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DEVELOPMENT AND NEUROGENIC POTENTIAL OF MÜLLER GLIAL CELLS IN THE VERTEBRATE RETINA

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Abstract

Considerable research on normal and diseased states within the retina has focused on neurons. Recent research on glia throughout the central nervous system, including within the retina where Müller glia are the main type of glia, has provided a more in depth view of glial functions in health and disease. Glial cells have been recognized as being vital for the maintenance of a healthy tissue environment, where they actively participate in neuronal activity. More recently, Müller glia have been recognized as being very similar to retinal progenitor cells, particularly when compared at the molecular level using comprehensive expression profiling techniques. The molecular similarities, as well as the developmental events that occur at the end of the genesis period of retinal cells, have led us to propose that Müller glia are a form of late stage retinal progenitor cells. These late stage progenitor cells acquire some specialized glial functions, but do not irreversibly leave the progenitor state. Indeed, Müller glia appear to be able to behave as a progenitor in that they have been shown to proliferate and produce neurons in several instances when an acute injury has been applied to the retina. Enhancement of this response is thus an exciting strategy for retinal repair.

INTRODUCTION

The vertebrate retina is an exquisitely light sensitive tissue lining the eye, capable of transforming the signal from a single photon into a neural stimulus (Rodieck, 1998). The retinal circuitry performs extensive computations of these signals such that highly processed information is delivered to the higher order visual centers, reducing their need for information processing. Various systemic and eye-specific diseases can compromise vision. Approximately 9 million Americans suffer from some stage of age related macular degeneration. Diabetic retinopathy is another common cause of vision loss and currently affects about 40% of the 14 million diabetics (Sieving, 2004). The economic burden of eye related disorders in America is estimated by the National Eye Institute to be 22 billion dollars. An understanding of normal retinal development and function, as well as the responses of retinal cells to disease, will enable the design of potential therapeutic approaches.

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The mature retina consists of seven major cell classes, including six neuronal cell classes and one glial cell class, deriving from retinal progenitor cells. Rod and cone photoreceptors are the sensory or input neurons that connect to interneurons (bipolar cells and horizontal cells). Following further synaptic interactions with amacrine cells, the bipolar cells eventually relay information to the output neurons, the retinal ganglion cells. Relatively recently, it has been begun to be appreciated that Müller glial cells, the single class of glial cells originating from the retina progenitor cells, play an active role in ensuring healthy retinal function. Several decades of research have established a clear role for Müller glial cells in maintaining retinal homeostasis and trophic support for the neurons (Bringmann et al., 2006). Aberrant Müller glial cell function, on the other hand, may contribute to various disease pathologies, including diseases of anomalous blood vessel formation (e.g. retinopathy of prematurity, diabetic retinopathy) and retinal degeneration. Other Müller glial cell activities, such as reactive gliosis, are of unclear significance and may alternatively play a supportive or detrimental role. Of particular interest is the recent recognition that Müller glial cells can contribute to adult neurogenesis.

This review aims to consider the literature regarding Müller glial cells as they develop from multipotent retinal progenitor cells, and the conditions under which Müller glial cells can contribute to adult neurogenesis.

GLIAL DEVELOPMENT

A common retinal progenitor gives rise to both Müller glia and neurons

Müller glial cells comprise approximately 4–5% of the cells of the mature murine retina (Jeon et al., 1998; Young, 1985), and a similar percentage exists among other mammals (Martin and Grunert, 1992; Strettoi and Masland, 1995). They emerge during retinal development from a pool of multipotent retinal progenitor cells. A hallmark of vertebrate central nervous system (CNS) development is the conserved order in which the various cell types are produced. Comprehensive birthdating studies in some mammals (rats, monkeys and quokka wallaby) and partial studies in other mammals (mouse, hamster, gerbil, cat) as well as non-mammals (chick, goldfish, zebrafish) have all revealed the following conserved order of retinal cell genesis: retinal ganglion cells, horizontal cells, cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells and Müller glial cells (reviewed by (Altshuler et al., 1991)). As in other parts of the CNS, gliogenesis in the retina occurs towards the end of development. In the rat, for example, retinal cell production initiates at E10 and terminates around P12, whereas Müller glial cell development occurs predominantly between E18–P12 (Rapaport et al., 2004). Lineage studies in the postnatal rat have demonstrated the existence of two cell clones consisting of a rod and a Müller glial cell, but no clones of more than one cell containing **only** Müller glia have been observed (Turner and Cepko, 1987). Together these findings argue against the existence of a restricted progenitor for producing only glia, i.e. a glioblast, and suggest instead that a highly specialized sensory neuron and a non-neuronal cell type can be generated from the terminal division of a common progenitor. This lack of a retinal glioblast is in contrast to observations made elsewhere in the CNS, where, for example, multicellular clones of only glia have been observed (Cai et al., 2000; Levison et al., 1993). In addition, Müller glia contrast with the radial glia found elsewhere in the CNS. Although radial in morphology, Müller glia do not share the cardinal features of radial glia in, e.g. the cerebral cortex (Weissman et al., 2003). In the cortex, radial glia are present early in development, serve as the progenitors for neurons throughout development, and serve as guides for neuronal migration. By virtue of their late appearance during the neurogenic period, Müller glia are obviously not the progenitor cells for retinal neurons, though as argued below, they can be neurogenic under certain conditions, in the adult. They do not serve as guides for the migration of early born retinal neurons, as retinal ganglion cells and amacrine cells are in

their inner retinal locations prior to the appearance of Müller glia. It is not clear if they serve as migration guides for late born cells.

Determinants of the Müller glial cell fate

Several paradigms of cell fate determination, which are not mutually exclusive, could be considered regarding Müller glial cell development:

1. A single 'master gene' regulator acts in a manner that is both necessary and sufficient for the glial fate.
2. Various factors act in concert to both suppress the neuronal fate and instruct the glial fate.
3. Retinal progenitor cells that fail to differentiate as neurons earlier in development adopt the glial fate, the last available fate, as a type of "default" fate.

The concept of a master gene regulator was first proposed for *Drosophila* glial cell development after the discovery of the transcription factor *glial cells missing* (*gcm*). As in the case of retinal development, *Drosophila* neurons and glia can derive from a common progenitor cell. A loss-of-function mutation in *gcm* eliminates glial cell production, thereby producing an excess of neurons, whereas misexpression of *gcm* in progenitor cells leads to an almost exclusive production of glial cells (Akiyama et al., 1996; Akiyama-Oda et al., 1998; Akiyama-Oda et al., 1999; Akiyama-Oda et al., 2000). This has led to the proposal that *gcm* acts as a binary switch between the neuronal and glial cell fate. Although there may be certain conditions under which the mammalian homologues, *Gcm1* and *Gcm2*, can promote the glial features in the mouse brain (Iwasaki et al., 2003), misexpression of these genes in the developing mouse retina does not appear to be sufficient for Müller glial cell formation (Hojo et al., 2000).

While the role of *Gcm* in vertebrate retinal development remains unclear, other transcription factors that influence Müller glia development have been identified. Misexpression of the negative bHLHs, *Hes1*, *Hes5* and *Hey2*, in the postnatal day 0 rodent retina can promote glial features and suppress neuronal production (Furukawa et al., 2000; Hojo et al., 2000; Satow et al., 2001). *Hes1* null mice produce all six neuronal cell classes of the retina but lack Müller glial cells (Tomita et al., 1996). *Hes5* null retinas have a 30–40% reduction in Müller glial cells without perturbations in other cell types (Hojo et al., 2000). Expression levels of the negative bHLH transcription factors are most likely controlled in large part by signaling through the Notch pathway. Gain-of-function experiments support a role for Notch in regulating the glial versus neuronal cell fate since introduction of a constitutively active form of Notch (NICD) can promote glial features in the fish and rodent retina (Bao and Cepko, 1997; Furukawa et al., 2000; Scheer et al., 2001). Conversely, loss-of-function experiments have demonstrated a requirement for Notch1 in the production of Müller glia in the mouse retina (Jadhav et al., 2006a; Yaron et al., 2006). Together, these activities would suggest that Notch activity may be a key regulator of the glial fate, but likely does not promote the glial fate in a simple model of a master regulator, as Notch1, *Hes1*, *Hes5* and *Hey2* are all expressed throughout retinal development, but Müller glial cell production only occurs during the later stages. Furthermore, overexpression of NICD is not sufficient to promote glial features early in mouse retinal development, suggesting the requirement for additional factors (Jadhav et al., 2006a).

While Notch, and its downstream bHLH target genes, may not be the master regulators of the Müller glial cell fate, the afore-mentioned genetic studies have demonstrated a critical role for Notch signaling in regulating glial production. One interpretation of these data is that Notch simply acts to suppress the neuronal fate. Indeed, the negative bHLH family of transcription factors is known to antagonize the activity of the proneuronal bHLHs. Null

mutations in the proneuronal bHLH genes, Mash1, Ngn2, Math3, NeuroD1 and Math5, influence neuronal cell fates to varying extents. Interestingly, mutations in any of these genes results in an increase in Müller glial cell production (Akagi et al., 2004; Brown et al., 2001; Inoue et al., 2002; Tomita et al., 1996; Wang et al., 2001). Misexpression of all proneuronal bHLHs tested so far in the postnatal day 0 rodent retina promotes neuron production at the expense of Müller glial cells. These experiments identify the proneuronal bHLHs as not only important regulators of individual neuronal cell fates, but also more broadly as inhibitors of gliogenesis. Together these data suggest that a balance between proneuronal bHLHs and negative bHLHs plays a critical role in ensuring the proper generation of neurons and glia. Notch likely regulates this balance.

Since glial cells derive from multipotent progenitor cells towards the end of retinal development, it is also possible that the glial cell fate is simply assigned to the last remaining progenitor cells that fail to acquire an earlier neuronal cell fate. In support of this, two of the homeodomain transcription factors that define retinal progenitor status, Rax and Chx10, can promote glial features when misexpressed in late retinal progenitor cells. A simplified view would be that prolonging the progenitor state simply drives an excess number of cells towards a terminal fate, one of which is the glial fate. One way that the progenitor cell state might be prolonged is through inhibition of the production of one type, or several types, of neurons. It has been shown that Chx10 most likely functions to inhibit rod photoreceptor production, and so the increased production of inner nuclear layer cells seen after misexpression of Chx10, which includes Müller glial cells, may be secondary to this activity (Hatakeyama et al., 2001). However, glial production does not appear to be reduced in *orJ* mice that harbor a null mutation in Chx10 (Burmeister et al., 1996). This rules out a simple model of a requirement for Chx10 regulating glial production through its activity(ies) on neuronal cell production. Viral misexpression of Rax, a progenitor gene with a paired type homeobox, promotes glial features in most late retinal progenitor cells (Furukawa et al., 2000). Experiments in a tissue culture cell line have demonstrated that Rax can upregulate the expression of a reporter fused to either the Notch1 or Hes1 promoter, suggesting that perhaps Rax promotes glial features through upregulation of these genes (Furukawa et al., 2000). The requirement for Rax in glial formation has not been directly tested.

As mentioned above, clones that contain a Müller glial cell are never composed of only Müller glia. These data demonstrate that there is not a mitotic committed progenitor cell that makes only glia, even in a terminal division where 2 glial cells would result. This implies that the decision to become a glial cell occurs during or after a final division of a multipotent progenitor cell. It is not clear whether the decision occurs in the cell as it exits cell cycle or whether a post-mitotic retinal precursor cell itself is still receptive to extrinsic cues that might direct its fate. Interestingly, overexpression of NICD in late rodent retinal progenitor cells promotes the formation of glial features, but these cells are slightly dysmorphic and do not appear to be normal glia (Furukawa et al., 2000). Dissociation and placement of NICD-activated cells in culture leads to neurosphere formation, a property of stem cells, whereas normal Müller glial cells do not exhibit this property when placed in similar conditions (Jadhav et al., 2006a; Tropepe et al., 2000). Overexpression of NICD in at least a subset of postmitotic retinal precursors, on the other hand, promotes Müller glial formation in the absence of stem cell features (Jadhav et al., 2006a). Together these data suggests that Notch activity must be downregulated in order for both neurons and glia to properly form. Re-initiation of Notch activity in the postmitotic retinal precursor cells is then necessary to promote the glial fate. The fact that Notch1 and Hes gene family members are expressed in mature Müller glia is consistent with this interpretation.

Additional Müller glial cell determinants also have been uncovered. In amphibians, it has been shown that the CDK inhibitor p27Xic1 can promote Müller glial cell formation when overexpressed in the developing retina (Ohnuma et al., 1999). Mutational analysis revealed a discrete region that was essential for the gliogenic activity but dispensable for kinase inhibition. Furthermore, it appears that this activity is sensitive to the Notch/Delta pathway. Interestingly, overexpression of mouse p27kip1 or *Xenopus* p27Xic1 does not promote Müller glia formation in the developing mouse retina (Dyer and Cepko, 2001). In mouse, one set of studies has suggested that EGF signaling is a limiting factor in glial cell production. Expression analysis of the EGF receptor demonstrated a low level of receptor in early progenitor cells and higher levels in later progenitor cells. Further increasing receptor levels by viral misexpression increased glial cell production in late progenitor cells and not early progenitor cells (Lillien, 1995; Lillien and Wancio, 1998). Müller glial production has also recently been shown to be dependent upon Sox 9 (Poche et al., 2008). A related SoxB1-box transcription factor, Sox2, appears to be sufficient for Müller glial production when introduced via a retrovirus into E17 mouse retinal explant cultures, though it was also shown in this same study that amacrine formation was even more favored by overexpression of Sox2 (Lin et al., 2009). As Sox2 can act upstream of Notch 1 (Taranova et al., 2006), and loss of Sox2 leads to anophthalmia and microphthalmia (Fantes et al., 2003), it is likely that Sox2 overexpression can drive Müller glial formation through upregulation of Notch 1. Again, however, the situation must be more complex given the overproduction of amacrine cells following overexpression of Sox2, which might be in part due to Sox 2 being upstream of Pax 6 (Lin et al., 2009). It is also of interest that Sox 2 and Sox 9, which are both expressed in progenitor cells and Müller glia, cannot compensate fully for each other in either progenitor cell maintenance or Müller glial production.

In summary, the evidence to date supports a model of Müller glial cell production that most likely relies on a combination of several mechanisms. Activity of transcription factors such as Notch1, Rax and Chx10 permits a subset of progenitor cells to remain uncommitted until a time during development when glial cell formation is possible. Why retinal progenitor cells can form glial cells later in development and not earlier in development is unknown, as all of these genes are expressed within progenitor cells throughout development. An unidentified glial cell competence factor may not be expressed until later stages of development. Once progenitor cells become competent to form Müller glia, a balance between negative and positive bHLH transcription factors informs the neuronal versus glial fate decision. Notch activity likely regulates this balance, and a complex network among positive and negative bHLH genes likely plays a part as well. The decision to be a glial cell probably can occur as late as the postmitotic retinal precursor stage. Clearly other mechanisms are at play as well and further experiments will be necessary to identify additional factors and further clarify the precise role of Sox genes, p27Xic1 and EGF signaling. Development of other vertebrate glial cell types, including astrocytes and oligodendrocytes, involves the activity of the JAK/STAT signaling pathway and the Olig family of bHLH transcription factors, respectively (Ross et al., 2003). The involvement of these molecules in Müller glial cells remains an outstanding question, although the simple addition of CNTF or LIF, which stimulates the JAK/STAT pathway, to developing retinal progenitor cells does not yield more Müller glial cells (Ezzeddine et al., 1997).

The maturation of Müller glial cells

The study of cell fate determination naturally raises the question of the defining features of a mature differentiated cell type that distinguish it from the progenitor cell type that gives rise to it. The maturation process of retinal progenitor cells to Müller glial cells can be observed morphologically. Both retinal progenitor cells and Müller glia share a radial morphology. Progenitor cells have processes extending from the scleral to the vitreal side of the INBL.

Their soma migrate between the scleral and vitreal side in a cell cycle dependent fashion. The position of the soma depends on the cell cycle such that the soma is in the vitreal side of the outer neuroblastic layer during S phase, the scleral side during M phase and in between in G1 and G2. After Müller glia are produced, the soma becomes non-migratory and resides in the inner nuclear layer. Müller glial scleral or apical processes project to the outer limiting membrane with microvilli extending into the subretinal space. The vitreal or radial processes that project towards the inner border terminate in a basal endfoot that forms the inner limiting membrane. Together these processes allow Müller glial cells to span the entire retina. Numerous additional processes originate from the trunk and cover neuronal cell bodies and processes (Reichenbach and Reichelt, 1986).

Concomitant with these morphological changes are a series of changes in their electrophysiological properties. For example, mature Müller glial cells require high potassium permeability that is achieved through several channels. The inward rectifying potassium channel (Kir4.1) is one such channel and its activity is known to greatly increase during the first few weeks in the postnatal retina (Kofuji et al., 2002; Pannicke et al., 2002). This allows for higher amplitude potassium currents and contributes to the mature Müller glial resting membrane potential.

These aforementioned morphological and physiological differences between retinal progenitor cells and Müller glial cells are likely necessary for proper mature glial function. However, it is interesting to note that there remains a great deal of overlap between genes expressed in Müller glia and those expressed in retinal progenitor cells. This relationship was first shown in a systematic way through a cDNA microarray analysis of retinal progenitor cells and other retinal cells. It was observed that the progenitor cells shared a significantly higher proportion of their expressed gene repertoire with that of glial cells (43%) than with that of neuronal cells (Livesey et al., 2004). A subsequent SAGE study, which covered a more complete set of transcripts, and was coupled with extensive *in situ* hybridization for validation, identified 63 transcripts as expressed in mature Müller glial cells, but not in other mature retinal cell types. Sixty one of these transcripts were also detected in retinal progenitor cells (Blackshaw et al., 2004). The most recent study which systematically examined gene expression in Müller glia and other retinal cells was carried out on 167 individual retinal cells from developing and mature mouse retinas (Roesch et al., 2008; Trimarchi et al., 2008). Included among these data were profiles from single Müller glia and single progenitor cells from several ages across development. These data showed that Müller glia expressed even more progenitor genes than previously known, such as the transcription factor Pax6 (Roesch et al., 2008) (although it should be noted that Pax6 had been observed immunohistochemically in Müller glia in mature zebrafish retina (Bernardos et al., 2007)). Included among the genes expressed at high levels in mature Müller glia and progenitor cells were the two transcripts thought not to be in progenitor cells in the aforementioned SAGE study, clusterin and carbonic anhydrase 2, likely due to a greater sensitivity of the single cell study. Comparison of the genes expressed in Müller glia and progenitor cells of different ages revealed an intriguing temporal correlation. The single cell profiles of early and late retinal progenitor cells were examined for temporal patterns, i.e. genes that were commonly expressed by early, but not late, progenitor cells, and vice versa. Late progenitor cells were seen to frequently express a cluster of genes that was not commonly observed among the expression profiles of early progenitor cells. This late cluster of genes included genes that were fairly high in Müller glia, e.g. mu crystalline, and tweety 1 (Figures 1) retinaldehyde binding protein 1 (Figure 2).

The molecular similarities revealed by all of the expression studies lead to two questions: (1) Why do Müller glia express the progenitor cell repertoire, and (2) Why do progenitor cells express Müller glial genes? In answer to the first question, one can speculate that Müller glia

are a form of retinal progenitor cell. Late retinal progenitor cells might become Müller glia without undergoing an irreversible cell fate determination event. In other words, most cells that become neurons irreversibly exit cell cycle, turn off most progenitor genes, enter a differentiation program, and appear to be completely locked into their chosen fate. Progenitor cells becoming Müller glia retain much of the progenitor gene expression program, particularly those genes of the late retinal progenitor cells. They do not irreversibly exit cell cycle, as shown by mitotic activity under certain conditions (discussed below). It also appears that they do not irreversibly differentiate, as the genes expressed at high levels that appear to be necessary for Müller glial function can be turned off under certain conditions (again, see below). These observations argue that late progenitor cells that give rise to Müller glial cells do not irreversibly leave the progenitor cell state, but simply add the expression of genes that are required for glial function to the repertoire expressed by late retinal progenitor cells. In answer to the second question, it is interesting to consider the types of Müller glial genes expressed in progenitor cells.

Examination of Figures 1 and 2 reveals at least two patterns of expression of genes expressed in Müller glia exclusively, or nearly so, in the mature retina. One pattern is of genes expressed at high levels in Müller glia, and at lower levels in progenitor cells. A second pattern is of genes expressed at high levels in mature Müller glia, but not detected in progenitor cells. Examples of the first pattern are Kir4.1, S100 calcium binding protein, and integrin alpha V, which are all expressed at low levels in progenitor cells, and at high levels in mature Müller glia (Figure 2). Examples of the second pattern are aquaporin 4 and apolipoprotein E, both previously shown to be expressed specifically in Müller glia in the mature retina (Amaratunga et al., 1996; Nagelhus et al., 1998), and not seen in retinal progenitor cells by the single cell microarray (Figure 2). These latter genes might represent a class of genes that are turned on as Müller glia begin to differentiate from late progenitor cells. However, as methods continue to gain in sensitivity, it might be the case that these genes will be found to be expressed at low levels in progenitor cells. Nonetheless, one can appreciate from the data shown in Figures 1 and 2 that not only are classical progenitor genes expressed in Müller glia, but some classical Müller glial genes are expressed in late progenitor cells. While it is possible that these classical Müller glial genes that are expressed in progenitor cells might provide the progenitor cells with particular functions, we favor a different interpretation. The Müller glial genes expressed in late progenitor cells likely do not function in these cells, but instead reveal a competency state, showing that late progenitor cells are competent to make or become Müller glia. The molecular underpinnings of such a competency state might be the expression of a network of transcription factors that have as some of their target genes the Müller glial genes. This network might allow a cell to interpret, e.g. high Notch signal, as a signal to make a Müller glial cell. The expression of some of these Müller glial genes prematurely in progenitor cells is then not an indication of their function in progenitor cells, but rather is an indication of the competence of a cell to make a Müller glial cell. This competency state must be reversible, i.e. does not indicate that any particular late progenitor cell will indeed produce Müller glia. In fact, the majority of late retinal progenitor cells **do not** make Müller glia, but instead produce rod photoreceptors and bipolar interneurons (Morrow et al., 1999; Turner and Cepko, 1987; Young, 1985). We proposed a similar model to explain the expression of other neuronal genes, such as neurofilament (Austin et al., 1995), and amacrine genes (Alexiades and Cepko, 1997), in progenitor cells. As with the correlated expression of Müller glial genes in late progenitor cells, which make Müller glia, there was a temporal correlation for the expression of neurofilament and amacrine genes. That is, early progenitor cells make ganglion and horizontal cells, which express neurofilament, and neurofilament was made only in early, and not late, progenitor cells. Similar observations were made for amacrine genes. The expression of Müller glial genes in late, but not early, progenitor cells is thus in keeping

with this same notion of a competency state among late progenitor cells for Müller glial production.

GLIAL FUNCTION IN THE HEALTHY RETINA

In the healthy retina, mature Müller glia are suggested to participate in a diverse set of functions critical for the function and structure of retinal neurons. Bringmann et al. (Bringmann et al., 2006) have provided an excellent and recent review of the roles of Müller glia in both health and disease, and thus only an update and summary of these functions in light of the recent molecular profiling studies and studies of retinal development will be given here.

Function follows form

In the mature retina, Müller cells are found throughout the retina and span the entire depth of the retina. Müller cell processes surround every neuronal cell body and processes and send out numerous branches within the two synaptic layers of the retina that surround neuronal synapses. The intimate structural relationship between Müller glial cells and retinal neurons predicts a major role for Müller glial cells in providing both a physical scaffold for retinal organization and physiological support for neuronal function. Müller glia form the outer and inner limiting membrane and the glia limitans of blood vessels. Developmental defects leading to a significantly reduced production of Müller glial cells, as in the case of Notch1 and Hes1 mutants, lead to a reorganization of retinal tissue into rosette formations (Jadhav et al., 2006b; Tomita et al., 1996; Turner and Cepko, 1987). Selective ablation of Müller glial cells in the postnatal retina also leads to massive retinal dysplasia and eventually retinal degeneration (Dubois-Dauphin et al., 2000). It has been shown that Sonic Hedgehog protein secreted by retinal ganglion cells is critical for maintaining Müller glial cell morphology and organization suggesting that normal retinal lamination is mediated indirectly by the neurons themselves (Wang et al., 2002). Next to these more typical glial cell functions, an elegant study by Franze et al. (Franze et al., 2007) has recently shown that the cylindrical shape and regular and parallel orientation of Müller glia within the retina, along with a high refractive index, enables them to serve as light pipes, directing light across the retinal layers and into the photoreceptor outer segment layer.

The molecular features of Müller glia

Electrophysiological and molecular studies of Müller glia have demonstrated the presence of numerous classes of molecules involved in neurotransmitter recycling, regulation of ion homeostasis and glia-neuronal communication. The best characterized of these include ion channels (Kir 4.1, ATP-binding K⁺ channel, Ca²⁺-activated K⁺ channel, fast inactivating K⁺ channel, L-type Ca²⁺ channel, Na⁺ channel), neurotransmitter receptors (AMPA, GluR4, NMDA receptors, GABA-A, dopaminergic, cholinergic, adrenergic receptors, neuroactive peptide receptors, bFGF-, EGF-, PDGF-, NGF-receptors, thrombin-receptor), neurotransmitter transporters and modulators (GLAST, glutamine synthetase, GABA transporter (GAT-3), GABA-transaminase) and various other transporters (Na⁺/HCO₃⁻ cotransporter, Na⁺/H⁺ exchanger, Cl⁻/HCO₃⁻ anion exchanger, carbonic anhydrase) (reviewed in Newman and Reichenbach (Newman and Reichenbach, 1996)).

As mentioned above, more recent efforts have been directed towards systematically describing the molecular character of Müller glial cells by genome-wide analysis. The comprehensive studies of the Müller glia transcriptome have confirmed the expression of many of the above mentioned genes encoding transporters and channels (e.g. Kir 4.1, GABA receptor, opioid growth factor receptor-like 1, Figure 2), and have also provided individual candidate genes for investigation into the functions of these cells. The IPA tool (Ingenuity

Systems, Redwood City, CA), which allows one to categorize microarray data for gene function and identifies pathways enriched in expression in microarray data sets, was applied to Müller glial transcriptome data. Only genes with average expression values > 10,000 on the Affymetrix chips used for single Müller glial cells were included in this analysis. Pathways with significant representation in Müller glia are listed in Table 1. The ratio provided by IPA, which indicates significance, is calculated as “number of molecules in a given pathway divided by the total number of molecules that make up the pathway”. Some of the IPA signaling pathways with the most significance in Müller glia were Notch signaling, GABA receptor signaling, glutamate receptor signaling, Wnt signaling, PDGF signaling and Ca²⁺ signaling.

As has been shown for astrocyte and neuron interactions in several regions of the CNS (Pellerin et al., 2007), Müller glia are likely to be involved in nourishing retinal neurons. Indeed, the energy requirements for neurotransmission in the retina are very high, particularly for photoreceptors, whose rate of energy consumption has been found to be higher than that of any other tissue/cell type (Laughlin et al., 1998). As was first shown in honeybees, glia provide lactate to photoreceptors (Tsacopoulos et al., 1987). An in vitro study of lactate production by Müller glia, and lactate uptake and metabolism by photoreceptors, led to the suggestion that such a role is also played by Müller glia in mammals (Poitry-Yamate et al., 1995). In keeping with such activities, genes for such metabolic activities as oxidative phosphorylation and glycolysis are all found to be present in Müller glia, and in higher IPA ratios than are found in neurons (Table 1). In addition to Müller glial activities relating to energy metabolism, other genes enriched in Müller glia fall into the category of lipid metabolism and transport (glycosphingolipid, glycerolipid, sphingolipid, phospholipids), with specific genes such as the lipid transporter ApoE, and the Diazepam binding inhibitor, Dbi. A recent expression analysis of astrocytes and oligodendrocytes in the brain also showed enrichment for specific metabolic and lipid pathways in astrocytes versus oligodendrocytes and neurons. One of the genes also highly expressed in astrocytes is ApoE (Cahoy et al., 2008). Glycosphingolipids are also implied to modulate cell signal transduction events and gangliosides are considered trophic factors in neural development, survival and pathology. Altered sphingolipid and glycosphingolipid metabolism has been shown to cause several retinal diseases (Fox et al., 2006). Two other pathways specifically expressed in Müller glia are N-glycan degradation and O-glycan biosynthesis. A partial listing of these genes and others mentioned here is shown as a heatmap in Figure 2.

Glia outside of the retina carry out additional functions such as providing scaffolding for migrating neurons and providing immune cell functions. The latter of these activities has more recently also been attributed to Müller glial cells. Modulators of interest in this context are the chemokines. Retinal glial cells have been shown to be the major source of cytokines, in particular, MCP-1, after retinal detachment (Nakazawa et al., 2006), and other cytokines as well in human and rabbit models of proliferative vitreoretinopathy (Goczalik et al., 2008). Interestingly, recent work by Stevens et al. (2007) has shown upregulation of complement components at synapses early on in a model of retinal disease (glaucoma). The role of Müller glia in such functions is not yet known, but the single cell expression study was extended to include Müller glia from a retinal degeneration model, which revealed that Müller glia upregulate some components of the complement pathways, as well as chemokines, during retinal degeneration (Roesch et al, unpublished).

Müller glia, in addition to the retinal pigment epithelium, facilitate the recycling of photo pigments via cellular retinal binding protein (CRALBP) mediated uptake and conversion of all-trans-retinal to 11-cis-retinol (Das et al., 1992). Müller glia have also been suggested to carry out the trans to cis isomerization of 11-trans-retinol, particularly for cones (Mata et al.,

2002). In zebrafish, which have a cone-dominated retina, it was recently found that there are two paralogues of CRALBP, designated CRALBP a and b) (Collery et al., 2008; Fleisch et al., 2008). The a form is expressed only in the RPE and the b form is preferentially expressed in Müller glial cells. Morpholino knock-down of the b form led to a reduction in 11 cis-retinol levels, in light sensitivity, and in visual behavior. In keeping with a role in retinol metabolism, retinol metabolism was found as one of the pathways enriched in Müller glia, in comparison with ganglion cells, amacrine cells and bipolar cells using the IPA (Table 1).

The Müller glial profiling study not only provided insights into the function of Müller glia, but also showed a heretofore unknown degree of heterogeneity in gene expression among individual Müller glia. Although the transcription factor, Chx10, had been previously observed to be expressed in a subset of Müller glia (Rowan and Cepko, 2004), it was not clear if additional genes of potential functional significance also were unevenly expressed. The microarray findings showing differences among Müller glia were confirmed for a subset of such genes by dissociated in situ hybridization (for an example see Figure 3), which also showed that some genes were expressed in only a subset of Müller glia. Further in depth characterization will be needed to help clarify the diversity of Müller glial cells, particularly with regards to their ability to produce neurons (discussed below).

Heterogeneity among different types of glia from elsewhere in the CNS also has been revealed by microarray profiling. Expression analysis of astrocytes that were first cultured in vitro revealed molecular heterogeneity among those cells (Bachoo et al., 2004). Transcriptional profiles of astrocytes and oligodendrocytes purified from the mouse forebrain showed huge differences among these cells, despite the fact that they are typically lumped together as glia in classification schemes (Cahoy et al., 2008). When the Müller glial profiles were compared with the profiling data from other types of CNS glia, some common pathways were observed. For example, the Notch, Wnt and TGF- β pathways were shared with astrocytes and the PDGF signaling pathway with oligodendrocytes. Interestingly, both astrocytes and Müller glia express some genes involved in phagocytosis, suggesting that these cell types can phagocytose *in vivo*.

GLIAL FUNCTION IN THE DISEASED RETINA

Glial response to ischemia and injury

Relative to our understanding of normal glial function, much less is known about the nature and significance of changes that glial cells undergo in response to diseases of the nervous system. These responses, termed reactive gliosis, include changes in morphology, upregulation of various markers, de-differentiation and proliferation. Reactive gliosis appears to be a fairly non-specific and ubiquitous response to a variety of disease processes both in the retina and throughout the rest of the nervous system (Bringmann and Reichenbach, 2001; Pekny and Nilsson, 2005). Offending agents can range from hypoxia to chemical toxins. As discussed below, some Müller glia can respond to loss of retinal neurons by producing more neurons.

Alterations in potassium conductance, which correspond to a decreased activity of the Kir channel, are often present as an early feature in disease states. This initial state has been referred to as 'conservative' gliosis since there is no proliferative activity (Bringmann et al., 2000). Proliferative gliosis has been characterized by a further decrease in Kir channel activity due to mislocalization of the Kir protein, which in turn leads to a change in the resting membrane potential (RMP) (Ulbricht et al., 2008). The depolarization of the gliotic Müller glial cell reverts these cells to an RMP reminiscent of immature glial cells. A depolarized RMP allows higher activation of particular channels only active in immature

Müller glia such as the calcium-activated K⁺ channel of big conductance (the BK channel). Thus gliosis results in changes of Müller glial cells that result in an electrophysiological state similar to that of the immature glial state.

The first known gene expression change to precede proliferative gliosis in the mouse retina is a downregulation of the cyclin kinase inhibitor, p27kip1 (Dyer and Cepko, 2000). This is followed by re-entry of the reactive glial cells into cell cycle. Proliferation is limited to a few cell cycles most likely due to a subsequent downregulation of cyclin D3. The electrophysiological studies would suggest that changes in glial cell RPM might itself lie upstream of p27kip1 downregulation and cell cycle re-entry. Downregulation of p27kip1 appears to be an important checkpoint in regulating gliosis, since mice lacking p27kip1 undergo gliosis in the absence of injury (Dyer and Cepko, 2000). Direct visualization of mitotic figures in the injured retina has suggested that Müller glia nuclei migrate from the INL to the outer limiting membrane where they round up and divide in a manner similar to that of progenitor cells during normal retinal development.

The upregulation of various intermediate filaments, most notably glial fibrillary acidic protein (GFAP) and vimentin, is a prominent and ubiquitous feature of gliotic cells (Pekny and Nilsson, 2005). Although glial scars comprised of significant amounts of intermediate filaments are prominent in various brain regions following many types of injury, they are not typically found at such high levels in degenerating retinal tissue, despite the fact that these intermediate filaments proteins are upregulated in Müller glia following disease or injury.

Does gliosis reduce or increase neural damage?

Reactive gliosis in the central nervous system forms a physical and diffusion barrier that protects the tissue from further damage, and might limit invasion by immune system cells. The functional consequences of gliosis on neuronal cell loss in the retina are unknown. A beneficial role for gliosis in the retina has been suggested by the finding that animals that undergo less gliosis appear to suffer more extensive and rapid neuronal degeneration. Squirrels exhibit minimal glial activation or gliosis in a retinal detachment model as compared to cats (Linberg et al., 2002). However, it is unclear whether other differences, such as the ratio of rod and cone photoreceptors, account for this variation in response to injury. Additionally, non-reactive Müller glial cells in mouse, squirrel and rabbit express vimentin in high levels across the cells, whereas in feline and human cells vimentin and GFAP are only expressed in their endfeet. A higher level of GFAP, as well as nestin, in Müller glial endfeet has been suggested to have a neuroprotective role, especially against neuronal cell loss due to increased intraocular pressure (Xue et al., 2006).

Genetic removal of the two hallmark intermediate filament proteins, GFAP and vimentin, does not significantly alter the morphology of Muller glia. However, under stress, compressed, thinner Müller glial morphology was observed, often accompanied with endfoot shearing (Verardo et al., 2008). In addition, reactive gliosis is attenuated and reduced photoreceptor cell loss can be observed (Nakazawa et al., 2007). Reactive Muller glial cells also induce MCP-1 production and release, which, interestingly, was lowered in these animals as well.

In the opposite direction, the rate of neuronal cell death might modulate the extent of gliosis. An atypical form of gliosis takes place in slow degenerating retinas that show expression of GFAP, but no downregulation of Kir currents, nor redistribution of the channels or membrane potential changes (Iandiev et al., 2006). Much more work will need to be done, particularly in vivo in relevant animal models to properly place the significance of the Muller glial response in different diseases.

Cell cycle re-entry and lack of glioma formation

The ability of mature cell types to re-enter cell cycle in the event of injury raises a concern regarding transformation and tumor formation. Indeed there appears to be a correlation between the ability to re-enter cell cycle and form tumors. An overwhelming majority of the cancers of the nervous system are of glial origin (reviewed by Stiles and Rowitch, 2008 #79). Neurons, on the other hand, typically appear to respond to oncogenic stress by undergoing apoptosis and rarely are the cell of origin in cancer. In the retina, forced cell cycle re-entry of photoreceptor cells leads to cell death, whereas amacrine cells and horizontal cells can apparently re-enter cell cycle and this may possibly be the basis for retinoblastoma formation (Ajioka et al., 2007; Chen et al., 2007; MacPherson et al., 2007). In fact, a recent mouse model of retinoblastoma showed that horizontal cells with extensive neuronal processes were able to re-enter cell cycle and form tumors, without losing their differentiated morphology. Curiously, there are no documented cases of tumors of Müller glial cell origin, even in these mouse models of retinoblastoma. In addition, another recent study examined the retina following expression of an allele of p27kip1 which cannot bind cyclin or cyclin dependent kinase (Besson et al., 2007). This allele caused progenitor hyperproliferation, and Müller glia showed signs of reactive gliosis, but they did not contribute to the observed hyperplasia. These studies in mice are in keeping with data from humans, as there are no documented cases of tumors of Müller glial cell origin. The reasons for this are presently unclear. A purely statistical explanation is that Müller glia are much less abundant than other glial cell types, though this could not be the explanation in the murine models of retinoblastoma as horizontal cells are approximately 10 fold less abundant than Müller glia. It would appear that the explanation involves a tight level of regulation of Müller glial cell proliferation. For example, overexpression of Notch1 NICD leads to only limited proliferation (Bao and Cepko, 1997; Furukawa et al., 2000), though other NICDs can lead to tumor formation in other areas of the CNS (Pierfelice et al., 2008). Similarly, reactive gliosis in the retina does not lead to the hyperproliferation of Müller glia, in contrast to glial proliferation in other areas of the CNS. It is likely that there are several safeguards that protect the retina from unchecked proliferation of Müller glial cells.

Müller glia as a source of neurons

Retinal progenitor cells cannot proliferate indefinitely and do not appear to be totipotent at all timepoints (Cepko et al., 1996). This population is generally exhausted by the end of development and is not considered a stem cell population, but nonetheless there is a varying degree of adult neurogenesis in the retina depending on the species. The capacity for adult neurogenesis in the retina is greatest in fish and amphibians. In these cases, an actively proliferating population of cells in the peripheral retina, the ciliary margin zone (CMZ), contributes to retinal growth throughout the animal's life (Hollyfield, 1968; Johns, 1977). The observation of the fish CMZ led to the search for a similar region in birds and mammals. Retinal neurogenesis in the chick is completed by E12, but it was recently found that the posthatch chick also has a CMZ with the capacity to proliferate and produce mature neurons (Fischer and Reh, 2000). This ability is lost after the first few weeks posthatch. Multiple avian species appear to have a CMZ, but there is no evidence for a CMZ in mammals, with the possible exception of the opossum (Kubota et al., 2002).

Recently, a population of cells cultured from the pigmented ciliary margin (PCM) of the peripheral retina of multiple mammalian species was shown to proliferate indefinitely *in vitro* (Coles et al., 2004; Tropepe et al., 2000). The cells formed spheres and exhibited expression of retinal progenitor cell genes, as well as some neuronal markers, and were thus interpreted to be retinal stem cells. These cells are either rare or hard to culture as they were detected at a frequency of 0.6% of the PCM. In addition, such cells have been isolated from the iris (Haruta et al., 2001). The *in vivo* significance of these populations is unclear. It is

also unclear how these peripheral cells develop, and how they are lineally related to retinal progenitor cells, if at all. Interestingly, the frequency of the PCM stem cells was higher in the adult retina as opposed to the embryonic day 14 retina, suggesting that these cells may be formed at the end of development and not the beginning (Tropepe et al., 2000). Recently, the identity of these cells as retinal stem cells has been called into question (Cicero et al., 2009). They were shown to be PCM cells which express some markers of retinal cells upon exposure to the growth factors within the culture medium, but were not found to exhibit the properties of true retinal stem cells. When pushed to produce photoreceptors, for example, they failed to do so, while they maintained the markers of PCM cells. Their status as retinal stem cells is thus unclear.

In a study of retinal progenitor cells and their response to NICD, it was observed that progenitor cells overexpressing NICD traversed through retinal development without perturbing the temporally-appropriate expression profile (Jadhav et al., 2006a). Early progenitor cells overexpressing NICD appropriately expressed early progenitor genes without precocious expression of late progenitor or glial genes. Similarly, late progenitor cells overexpressing NICD appropriately downregulated the early progenitor genes and demonstrated expression of late progenitor genes. Ultimately, these cells initiated glial marker expression. Surprisingly, these cells can also could form neurospheres and be serially passaged, which is not typically of normal Muller glial cells (Jadhav et al., 2006a). These data suggest a progression wherein early progenitor cells produce or become late progenitor cells, which then become or produce Müller glial cells, which might in some cases also become or produce retinal stem cells. Additional lineage studies will be necessary to establish this relationship.

Müller glial cells can participate in adult neurogenesis: from home maker to cell maker

The long-standing view of Müller glial cells as passive support cells of the retina has been challenged by the discovery of conditions in which Müller glial cells of the zebrafish, chick, and mouse can exhibit neurogenic properties. To put these findings in context, it is again worth considering the observations of neurogenesis in fish. After the major phase of neurogenesis in the fish retina is complete, there is a late phase of rod photoreceptor neurogenesis by progenitor cells located within the INL, throughout the retina and not just in the periphery (reviewed in (Amato et al., 2004). The molecular and cellular characterization of this progenitor have not been carried out, so it is not certain what its relationship is to normal multipotent retinal progenitor cells, and to normal Muller glia. However, it might be a late progenitor cell of the type that makes Muller glia, or it might be Muller glia themselves. This possibility is raised by observations made on injured fish retinas. An INL progenitor cell in mature regions of the retina has been described that expresses Pax6, Notch3 and N-cadherin in response to injury (for review see Hitchcock et al., 2004). Yurco and Cameron showed that cells with Müller glial properties in the vicinity of a local mechanical ablation can re-enter cell cycle and produce neurons that populate the ablated region (Yurco and Cameron, 2005). The newly generated neurons appeared to use local Müller glia for migration to form the appropriate layers. Fausett and Goldman generated transgenic fish with a GFP transgene driven by an alpha 1 tubulin promoter fragment whose retinal expression was specific to Müller glia in injured retinas (Fausett and Goldman, 2006). They found that GFP⁺ cells divided and were the source of neurons in all laminae of the regenerated retina. Similarly, using a GFAP-GFP transgenic fish, which labels Müller glia only in the adult fish, Bernardos and Raymond (Bernardos et al., 2007) showed that Müller glia responded to the bright light ablation of photoreceptors by dividing and producing both rod and cone photoreceptor cells. The response was only in the ablated area and the regenerated area of the retina looked remarkably normal. Analysis of the molecular response to these injuries is ongoing (Fausett et al., 2008).

In vitro and *in vivo* studies have defined conditions in which Müller glial cells of the peripheral chick retina can proliferate and re-initiate expression of progenitor markers (e.g. Chx10, Pax6) (Fischer et al., 2002b; Fischer and Reh, 2000; Fischer and Reh, 2001). For example, visual deprivation or addition of insulin, insulin-like growth factor, or epidermal growth factor could induce proliferation of the peripheral Müller glial cells. Peripheral Müller glial cells were initially immunoreactive for the Müller marker, glutamine synthetase, but were not proliferating (GS+, BrdU-, GFAP-). Following one of the various treatments, approximately 35% of these cells initiated GFAP expression, whereas the remaining 65% did not become GFAP+, but were able to incorporate BrdU. An overwhelming majority of the BrdU+ cells initiated the expression of the progenitor markers, Chx10 and Pax6. A smaller percentage appeared to revert to the expression of the original set of markers, and thus perhaps revert to normal Müller glia. Most intriguing, a small fraction of cells (4%) expressed retinal neuron markers in the absence of glial or progenitor features. Additional experiments suggested that specific neuronal cell fates could be enhanced by pretreating the retina with particular toxins or introducing transcription factors that are known to promote particular cell fates (Fischer et al., 2002a; Fischer and Reh, 2002). The *in vivo* neurogenic potential of these Müller glial cells has thus been demonstrated, but has been noted to be of low efficiency. Furthermore, it is not known whether the Müller glial cell-derived neurons have functional capacity.

In order to address whether these observations in zebrafish and chicks are predictive of Müller glia behavior in the mammalian retina, several studies have now investigated the neurogenic potential of rodent Müller glial cells. As mentioned above, murine Müller glia do retain expression of the classic retinal progenitor cell genes, such as Sox2, Notch pathway genes, and cell cycle genes. In addition, at least some of them express Pax6 and Chx10. Interestingly, Chx10, as well as several Müller glial genes, are not expressed in all Müller glia, suggesting that there is heterogeneity among Müller glia, which might extend to variation in their abilities to generate retinal neurons (Roesch et al., 2008). All of these data point to Müller glia as a logical source of retinal neurons in mammals. Curiously, however, as summarized above, early reports on the formation of retinal neurospheres, the assay used for isolation of CMZ stem cells from mammals, did not report the production of neurospheres from the central retina (Tropepe et al., 2000). Non-activated Müller glia taken straight from an uninjured retina thus may not have self-renewal properties, and/or may not have the response that stimulates cell division under the neurosphere culture conditions. However, manipulations wherein the Müller glia are first stimulated to divide in a particular culture condition, and then cultured in neurosphere conditions, allows them to form neurospheres (Das et al., 2006). Transplantation of these cells *in vivo* has demonstrated their ability to produce some neurons with retinal antigens, with expression appropriate to the location of the engrafted cells. Interestingly, when injured retinas are analyzed and their cells sorted by FACS prior to culture, cells with Müller glial markers could be identified by the Hoechst dye efflux assay (i.e. they appear as a side population (SP) in the FACS). This assay has been used to identify SP cells as stem cells in multiple tissues, and suggests that the Müller glia isolated following injury have been induced to a stem cell state. Very few Müller glia from non-injured retinas were recovered as SP cells. Stimulation or inhibition of either the Notch or Wnt pathways showed that SP cells were increased by Notch and Wnt pathway activation, and decreased by inhibition of these pathways. Neurosphere generation was similarly effected. Again, the response to these factors suggests that Müller glia have features in common with stem cells from other tissues.

More important than the *in vitro* behavior of Müller glia is their ability to divide and produce neurons *in vivo*. The Takahashi laboratory treated rat retinas *in vivo* with the neurotoxin, N-methyl-D-aspartate (NMDA), at doses that kill ganglion cells and some cells of the INL (Ooto et al., 2004). They found cells with Müller glial properties entered the cell

cycle and produced neurons that migrated to various retinal laminae. The number of such neurons was quite small. However, they could stimulate the production of different types of neurons using either environmental cues or transcription factors delivered by retroviruses. The Chen lab found that treatment of retinas with an alpha amino adipate, which can mimic glutamate in terms of binding to glutamate receptors and transporters, led to the proliferation of Müller glia (Takeda et al., 2008). This drug apparently acts directly only on Müller glia, and it was administered at a dose that did not lead to Müller glia death, only Müller glia proliferation. The BrdU+ cells moved into the ONL and differentiated into cells expressing rod photoreceptor markers, as well as exhibited the nuclear morphology of rod photoreceptor cells. The amount of proliferation was quite substantial, yet was limited in that it did not lead to an overproduction of cells, or dysplasia. The laboratory of Takahashi further explored regeneration from Müller glia in mammals (Osakada et al., 2007). They stimulated Müller glia proliferation using Wnt3A, or a Wnt agonist. This led to the production of photoreceptor cells, particularly if they introduced the Crx gene, which, as shown in previous studies (Haruta et al., 2001), led to the production of more cells that expressed photoreceptor specific genes.

The recent studies of regeneration by Müller glia suggest that the stimulation of Müller glia proliferation is a promising avenue for the development of therapies for the replacement of retinal neurons. In the many cases where a genetic defect leads to degeneration, a repair strategy can be undertaken. The Müller glia can be genetically treated such that their progeny would be repaired e.g. for defective photoreceptor genes, and thus the photoreceptor cells so produced would presumably be functional and perhaps able to participate in retinal circuitry. Stimulation of neuronal replacement by endogenous Müller glia would also bypass two problems faced by the engraftment of cells into the subretinal or vitreal space. Many engrafted cells do not enter the retina proper, but remain in the subretinal space or vitreous body (e.g. (Singhal et al., 2008)). This may be in part due to an environment that blocks their integration, which might include Müller glial processes with IF proteins (Kinouchi et al., 2003). The production of retinal neurons from within the retina by Müller glia would presumably not lead to this type of problem, though such neurons would still need to migrate to their proper position and, ideally, form proper synapses. Migration was demonstrated in the afore-mentioned studies, though synapse formation has not been demonstrated. An additional problem faced by engrafted cells is the problem posed by the immune system. It is not clear if there is long-term immune privilege in the retina, and thus it is not clear whether autologous tissue would be needed for engraftment. Again, regeneration from endogenous Müller glia would bypass this problem.

Gliosis versus neurogenesis

While Müller glial cells may have the potential to be neurogenic in some conditions, there is no evidence for significant Müller glial-derived cell replacement in the setting of mammalian eye disease. Instead, most disease processes result in reactive gliosis. Interestingly both neurogenesis and gliosis are characterized by a retinal insult followed by glial cell de-differentiation, proliferation and often migration of their nuclei to the apical surface. This raises the question of whether these two processes originate from a common initial pathway which then diverges to serve different responses to neuronal injury.

Interestingly, injured Müller glial cells in the zebrafish retina have been shown to have features of both gliosis (upregulation of GFAP) and neurogenesis (upregulation of progenitor genes) (Hitchcock et al., 2004). In the chick retina, it appears that the addition of CNTF is sufficient to induce GFAP expression in normal Müller glia (Fischer et al., 2004). In contrast, the addition of FGF2 and insulin does not promote GFAP expression, but instead induces the expression of neuronal markers (e.g. neurofilament) (Fischer et al., 2004). Furthermore, only young (2–3 weeks post-hatch) and peripheral Müller glial cells

appear to respond by neurogenesis whereas older and more centrally located cells do not (Fischer and Reh, 2000; Fischer and Reh, 2001). These experiments would suggest that perhaps younger Müller glia are more similar to the progenitor state and therefore more receptive to neurogenic cues, or perhaps that peripheral cells are proximally located to a necessary progenitor factor secreted by the peripheral retina.

Together, these studies support the possibility of a normal developmental switch that allows gliosis vs. neurogenesis, or induction of one vs. the other in subpopulations of Müller glia. Understanding the molecular profile of maturing Müller glia, central versus peripheral Müller glia, and Müller glia in a compromised environment would help highlight the differences among these various glial states. It is also of great interest to understand whether simply re-introducing progenitor genes, such as *Rax*, *Pax6*, *Chx10* or *Notch*, or antagonizing *Notch* just after its activation, would be sufficient to switch a gliotic response to a neurogenic response, or induce a neurogenic response. Having a catalogue of genes expressed in normal Müller glia, as well as in Müller glia in the various locations and states, might suggest more targeted strategies towards specific manipulations in specific disease states.

CONCLUSION

Glial cells as a therapeutic target and tool

In summary, studies to date have established an integral role for normal glial function in ensuring the necessary environment for proper retinal activity. An examination of Müller glial cell development has not only defined some of the factors that govern glial cell identity, but it has also revealed striking similarities between Müller glial cells and retinal progenitor cells. The *in vivo* potential of mammalian retinal stem cells and Müller glial cells to contribute to adult neurogenesis is unclear, although there do appear to be certain conditions under which glia can de-differentiate, proliferate and produce cells with neuronal features.

Further experiments will be necessary to define the nature and significance of gliosis. In particular, it will be important to understand how and if this process relates to neurogenesis. Therapies targeted at minimizing gliosis or diverting it to neurogenesis may be relevant to disease processes adversely affected by gliosis, such as diabetic retinopathy, or for purposes of cell replacement in the event of retinal degeneration and glaucoma.

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Figure 1. Similarities between Müller glia and progenitor cell gene expression profiles

A subset of genes with their Affymetrix signals from 21 immature neurons and 2 adult rods (Trimarchi et al., 2007) and 21 retinal progenitor cells (Trimarchi et al., 2008) are illustrated in this heatmap. Genes considered to be classic progenitor genes (Dkk3, Chx10, Pax6, Notch1, Hes1), as well as other genes expressed in common in Müller glia and retinal progenitor cells, have been grouped together. Late progenitor cells, but not early progenitor cells, have a group of genes in common with Müller glia (Trimarchi et al., 2008), as indicated. Rows correspond to different Affymetrix probe sets for the indicated genes; columns represent the signals from single cell probes. Bright red represents signal over 10,000, black as <1,000 and graded is in between.

Figure 2. Gene expression in Müller glia
This heat map represents the expression levels of the Müller glia markers Rlbp1, Kir4.1, S100 calcium binding protein and Aqp4, and also includes other genes, such as Dbi and Opioid growth factor receptor-like 1, with potentially important roles in Müller glia function. Rows correspond to different Affymetrix probe sets for the indicated genes; columns represent the signals from single cell probes. Bright red represents signal over 10,000, black as <1,000 and graded is in between.

Figure 3. Müller glia heterogeneity

Dissociated cell ISH shows heterogenous expression of *Rlbp1* in only a subset of Müller glial cells. A–I: Retinas from mature mice were dissociated, plated on slides, and processed for detection by ISH for the indicated probes (*Clu*, *Itm2b*, *Rlbp1*). In a second step immunostaining for *Glul* (a Müller glial marker, glutamine synthase) and DAPI was performed. A heat map showing expression levels in the single Müller glia cells is shown on the right, with bright purple signal representing signal >10,000. From (Roesch et al., 2008).

Figure 4. A model of Müller glial cell genesis and response to retinal injury

Retinal progenitor cells (RPC) express characteristic genes including Rax, Pax6, and Chx10, and appear to remain proliferative and undifferentiated, at least partially, under the influence of Notch activity. Early during development, a subset of progenitor cells downregulate Notch activity, exit cell cycle and give rise to early born neurons, including retinal ganglion cells, cone photoreceptor cells, amacrine cells and horizontal cells. Under the influence of Notch, Rax, Chx10 and other progenitor genes, a fraction of the RPCs remain undifferentiated and reach a later stage of development during which both neurogenesis and gliogenesis is possible. The late RPCs downregulate Notch activity, exit cell cycle and are then able to commit to a neuronal or glial fate. High levels of positive bHLHs favor the neuronal fate. A reinitiation of the Notch pathway and concomitant upregulation of negative bHLHs favors the glial fate. Other pro-gliogenic factors, such as p27, Sox9, and EGF signaling, are most likely also involved. Neuronal insult in the form of injury, disease or hypoxia, leads to various changes including increased permeability of the blood retinal barrier and subsequent release of inflammatory mediators. These factors trigger glial activation, which initially manifests as a downregulation of the Kir channel activity. During this phase, proliferation is not observed. Decreased Kir channel activity along with activation of the BK channel characterizes proliferative gliosis. Downregulation of p27 appears to be the initial molecular alteration leading to cell cycle re-entry. At this time, intermediate filaments such as vimentin and GFAP are upregulated. Subsequent downregulation of cyclin D3 appears to limit the number of cell cycles that Müller glia undergo. Production of neurons might occur in only a subset of Müller glia, using signals that might be distinct from those that induce gliogenesis.

Table 1
Enriched molecular features of mature Müller glial cells

The IPA tool (Ingenuity Systems) was used to systematically analyze signaling and metabolic pathways present in mature Müller glia. Only genes with average expression values in all Müller glia of > 10,000 on the microarray were included for this analysis. Genes expressed in Müller glial cells also were compared to genes with > 10,000 averaged gene expression values in immature retinal amacrine and ganglion cells, as well as adult bipolar cells. The ratio is calculated as “number of molecules in a given pathway divided by the total number of molecules that make up the pathway”. A summarized comparison of Müller glia to the other cell types is shown in a color-coded manner, where “Red” represents a pathway only expressed in Müller glia, “Orange” highly enriched in Müller glia and “Yellow” enriched in Müller glia. From (Roesch et al., 2008).

Signaling Pathway	Ratio	Metabolic Pathway	Ratio
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	1.40E-01	Oxidative Phosphorylation	1.77E-01
Chemokine Signaling	1.20E-01	Inositol Metabolism	1.60E-01
Mitochondrial Dysfunction	1.09E-01	Ubiquinone Biosynthesis	1.06E-01
Hypoxia Signaling in the Cardiovascular System	9.86E-02	Glycolysis/Gluconeogenesis	7.80E-02
Regulation of Actin-based Motility by Rho	9.78E-02	N-Glycan Degradation	6.67E-02
Notch Signaling	9.76E-02	Citrate Cycle	5.08E-02
NRF2-mediated Oxidative Stress Response	9.44E-02	Glycosphingolipid Biosynthesis - Globoseries	4.88E-02
GABA Receptor Signaling	9.43E-02	Glutathione Metabolism	4.81E-02
Ephrin Receptor Signaling	9.24E-02	Fructose and Mannose Metabolism	4.32E-02
Glutamate Receptor Signaling	8.96E-02	Purine Metabolism	4.31E-02
PI3K/AKT Signaling	8.87E-02	Pyrimidine Metabolism	3.95E-02
IGF-1 Signaling	8.70E-02	Aminosugars Metabolism	3.85E-02
SAPK/JNK Signaling	8.60E-02	Glycerolipid Metabolism	3.47E-02
ERK/MAPK Signaling	8.33E-02	Glycosphingolipid Biosynthesis - Ganglioseries	3.45E-02
Integrin Signaling	8.33E-02	Glycosaminoglycan Degradation	3.28E-02
Tight Junction Signaling	8.18E-02	Sphingolipid Metabolism	3.26E-02
Wnt/beta-catenin Signaling	7.27E-02	Linoleic Acid Metabolism	3.20E-02
Cardiac α -adrenergic Signaling	6.92E-02	Starch and Sucrose Metabolism	3.12E-02
Estrogen Receptor Signaling	6.78E-02	Methane Metabolism	3.08E-02
PDGF Signaling	6.76E-02	Phenylalanine, Tyrosine and Tryptophan Biosynthesis	3.08E-02
Aryl Hydrocarbon Receptor Signaling	6.58E-02	Phospholipid Degradation	3.03E-02
Axonal Guidance Signaling	6.48E-02	Nitrogen Metabolism	3.01E-02
Sonic Hedgehog Signaling	6.45E-02	Arachidonic Acid Metabolism	2.82E-02
Ceramide Signaling	6.33E-02	Pyruvate Metabolism	2.76E-02
Leukocyte Extravasation Signaling	6.28E-02	Galactose Metabolism	2.68E-02
Huntington's Disease Signaling	6.03E-02	Stilbene, Coumarine and Lignin Biosynthesis	2.67E-02
Actin Cytoskeleton Signaling	5.96E-02	One Carbon Pool by Folate	2.63E-02
Protein Ubiquitination Pathway	5.94E-02	Glutamate Metabolism	2.56E-02
Parkinson's Signaling	5.88E-02	Aminoacyl-tRNA Biosynthesis	2.35E-02
Nitric Oxide Signaling in the Cardiovascular System	5.88E-02	O-Glycan Biosynthesis	2.33E-02
Amyotrophic Lateral Sclerosis Signaling	5.83E-02	Metabolism of Xenobiotics by Cytochrome P450	2.33E-02
Nucleotide Excision Repair Pathway	5.71E-02	Alanine and Aspartate Metabolism	2.30E-02
Neuregulin Signaling	5.49E-02	N-Glycan Biosynthesis	2.30E-02

Signaling Pathway	Ratio	Metabolic Pathway	Ratio
VEGF Signaling	5.43E-02	Glycerophospholipid Metabolism	2.25E-02
Synaptic Long Term Potentiation	5.41E-02	Pentose Phosphate Pathway	2.25E-02
cAMP-mediated Signaling	5.03E-02	Fatty Acid Metabolism	2.12E-02
Synaptic Long Term Depression	4.94E-02	Fatty Acid Biosynthesis	2.04E-02
TGF-beta Signaling	4.82E-02	Aminophosphonate Metabolism	1.96E-02
Xenobiotic Metabolism Signaling	4.80E-02	Phenylalanine Metabolism	1.87E-02
PTEN Signaling	4.35E-02	Pantothenate and CoA Biosynthesis	1.59E-02
PPAR Signaling	4.21E-02	Retinol Metabolism	1.59E-02
p38 MAPK Signaling	4.21E-02	Lysine Biosynthesis	1.56E-02
Neurotrophin/TRK Signaling	4.11E-02	Glycosphingolipid Biosynthesis -Neolactoseries	1.49E-02
Glucocorticoid Receptor Signaling	4.00E-02	Androgen and Estrogen Metabolism	1.46E-02
T Cell Receptor Signaling	3.92E-02	Selenoamino Acid Metabolism	1.45E-02
G-Protein Coupled Receptor Signaling	3.52E-02	Glycine, Serine and Threonine Metabolism	1.38E-02
Acute Phase Response Signaling	3.49E-02	Urea Cycle and Metabolism of Amino Groups	1.25E-02
p53 Signaling	3.45E-02	Tyrosine Metabolism	1.08E-02
Apoptosis Signaling	3.45E-02	Glyoxylate and Dicarboxylate Metabolism	8.77E-03
B Cell Receptor Signaling	3.38E-02	Pentose and Glucuronate Interconversions	6.71E-03
Dopamine Receptor Signaling	3.30E-02		
TR/RXR Activation	3.26E-02		
GM-CSF Signaling	3.23E-02		
Phototransduction Pathway	3.17E-02		
LPS/IL-1 Mediated Inhibition of RXR Function	3.06E-02		
Insulin Receptor Signaling	3.01E-02		
Antigen Presentation Pathway	2.56E-02		
Calcium Signaling	2.46E-02		
Eicosanoid Signaling	2.38E-02		
PXR/RXR Activation	2.33E-02		