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TITLE PAGE
Title: Mechanisms of Müller glial cell morphogenesis

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Abstract:
Müller Glia (MG), the radial glia cells of the retina, have spectacular morphologies subserving their enormous functional complexity. As early as 1892, the great neuroanatomist Santiago Ramon y Cajal studied the morphological development of MG, defining several steps in their morphogenesis [1,2]. However, the molecular cues controlling these developmental steps remain poorly understood. As MG have roles to play in every cellular and plexiform layer this review discusses our current understanding on how MG morphology may be linked to their function, including the developmental mechanisms involved in MG patterning and morphogenesis. Finally, uncovering the mechanisms governing glial morphogenesis, using transcriptomics and imaging, may provide shed new light on the pathophysiology and treatment of human neurological disorders.

Highlights:
- MG have intricate morphologies amongst the neural layers of the retina
- MG are patterned in a “tiled” configuration amongst the neurons
- The mature MG morphology requires contact inhibition via homotypic interactions
- Imaging and transcriptomics are key to identifying mechanisms of MG development

Introduction
Glial cells are a morphologically diverse group of cells in the nervous system. Each glial cell type is morphologically specialised to perform critical roles in the regulation of the structure, development, and physiological function of the nervous system [3]. Glial cell dysfunction has been associated with several neurological disorders [4] and may even precede cognitive dysfunction in neurodegenerative disease [5,6]. Radial glia are mostly known for their role in cortical development where they serve as primary progenitor cells capable of generating neurons, astrocytes, and oligodendrocytes [7]. In 1851, Heinrich

Müller observed “radial fibers” in the retina. These turned out to be the first images of a neuroglia and were later renamed Müller glia (MG) in his honour [8]. MG have a variety of retinal specific functions, including developmental [9], physiological [10-14], structural [12,15,16] and even optical [12,17-19]. MG have also been implicated in many retinal diseases [20,21] and have the ability to regenerate the retina in some vertebrate species [22,23]. Despite the abundance of knowledge about MG, we know surprisingly little about the developmental processes that govern their morphology and pattern in the retina.

The genesis of the MG

During embryogenesis, the retina is generated from radial cells called retinal progenitor cells (RPCs) [24]. Retinogenesis culminates with these RPCs being depleted after producing five main types of neuron and the MG. MG are specified near the end of RPC lineages [24,25]. In fact, MG begin their postmitotic lives greatly resembling RPCs with simple apical and basal process attached to the outer and inner limiting membranes of the retina, respectively **(Figure 1)** [15,26]. It seems possible that after the RPC lineage, MGs arise as a “default” without undergoing irreversible cell fate determination [9], and thus inherit the bipolar radial morphology from the parent RPC. This idea is supported by the remarkably similar transcriptional profile between RPCs and MG [27,28] and time-lapse observations of single RPCs in a clone, in which one cell will up-regulate Notch signaling, maintain the radial morphology and differentiate into a MG cell [15].

While the radial morphology of MG is striking, perhaps even more impressive are the extensive specialised processes that emanate from the central stalk to make connections with neurons and blood vessels. Once the MG is specified to the glial fate the nucleus undergoes an apical to basal migration to its’ characteristic position between bipolar and amacrine cells in the inner nuclear layer [15]. Then, MG cells begin to elaborate processes into several retinal layers. Recently, Wang et al. [26] carried out a high-resolution analysis of MG morphology in the mouse retina using mosaic analysis of membrane bound proteins. This included the characterisation of the fine processes of MG cells. In this analysis it was shown that MG have symmetric branching from the central stalk in each layer and feature differing morphologies between layers. These domains are specialised for specific functions related to the specific neurons and synapses in each layer, as well as the blood vessels and other glial cells that they contact in each retinal layer [29]. Each MG cell has at least five domains within the retina [26] (**Figure 1**): 1) a basal oriented process that forms an endfoot on the extracellular matrix of the inner limiting membrane, 2) fine processes emanating from the MG core that contact synapses of the inner plexiform layer, 3) a cell body that is positioned amongst bipolar and amacrine cell neurons in the inner nuclear layer, 4) additional processes that contact synapses in outer plexiform layer, 5) an apical stem process that elaborates around photoreceptor cell bodies with the apical microvilli extending to photoreceptor inner segments. These domains can vary greatly between vertebrate species depending on scotopic or photopic specialisations of the retina supporting the idea that distinct morphologies are required for different functions [11]. Cajal showed that there are many species-specific

differences in these domains (**Figure 2**), while the overall MG morphology remains conserved [1,2].

The radial morphology and supportive functions of the MG cell are reliant on the establishment and maintenance of apicobasal polarity during development. Polarity is set up in the retina during development from signals conferred by the basement membranes, where initially RPCs and later the MG are attached [30]. Defects in these polarizing signals, such as laminins, result in disruption of MG polarity and lead to abnormal physiology due to the misexpression of functional enzymes within MG domains [30,31]. Once polarity is established in the MG cell, the localization of intrinsic polarity factors play an important role in maintaining this radial morphology. For example, the zebrafish P50 *dynactin* mutant results in a mislocalisation of apical determinants leading to a loss of MG apical processes and displaced cell bodies [32]. Furthermore, loss of Crumbs1, a protein necessary for the formation of adherens junctions at the apical pole, with consequences for the surrounding neurons and blood vessels reminiscent of the human disease Retinal Telangiectasia [33,34].

Patterning of MG

One of the most obvious features of MG anatomy is their even spacing or "tiling" within the retina [26,35] (**Figure 3**). This tiling results in specific MG domains containing minimally overlapping processes around the surrounding neurons. Tiling is seen in astrocyte domains in many regions of the mammalian brain [36,37]. Interestingly, tiling differs between brain regions and species, as different classes of astrocytes have varying levels of overlap between their spatial domains [38]. "Brainbow" studies in the mouse retina have shown there is minimal overlap between MG cell processes at several retinal layers, including in the plexiform layers, with each MG occupying unique territories [26]. Repulsive homotypic interactions between glia have been described for mammalian astrocytes [36] and oligodendrocytes [39], leading to the idea that these interactions may be a patterning mechanism employed by many types of glia during tiling. To test if this mechanism exists in MG, Williams et al. used laser ablation to remove a small number of MG cells from the embryonic zebrafish retina [35]. This ablation resulted in a gap in the tiled pattern, including a loss of glial processes within the IPL. Shortly after ablation the neighbouring MG processes move into the voided territory and filled the hole [35]. This indicates that neighbouring MG cells actively repel each other via contact inhibition and carve out their respective spatial territories via homotypic interactions (i.e. MG to MG). While the positioning of MG processes clearly relies on specific interactions, the MG cell body does not seem to conform to the same constrains. Wang and colleagues used cell labelling and modelling to show that the spatial organisation of MG cell bodies appears to be random, with no extrinsic input from neighbouring MG other than the physical restrictions inferred by the surrounding cells [26]. Thus, it remains unclear whether and how homotypic interactions are acting to sculpt overall MG morphology and tiling in the retina during development. The signals conferring this repulsive interaction between neighbouring MG cells are also completely unknown. In fly astrocytes, FGFs and their receptors regulate glial morphology amongst neuronal synapses and overall

morphology by controlling dynamic homotypic interactions between them to establish their tiled domains [40]. It is unknown if similar mechanisms translate to vertebrates.

Emergence of processes

In the 1890s, Cajal described the morphogenesis of MG as an inside to outside process, as if the MG were splitting apart retinal elements repeatedly in this direction [1,2]. As development proceeds, processes first emerge from the main shaft of the MG in the inner layers of the retina, later emerging in the plexiform and outer nuclear layers (**Figure 4**). Finally, processes associated with the outer limiting membrane appear and send descending sheaths of process between the outer segments of the photoreceptors [1]. The emergence of these different functional domains is one of the least explored areas of MG morphogenesis. It is known that MG processes are guided directly to the plexiform layers during development [35], where they are precisely positioned amongst sublaminae indicating active guidance by specific neurons [26]. Synaptic activity may play a role in the tiling of MG processes within the plexiform layers. In rabbits, MG elaborate their processes in the presence of retinal activity [41]. Similarly in fish, MG processes elaborate after retinal specification is complete but just before robust vision commences [35]. Prior to the completion of synaptogenesis calcium transients are propagated within the MG cell by neurotransmitter spillover at the synaptic cleft. As the retina matures and neural connections are strengthened these transients recede [42]. This data suggests that MG and neurons communicate during development and potentially refine their processes controlled by neural activity. This has been noted in the hippocampus as astrocytes change their fine processes in response to glutamate release and calcium [43]. These data point to neural activity playing an active role in MG process sublamination, although the inverse scenario doesn't seem to be true as MG are not required for neurons to sublaminate within the plexiform layers [44].

Imaging MG development *in vivo*

As described above, the morphology and pattern of MG cells may be sculpted by cellular interactions between neurons and glia during development. However, the progression of MG morphogenesis and patterning has largely been studied in fixed tissues at defined time points [15,26,35,41]. This is insufficient to capture any highly dynamic cell contacts that may mould the exact position of MG processes in the cellular environment. To study these cell interactions in real developmental time requires the use of specific cellular markers or vital dyes paired with time-lapse imaging. For example, i*n vivo* time-lapse imaging in the zebrafish retina showed that MG are highly dynamic both in terms of cell body position and branching [15,35]. Time-lapse studies of cultured retina are also fruitful for uncovering these interactions, however MG have been reported to lose their morphology and rapidly de-differentiate in culture experiments [45-47]. More promising *in vitro* studies come from the advances in retinal organoid cultures that now provide a promising platform for both morphological analysis and genetic manipulations in chick, zebrafish, mammal and possibly human retinas [48-51]. Pairing one or more of these models to advanced imaging, such as confocal or light sheet microscopy, will

allow for the characterisation of the specific cell-cell interactions during the process of glial patterning and morphogenesis.

Molecular analysis of MG morphogenesis

There have been several molecular pathways identified as key regulators of MG fate, including Notch signalling [9]. While Notch is critical for MG fate, it does not appear to have any role in overall MG morphogenesis or patterning after the cell is specified in the zebrafish [15]. It is therefore necessary to begin to uncover the specific postmitotic pathways that guide the various aspects of MG morphogenesis. Many novel markers and potential molecules involved in MG morphogenesis are being discovered through recent boom in sequencing and proteomic technologies. For example, a recent study used single cell RNA sequencing to match molecular classification with cell morphology and successfully identified several novel markers for each unique bipolar subtype in the retina [52]. There are already multiple readily available transcriptome data sets for MG [27,28,53-56]. Furthermore, miRNA expression profiling and proteomics of have been used to identify a number of new MG specific markers [47,57,58]. Microarray studies on developing MG have provided large lists of genes that can be used to identify novel markers of these cells during development [28,59] and regeneration [53,54]. In many of these cases, transcriptomics were carried out at several stages during early MG development. However, the time scale of cells selected has not yet been correlated to specific stages of MG morphogenesis, which would help suggest which molecular pathways are involved in which aspects of MG morphogenesis. Importantly, in these studies MG have been treated as a single uniform population, however, previous studies have shown that central MG, express Pax2 while peripheral MG do not [60,61]. Moreover, the recently identified retinal glia of the *Drosophila* eye share striking morphological and molecular characteristics with MG and these too are sub-classed by Pax2 expression [62,63]. Thus, it will also be beneficial to isolate and compare these sub-populations in future studies.

Combining the power of genetics, imaging, transcriptomics and genome editing capabilities will facilitate great strides in the understanding of glial development, including morphogenesis. Transcriptome datasets provide many candidate genes or pathways but without functional testing, this knowledge is of limited value. The advent of CRIPSRs to conduct reverse genetic screens provides a promising tool for future large-scale reverse genetic screens in vertebrate models [64]. Any gene shown to have a MG morphological defect will almost certainly need to be analysed with time-lapse imaging to fully characterise the candidates' role in glial development.

Conclusions: MG, Astrocytes and neuropathology

MG have been described as the molecular and functional homologues to both radial glia and astrocytes [10,21], leading to the intriguing potential that the molecular mechanisms regulating MG shape and patterning may have broader implications for the development of many different glia types. An important consideration is not only how glial cell morphology is instructed during development, but also how is it maintained throughout life and altered in the pathology of neurological disease. With age, it has been shown that astrocyte domains more than doubled their process territory overlap with their

neighbours [65]. Region specific glial morphology and functional defects have been noted in several neurological diseases [4,6]. For example, alterations in glial cell morphology and organization have been noted in the epileptic brain [66]. Intriguingly, after drug treatment the astrocyte domains recede and the seizures are reduced [66], but it is unclear which one begets the other. It is becoming increasingly accepted the glia may provide opportunities for pharmacological intervention in neurodegeneration [5,6]. So perhaps treating or preventing glia morphology changes is an important step in limiting or reversing the pathology of disease. Thus, the MG cell provides an attractive model to identify and understand the molecular mechanisms regulating glial cell shape in development and may lead to a better understanding and treatment of human disease.

Figure 1. The pattern and morphology of the Müller glia in the retina. A) Development of MG (green) from RPCs **B)** Schematic showing the general organization of the vertebrate retina. MG span the entirety of the three neural layers from the apical outer limiting membrane (OLM) to the basal inner limiting membrane (ILM). The morphology of a single MG (example from zebrafish) can be separated into **five** distinct domains: **1**) The basal endfoot forming the ILM of the retinal ganglion cell (RGC) layer; **2**) the fine processes contacting synapses in the inner plexiform layer (IPL); **3**) the cell body amongst neurons in the inner nuclear layer (INL); **4**) the fine processes contacting synapses in the outer plexiform layer (OPL); **5**) an apical process around photoreceptors.

Figure 2. Species specific Müller glia morphologies. Cajal's drawings [2] of the structural differences between MG from frogs, carp, lizards, chickens and cows.

Figure 3. Müller glia "tile" to form an extensive glial network. MG cells have their own unique spatial domains with little to no overlap of glial processes in any of them.

Figure 4. Müller glia development. Schematic representation of the morphological changes that occur over the course of zebrafish MG development [15, 35].

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