Glia-Mediated Neurodegeneration in the Drosophila melanogaster CNS

Ivan J. Santiago
CUNY City College

How does access to this work benefit you? Let us know!

Follow this and additional works at: http://academicworks.cuny.edu/cc_etds_theses

Part of the Biology Commons

Recommended Citation
http://academicworks.cuny.edu/cc_etds_theses/152

This Thesis is brought to you for free and open access by the City College of New York at CUNY Academic Works. It has been accepted for inclusion in Master's Theses by an authorized administrator of CUNY Academic Works. For more information, please contact AcademicWorks@cuny.edu.
Glia-Mediated Neurodegeneration in the *Drosophila melanogaster* CNS

Ivan J. Santiago

Mentor: Dr. Tadmiri R. Venkatesh
<table>
<thead>
<tr>
<th>Index</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>3</td>
</tr>
<tr>
<td>Introduction</td>
<td>4 - 17</td>
</tr>
<tr>
<td>Overview of the glia types in the mammalian central nervous system</td>
<td>4 - 8</td>
</tr>
<tr>
<td>Astrocytes in health and disease</td>
<td>8 - 10</td>
</tr>
<tr>
<td>Drosophila melanogaster ad a model organism</td>
<td>10 - 13</td>
</tr>
<tr>
<td>The Anaphase Promoting Complex/Cyclosome</td>
<td>13 - 17</td>
</tr>
<tr>
<td>Methods</td>
<td>17 - 20</td>
</tr>
<tr>
<td>Results</td>
<td>20 - 29</td>
</tr>
<tr>
<td>Loss of glia cells, induced by the ectopic expression of Rap/Fzr, results in neurodegenerative phenotypes</td>
<td>20 - 22</td>
</tr>
<tr>
<td>Glia subtype specificity of GAL4 drivers</td>
<td>23 - 24</td>
</tr>
<tr>
<td>Astrocyte-like glia-specific ectopic expression of Rap/Fzr perturbs development, and results in neurodegenerative phenotypes</td>
<td>24 - 29</td>
</tr>
<tr>
<td>Discussion</td>
<td>29 - 34</td>
</tr>
<tr>
<td>On the role of APC/C in astrocyte-like glia development</td>
<td>30 - 33</td>
</tr>
<tr>
<td>On the involvement of APC/C in neurodegenerative disorders</td>
<td>34 - 35</td>
</tr>
<tr>
<td>References</td>
<td>35 - 41</td>
</tr>
</tbody>
</table>
ABSTRACT

Proper development, function and maintenance of the central nervous system (CNS) are reliant on the intricate relationship between glia and neurons. Glia cells possess a variety of key functions such as maintaining homeostasis, providing neurons with trophic support and the uptake and recycling of neuronal debris. Disruptions in glial function have been implicated in neurological disorders. Despite their obvious importance in the CNS, glia cells remain much less characterized than their neuronal counterparts. With its wide array of genetic tools and its low glia-to-neuron ratio, Drosophila melanogaster presents itself as a useful model organism in studies of glial characterization. Rap/Fzr, an activator of the Anaphase Promoting Complex/Cyclosome (APC/C), a E3 ubiquitin ligase, is the Drosophila homolog of the mammalian Cdh1. Previous studies have shown that proper function of the APC/C regulates glial differentiation in the CNS (Kaplow et al., 2008). Our studies show that targeted pan-glial expression of Rap/Fzr/Cdh1 results in the reduction of glia number in the CNS of 3rd instar larvae. Adult Drosophila with reduced number of glia cells exhibit progressive age-dependent phenotypes such as temperature-sensitive paralysis and vacuolization of the brain, as well as significant lifespan reduction. Employing glia subtype specific GAL4 drivers, we have identified astrocyte-like glia as a critical cell type in neuroprotection. Targeted expression of Rap/Fzr to astrocyte-like glia results in temperature-sensitive paralysis and lifespan reduction. These neurodegenerative phenotypes suggest a vital role for astrocyte-like glia in neuroprotection.
Introduction

The interactions between glia cells and neurons are crucial for proper development, maintenance and function of the central nervous system (CNS). Disruptions in glial function have been widely implicated in several neurodegenerative diseases. Although glia cells have been shown to possess critical functions, the mechanisms that regulate their development are largely unknown. Thus, a more detailed characterization of glia cells would enable a greater understanding of their role in mediating these disorders, as well as their roles in normal function of the CNS.

Overview of the glia types in the mammalian central nervous system

Glia cells of the mammalian central nervous system are divided into two groups – microglia and macroglia. Microglia are a unique class of glia cells, in that they are derived from hematopoietic precursors and act as the resident macrophages of the mammalian brain. As such, they are largely responsible for the uptake of neural debris and they are key mediators of immune responses in the brain (Harry, 2013). Recent studies have implicated microglia in the engulfment of synapses during development of the visual system. These glia cells mediate the ocular dominance that is observed in the lateral geniculate nucleus by use of the complement system (Schafer et al., 2012; Figure 1). The importance of microglia in synaptic development has inspired the new term “quad-partite synapse” (Schafer et al., 2013).
Macroglia are comprised of the oligodendrocytes and astrocytes. Oligodendrocytes are of critical importance to the central nervous system as they are the principle myelinating cells for neurons. Myelination is crucial for neuronal function as it promotes saltatory conduction of a signal being transmitted down the axonal process. Axon potentials would dissipate through the axolemma if not for the myelin sheath, and it would be very difficult for neurons to convey signals, especially cells of particularly long length. Oligodendrocytes are also critical for neuronal health. By covering much of the cell, they create a microenvironment in which the axonal portion does not have much contact with the extracellular space (Figure 2). As such, the oligodendrocyte is the key link between the nerve process and the extracellular environment (Bankston, et al. 2013).

Figure 1. Model for the complement-dependent role of microglia in synaptic pruning

Annu. Rev. Neurosci. 35:369–89
Astrocytes are another major class of macroglia in the mammalian brain. Astrocytes possess several functions that are critical for neuronal functions. Their processes are located in close proximity to synapse, creating the “tri-partite” synapse (Figure 3). This positioning allows astrocytes to uptake neurotransmitters from the synaptic cleft. This ability serves three functions. First, it prevents an abundance of neurotransmitters from accumulating at the cleft, which can lead to excitotoxicity. Second, it allows astrocytes to recycle neurotransmitters and convert them into a molecular form that can be utilized by the neurons again. Third, this allows astrocytes to supply nearby neurons with chemicals that can be used for energy, such as lactate.
In addition to being able to uptake chemicals, astrocytes have also been shown to release chemicals into the synaptic cleft. These chemicals have come to be known as gliotransmitters. These chemicals include glutamate and D-serine. Astrocytes have been implicated in playing a role in regulating cognitive functions such as learning and memory, as both of these molecules can bind and open NMDA receptors, which are critical for long term potentiation (LTP) (Ben Achour and Pascual, 2012).

Astrocytes are also proximal to blood vessels, allowing them to be a critical component of the blood brain barrier. These cells’ processes form “end-feet” around blood vessels (Figure 4). This allows for the coupling of the neural circuitry to the circulatory system. This astrocytic bridge allows the astrocytes to play a key role in mediating the transfer of nutrients from blood stream to nerve cell. Also, since astrocytes could act as a read-out of neural transmission, they
can regulate the dilation and contraction of blood vessels, mediating the amount of blood flow to particular areas of the brain (Wolburg et al., 2012).

**Figure 4. Astrocyte endfeet establish blood-brain barrier**

AMIL

*Astrocytes in health and disease*

Considering their numerous functions in the CNS, it is not surprising that malfunctions in astrocytes have been implicated in neurodegenerative and neurodevelopmental disorders (Maragakis and Rothstein, 2006; Molofsky et al., 2012).

Amyotrophic lateral sclerosis (ALS) is a debilitating disease in which the motor neurons degenerate. As a consequence, voluntary motor control is severely impaired. Mutations in the superoxide dismutase 1 (SOD1) gene have been heavily implicated in this disease. Recent
evidence has shown that astrocytes harboring the mutated gene secrete neurotoxic factors that induce hyperexcitability of nearby neurons, which leads to neuronal cell death (Nagai et al., 2007; Fritz et al., 2013).

Astrocytes have also been implicated in Alexander’s disease, a neurodegenerative disease in which Rosenthal fibers are formed near astrocyte end-feet (Messing et al., 2012). Glial fibrillary acidic protein (GFAP) is an intermediate filament protein that is thought to be specific to astrocytes, although its function is unknown (Maragakis and Rothstein, 2006). This protein is implicated in Alexander’s disease, as overexpression of the human wild-type protein in astrocytes in the mouse, and astrocyte-like glia in the fly, results in pathology of the disease (Eng et al., 1998; Wang et al., 2011).

GFAP is upregulated in astrocytes during formation of the glial scar, which is formed by astrocytes in response to neural injury. Although the glial scar prevents toxic substances that are released from the site of injury to influence surrounding tissue, it also prevents axonal regeneration. Efforts to promote axonal injury would involve circumventing this astrocytic blockade.

Astrocytes have also been implicated in neurodevelopmental disorders, such as Rett Syndrome, which is one of the severe causes of mental retardation (Bahi-Buisson, 2013). One of the genes implicated in Rett Syndrome is methyl-CpG-binding protein 2 (MeCP2), which has been shown to regulate gene expression in astrocytes (Yasui et al., 2013). In addition, astrocytes deficient in MeCP2 have been shown to perturb dendritic arborization (Ballas et al., 2009) and astrocytes expressing functional MeCP2 have been shown to have the capacity to rescue defects observed in MeCP2 mutant mice (Lioy et al., 2011).
Taken together, this evidence supports the need to study astrocytes more carefully, especially in the context of the mutations discussed above.

*Drosophila melanogaster as a model organism*

*Drosophila melanogaster* presents itself as an ideal model organism for studying glial development. While the Drosophila CNS includes a complex population of glia cells, the ratio of glia to neurons in the fly brain is much smaller than that of mammals. In addition, the Drosophila life cycle is relatively short, and the fly is particularly amenable to genetic manipulations. For these reasons, the fruit fly has long been established as a strong model organism in developmental neurobiology.

In particular, fly geneticists employ the GAL4/UAS system for targeted gene expression. This is a binary system in which the GAL4 gene, which is exogenous to the fly, is transcribed downstream of a tissue-specific promoter, thus allowing for the expression of GAL4 in certain classes of tissues and not others. The gene of interest is located downstream of an upstream activating sequence (UAS), which is the binding site for the GAL4. In addition, a temperature-sensitive GAL80 can be used to direct expression in a temporally regulated manner, as GAL80 inhibits GAL4 at room temperature, but is permissive at elevated temperatures (Brand and Perrimon, 1993; Suster et al., 2004; Figure 5).
Glia cells are divided into three categories in the Drosophila CNS, of which include surface, cortex and neuropil glia. Surface glia cells form a double cell layer on the brain surface. Perineurial glia cells constitute the top layer, while subperineurial glia cells constitute the bottom layer and are a key component of the blood-brain barrier (BBB). Cortex glia cells form an intricate network that surrounds neuronal cell bodies, providing neurons with trophic support (Hartenstein, 2011). Neuropil glia cells are located in the neuropil regions of the brain, which are dense with synaptic connections. Neuropil glia cells are further divided into two subclasses: astrocyte-like and ensheathing glia. Astrocyte-like glia cells have a similar morphology to mammalian astrocytes and are responsible for neurotransmitter uptake at the synaptic cleft (Parker and Auld, 2006). Astrocyte-like glia cell bodies reside on the periphery of the neuropil region and their processes infiltrate the neuropil. Ensheathing glia cells have been shown to
possess a phagocytic function and are involved in the clearing of neuronal debris (Doherty et al., 2009). Ensheathing glia reside on the periphery of the neuropil region and extend into the neuropil upon axonal damage (Figure 6 & 7).

Figure 6. The types of glia found in the Drosophila CNS
The Anaphase Promoting Complex/Cyclosome ( APC/C)

The Anaphase-Promoting Complex/Cyclosome (APC/C), an E3 ubiquitin ligase, is a key player in the ubiquitin proteosome system (UPS). Proteins poly-ubiquitinated by the APC/C are eventually degraded by the 26S proteasome (Figure 8). The APC/C has been shown to regulate important processes such as the mitotic cell cycle (Primorac and Musacchio, 2013) and neuroblast differentiation (Kaplow et al., 2008). The APC/C is a multi-subunit protein comprised of about 13 subunits. One critical subunit is the activating subunit, of which there are two types: Cdc20 and Cdh1. Cdc20 and Cdh1 are responsible for the substrate specificity of the APC/C, as they are the subunits which bind the substrate. This interaction is possible when a molecule
contains a D-Box or KEN Box region, the binding sites for APC/C (Pfleger and Kirschner, 2000). Mitotic cell cycle progression is promoted by APC/C$^{\text{Cdc20}}$, as it ubiquitinates securin, allowing for separase to cleave the bound sister chromatids at the metaphase plate (van Leuken et al., 2008).

Retina aberrant in pattern/Fizzy-related (Rap/Fzr) is the Drosophila homolog to the mammalian Cdh1 (Figure 9). APC/C$^{\text{Rap/Fzr/Cdh1}}$ is crucial for mitotic cell exit. Several substrates of the APC/C$^{\text{Rap/Fzr/Cdh1}}$ have been shown to include positive regulators of the cell cycle such as mitotic cyclins and negative regulators of Cdk inhibitors (Hu et al., 2011). The APC/C$^{\text{Rap/Fzr/Cdh1}}$ has also been shown to promote endoreplication in cells (Zielke et al., 2008).

The role of the Ubiquitin Proteosome System in glial development, and more specifically the role of the APC/C$^{\text{Rap/Fzr/Cdh1}}$, has not been examined in great detail. In the adult human brain, astrocytes have been implicated as progenitor cells for olfactory bulb-bound neurons in the subventricular zone (SVZ) (Doetsch et al., 1999). In the developing Drosophila brain, astrocyte-like glia have been shown to proliferate (Awasaki et al., 2008). A closer inspection of the roles of cell cycle regulators like the APC/C$^{\text{Rap/Fzr/Cdh1}}$ would thus be critical in our advancement in knowledge of glial development, especially astrocytes.
Figure 8. The Ubiquitin Proteosome System

Figure 10. The Anaphase Promoting Complex/Cyclosome
Our previous studies have implicated the (APC/C) in regulating differentiation of neuroblasts. Ectopic expression of Rap/Fzr, by use of the pan-glial GAL4 driver repo-GAL4, resulted in a decrease of glia cells in the 3rd instar larval brain. The APC/C was shown to target Loco, a regulator of G-protein signaling (RGS), which prevented differentiation of neuroblasts into glia (Kaplow et al., 2008; Figure 10). The consequences of this glia reduction in the adult fly remain to be determined.

![Image](image-url)

**Figure 10. Rap/Fzr regulates differentiation of ganglion mother cells**

In this study we have assessed the consequences of perturbation of glia cell development in the adult fly. Our results show that adult flies exhibit several neurodegenerative phenotypes, such as lifespan reduction, temperature-sensitive paralysis and vacuole formation. In using glia subtype GAL4 drivers to ectopically express Rap/Fzr, we have identified astrocyte-like glia as a
critical glial subtype in neuroprotection. Targeted expression of Rap/Fzr to astrocyte-like glia also results in lifespan reduction and temperature-sensitive paralysis. Interestingly, astrocyte-like glia cells show no evidence of disruption at the 3\textsuperscript{rd} instar larval stage but are vastly disrupted in the adult brain. This implicates the ubiquitin-proteosome system in regulating astrocyte-like glia development in the Drosophila CNS.

**Methods**

*Fly strains*

The following GAL4 drivers were used to target ectopic expression in the tissue listed:

<table>
<thead>
<tr>
<th>GAL4 Driver</th>
<th>Tissue Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP6293-GAL 4</td>
<td>Perineurial</td>
</tr>
<tr>
<td>NP2276-GAL 4</td>
<td>Subperineurial</td>
</tr>
<tr>
<td>NP2222-GAL 4</td>
<td>Cortex</td>
</tr>
<tr>
<td>NP577-GAL 4</td>
<td>Cortex</td>
</tr>
<tr>
<td>NP1243-GAL 4</td>
<td>Astrocyte-like</td>
</tr>
<tr>
<td>NP3233-GAL 4</td>
<td>Astrocyte-like</td>
</tr>
<tr>
<td>NP6520-GAL 4</td>
<td>Ensheathing</td>
</tr>
<tr>
<td>repo-GAL4</td>
<td>Pan-glia</td>
</tr>
<tr>
<td>elav-GAL4</td>
<td>Pan-neuronal</td>
</tr>
</tbody>
</table>

Awasaki et al., 2009

**Table 1. Tissue specificity of GAL4 drivers**

The following UAS constructs were used to ectopically express the protein listed:
Canton-s was used as a wild-type control. *tub-GAL80ts* was used for temporal control of GAL4 expression. The recombinant strain *NP3233-GAL4, UAS-mcD8::GFP* was kindly provided by the Hartenstein Lab (UCLA).

**Lifespan**

Flies were collected on the day of eclosion and kept in a temperature-controlled incubator. No more than 20 flies were kept in a vial, with all vials containing both males and females. Flies were transferred into fresh vials twice a week to reduce death due to bad fly food. The number of flies found dead was recorded during each transfer.

*For the GAL80 experiments:* 

Flies were treated as all the others involved in the lifespan assay. On day one of eclosion these flies were heat-shocked at 37°C for ½ hour to allow for GAL4 activity. Fly death was then recorded as detailed above.

<table>
<thead>
<tr>
<th>UAS</th>
<th>Protein type</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAS-rap/fzr</td>
<td>Activating subunit of APC/C</td>
</tr>
<tr>
<td>UAS-highwire</td>
<td>E3 ubiquitin ligase</td>
</tr>
<tr>
<td>UAS-APC2</td>
<td>Catalytic subunit of APC/C</td>
</tr>
<tr>
<td>UAS-mcD8::GFP</td>
<td>Membrane – tethered green fluorescent protein</td>
</tr>
</tbody>
</table>

**Table 2. UAS constructs used and the protein types expressed**
Temperature-sensitive paralysis

Bottles were emptied of all adult flies in the morning and collected from in the evening of the same day. Flies were kept in vials at no more than 20 per vial. Flies were transferred 1-2 times a week and kept in a temperature-controlled incubator. Males and females were kept in the same vials.

On the day of the experiment flies were exposed to CO$_2$ and sorted into pre-heated glass vials at a volume of 10-15 to avoid over-crowding. Flies were given 2-3 hours to recover after exposure to CO$_2$. Flies were tested at 37°C for paralysis for increments of 4 minutes totaling 12 minutes (data shown is for 12 minutes). The same fly was not tested at multiple temperatures in one experiment. Flies were considered paralyzed if they were found immobile at the bottom of the vial (flies that died during the experiment were discarded from the study).

Note: Different flies were used for the lifespan and temperature-sensitive paralysis experiments.

Immunohistochemistry

Third instar larval brains and adult brains were dissected in PBS and fixed in 4% PFA for 20 minutes. 3 x 15 minute washes in PBST were followed by placing dissected brains in primary antibody at 4°C overnight. The dissected brains were placed in 3 x 15 minute washes in PBST the following day, after which they were placed in the secondary antibody for 1.5 hours at room-temperature shaking. Brains were then placed in 3 x 15 minute washes in PBST followed by mounting on vectashield.
The primary antibody used was mouse anti-repo (8D12, DHSB) at a dilution of 1:5. The secondary antibody used was TRITC anti-mouse (DHSB) at a dilution of 1:100.

Results

Loss of glia cells, induced by the ectopic expression of Rap/Fzr, results in neurodegenerative phenotypes

To assess the consequences of Rap/Fzr-induced loss of glia cells we performed lifespan and temperature-sensitive paralysis assays. Reduction in longevity is commonly associated with neurodegeneration mutants, and temperature-sensitive paralysis is a widely used marker for neuronal dysfunction (Reenan and Rogina, 2007). In our previous studies, UAS-rap/fzr;repo-GAL4 flies exhibited a drastic decrease in glia cells at the 3rd instar larval stage (Kaplow et al., 2008). Adult UAS-rap/fzr;repo-GAL4 flies also exhibited a significant reduction in longevity (Figure 11 A & B, p < .0001). This phenotype was specific to pan-glial overexpression of Rap/Fzr, as the pan-neuronal overexpression did not result in lifespan reduction (Figure 11 B). Similarly, UAS-rap/fzr;repo-GAL4 flies exhibited an age-dependent paralytic phenotype. This was not observed in elav-GAL4;UAS-rap/fzr flies (Figure 11 D).

These data indicate that pan-glial ectopic expression of Rap/Fzr may adversely affect the neuronal population. To assess this, we performed light and electron microscopy. UAS-rap/fzr;repo-GAL4 fly brains formed vacuoles in the lamina and medulla regions of the optic lobe (Figure 11 E, black arrows). Similarly, UAS-rap/fzr;repo-GAL4 flies formed vacuoles in the central brain neuropil regions (Figure 11 F, red arrows). The severity of both phenotypes worsened as the flies aged, which may be causal to the age-dependent paralysis experienced by
these flies. Taken together, these data indicate that flies experienced neurodegeneration as a result of loss of glia cells or perturbation of existing glia by the ectopic expression of Rap/Fzr.
Figure 11. Pan-glial ectopic expression of Rap/Fzr results in neurodegenerative phenotypes. repo-GAL4 and elav-GAL4 were used to overexpress UAS-rap/fzr in glia and neurons, respectively. (A-C) Lifespan reduction. Survival curves illustrate a significant lifespan decrease in UAS-rap/fzr;repo-GAL4 flies (A, p < .0001), but not in elav-GAL4;UAS-rap/fzr flies (B). (C) illustrates the average lifespan of the genotypes tested. (D) Temperature-sensitive paralysis. Flies were placed in 37°C water for intervals of 4 minutes (12 minutes total) on a weekly basis. Data shown are results for 12 minutes. UAS-rap/fzr;repo-GAL4 flies exhibited an age-dependent paralytic phenotype. This phenotype was not observed in the parental controls or elav-GAL4;UAS-rap/fzr flies (D). (E & F) Vacuole formation. Light microscopy images illustrate vacuoles in the lamina and medulla region of UAS-rap/fzr;repo-GAL4 fly brains (E, K-O, black arrows). Electron micrographs illustrate progressive vacuole formation in the neuropil regions of the central brain of UAS-rap/fzr;repo-GAL4 flies (F, A’-E’, red arrows). Sample sizes for lifespan assay: repo-GAL4 (188), UAS-rap/fzr;repo-GAL4 (476), elav-GAL4 (154), elav-GAL4;UAS-rap/fzr (283). Sample size for temperature-sensitive paralysis: repo-GAL4 (107), UAS-rap/fzr;repo-GAL4 (102), elav-GAL4 (53), elav-GAL4;UAS-rap/fzr (70).
Glia subtype specificity of GAL4 drivers

Although our results indicate the critical importance of glia cells in the healthy fly brain, they do not shed light on the glia subtypes responsible for the phenotypes observed. Ectopic expression of Rap/Fzr in individual types of glia cells would allow for the identification of the causal glia cells. To this end, we utilized GAL4 drivers specific to different subtypes of glia cells. We first characterized the expression patterns of these GAL4 drivers in the 3rd instar larval brain. To accomplish this we crossed each GAL4 driver with the reporter gene UAS-\textit{mcD8::GFP}. \textit{mcD8::GFP} is a membrane-tethered protein that would afford the visualization of the cell’s morphology. We immunostained with anti-GFP and anti-repo, a glia specific nuclear antibody. The following criteria were used to characterize the glia cells: repo-positive, GFP-positive and exhibition of previously published morphologies (Haretnstein, 2011; Stork et al., 2013). Our results showed that several GAL4 lines were specific to their respective subtype, while others exhibited cross expression in another subtype (Figure 12).

Two lines that exhibited this cross expression were \textit{NP1243-GAL4} and \textit{NP2222-GAL4}. Though \textit{NP1243-GAL4} is published as an astrocyte-like glia driver, it also labeled cortex glia (Figure 12 E and F). \textit{NP2222-GAL4} is published as a cortex glia driver but labeled other morphologically distinct glia cells (Figure 12 C). These data call into question the use of these GAL4 drivers in experiments which necessitate the targeted expression of a gene of interest in one type of glia.

In addition, all of these GAL4 drivers showed cross expression in a subset of neurons (Awasaki et al., 2008). That said, our lifespan analysis and paralysis results do not indicate that ectopic expression of Rap/Fzr in neurons causes neurodegenerative phenotypes. Thus, we used
these GAL4 drivers to assess the effects of Rap/Fzr overexpression in distinct subtypes of glia, neglecting the simultaneous expression in neurons.

Astrocyte-like glia-specific ectopic expression of Rap/Fzr perturbs development, and results in neurodegenerative phenotypes.

These GAL4 drivers were crossed into UAS-rap/fzr, and the progeny were tested for lifespan reduction and temperature-sensitive paralysis. As our results show, ectopic expression of Rap/Fzr in astrocyte-like glia showed the most severe neurodegenerative phenotypes (Figure
13 and 14). The phenotypes were much more severe when using \textit{NP3233-GAL4} than \textit{NP1243-GAL4} but as previously stated \textit{NP1243-GAL4} is not astrocyte-like glia-specific (Figure 12 E and F). Targeted expression of Rap/Fzr in astrocyte-like glia cells, driven by the GAL4 driver \textit{NP3233-GAL4}, resulted in severe lifespan reduction (Figure 14 A & B) and temperature-sensitive paralysis (Figure 14 C). Interestingly, these phenotypes were more severe than those observed in \textit{UAS-rap/fzr;repo-GAL4} flies, which may be explained by the varying strength of different GAL4 drivers. Another possibility is that ectopic expression of Rap/Fzr in another subtype of glia buffered the pan-glial phenotype.

We questioned whether the effects we observed were specific to the APC/C$^{\text{Rap/Fzr/Cdh1}}$. To assess this, we used \textit{repo-GAL4} to drive expression of \textit{UAS-highwire}, another E3 ubiquitin ligase. We saw no change in lifespan (Figure 13 G) no paralytic phenotype (Figure 13 J). In addition, we used \textit{repo-GAL4} to drive expression of \textit{UAS-APC2}, which is the catalytic subunit of the APC/C. We did see a mild reduction in lifespan (Figure 13 H). This phenotype was not nearly as severe as the ectopic expression of Rap/Fzr, which can be explained by APC2’s function during all APC/C activity, including while Cdc20 is bound to the APC/C rather than Cdh1.
Figure 14. Astrocyte-like glia-specific ectopic expression of Rap/Fzr results in lifespan reduction and temperature-sensitive paralysis. NP3233-GAL4 was used to overexpress UAS-rap/fzr in astrocyte-like glia. (A-B) Lifespan reduction. UAS-rap/fzr;NP3233-GAL4 flies exhibited a significant lifespan reduction (A, p < .0001). (B) illustrates the drop in average lifespan of the experimental genotype compared to the control. (C) Temperature-sensitive paralysis. Adult flies were tested for temperature-sensitive paralysis as in Figure 1. UAS-rap/fzr;NP3233-GAL4 flies also exhibited an age-dependent paralytic phenotype (C). Sample sizes for lifespan assay: NP3233-GAL4 (188), NP3233-GAL4;UAS-rap/fzr (309). Sample sizes for temperature-sensitive paralysis: NP3233-GAL4 (89), NP3233-GAL4;UAS-rap/fzr (67).
Considering the APC/C’s role in regulating neuroblasts differentiation, we hypothesized that the astrocyte-like glia-targeted overexpression of Rap/Fzr was resulting in loss of astrocyte-like glia cells in the 3rd instar larval brain. To explore this idea we used NP3233-GAL4 to simultaneously express Rap/Fzr and UAS-mcD8::GFP. Unexpectedly, astrocyte-like glia cells were not disrupted in the 3rd instar larval brain, as they exhibited wild-type morphology, location and cell number (Figure 15 A & B). There was an increase in average nucleus size in these glia cells (Figure 15 C), which could be explained by the APC/C’s role in endoreplication (Zielke et al., 2008). Interestingly, astrocyte-like glia cells were greatly disrupted in the brains of one-day-old adults (Figure 15 A).

These data indicate that ectopic expression of Rap/Fzr in astrocyte-like glia cells disrupts their development beyond the 3rd instar larval period. We hypothesized that a delayed expression of the GAL4 beyond the adult stage would allow for normal development of astrocyte-like glia, and prevent the neurodegenerative phenotypes. To this end, we employed a temperature-sensitive GAL80 construct. GAL80 inhibits GAL4 at room temperature, but allows GAL4 activity when placed at an elevated temperature (Suster et al., 2004). Tub-GAL80ts;NP3233-GAL4/UAS-rap/fzr flies were heat-shocked at 37°C on the day of eclosion, and their longevity was monitored. These flies did not show a reduction in lifespan (Figure 15 D). These data indicate that directed ectopic expression of Rap/Fzr in astrocyte-like glia disrupts their development during the pupal period, thus resulting in lifespan reduction and possibly temperature-sensitive paralysis.
Figure 15. Overexpression of Rap/Fzr disrupts astrocyte-like glia development. (A-C) NP3233-GAL4 was used to target ectopic expression of UAS-rap/fzr and UAS-mcd8::GFP in astrocyte-like glia. 3rd instar larval and Day 1 adult brains were dissected and immunostained with anti-repo and anti-GFP. 3rd instar larval experimental brains exhibited wild-type morphology and location (A) and cell count (B), while also exhibiting enlarged nuclei (A, arrows, C, p < .0001). In contrast, astrocyte-like glia of the adult central brain and optic lobe exhibit severe perturbations (A). Flies carrying the temperature-sensitive tub-GAL80 transgene were heat-shocked (HS) on the day of eclosion GAL80°;UAS-rap/fzr/3233-GAL4 flies did not exhibit lifespan reduction (D, red line). Scale bars in A measure 50 microns. Sample sizes for lifespan assay: tub-GAL80°;UAS-rap/fzr HS (155), tub-GAL80°;UAS-rap/fzr/3233-GAL4 HS (193), tub-GAL80°;UAS-rap/fzr/3233-GAL4 no HS (225).
Discussion

On the role of $APC/C^{Rap/Fzr/Cdh1}$ in astrocyte-like glia development

There has been an increasing interest in the function of glia cells in the context of both normal brain function and disease. No other glia cell has garnered more interest than the astrocyte. Possessing critical functions such as reuptake and recycling of neurotransmitters and establishing the blood brain barrier, the astrocyte is truly an essential cell of the nervous system. Indeed, astrocytes have been implicated in several neurodevelopmental and neurodegenerative disorders. That said, there is much to be learned about the development of astrocytes. Here we have shown evidence that the Ubiquitin Proteasome System plays a role in developing astrocyte-like glia in the Drosophila central nervous system.

The astrocyte-like glia targeted ectopic expression of Rap/Fzr resulted in severe neurodegenerative phenotypes (lifespan reduction and temperature-sensitive paralysis). This led to the unexpected result that astrocyte-like glia were still intact in the 3rd instar larval brains. These results now raise two questions. First, does the $APC/C^{Rap/Fzr/Cdh1}$ regulate differentiation of ganglion mother cells into astrocyte-like glia? Second, are the glia observed at the 3rd instar larval stage functional?

On the matter of differentiation, this may be an issue of the onset of the GAL4 driver’s expression. GAL4 drivers are limited in expression by the activity of the promoter region. The repo-GAL4 driver is turned on very early in development due to the transcriptional regulatory function of Repo during gliogenesis. In contrast, the earliest we have observed expression of NP3233-GAL4 is during the 3rd instar larval stage (this is the earliest we have looked). Unfortunately, the promoter associated with NP3233-GAL4 is currently unknown. That said, a
GFP expression profile of *NP3233-GAL4, UAS-mcD8::GFP* flies throughout development would give an indication as to the onset of this drivers activity. If *NP3233-GAL4* turns on after the differentiation decision has been made, our results do not rule out the role of the APC/C<sup>Rap/Fzr/Cdh1</sup> in regulating the differentiation of these cells.

The lineages of adult astrocyte-like glia are currently unknown. That is, we are not sure if the cells that populate the adult brain are derived from pre-existing astrocyte-like glia, or if these are cells that have differentiated from other ganglion mother cells (there is evidence that the former is the case, as is discussed below). This is clearly a gray area that must be cleared. Recently developed tools such as photo-convertible fluorescent proteins could lend insight into this aspect of development. These proteins initially fluoresce at a particular wavelength but change when exposed to UV light (Hoi et al., 2010). Utilization of this powerful tool could help us identify the adult astrocyte-like glia that came from previous astrocyte-like glia and the alternative.

On the issue of functionality, we are currently limited in assessing the functionality of these cells at this particular stage. Many studies of astrocyte-like glia have centered on their role in the adult brain, not the larval stages (Awasaki et al., 2008; Ng et al., 2011). Thus, the known roles for astrocyte-like glia during the 3<sup>rd</sup> instar period are limited. It is known that astrocyte-like glia cells are expressing the extracellular amino acid transporter 1 (EAAT1) this early in development (GFP labeled astrocyte-like glia are observed in the brains of *EAAT1-GAL4, UAS-mcD8::GFP* larvae). One approach would be to stain for EAAT1, of which there is an antibody available. Loss of expression of this protein would clearly indicate malfunction, whereas its expression would be an indirect indication that these glia cells are retrieving neurotransmitters from the synaptic cleft.
Recent work has examined the importance of calcium signaling in astrocyte-like glia function. Genetically encoded calcium indicators (GECI’s) have recently been developed (Tian et al., 2012), and there are fly strains in which genes for these GECI’s are downstream of UAS sequences. In addition, the transparency of larvae lends itself to live imaging. Thus, the tools are available to study calcium signaling in Drosophila in a tissue specific manner. Assessment of calcium imaging in these mutant flies could also shed light on the functionality of their astrocyte-like glia.

Although astrocyte-like glia exhibited wild-type phenotypes (location, morphology, number and repo-positive) there was one difference – their nucleus size. Although the APC/C\textsuperscript{Rap/Fzr/Cdh1} is well characterized for it’s role in stabilizing G0 phase, and preventing entry into S phase, it has also been shown to promote endoreplication. We have not yet observed any poly-nucleated astrocyte-like glia in 3\textsuperscript{rd} instar larval brains but it would be interesting to assess this in later stages of development (we have observed poly-nucleated cortex glia of \textit{UAS-rap/fzr;repo-GAL4} larvae). This question ties into the next topic at hand, that is the apparent failure of these astrocyte-like glia cells to proliferate during the pupal stage.

We saw an astonishing disruption in the astrocyte-like glia population in one-day-old adults, compared to their normalcy during the larval stage. There are currently two prevailing hypotheses about this phenomenon. First, that astrocyte-like glia cells are dying after the 3\textsuperscript{rd} instar stage and do not make it beyond eclosion. Second, that ectopic expression of Rap/Fzr is causing a proliferative defect of the glia cells present in 3\textsuperscript{rd} instar larvae.

With respect to the first hypothesis, prevailing evidence suggest that the APC/C\textsuperscript{Rap/Fzr/Cdh1} is involved in promoting cell survival. Modulator of apoptotic protein 1 (MOAP1), a pro-
apoptotic factor, is a substrate of the APC/C<sup><i>Rap/Fzr/Cdh1</i></sup>. APC/C<sup><i>Rap/Fzr/Cdh1</i></sup> is also inhibited by TRIM39, another E3 ubiquitin ligase that promotes cell death (Huang et al., 2012). In addition, the APC/C<sup><i>Rap/Fzr/Cdh1</i></sup> has also been implicated in neuronal survival (Almeida, 2012). That said, these studies were not focused on glial biology specifically. Thus, we cannot rule out the promotion of cell death in these glia cells. This could be assessed by simply tracking the progression of the pupal formation while staining for both anti-repo and anti-cleaved caspase 3. Co-localization of these two antibodies would be direct evidence that glia cells are dying, which would support the hypothesis.

With respect to the second hypothesis, the APC/C<sup><i>Rap/Fzr/Cdh1</i></sup> is a well-known regulator of the cell cycle. During G1, the APC/C<sup><i>Rap/Fzr/Cdh1</i></sup> targets Skp2, a component of the SCF ubiquitin ligase, and Ets2, a transcription factor, for ubiquitination (Wei et al., 2004; Li et al., 2008). Degradation of Skp2 and Ets2 prevent entry into S phase (Li and Zhang, 2009). Recent studies have shown that astrocyte-like glia proliferate during the 3<sup>rd</sup> instar larval and pupal periods. Thus, ectopic expression of Rap/Fzr may be perturbing this proliferation from occurring. We have shown evidence that inducing Rap/Fzr expression in the adult brain does not result in lifespan reduction. This supports the proliferative defect hypothesis, as Rap/Fzr is being induced after the proliferation occurs and the glia cells have already developed normally. A more direct approach would be to knock out Skp2 or Ets2 and monitor development of these glia cells. If a dominant negative of these proteins is available downstream of a UAS, then it’s expression can be induced prior to 3<sup>rd</sup> instar stages by use of the <i>NP3233-GAL4</i> driver. If the hypothesis is correct, this should lead to the perturbation of astrocyte-like glia in the adult, as well as lifespan reduction and temperature-sensitive paralysis.
On the involvement of APC/C^{Rap/Fzr/Cdh1} in neurodegenerative disorders

There is precedent for the involvement of the Ubiquitin Proteosome System in neurodegenerative diseases. In Parkinson’s, one of the most prominent neurodegenerative disorders in which death of dopaminergic neurons in the substantia nigra leads to motor deficits, one of the genes involved is parkin, which encodes for an E3 ubiquitin ligase (Worth, 2013).

The APC/C^{Rap/Fzr/Cdh1} has been implicated in neurodegenerative diseases such as Alzheimer’s because of it’s regulation of the cell cycle. The APC/C^{Rap/Fzr/Cdh1} is found in post-mitotic neurons, as it is involved in synapse formation and axon growth. Malfunction of the APC/C^{Rap/Fzr/Cdh1} could lead to reentry of post-mitotic neurons into the cell cycle, which would lead to apoptosis (Aulia and Tang, 2006) In fact, high expression of APC/C^{Rap/Fzr/Cdh1} in neurons has been shown to be responsible for their ability to maintain an antioxidant status. In contrast, low expression of APC/C^{Rap/Fzr/Cdh1} in surrounding astrocytes allows their processing of glucose, enabling them to supply neurons with energy (Herrero-Mendez et al., 2009). Whether or not this system is occurring in the Drosophila brain remains unknown but we may be causing an imbalance in this bioenergetic relationship.

There has recently been a model of Alexander’s disease established in the fly (Wang et al., 2011). Overexpression of the mammalian mutant GFAP gene in fly glia causes seizures and Rosenthal fiber-like formations within the glia. These phenotypes are ameliorated by the simultaneous expression of Hsp70. Hsp70 is downstream of heat shock factor 2 (HSF2), which is a transcription factor. HSF2 is a known target of the APC/C^{Rap/Fzr/Cdh1} (Ahlskog et al., 2010). This implicates the APC/C^{Rap/Fzr/Cdh1} in development of Alexander’s disease.
These first three examples examine the consequence of lost APC/C\textsuperscript{Rap/Fzr/Cdh1} function, while the third implies a gain-of-function phenotype. In our current model, the increased activity (or untimely activity) of the APC/C\textsuperscript{Rap/Fzr/Cdh1} is responsible for the observed phenotypes. APC/C\textsuperscript{Rap/Fzr/Cdh1} has commonly been referred to as a tumor suppressor because of it’s halting of the cell cycle (Jackson, 2004). It seems that the APC/C\textsuperscript{Rap/Fzr/Cdh1}’s tumor suppressive function is inhibiting normal astrocyte-like glia growth. It would be interesting to see if in a loss-of-function background these astrocyte-like glia actually over-proliferate, causing a cancerous glial population. This could easily be accomplished by crossing \textit{NP3233-GAL4, UAS-mcD8::GFP} into a mutant background for Rap/Fzr and observing the cells’ development and growth in the adult brain.

References


Harry GJ, 2013, Microglia during development and aging, Pharmacology & Therapeutics.


Ng FS, Tangredi MM, and Jackson FB, 2011, Glial Cells Physiologically Modulate Clock Neurons and Circadian Behavior in a Calcium-Dependent Manner, *Current Biology*, v. 21, p. 625-634.


