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***Lhx9* Is Required for the Development of Retinal Nitric Oxide-Synthesizing Amacrine Cell Subtype**

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Abstract

Amacrine cells are the most diverse group of retinal neurons. Various subtypes of amacrine interneurons mediate a vast majority of image forming and non-image forming visual functions. The transcriptional regulation governing the development of individual amacrine cell subtypes is not well understood. One such amacrine cell subtype comprises neuronal nitric oxide synthase (nNOS/bNOS/NOS1)-expressing amacrine cells (NOACs) that regulate the release of nitric oxide (NO), a neurotransmitter with physiological and clinical implications in the retina. We have identified the LIM-homeodomain transcription factor LHX9 to be necessary for the genesis of NOACs. During retinal development, NOACs express *Lhx9*, and *Lhx9*-null retinas lack NOACs. *Lhx9*-null retinas also display aberrations in dendritic stratification at the inner plexiform layer. Our cell lineage-tracing studies show that *Lhx9*-expressing cells give rise to both the GAD65 and GAD67 expressing sub-populations of GABAergic amacrine cells. As development proceeds, *Lhx9* is downregulated in the GAD65 sub-population of GABAergic cells and is largely restricted to the GAD67 sub-population of amacrine cells that NOACs are a part of. Taken together, we have uncovered *Lhx9* as a new molecular marker that defines a subset of amacrine cells and show that it is necessary for the development of the NOAC subtype of amacrine cells.

Keywords

Retina; LIM-homeodomain transcription factor; Amacrine cell subtype; Retinal lamination

Introduction

The complex architecture of the mammalian retina is in part due to the degree of morphological diversity exhibited by retinal neurons. This diversity further extends to each

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

retinal neuron type, which are classified into different subtypes based on their cellular morphology, dendritic stratification, and/or neurotransmitter identity [1, 2]. Amacrine neurons in the retina are the most diverse type of retinal cells with about 33 subtypes identified so far [3, 4]. Recently, we have come to understand that several visual functions—both image forming and non-image forming—are performed at the level of the retina and are largely regulated by amacrine cell subtypes [5–10]. While several studies have identified the transcriptional network responsible for the development of amacrine cell class as a whole [11–16], we are only now beginning to decipher the transcriptional codes underlying amacrine cell subtype development [17–20].

Amacrine interneurons express inhibitory neurotransmitters and are broadly classified as GABAergic or glycinergic. GABAergic amacrine cells are broadly categorized as cells that express GAD65 or GAD67, the two isoforms of glutamic acid decarboxylase (GAD). Most GABAergic amacrine cells, however, express both isoforms of GAD [21]. Previous studies have suggested that the dopaminergic population of amacrine cells are GAD65+/GAD67– [22] and NOACs are GAD67+/GAD65– [21, 22]. In addition, GAD65 and GAD67 are expressed at different developmental time points, vary in their sub-cellular localization, and have functionally different properties in the rat brain [23]. It is currently unknown if there is a difference in the developmental paradigm leading to the differentiation of distinct GAD65 and GAD67 populations of GABAergic amacrine cells.

NOACs are a distinct subtype of amacrine cells that regulate the release of nitric oxide (NO), a neurotransmitter molecule modulating physiological functions of other retinal neurons [24–26]. In the retina, NO can selectively stimulate the release of GABA while inhibiting that of glycine [27]. Moreover, NO is an important regulator of ocular blood flow and abnormal NO activity has been implicated in the pathology of several ocular diseases such as diabetic retinopathy and glaucoma [28, 29]. NOACs are distributed in the inner nuclear layer (INL) and ganglion cell layer (GCL). They arborize in the center of the inner plexiform layer (IPL), receive excitatory inputs from both ON and OFF bipolar cells, and synapse onto other amacrine cells or ganglion cells [26]. To date, the genetic basis of NOAC subtype specification has not been described.

Previously, we have shown that LIM-HD transcription factor LHX9 is expressed in the developing retina starting at around embryonic day (E)13.5 and continues to be expressed in cells in the INL and GCL of the adult retina [30]. We also created an *Lhx9-GFPCreER* (*Lhx9-GCE*) mouse line allowing the lineage study of *Lhx9*-expressing cells [31]. In this current study, we show that LHX9 is primarily expressed in GAD67+/GAD65– GABAergic cells. Consistently, our lineage-tracing studies reveal that *Lhx9* is initially expressed in both the GAD65 and GAD67 sub-population of amacrine cells, but at later time points becomes restricted to the GAD67 sub-population of amacrine cells. We show that NOACs express LHX9 during development and that targeted deletion of *Lhx9* in mice results in the loss of NOACs, demonstrating that *Lhx9* is necessary for the development of NOACs. Furthermore, *Lhx9*-null retinas display aberrations of amacrine cell dendritic projections in the inner plexiform layer and disruption in organization of the IPL. These data demonstrate that *Lhx9* plays a major role in the development of NOACs and in the specification of a sub-population of GABAergic amacrine cells.

Results

Lhx9 Expression in the GAD67 Population of GABAergic Amacrine Neurons

Previously, we showed that *Lhx9* is expressed in a population of amacrine cells in the INL and GCL [30, 31]. We first sought to determine the subtype identity of the *Lhx9* population of amacrine cells using immunohistochemistry at P18 when amacrine subtypes are identifiable and before the downregulation of LHX9 expression at later time points. We co-labeled LHX9 with GAD65 and GAD67, two isoforms of GAD that together mark the entire population of GABAergic amacrine cells (Fig. 1a, b). We found that most (87.27%) LHX9+ cells co-expressed GAD67 and a small population of cells (6.47%) expressed GAD65. Upon co-labeling of LHX9 with GlyT1, a pan-glycinergic cell marker, we found that most LHX9+ cells did not co-express GlyT1 (Fig. 1c). To further identify the retinal cell subtype, we co-labeled LHX9 with ChAT, TH, bNOS, and calretinin (Fig. 1d – g) markers, which identify different types of GABAergic cells. None of the LHX9+ cells in the INL were cholinergic (ChAT+) or dopaminergic (TH+). Most (93.54%) NOACs identified with bNOS antibody were LHX9+ at this time point and 10.11% population of calretinin+ cells in the INL were LHX9+ as well. Thus, LHX9+ cells are GABAergic amacrine cells mostly of the GAD67+ subgroup and include most NOACs.

Loss of NOACs, Disruption of S3 Sublamina, and Aberrant Dendritic Targeting in Lhx9-Null Retinas

To assess the role of *Lhx9* in amacrine cell subtype development and particularly that of NOACs, we used the *Lhx9-GCE* knock-in mouse line that has been previously described and characterized as a null mutation of *Lhx9* [31]. We observed that bNOS immunostaining was eliminated in the *Lhx9-null* retinas (Fig. 2a). Quantification of bNOS+ cell numbers ($n = 4$) displayed a dramatic loss (96.15%) of bNOS-expressing cells in the nulls (Fig. 2b). Thus, *Lhx9* is necessary for the development of retinal NOACs and for the expression of bNOS.

Previous studies have shown that NOACs arborize in the center of IPL [26]. To investigate whether the loss of NOACs affect the IPL, we assessed aberrations in retinal lamination in *Lhx9-null* retinas at an adult time point (P30). Compared to control retinas, *Lhx9-null* retinas displayed a specific loss of middle band of anti-calretinin immunostaining in the IPL corresponding to sublamina S3 whereas S2 and S4 sublaminae ($n = 8$) marked by the expression of ChAT and S1 sublamina highlighted by the expression of TH were unaltered (Fig. 2c – e). The expression of substance P, although relatively undistinguishable in the cell soma as compared to other markers, provided clear staining in sublaminae S1, S3, and S5 of the IPL. While some sparse and punctate expression of substance P was observed in S1 and S5 sublaminae, this expression was not observed in S3 of *Lhx9-null* retinas (Fig. 2f – h), further confirming that targeted deletion of *Lhx9* disrupts the sublamina S3 in the IPL.

To better visualize the dendritic ramifications of LHX9+ amacrine cells, we crossed the *Lhx9-GCE* mouse line to a *Rosa26-tdTomato* reporter line. Cre-mediated recombination was induced with tamoxifen starting at P30 over five consecutive days and animals were sacrificed at P40. *Lhx9-control* retinas displayed strong tdTomato staining in S3 and below the ChAT+ S4 sublamina (S4/S5), clearly avoiding the narrow CHAT+ S4 neuropil layer

(Fig. 2i, j). In contrast, *Lhx9-null* retinas displayed stronger tdTomato staining in S1, but the thickness of S3 and S4/S5 laminae was markedly reduced (Fig. 2i, j). We quantified the number of tdTomato+ cells in both the control and mutant retinas to exclude the possibility that the variability in dendritic staining was not due to a difference in cell numbers (Fig. 2k). We also quantified the total number of calretinin positive cells and the percent of tdTomato+ cells that were calretinin+ in the control and mutant retinas to ascertain that the loss of the calretinin S3 band was not attributable to calretinin numbers themselves (Fig. 2k, l). In sum, the loss of *Lhx9* leads to (i) the loss of NOACs, (ii) the loss of the S3 calretinin band in the IPL, and (iii) ectopic dendritic projections to S1 sublamina of the IPL.

To address the aforementioned phenotypic characteristics, we first quantified and compared overall numbers of retinal ganglion cells, amacrine cells, and bipolar cells in the adult *Lhx9* null retinas to those of controls. We did not find any significant changes in cell numbers of POU4F1+, POU4F2+ population of retinal ganglion cells, PAX6+ population of amacrine cells or CHX10+ population of bipolar cells ($n = 3$) (Fig. 3a–c, j). To determine if loss of *Lhx9* affected the survival of NOACs, we performed anti-activated caspase 3 immunostaining (Fig. 3d, e, j) to identify cells undergoing apoptosis at late developmental stages P3 and P5 when NOACs are first observed (Fig. 3f, j) and were unable to find a significant change in the number of cells expressing caspase 3 in *Lhx9-null* retinas as compared to the controls suggesting that the loss of bNOS expression might not be a result of apoptosis of the developing population of bNOS-expressing amacrine neurons.

We then turned our attention to the aberrant dendritic stratification in the *Lhx9*-null retinas. The loss of S3 sublamina displayed by the loss of calretinin staining in the narrow band of S3 could be attributed to the absence of bNOS-expressing amacrine neurons. The reduction in thickness of S4/S5 sublamina could potentially be due to aberrant substance P projections to this layer of the IPL. However, ectopic projections to sublamina S1 in *Lhx9-null* retinas prompted us to look at cell numbers and dendritic projection of dopaminergic amacrine neurons that normally project to S1. Immunostaining of sections for TH previously showed no gross changes in dendritic projections of dopaminergic cells (Fig. 2e) and a quantification of whole mount tissue stained for TH did not display changes in cell number (Fig. 3g, j). It is hence unlikely that *Lhx9* lineage cells were switching to a dopaminergic cell fate. We also quantified starburst amacrine cell numbers on whole mount retinal tissue stained for ChAT and were unable to find changes in ChAT cell numbers in the INL or GCL (Fig. 3h, i, j).

Lhx9-Expressing Cell Lineage of Both GAD65 and GAD67 Population of GABAergic Amacrine Cells

To determine the fate of *Lhx9*-expressing cells, we used *Lhx9-GCE* and *Rosa26-tdTomato* reporter mice to perform cell lineage-tracing experiments in the control and the *Lhx9-null* retinas. We first induced Cre-recombination at an early embryonic time point (E13.5) upon the onset of *Lhx9* expression in the developing retina and harvested the retinas at postnatal day P21, a time point at which retinal cells have matured. We found that *Lhx9*-expressing cells gave rise to GAD65 (Fig. 4a, m) sub-population (44.76% in control; 47.61% in null; $n = 3$) as well as GAD67 (Fig. 4b, m) sub-population (37.46% in control; 39.22% in null; $n = 3$) of GABAergic cells, suggesting that *Lhx9* should be expressed prior to the specification

of GABAergic amacrine subtypes. However, in agreement with the non-overlapping expression of LHX9 and ChAT (Fig. 4c), no cholinergic cells in the INL originated from *Lhx9*-expressing cells. Consistent with the expression of LHX9 in bNOS+ cells (Fig. 1f) and with the absence of bNOS+ cells in the *Lhx9-null* retina (Fig. 2a, b), we observed that all bNOS+ cells expressed tdTomato and accounted for 4.58% of tdTomato+ cells in the *Lhx9-control* retinas (Fig. 4d), indicating that NOACs arise exclusively from *Lhx9*-lineage and that *Lhx9* is cell-autonomously necessary for bNOS cell development. We again observed a marked disruption in substance P lamination in *Lhx9-null* retinas (Fig. 4e) and did not observe any dopaminergic cells originating from *Lhx9*-expressing cells (Fig. 4f).

The amacrine cells are specified over a protracted range of developmental time points with an early peak occurring between E14–16 and a later peak occurring at around P1 [32]. We hence induced Cre-recombination at the midway time point (P0). Similar to the induced Cre-recombination at E13.5, we again observed that *Lhx9*-expressing cells gave rise to both the GAD65 (35.83% in control; 37.93% in null; $n = 3$) and GAD67 (47.43% in control; 43.81% in null; $n = 3$) sub-population of GABAergic amacrine cells (Fig. 4g–h, n) without a significant difference in numbers between the *Lhx9-control* and *Lhx9-null* retinas when Cre-recombination was induced at P0. Similar to the E13.5 induced recombination, we did not find any cholinergic cells originating from *Lhx9*-expressing cells (Fig. 4i). However, we noticed a slight change in the number of bNOS+ cells co-expressing tdTomato (Fig. 4j) in the *Lhx9-control* animals. A small portion of bNOS+ cells (1.59%) did not express tdTomato at this stage indicating that while needed for the formation of NOACs, *Lhx9* expression might be down-regulated in NOACs after they are specified. As with E13.5 induced recombination, we noted an aberrant lamination of substance P neurons in the *Lhx9-null* and no dopaminergic cells originating from *Lhx9*-expressing cells (Fig. 4k–l). Our embryonic and neonatal lineage-tracing results show that *Lhx9* is expressed in more GAD65+ cells as compared to GAD67+ cells during embryonic stages, and as development proceeds, *Lhx9* expression in GAD65+ cells declines in comparison to GAD67+ cells (Fig. 4m, n).

We further examined the identity of *Lhx9*-expressing cells in mature retinas by inducing recombination via injection of tamoxifen at P30 over 5 consecutive days and collecting retinas at P40. Compared to the above embryonic cell and neonatal lineage-tracing results, we observed a drastic reduction in the percent of GAD65+ sub-population cells expressing tdTomato (16.05% in controls; 17.29% in nulls; $n = 3$) while the percent of GAD67+ sub-population cells expressing tdTomato increased (85.29% in controls; 83.87% in nulls; $n = 3$) (Fig. 5a, b, i). GAD65 and GAD67 populations in *Lhx9-control* and *Lhx9-null* display no significant changes in cell numbers (Fig. 5h). Co-labeling of tdTomato with ChAT and TH showed no difference between that in the INL of *Lhx9-null* as compared to the *Lhx9-control* (Fig. 5c, f). Interestingly, while NOACs originated from *Lhx9*-expressing cell lineage during development and we noted the absence of bNOS-expressing cells in the *Lhx9-null*, we did not observe expression of tdTomato in bNOS-expressing cells at P40 in the control retina, suggesting that *Lhx9* could be transiently expressed in and be required for the formation of NOACs, and that its expression might be downregulated in NOACs in the adult retina by P30 (Fig. 5d). We observed similar lamination defects in substance P-expressing neurons at P40 as previously shown in embryonic and neonatal lineage tracing (Fig. 5e). Lastly, we

compared the expression of SATB2—a marker for a third of glycinergic amacrine cells and all nGnG (neither glycinergic nor GABAergic) amacrine cells in the retina [33], in *Lhx9*-control and *Lhx9*-null retinas and noted the lack of difference in SATB2 cell number in the INL (Fig. 5g, h).

Discussion

In this study, we found that *Lhx9* is necessary for the development of NOACs—a subtype of amacrine cells that express nitric oxide synthase. We also found that in the absence of *Lhx9*, retinas show aberrant retinal lamination, suggesting that LHX9 might regulate the development of retinal lamination. We also showed that *Lhx9* is expressed in both GAD65 and GAD67 isoform types of GABAergic amacrine cells during development, with subsequent down-regulation of expression in the GAD65 population as development proceeds, indicating that *Lhx9* might be part of a dynamic regulatory mechanism with other transcription factors in controlling the differentiation of various amacrine cell subtypes. Overall, we have identified LHX9 as a transcription factor that plays an important role in determining amacrine cell subtype fate and serves as a molecular marker for amacrine cell subtypes that can be exploited to further our understanding of amacrine cell diversity and function.

Lhx9 Is Necessary for the Development of NOACs

Loss of nearly all NOACs in *Lhx9*-nulls and the *Lhx9*-lineage study in which all NOACs are originated from *Lhx9*-cell lineage, strongly argue for the role of *Lhx9* in normal development of all NOACs. Interestingly, our immunohistochemical analyses of four whole-mount retinas, we detected only one cell that weakly expressed bNOS in the *Lhx9*-null animals. It should be noted that we did not find any LHX9 expression in the *Lhx9*-null retinas. Thus, the absence of a complete loss of bNOS in *Lhx9*-null retinas could be attributed either to random activation of NOAC differentiation pathway downstream of *Lhx9* or to a random cross-activation of a transcription factor closely related to *Lhx9* such as *Lhx2* which could potentially impact common downstream targets as that of *Lhx9*. Furthermore, *Lhx9*-nulls display a clear loss of S3 sublamina as displayed by the loss of middle calretinin band in the IPL (Fig. 2). A previous study noted that NOACs project to the middle of the IPL and our results are in agreement with their finding [26]. Taken together, our study has shown that *Lhx9* is necessary for the genesis of NOACs. The sufficiency of *Lhx9* in the genesis of NOACs still remains to be tested.

Another significant finding of this study is that the expression of *Lhx9* in NOACs is dynamically regulated during retinal development. NOACs express LHX9 at P18 and the *Lhx9*-lineage-tracing results show that all NOACs express tdTomato reporter protein when *Lhx9*-GCE activity is induced at E13. Interestingly, activation of tdTomato reporter expression by *Lhx9*-GCE at P0 and later at P30 increasingly fails to label NOACs, indicating that *Lhx9* expression is downregulated in NOACs later in development. Taken together, our study has shown that *Lhx9* is expressed in and is necessary during the early development of NOACs and that its expression is down-regulated later in NOACs.

Some NOACs have been previously shown to exhibit highly sensitive ON-OFF light responses. A small population of NOACs is also coupled to retinal ganglion cells [26]. This indicates that NOACs may be able to relay highly sensitive ON-OFF responses to retinal ganglion cells. While the functional relevance of NOACs and thus *Lhx9* in image forming or non-image forming vision is currently unknown, future experiments with electrophysiological testing combined with behavioral studies of *Lhx9*-null mice will help delineate the importance of NOACs in visual function.

Loss of *Lhx9* Alters Retinal Lamination at the Inner Plexiform Layer

Retinal lamination in the IPL depicts the organization in dendritic arborization of various amacrine cell subtypes in distinct layers where they make connections with other amacrine cells and/or retinal ganglion cells. During our analyses of *Lhx9*-nulls, we found that the S3 calretinin+ neuropil layer between the anti-ChAT labeled S2 and S4 sublaminae was completely ablated in the nulls. We attributed this loss to the loss of NOACs that typically project to the middle of the IPL. Moreover, substance P+ amacrine cells also project to the center of IPL. At this point, we are unable to determine if the substance P-expressing amacrine cells are affected as well. We were unable to analyze co-expression of LHX9 with substance P since the dendritic expression of substance P does not afford us the resolution to visualize co-localization of LHX9 and substance P.

Our lineage-tracing results showed that in addition to the loss of S3 calretinin+ sublamina in the absence of *Lhx9*, there was also a thinning of the S4/S5 sublamina marked by the area below S4 cholinergic band. This could be attributed to aberrations seen in substance P arborization that typically projects to S4/S5, S3 and minimally to S1. In addition to this, the increase in tdTomato expression in the area above the S2 cholinergic band suggests that loss of *Lhx9* could result in a cell fate change to cells that project their dendrites to the S1 lamina. Our analysis of dopaminergic amacrine cell number and stratification indicated that the ectopic S1 projections were not a result of fate switch into dopaminergic cells. However, limitations imposed by the availability of cell type markers and the relatively small cell numbers of some amacrine cell subtypes makes it hard to identify a cell fate switch among amacrine cell subtypes. Alternatively, *Lhx9* function might be directly or indirectly required for the development of proper dendritic projections of amacrine subtypes in the IPL, resulting in ectopic dendritic projections to S1 when *Lhx9* activity is lost. Previously, cell adhesion molecule DSCAM was identified to be required for the spacing and arborization of bNOS amacrine cells [34, 35]. The lamination of bNOS amacrine cells are also disrupted in the absence of DSCAM [34, 36]. Moreover, type II dopaminergic amacrine (type II DACs) cells also project to the middle of the IPL [37] and DSCAMs guide the targeting of these type II DACs [36]. Probing interactions of *Lhx9* with DSCAMs might reveal if *Lhx9* does indeed affect retinal lamination by influencing the development of GABAergic amacrine cell projections to the IPL. Plexin-semaphorin interactions have also been identified to be crucial for normal retinal circuit assembly at the IPL [38]. Analyses of plexin and semaphorin family expression in the *Lhx9*-null animals might shed further light on the role of *Lhx9* in laminar organization. Lastly, cadherins are known to be critical players in determining bipolar cell connectivity at the IPL [39]. It will be interesting to know if the loss of *Lhx9* in a subset of amacrine cells could impede the cadherin based targeting at the IPL.

Lhx9 Dynamically Regulates the Differentiation of Amacrine Cell Subtypes

The expression of LHX9 being largely restricted to the GAD67 population of amacrine cells prompted a closer look at the GAD67+/GAD65- population of amacrine cell subtypes. We showed that at P18, most LHX9 cells expressed GAD67, LHX9 expression was identified in NOACs and none of TH+ cells expressed LHX9. We were unable to analyze co-expression of LHX9 with substance P since the antibody against substance P, although marks dendritic projections into the IPL well, does not afford us the resolution to visualize co-localization within the cell soma. However, our analysis of substance P expression in the IPL of *Lhx9*-nulls suggests that the remaining population of GAD67+ LHX9+ cells is likely to be substance P+.

Using lineage-tracing methods, we observed that *Lhx9* expressing amacrine progenitors gave rise to both the GAD65 and GAD67 sub-population of amacrine cells. However, the expression of *Lhx9* in GAD65+ cells waned with advancement in development and was undetectable by adult time points. This dynamic regulation of *Lhx9* expression suggests that *Lhx9* might bias the acquisition of GAD67+ fate over the GAD65+ population. GAD65 and GAD67 isoforms of GABA have unique functions during development. While GAD67 is thought to be important for synthesizing GABA during synaptogenesis, GAD65 is more likely to synthesize GABA for neurotransmission [23]. Curiously enough, our lineage-tracing studies suggest that while *Lhx9* is downregulated after the specification of bNOS cells, it continues to be expressed in GAD67 and GAD65 population of cells even at neonatal time points, suggesting its role in retinal lamination and synaptogenesis that peaks at early postnatal stages. Whether the absence of *Lhx9* influences synaptogenesis and retinal lamination by regulating GAD67 and DSCAM is currently unknown.

Our understanding of retinal amacrine cell subtypes, their retinal circuitry and contributions to visual function can be greatly enhanced by identifying molecular markers for types of amacrine cells. Our study has indeed identified one such molecular marker LHX9 for the development of subgroup(s) of amacrine cells. LHX9 belongs to the LIM-HD transcription factor family that has been previously described to have several roles in development and differentiation of various central nervous system structures. Further, the importance of combinatorial expression of LIM-HD factors LHX9 and LHX2 has been demonstrated during the differentiation of caudal forebrain thalamus neurons [40, 41], development of thalamocortical axon guidance [42], development of chick olfactory bulb, amygdala, and entorhinal cortex [43], and the development of axonal projections of dorsal spinal cord relay commissural neurons [44] to cite a few. It is thus plausible that LHX9 and LHX2 also have combinatorial roles during retinal cell subtype differentiation and/or in influencing their axonal guidance. Some of our ongoing studies have suggested that LHX2 and LHX9 have a significant degree of overlapping expression in amacrine cell subtypes. Whether LHX2 has an adjunct supportive or redundant role along with LHX9 in aiding the differentiation of amacrine cell subtypes is yet to be determined. Moreover, recent studies indicate that *Lhx2* and *Lhx9* positively regulate *Pax6* α -enhancer activity and could influence GABAergic amacrine cell development and maintenance [45]. Future studies elucidating the LIM-combinatorial code will shed more light on the regulation of amacrine cell subtype development.

Methods

Animals

All animal work was performed in accordance with protocol approved by the University Committee of Animal Resources at the University of Rochester. The generation and characterization of *Lhx9-GCE* mouse line have been previously described [31]. In the remainder of the manuscript, *Lhx9^{+/+}* and *Lhx9^{GCE/+}* mice are referred to as *Lhx9* controls and *Lhx9^{GCE/GCE}* mice are referred to as *Lhx9* nulls. *Rosa26-tdTomato* reporter (*Gt(ROSA)26Sor^{tm14}(CAG-tdTomato)Hze*) mice were obtained from Jackson Laboratory (Stock number: 007908). Male and female *Lhx9^{GCE/+}; Rosa^{tdTomato/+}* mice were crossed to generate both *Lhx9^{GCE/+}; Rosa^{tdTomato/+}* (*Lhx9 control; Tomato*) and *Lhx9^{GCE/GCE}; Rosa^{tdTomato/+}* (*Lhx9 null; Tomato*) mice with the former genotype used as controls. Mice were obtained in the typical Mendelian ratio and haploinsufficiency was not observed.

To induce recombination in mice older than postnatal day 30 (P30) to adult, tamoxifen was prepared in corn oil and administered intraperitoneally (IP) at a dose of 5 mg/40 g body weight over five consecutive days. To induce recombination starting at P0, pups were intraperitoneally injected with tamoxifen (0.2 mg) on days P0 and P1. To obtain embryos, timed matings were setup between heterozygous mice as previously described. Noon of the day a vaginal plug was observed was designated as E0.5. Embryonic recombination was induced by injecting time-mated pregnant females with tamoxifen once at a dose of 5 mg/40 g body weight at the pre-determined time point, if embryos were to be harvested prior to E18.5. Since administration of tamoxifen to pregnant females interfered with labor and was detrimental to birth of pups, for analyses at postnatal stages, pregnant females were injected with tamoxifen at a dose of 1.5 mg/40 g body weight and pups were delivered via C-section and fostered with another dam, for analyses at postnatal time points.

Immunohistochemistry

Mice were euthanized and eye cups were harvested in ice cold PBS. Eyes were fixed in 4% (w/v) PFA for 2 h. Anterior segments of eyes were dissected out to remove the lens and expose the retina in the optic cup. The retina was then equilibrated in 30% (w/v) sucrose and embedded in tissue freezing medium (TFM). Ten- to twenty-micrometer-thick horizontal sections were collected on slides using a freezing cryostat. Slides were dried on a slide warmer and washed three times in PBS for 5 min each followed by three washes in 0.3% (w/v) PBST for 5 min each. Sections were incubated in blocking solution (10% Horse serum in 0.3% PBST) at room temperature for 1 h. Blocking solution was then removed and replaced with primary antibodies dissolved in blocking solution, and allowed to stand overnight. Slides were washed briefly in PBST and secondary antibody was added to the slides for 2 h at room temperature. Slides were then washed and cover-slipped for imaging. All imaging was performed with a Zeiss LSM 510 confocal microscope. Images were processed with ImageJ and Adobe Photoshop. Antibodies used in this study are listed in Table 1. All experiments were repeated for a minimum of three times.

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Fig. 1. Expression of LHX9 in GABAergic amacrine cells

a–c LHX9 is expressed in GABAergic but not glycinergic amacrine cells. Co-immunolabeling of LHX9 with GABAergic and glycinergic markers reveals the expression of LHX9 in GAD67 isoform-expressing GABAergic amacrine cells (**b**) but not in GAD65 isoform-expressing GABAergic amacrine cells (**a**) or the GlyT1-expressing glycinergic amacrine cells (**c**) at P18. **d–g** Amacrine cell subtype characterization of LHX9 expressing cells. LHX9 expressing amacrine cells do not co-localize with ChAT, a marker for cholinergic amacrine cells (**d**) or TH, a marker for dopaminergic amacrine cells (**e**). LHX9 co-localizes with bNOS, a marker for nitric-oxide expressing amacrine cells (**f**). A small

population of LHX9 expressing amacrine cells co-localizes with Calretinin at P18 (**g**). *Scale bar* equals 200 μm

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Fig. 2. bNOS expression in amacrine cells and S3 lamina phenotype in *Lhx9*-null retinas

a Anti-bNOS immunolabeling shows that bNOS is expressed in control retinas and is absent in *Lhx9*-null retinas. **b** Quantification of bNOS cell number per 0.4 mm^2 center area of retinal whole-mount samples in the *Lhx9*-null as compared to *Lhx9*-control retinas ($n = 4$, **** $p < 0.0001$). **c–h** S3 lamina is missing in *Lhx9*-null retinas. ChAT labeling of cholinergic cells and their dendrites projecting to S2 and S4 lamina (arrowheads) of the IPL is unaltered in *Lhx9*-null retinas as compared to control (**c**, **f**). Calretinin labeling of S2 (arrowhead), S3 (arrow), and S4 (arrowhead) sublaminae in the IPL of controls is altered in the mutants, which lacks the S3 sublamina (**d**, **g**). TH labeling of dopaminergic amacrine

cells and their dendrites projecting to S1 sublamina is not changed in the mutants as compared to controls (**e**). Substance P staining in S3 and S4/5 sublamina in the control is aberrant in the mutants with sparse staining visible in the S4/S5 lamina and loss of staining in the S3 sublamina (**h**). **i, j** Altered retinal lamination at the IPL in *Lhx9-null* mutants. LHX9 expressing cells project to S3 and S4/S5 lamina of the IPL in the control retina while they project ectopically to S1 and display aberrations in S3, S4/S5 in the null retina (**i**). Comparisons for the S2, S3, S4 bands of the IPL are shown with calretinin staining (**j**). Reporter expression was induced by tamoxifen injection at P30 and tissues were harvested at P40. **k-l** Quantification in **i, j** shows no difference in cell numbers between *Lhx9*-control and *Lhx9*-null retinas. *Scale bars* equal 800 μm (**a**) 200 μm (**c-j**)

Fig. 3. Impact of *Lhx9*-null mutation on other retinal neurons

a A comparison of POU4F1 and POU4F2 staining in *Lhx9-control* and *Lhx9-null* retinas showed no overt difference in retinal ganglion cell numbers at P30. **b–c** A comparison of PAX6 (**b**) and CHX10 (**c**) staining in *Lhx9-control* and *Lhx9-null* retinas showed no overt difference in amacrine cell and bipolar cell numbers at P30. **d–e** A comparison of anti-activated caspase 3 staining at P3 and P5 showed no overt difference in number of cells undergoing cell death. **f** bNOS expression appears in the *Lhx9-control* P5 retina but is absent in *Lhx9-null* retina. **g** Anti-TH immunolabeling of retinal whole-mounts reveals no change in dopaminergic cell projections and cell numbers in *Lhx9-control* and *Lhx9-null* retinas. **h–**

i Anti-ChAT immunolabeling of retinal whole-mounts reveals no change in dopaminergic cell projections and cell numbers in *Lhx9-control* and *Lhx9-null* retinas in the GCL (h) or INL (i). Quantification of cell numbers in **a–e** and **g–i** shows no difference between *Lhx9-null* as compared to *Lhx9-control* retinas ($n = 3$) (**j**). *Scale bars* equal 200 μm (**a–c**), 100 μm (**d–f**), and 800 μm (**g–i**)

Fig. 4. Lineage analysis of *Lhx9*-expressing retinal neurons during development

a–g Lineage-tracing analysis in early born amacrine cells with Cre-recombination induced at E13.5. Co-expression of reporter tdTomato with GAD65 and GAD67 shows significant co-expression at P21 in *Lhx9*-expressing neurons both in the control and null mutants (**a, b**). *Lhx9*-expressing cells do not give rise to ChAT+ amacrine cells in the INL in the control or the null mutants (**c**). All bNOS+ cells express tdTomato reporter in the control and bNOS expression is absent in the *Lhx9*-null retinas (**d**). Substance P expression delineates a lamination defect in the IPL between *Lhx9*-control and *Lhx9*-null retinas (**e**). *Lhx9*-expressing cells do not give rise to TH+ dopaminergic neurons in the control or mutant retinas (**f**). Quantification of cell numbers in **a, b** shows no difference between *Lhx9*-control and *Lhx9*-null retinas ($n = 3$) (**g**). **h–n** Lineage-tracing analysis in late-born amacrine cells with Cre-recombination induced at P1. Co-expression of tdTomato with GAD65 and GAD67 shows significant co-expression at P30 in *Lhx9*-expressing neurons both in the control and null mutants (**h, i**). *Lhx9*-expressing cells do not give rise to ChAT+ amacrine cells in the INL in the control or the null mutants (**j**). Some bNOS+ cells do not co-localize expression with *Lhx9*-reporter. bNOS expression is absent in the *Lhx9*-null retinas (**k**). Substance P expression delineates a lamination defect in the IPL between *Lhx9*-control and *Lhx9*-null retinas (**l**). *Lhx9*-expressing cells do not give rise to TH+ dopaminergic neurons in the control or mutant retinas (**m**). Quantification of cell numbers in **h** and **i** shows no difference between *Lhx9*-control and *Lhx9*-null retinas ($n = 3$) (**n**). Scale bar equals 200 μm

Fig. 5.

a-i Lineage-tracing analysis after the developmental wave of amacrine birth with Cre-recombination induced at P30. Co-expression of tdTomato with GAD65 shows no significant difference at P40 in *Lhx9*-expressing neurons both in the control and null mutants (**a**). Co-expression of tdTomato with GAD67 shows no significant difference at P40 in *Lhx9*-expressing neurons both in the control and null mutants (**b**). *Lhx9*-expressing cells do not give rise to ChAT+ amacrine cells in the INL in the control or the null mutants (**c**). bNOS+ cells do not co-localize expression with *Lhx9*-reporter. bNOS expression is absent in the *Lhx9-null* retinas (**d**). Substance P expression delineates a lamination defect in the IPL between *Lhx9-control* and *Lhx9-null* retinas (**e**). *Lhx9*-expressing cells do not give rise to TH+ dopaminergic neurons in the control or mutant retinas (**f**). *Lhx9*-expressing cells do not give rise to SATB2+ population of amacrine neurons in the control or mutant retinas (**g**). Quantification of cell numbers in **a**, **b**, and **g** shows no difference between *Lhx9-control* and *Lhx9-null* retinas ($n = 3$) (**h**, **i**). Scale bar equals 200 μm

Table 1

Table lists all antibodies used in this study, their host species, source, and working dilution

Antibody	Host species	Working dilution	Source
AP2α	Mouse	1:200	DSHB
bNOS	Sheep	1:1000	Millipore
	Rabbit	1:500	Abcam
Calretinin	Rabbit	1:1000	Millipore
	Goat	1:1000	Millipore
Caspase3	Rabbit	1:500	R&D systems
ChAT	Goat	1:1000	Millipore
CHX10	Sheep	1:500	Exalpha biologicals
GAD65	Mouse	1:200	DSHB
GAD67	Mouse	1:500	Millipore
GlyT1	Goat	1:5000	Millipore
LHX9	Guinea Pig	1:20,000	Gift from Dr. Jane Dodd, Columbia University
POU4F1	Mouse	1:200	Santa Cruz Biotech
POU4F2	Goat	1:200	Santa Cruz Biotech
RFP	Rabbit	1:1000	Rockland Immunochemicals
Substance P	Guinea Pig	1:500	Abcam
TH	Rabbit	1:1000	Millipore
	Chick	1:1000	AVES