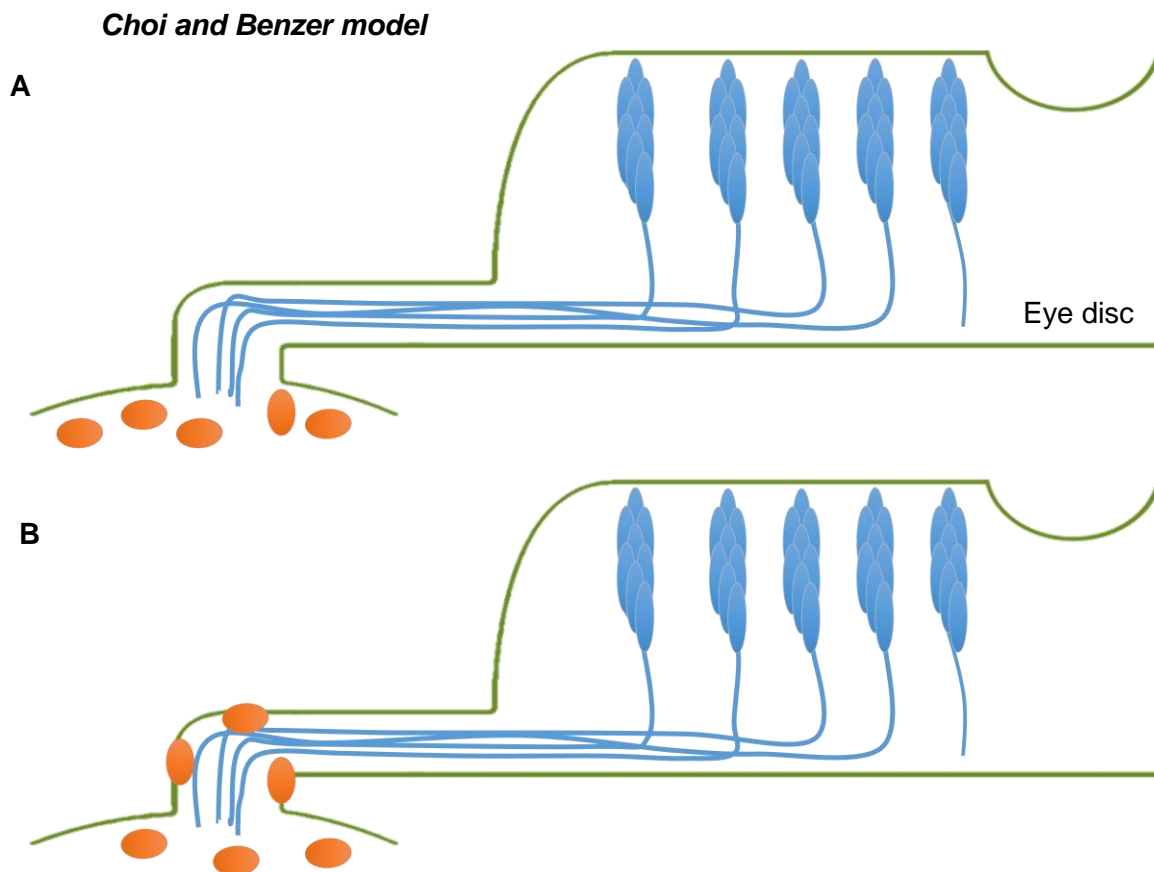
Figure 234 Choi and Benzer model for glial migration. In this model, Choi and Benzer suggested that the presence of established axons (blue) outgrown through the OS (**A**), would be required for starting up glial (orange) migration (**B**).

Surprisingly in this study there are evidences against the model suggested by Rangarajan *et al.* as it is shown that photoreceptor axons can target into the optic stalk even in the absence of glial cells, pointing out that glia do not work as guideposts. Rangarajan used omb-GAL4 which is expressed in glia but also in the lateral regions of the eye disc making its expression is not restricted to glia cells what might influence the result. Also they used a dominant negative form of Ras which can interfere with signalling pathways between glia and axons and maybe inhibit axonal pathfinding.

This work clearly shows axons going through the optic stalk even though in a much reduced number when compared with control (Figure 21). This can be explained just by a delay in eye disc development and photoreceptor differentiation, as can be seen in Figure 22. Conversely, the diminish number of axons in the absence of glia could be due to misroute and not only to problems of axogenesis, even though we have seen no evidence for this. Furthermore, axons that not contact with glia show an increased content in mitochondria, which points to oxidative stress and a possible degeneration. In βPS RNAi eye discs, carpet cells are not present in the eye disc to form the blood brain barrier to protect internal axons from pathogens and high concentration of potassium ions in the hemolymph which would largely abolish axon excitability. In addition Repo>Dicer2>βPS RNAi axons are not fasciculate and so, all these evidences suggest that they could be degenerating. While the misrouted and desfasciculated axons most likely result from loss of glia signals that control axonal pathway formation, some of the degenerated axon segments are possibly a consequence from loss of glial signals that sustain neuronal survival.

Therefore, in my model and according to the results of this study, if glia is not present in the eye disc, photoreceptor differentiation slows down due to lack of queues from glia. Therefore the migration of glia somehow regulates the differentiation of the eye disc. Concomitantly, the increase of differentiation drives the migration of glia into the eye disc (Figure 26).

Hummel et al. show a precocious migration of glial cells onto the eye field (Hummel, Attix et al. 2002). They suggested the existence of an early inhibitory signal from the disc epithelium that blocks glia migration. Since neuronal cells also point the direction of glial cells in the eye imaginal disc, the question that arises is how this migration is controlled and inhibited, and how migration is coupled with the sequential formation of photoreceptors in the eye disc. Perhaps, the existence of diffusible factors, as decapentaplegic (Dpp) and hedgehog (Hg), which have an important role in photoreceptor differentiation, superimposes the role of glia in the targeting of axons into the brain.
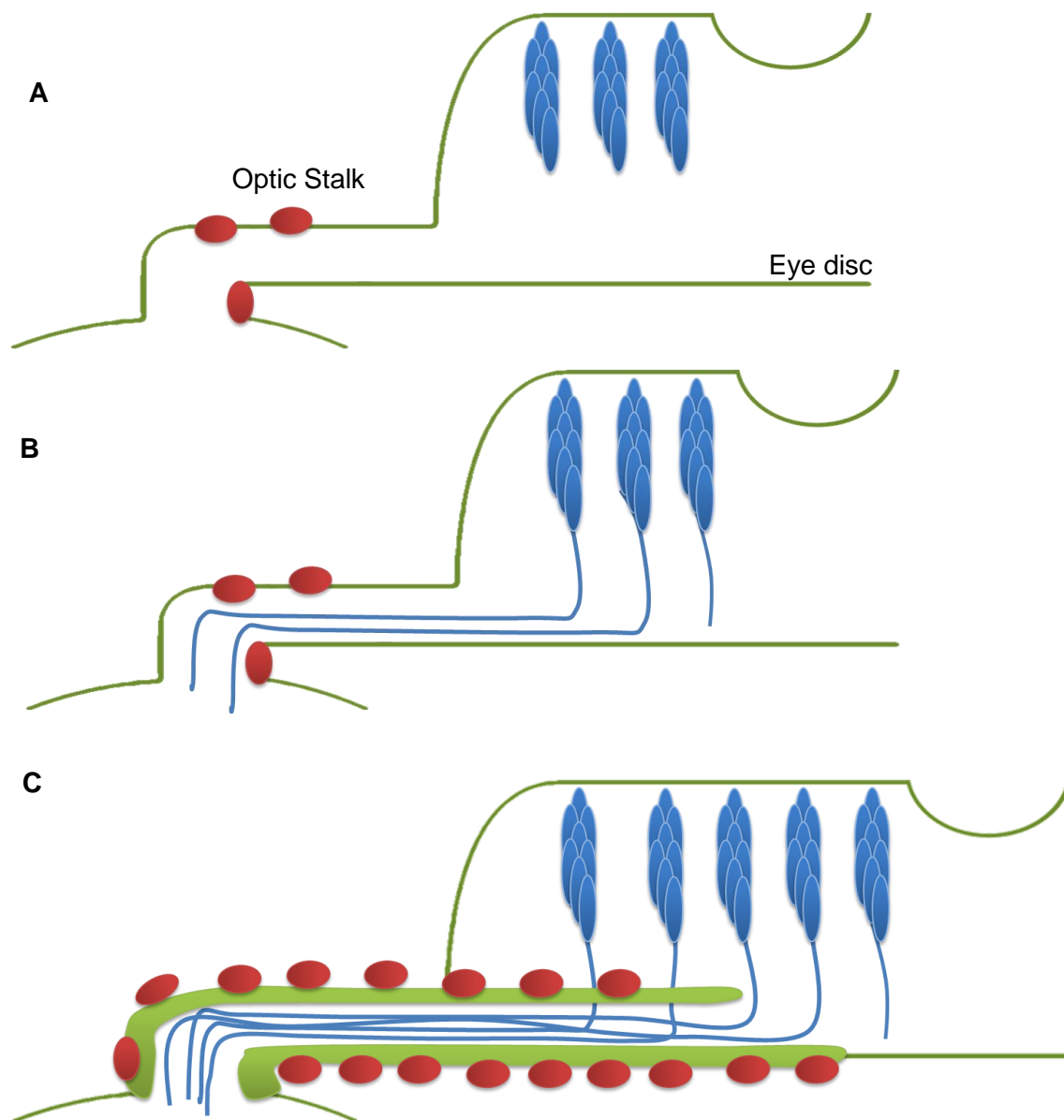
Figure 25 Our model for glial migration suggests that axons (blue) send attractive signals to drive glial (PG red and Carpet cells green) migration into the eye disc or block inhibitory signals for glia to migrate into the eye disc (**A**, **B**). Progressive differentiation of photoreceptors (blue) stimulates glial migration (**C**). Photoreceptors targeting into the OS is independent of the presence of glial cells.

4.4   Contributions of this study and future directions

Normal nervous system development needs both glia and neurons. In the *Drosophila* developing visual system, photoreceptor axons contact diverse types of glia prior to reach their destinations in the optic lobe. Some of them had been show to be needed for proper axon outgrowth and termination. For instance, glial cells in the optic lobe are

required for photoreceptor axon target selections (Poeck, Fischer et al. 2001). My study aims to better understood how axonal pathfinding occurs when axons exit the eye disc.

In summary my study has several interesting and surprising findings that shake some commonly accepted dogmas, I have shown that βPS and αPS2 integrin subunits are required for glial migration but that glia support is not required for axonal outgrowth. Ultrastructure analysis results show that glial cells integrins have a role in the organization and rearrangements of the extracellular matrix component. Also, glial cells are important regulators of axonal calibre, and for vital support of axons, that show stress signs when glial cells are nor present in the larvae eye imaginal disc.

In the future, it will be necessary to carry on electron microscopy assays for If RNAi to show that the phenotype is exactly the same as for βPS RNAi. It will also be important to understand the role of extracellular matrix proteins in glia migration. That could be done knocking down ECM matrix protein levels by RNAi or with the use of viable mutants. It would also be very interesting to use the Repo>Dicer2> βPS RNAi phenotype where glia cells show migration defects, to screen for proteins which would be able to reverse this phenotype. This would allow us to understand how migration happens in glial cells what would have a huge impact in the scientific and medical communities.

Migration is a very important process in some diseases, such as in cancer. Metastasis is the primary cause of mortality in most cancer patients (Friedl and Gilmour 2009). Also, glial migration is implicated in diseases as gliomas, multiple sclerosis and others (Lee, Jang et al. 2012). The results of this work may help create a model to address several questions regarding migration and disease.

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