

Development of Retinal Amacrine Cells and Their Dendritic Stratification

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Abstract The mammalian retina contains multiple neurons, each of which contributes differentially to visual processing. Of these retinal neurons, amacrine cells have recently come to prime light since they facilitate majority of visual processing that takes place in the retina. Amacrine cells are also the most diverse group of neurons in the retina, classified majorly based on the neurotransmitter type they express and morphology of their dendritic arbors. Currently, little is known about the molecular basis contributing to this diversity during development. Amacrine cells also contribute to most of the synapses in the inner plexiform layer and mediate visual information input from bipolar cells onto retinal ganglion cells. In this review, we will describe the current understanding of amacrine cell and cell subtype development. Furthermore, we will address the molecular basis of retinal lamination at the inner plexiform layer. Overall, our review will provide a developmental perspective of amacrine cell subtype classification and their dendritic stratification.

Keywords Retina · Amacrine cells · Retinogenesis · Transcription factors · Dendritic stratification

Introduction

The neural retina is broadly categorized into seven distinct cell types (Fig. 1a): retinal ganglion cells, amacrine cells, bipolar

cells, Müller glial cells, horizontal cells, and rod and cone photoreceptor cells. Anatomically, the mammalian retina is composed of three layers of nerve cell bodies (ganglion cell layer, inner nuclear layer, and outer nuclear layer) and two layers of synapses (inner plexiform layer and outer plexiform layer). The ganglion cell layer (GCL) contains cell bodies of ganglion cells and displaced amacrine cells. The inner nuclear layer (INL) contains cell bodies of amacrine cells, Müller glial cells, horizontal cells, and bipolar cells. The outer nuclear layer (ONL) contains cell bodies of cone and rod photoreceptors. Connections between bipolar cells, horizontal cells, and cone and rod photoreceptors occur in the outer plexiform layer (OPL), while that between bipolar cells, amacrine cells, and ganglion cells occur in the inner plexiform layer (IPL) [1, 2]. The diversity of retinal neurons goes further beyond retinal cell classification with each retinal cell type being further classified into many subtypes [3]. This neuronal diversity is brought about during retinogenesis by the changing competence of progenitor cells with time [4]. This leads to the specification and differentiation of different retinal cell classes and their subtypes and a distinction in their laminar stratification.

Recently, amacrine cells and their multitude of subtypes have been of particular interest, partly because amacrine cells display the highest degree of diversity in both morphology and function in the retina [5, 6]. So far, up to thirty distinct subpopulations of amacrine cells have been identified based on the neurotransmitter type they express, dendrite morphology, and/or functional characteristics [3, 5]. However, the list is still growing and the precise number of subtypes of amacrine cells is currently unknown.

Subtype Classification of Amacrine Cells

Amacrine cells have been categorized into at least 33 different subtypes based on their dendritic morphology and

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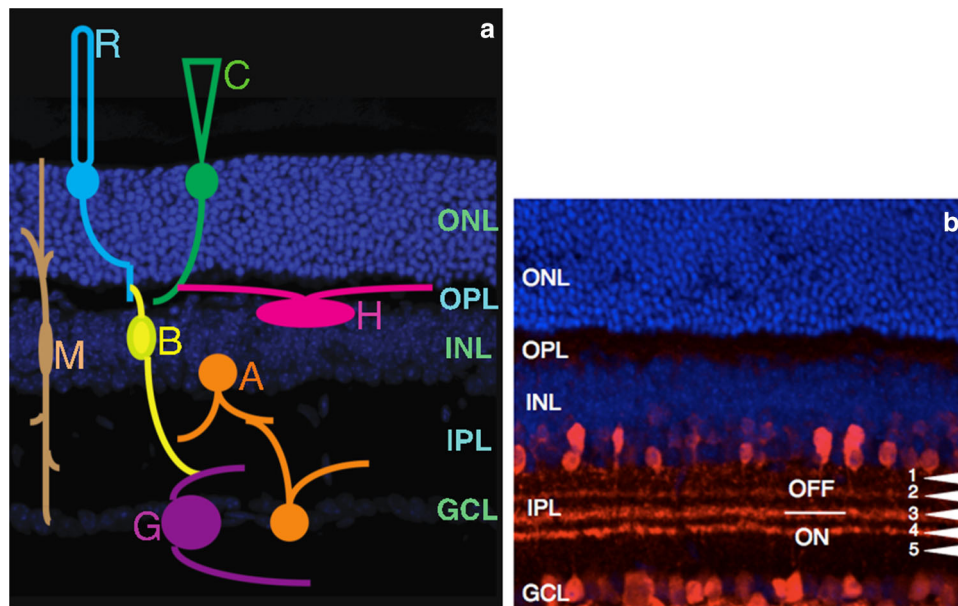


Fig. 1 Diagrammatic representation of retinal neurons in distinct retinal layers. **a** Localization of rod and cone photoreceptors and their cell bodies in the ONL; horizontal cells, bipolar cells, amacrine cells, and Müller glial cells in the INL; and retinal ganglion cells and displaced amacrine cells in the GCL. **b** Anti-calretinin staining (red) of the IPL marks 4 of the 5 sublaminae. S1 and S2 are in the OFF lamina, S4 and S5 are in the ON lamina, and S3 is a watershed region

between the ON and OFF lamina. Sections are counterstained with DAPI (blue). *R* rod photoreceptor, *C* cone photoreceptor, *M* Müller glial cell, *B* bipolar cell, *A* amacrine cell, *H* horizontal cell, *G* retinal ganglion cell, *ONL* outer nuclear layer, *OPL* outer plexiform layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *GCL* ganglion cell layer (Color figure online)

stratification. Pioneering studies by Masland, Vaney, Wässle, and others identified these subtypes of amacrine cells in the rodent retina [6–15]. Based on how proliferative their dendritic arbors are, they are categorized as narrow-field amacrine cells (dendritic arbor of around 70 μm in diameter), medium-field amacrine cells (dendritic arbor of around 170 μm in diameter), and wide-field amacrine cells (dendritic arbor of about 350 μm in diameter). Amacrine cells contribute toward majority of synapses in the IPL. Based on axonal projections of OFF and ON bipolar cells, the IPL is divided into an OFF lamina and an ON lamina, respectively. These laminae are further divided into five parallel sublaminae (S1–S5) based on the dendritic stratification of subtypes of amacrine cells [1, 2] (Fig. 1b).

Based on their ramification in the IPL, amacrine cells are classified into various subtypes. The details of individual subtypes categorized based on morphology will not be discussed here, an extensive review being available in [6, 10].

Amacrine cells are inhibitory interneurons that project their dendritic arborization to the IPL where they intercept retinal ganglion cells and/or bipolar cells. Based on the inhibitory neurotransmitter type they express, amacrine cells are classified into two major subpopulations: GABAergic and glycinergic types [6, 10]. A small population of

amacrine cells has also been found to be neither GABAergic nor glycinergic [16]. GABAergic amacrine cells are distributed in both the GCL as well as the INL, have mostly wide-field dendritic arbors, and can be further classified by their expression of neurotransmitters and neuropeptides such as choline acetyl transferase (ChAT), neuropeptide Y (NPY), tyrosine hydroxylase (TH), or nitric oxide synthase (NOS) [12, 17–21]. Glycinergic amacrine cells are largely restricted to the INL, have mostly narrow-field dendritic arbors, and can be further identified by the expression of Disabled-1 (*Dab1*), parvalbumin (PV), or vesicular glutamate transporter type 3 (VGLut3) [12, 13, 14, 22–25]. Developmentally, GABAergic amacrine cells are born first with their developmental peak at about E14, while glycinergic amacrine cells are born later with peak of birth at about P0 [26].

Neurotransmitters and neuropeptide markers are useful in distinguishing between the different cell subtypes of amacrine cells. Calcium binding proteins such as calbindin and calretinin have also been used to characterize amacrine cells and specifically to visualize their stratification in the IPL. Depending on the species, calretinin, for instance, marks a subclass of amacrine cells and retinal ganglion cells. It also labels three prominent strata of the IPL—S2, S3, and S4, with weak expression also seen in S1 (Fig. 1b). Calbindin marks horizontal cells, some amacrine cells and

ganglion cells and also labels S2, S3, and S4 strata of the IPL [12•].

A significant percentage of GABAergic amacrine cells that express ChAT (otherwise known as starburst amacrine cells) are present both in the INL and GCL. Cholinergic amacrine cells from the INL ramify in the OFF sublamina S2 of the IPL, while those from the GCL ramify in the ON sublamina S4 [12•]. Developmentally, cholinergic amacrine cells are among the first to be specified at around E11 [26•]. Cholinergic amacrine cells in the GCL are born first followed closely by cholinergic amacrine cells of the INL, lending credence to a theory that the position of soma in the retina might correlate with birthdates. Starburst amacrine cells are among the most extensively studied subset of amacrine cells, and it is well known that starburst amacrine cells play a major role in modulating the response of direction sensitive retinal ganglion cells and hence contribute to direction selectivity and detection of directional motion (reviewed in [27]).

NPY is expressed in a population of GABAergic amacrine cells that are located in both the INL and GCL. NPY cells in the INL project their dendrites mostly to the OFF sublamina S1, while those in the INL, to the ON sublamina S5 [18, 20]. A small population of NPY cells also expresses TH and projects to S3 sublamina. Interestingly, Voinescu et al. [26•] found coincidental expression of NPY in all cholinergic amacrine cells at P7. As development proceeds, NPY expression in cholinergic amacrine cells reduces. This indicates that not only do progenitor cells change their competence to produce different retinal types, but the same mechanism might be at play in specifying retinal cell subtypes as well. Functionally, NPY seems to have a role in mediating low-frequency spatial processing (large spatial patterns) by retinal ganglion cells [28].

Almost all dopaminergic amacrine cells (TH expressing) are GABAergic and are located at the boundary of INL and IPL and ramify in S1 lamina of the IPL. Studies that use a TH promoter-driven RFP/GFP line identify a group of GABAergic catecholaminergic cells (Type II cells) that do not contain dopamine and specifically stratify in S3 [21, 29]. It is possible these cells express NPY as reported by Oh et al. [18] who found a subpopulation of NPY cells that were immunoreactive for TH. Functionally, dopaminergic amacrine cells modulate light adaptation and circadian rhythm.

Another group of GABAergic amacrine cells express neuronal NOS. NOS-expressing amacrine cells have been categorized further based on the size of their soma and dendritic arborization. Types I and II are situated in the INL and ramify in S1 and mainly in S3. The displaced NOS amacrine cells in the GCL ramify mainly in S4 and S5 [17, 30]. NOS amacrine cells usually intercept and interact with other amacrine cells in the IPL, but some synapse with

retinal ganglion cells as well. Some cells also mediate input from bipolar cells projecting to the ON sublamina. Current studies seem to suggest that NO amacrine cells might play a role in visual adaptation since NO synthesis is largely dependent on stimulation by light [19, 30].

Glycinergic amacrine cells are not extensively characterized due to the lack of an extensive marker set to label the different subtypes of glycinergic cells. Glycinergic amacrine cells as a whole are marked by glycine transporter (GlyT1) [14]. One well-characterized subset of glycinergic amacrine cells is the AII amacrine cells [9, 24, 31]. AII amacrine cells are present primarily in the INL and are rarely found in the GCL. They can be marked by cytoplasmic protein DAB1, which is part of the reelin pathway that controls cell positioning during development in parts of the central nervous system. DAB1 is visibly expressed in AII glycinergic amacrine cells as early as P7 in the mouse retina [22]. PV is another marker for AII amacrine cells as PV-expressing cells are morphologically similar to AII amacrine cells and also co-express DAB1 [12, 22, 23]. In the adult mouse retina, the AII amacrine cells ramify primarily in the ON S5 sublamina. DAB1 immunoreactivity is also discernible as thick lobules at the distal part of the OFF sublamina. Studies have suggested that these lobules are sites of inhibitory interaction between AII amacrine cells, OFF bipolar cells, and OFF retinal ganglion cells. The inhibitory interaction between AII amacrine cells and OFF bipolar cells may be important for visual responses at high spatial frequencies and/or detecting motion of varying spatiotemporal contrasts [31].

Vesicular glutamate transporter type 3 (VGlut3) is expressed in a subset of glycinergic amacrine cells in the INL. VGlut3 amacrine cells ramify in the ON and OFF sublaminae (S2 and S3), filling in the gaps between the three calretinin bands and seem to make connections with OFF cone bipolar cells at S2, ON cone bipolar cells at S3, and rod bipolar cells at S4/S5 [13, 32]. VGlut3 cells seem to receive inputs primarily from amacrine cells and hence are thought to be involved in amacrine-to-amacrine cell crossover inhibition. A population of VGlut3 cells expresses circadian clock gene *Period 1* (*Per1*) suggesting that this population might have a role in generating circadian rhythms [25].

There is also a small population of amacrine cells that are neither GABAergic nor glycinergic (nGnG). Roughly 85 % of amacrine cells are accounted for, by staining with glutamic acid decarboxylase 65/67 (GAD65/67) for GABAergic cells or GlyT1 for glycinergic amacrine cells. The remaining ~15 % of amacrine cells express neither. The neurotransmitter type(s) expressed by these amacrine cells is currently unknown. nGnG amacrine cells ramify primarily to the OFF lamina (S1–S3) of the IPL with their dendritic arborization being morphologically narrow field.

Developmentally, these cells are born after glycinergic cells during the first few postnatal days [16•].

Amacrine Cells Contribute to the Majority of Synapses Formed in the IPL

Amacrine cells and bipolar cells project presynaptic dendrites to the IPL where they synapse with retinal ganglion cells. Various aspects of visual information encoded by retinal neurons are transmitted to retinal ganglion cells via specific synapses in the IPL, and hence it is pertinent that the specification of IPL occurs in a well-directed and timely manner. Yamagata et al. [33] first identified and elucidated the role of two homologous transmembrane immunoglobulin superfamily molecules (sidekick family)—*Sdk1* and *Sdk2*—in retinal lamination of the chick retina. They found that *Sdk1* and *Sdk2* are expressed in mutually exclusive populations of retinal neurons in the INL and GCL. While *Sdk2* expression is greater in sublamina S2 of the IPL and weaker in S4, the opposite is true of *Sdk1*. Over-expression of Sdks in cells that are devoid of it, rerouted these cells to the Sdk-positive layer without a change in cell identity. This suggests that Sdks play an important role in promoting laminar synaptic formation by promoting synapse formation between pre- and post-synaptic Sdk-positive neurons. Further, they have identified the presence of related adhesion molecules, *Dscam* and *DscamL*, in exclusive layers of the IPL. *Dscam* expression is restricted to S5, while *DscamL* is expressed in S1, S2, and S4 [34]. *Dscam* is also localized to the somata of the TH- and NOS-expressing amacrine cells. Loss- and gain-of-function studies demonstrated that the *Dscam* adhesion molecules along with Sdks regulate the development of sublaminal stratification across the IPL [34]. *Dscams* are also shown to influence mosaic spacing by promoting self-avoidance between different types of amacrine cells in the INL, which is essential to preserve functionality of amacrine neurons [35]. Another class of transmembrane signaling molecules—semaphorins, signaling via their receptors—plexins, display complementary expression patterns across the IPL and regulate retinal lamination during development [36, 37]. In *Sema5A* and *Sema5B* double null mutants, TH- and VGlut3-expressing amacrine cells display gross dendritic mistargeting in the IPL. Other amacrine types like AII amacrine cells, cholinergic amacrine cells, calretinin, and calbindin-expressing amacrine cells display prominent ectopic projections of dendrites toward the OPL. Double null mutants of receptors *PlexA1* and *PlexA3* phenocopy the anomalies in stratification displayed by *Sema5A* and *Sema5B* double mutants [37]. *Sema6A* ligand and *PlexA4* receptor combination are also expressed in a complimentary manner through IPL development. *PlexA4* is expressed in S1 and S2, and *Sema6A* is

expressed in S3, S4, and S5 laminae of the IPL. *PlexA4* mutants display aberrant dendritic projections of TH-expressing amacrine cells, calbindin-expressing amacrine cells, and intrinsically photosensitive retinal ganglion cells, with *Sema6A*-null mutants phenocopying the aberrant dendritic stratification, suggesting that *Sema6A* acts a functional ligand for *PlexA4* in regulating retinal lamination at the IPL [36]. Taken together, transmembrane guidance molecules have been shown to regulate neurite stratification and retinal lamination at the IPL by influencing the development of dendritic arborization of amacrine cells among others. Recent studies in Zebrafish have shown the role of transcription factor *Irx7* in regulating retinal lamination by affecting neurite outgrowth [38]. Another factor namely PTEN phosphatase also mediates the mosaic spacing, fasciculation, and dendritic arborization of TH amacrine cells and of horizontal cells, likely in a *Dscam/Sema*-independent manner [39]. Although we currently understand the role of these signaling molecules in IPL development, we are yet to assimilate the full network of molecules upstream and downstream of the signaling factors that play into this regulation.

Transcription Factors Influencing the Development of Amacrine Cell Types and Subtypes

During development, amacrine cells are specified early along with retinal ganglion cells, cone photoreceptors, and horizontal cells. Bipolar cells, rod photoreceptors, and Müller glial cells are specified later. In mouse retina, amacrine cells are born between embryonic day 8 (E8) and postnatal day 5 (P5), with the peak of competence between E14 and E16 [4, 26]. The competence of the early retinal progenitor pool changes with time and the expression of intrinsic factors such as transcription factors are instrumental in specifying the identity of the retinal cell class as a whole and of individual cell subtypes. Here, we discuss some of the molecular factors governing the differentiation and specification of amacrine cells and their subtypes.

Early factors such as *Pax6*, *Sox2*, *Bclaf1* influence the competence of retinal progenitors and significantly impact the development of amacrine cells from the early progenitor pool. Initial studies on *Pax6* showed that a conditional deletion of *Pax6* in the eye resulted in the production of only amacrine cells with a loss of most other neuron types and is hence necessary for the maintenance of progenitors in their multi-potent state [40, 41]. Furthermore, all amacrine cells born in the *Pax6* mutant were identified as GABAergic, hence further restricting the progenitor fate choice to a non-glycinergic amacrine cell type. *Bclaf1* has been recently identified as a factor that could potentially act along with *Pax6* in the differentiation of early retinal neuron population [42]. *Sox2* is expressed in Müller glial

cells and the cholinergic subset of amacrine cells in the adult retina. During development, over-expression of *Sox2* in explant cultures biases progenitor cells to an amacrine cell fate [43].

Conditional deletion and over-expression studies of *Foxn4* show that it is necessary and sufficient for the generation of amacrine and horizontal cells. *Foxn4* deletion greatly attenuated amacrine cell numbers, while an over-expression of *Foxn4* in progenitors greatly facilitated amacrine cell fate (both glycinergic and GABAergic) at the expense of rod photoreceptors [44, 45]. Recent studies in the chick retina have shown that *Foxn4* expression might be in part regulated by *Meis1*, which regulates the activity of a non-coding DNA fragment upstream of the *Foxn4* transcription start site [46]. *Foxn4* also interacts with its downstream targets—*Neurod4* (previously *Math3*) and *Neurod1*, and regulates the differentiation of multi-potent progenitors into amacrine cells [44]. A loss of *Neurod4* or *Neurod1* alone does not affect amacrine cell numbers, whereas a double mutant of *Neurod1* and *Neurod4* completely abolished amacrine cell generation. An over-expression of *Neurod4* or *Neurod1* alone does not restore amacrine cell numbers, but addition of *Pax6* to either *Neurod1* or *Neurod4* is sufficient to generate amacrine cells [47]. This signifies that *Neurod4* and *Neurod1* have mutually redundant functions in amacrine cell genesis, and act downstream of *Foxn4* and *Pax6*. While they are together essential for the generation of amacrine cells, they are not sufficient for amacrine cell genesis. Another factor acting downstream of *Foxn4* is *Ptf1a*, the loss of which biases precursors of amacrine and horizontal cells into retinal ganglion cell fate leading to greatly reduced amacrine cell numbers [48, 49]. Gain-of-function and loss-of-function studies in the *Xenopus* retina indicate that *Ptf1a* might be particularly essential for the development of GABAergic fate in amacrine cells [50].

Several transcription factors have been studied for their role in amacrine subtype specification. *Barhl2* is one such factor, which is expressed in bipolar cells, amacrine cells, and retinal ganglion cells. A forced expression study of *Barhl2* suggested that it might be an important regulator of the glycinergic subtype development [51]. Loss-of-function studies have shown that *Barhl2* attenuates both glycinergic and GABAergic amacrine cell numbers with a concomitant increase in cholinergic amacrine cells [52]. *Isl1* has been extensively studied for its role in cholinergic amacrine cell development. *Isl1* is expressed in retinal ganglion cells, cholinergic amacrine cells, and bipolar cell subtypes [53]. Loss of *Isl1* results in the loss of about 93 % of cholinergic amacrine cells and a concomitant disruption of the S2 and S4 stratification in the IPL [54]. *Bhlhb5* is expressed in GABAergic amacrine cells and cone bipolar cells in the adult. Loss of *Bhlhb5* expression results in a

loss of about a third of GABAergic cells. TH cells seem to be the most affected one with about a 90 % decrease in *Bhlhb5* mutants [55]. Lineage tracing studies have shown that *Bhlhb5*-expressing progenitors give rise to cholinergic, glycinergic, and GABAergic amacrine cell fates. The expression in the adult in cholinergic and glycinergic amacrine cells is downregulated but is retained in GABAergic amacrine cell subtypes [56]. *Lmo4* is expressed in a subset of GABAergic amacrine cells, and a conditional deletion of *Lmo4* leads to a reduction in the number of *Bhlhb5*-expressing GABAergic amacrine cells [57]. Analysis of specific expression of individual members of the Neurod1 transcription factor family showed the expression of *Neurod2* specifically in Dab1-expressing AII amacrine cells. Loss of *Neurod2* results in a partial loss of AII amacrine cells [58]. *Neurod6* expression in adult amacrine neurons was also identified and is expressed mostly in nGnG amacrine cells [16, 58]. Loss of *Neurod6* results in an increase in the number of glycinergic amacrine cells at the expense of nGnG amacrine cells [16]. Interplay between *Neurod6* and *Satb2/Ebf3*-expressing amacrine cells regulates the decision of the progenitor cells to differentiate into glycinergic amacrine cells versus the nGnG fate. *Ebf* class transcription factors have been studied for their role in the differentiation of AII class amacrine cells. Gain- and loss-of-function studies indicate that *Ebf* class transcription factors are both necessary and sufficient for specifying AII amacrine subtypes [59].

What Remains Unknown?

We are currently aware of the presence of the many subtypes of amacrine cells but unclear on their individual functions in processing visual information. We have elucidated some of the transcription factors that play an important role in the development of amacrine cells class as a whole, but our understanding of how these factors come together or compete with other factors responsible for specifying other retinal cell types is currently limited. Molecular mechanisms governing the development of individual amacrine cells subtypes and their specific laminar stratification are also currently limited. Identifying unique regulatory factor or a unique combination of factors responsible for amacrine subtype specification will be useful for answering some of these questions. We are now beginning to understand the molecular basis of laminar stratification and mosaic tiling of amacrine cells, but are yet to determine the influence of the complete synaptic laminar network involving retinal ganglion cells, amacrine cells, and bipolar cells on retinal function. Identifying the developmental factors responsible for laminar stratification has been immensely useful, allowing us to mark cells and their synaptic contributions to the IPL. More work in this

direction will help put together a developmental framework which will ultimately provide us with a deeper understanding of amacrine cells and their contribution to visual information processing.

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Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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