BARHL2 Differentially Regulates the Development of Retinal Amacrine and Ganglion Neurons

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Through transcriptional regulations, the BarH family of homeodomain proteins play essential roles in cell fate specification, cell differentiation, migration, and survival. *Barhl2*, a member of the *Barh* gene family, is expressed in retinal ganglion cells (RGCs), amacrine cells (ACs), and horizontal cells. Here, to investigate the role of *Barhl2* in retinal development, *Barhl2*-deficient mice were generated. Analysis of AC subtypes in *Barhl2*-deficient retinas suggests that *Barhl2* plays a critical role in AC subtype determination. A significant reduction of glycinergic and GABAergic ACs with a substantial increase in the number of cholinergic ACs was observed in *Barhl2*-null retinas. *Barhl2* is also critical for the development of a normal complement of RGCs. *Barhl2* deficiency resulted in a 35% increase in RGCs undergoing apoptosis during development. Genetic analysis revealed that *Barhl2* functions downstream of the *Atoh7*–*Pou4f3* regulatory pathway and regulates the maturation and/or survival of RGCs. Thus, BARHL2 appears to have numerous roles in retinal development, including regulating neuronal subtype specification, differentiation, and survival.

Introduction

In retina, visual stimuli are received by photoreceptor cells and transferred via bipolar cells to retinal ganglion cells (RGCs). The two types of interneurons, amacrine and horizontal cells, function to integrate and modulate visual signal processing within retina. Based on their unique morphological, physiological, neurotransmitter, and connectivity properties, retinal neurons are further divided into subtypes. For instance, amacrine cells (ACs), which reside in the amacrine cell layer (ACL), an inner layer of the inner nuclear layer (INL), and the ganglion cell layer (GCL), synapse with bipolar cells and RGCs. They constitute the most diverse cell type within the retina with more than 26 different subtypes based on their sublamina localization (e.g., displaced ACs), morphology (e.g., starburst), and neurotransmitter types (e.g., GABAergic, dopaminergic, and cholinergic) (Masland, 2001a,b). Similarly, RGCs, the output neurons of the retina, are divided into more than 10 different subtypes based on their morphology, physiological properties, and stratification patterns (Kong et al., 2005). Although we have made significant progresses understanding the molecules important in generating the broad classes of retinal cell types, currently, the molecules defining retinal subtypes are poorly understood.

Previous studies have demonstrated that many transcription factors (TFs) play important roles in retinogenesis. During the

development of RGCs, ATOH7 (MATH5), a basic helix–loop–helix (bHLH) TF, endows retinal precursors with the competency to form RGCs (Yang et al., 2003). In *Atoh7*-null retinas, >95% RGCs fail to form (Brown et al., 2001; Wang et al., 2001). POU4F2, a POU-homeodomain (HD) TF, and ISL1, an LIM-HD TF, are synergistically required for RGC differentiation and survival (Gan et al., 1999; Pan et al., 2008). Nevertheless, the loss of RGCs in mice lacking *Pou4f2* or *Atoh7* is not selective to any particular subtype (Lin et al., 2004), suggesting that they are general factors in RGC development. Among the key TFs in amacrine development, NEUROD4 and NEUROD1, both bHLH TFs, are redundantly required for amacrine genesis (Marquardt et al., 2001; Inoue et al., 2002), whereas retina-specific deletion of *Pax6* HD TF results in the formation of only ACs (Marquardt et al., 2001). Mice lacking the winged helix/forkhead TF FOXN4 have fewer ACs and no horizontal cells (Li et al., 2004). However, the above TFs regulate AC generation in a nonsubtype-specific manner. Recently, studies show that BHLHB5, a bHLH TF, and ISL1 are expressed in GABAergic and cholinergic ACs, respectively, and are necessary for their differentiation (Feng et al., 2006; Elshatory et al., 2007), suggesting that unique TFs determine amacrine subtype development.

Bar-class HD (BarH) proteins are evolutionarily conserved and play essential roles in organogenesis. In *Drosophila*, BarH1 and BarH2 are expressed in R1/6 photoreceptor cells and are required for their differentiations (Higashijima et al., 1992). The *Xenopus* BarH orthologue, Xbh1, promotes RGC differentiation (Patterson et al., 2000; Poggi et al., 2004). In mouse retina, BARHL2 expression is found in RGCs, ACs, and horizontal cells. Forced expression of BARHL2 promotes the differentiation of glycinergic ACs at the expense of bipolar and Müller cells (Mo et al., 2004). Thus, BARHL2 appears to be an important molecule in

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mammalian retinal development. To understand the role of BARHL2 in retinal development, we generated a nonfunctional allele of *Barhl2* in mice. In *Barhl2*-null retinas, there was a selective loss of RGCs at late embryonic stages. Moreover, there was an overall loss of ACs that included a significant loss of both glycinergic and GABAergic in *Barhl2*-null retinas. Interestingly, the number of cholinergic ACs was greatly increased. These data demonstrate that BARHL2 is an important factor for the maintenance of RGCs and ACs, as well as being critical for subtype specification of retinal neurons.

Materials and Methods

Generation and genotyping of *Barhl2*-lacZ and *Barhl2*-Cre mice. To generate *Barhl2*-null mice, we used *Barhl2*-coding sequences as a probe to isolate genomic sequences from mouse 129S6 BAC genomic library (CHORI). The *Barhl2*-lacZ reporter and *Barhl2*-Cre knock-in construct were generated by inserting 3.5 kb *Barhl2* 5' flanking sequences ending at the 20th nucleotide upstream of the initiation codon ATG and 3.5 kb 3' sequence starting from the 68th nucleotide after ATG into 5' and 3' multiple cloning sites of pKI-lacZ and pKII-Cre vectors (L. Gan, unpublished observation) at the *HindIII* and *EcoRI* sites, respectively. The insertion placed *lacZ* or *Cre* under the control of endogenous *Barhl2* regulatory sequences. To generate *Barhl2*-null mice, *Ascl*-linearized *Barhl2*-lacZ- and *Barhl2*-Cre-targeting constructs were electroporated into W4 mouse embryonic stem (ES) cells (Auerbach et al., 2000) and positive-targeted ES cells were obtained by drug selection with G418 and FIAU. The targeted ES cells were confirmed by Southern blotting genotyping and were injected into C57BL/6J blastocysts to generate mouse chimeras. *Barhl2*-lacZ and *Barhl2*-Cre mice were generated in 129S6 and C57BL/6J mixed background as described previously (Gan et al., 1996, 1999). PCR was performed to genotype mice from heterozygous mating. PCR primers used to identify the wild-type (WT) *Barhl2* allele are 5'-ATGACAGCACTGGAAGGGGCC-3' and 5'-ACGCTGGTGTCTAGGGTGCACA-3'; the *lacZ* allele 5'-AGGGCCGCAAACTATCC-3' and 5'-ACTTCGGCACCTTACGCTTCTTCT-3'; and the *Cre* allele 5'-GCAGTGTCAACGCTTTTGTAGTGC-3' and 5'-CCATGAGTGAA-CGAACCTGGTCG-3'. The Z/EG conditional green fluorescent protein (GFP) reporter mice were purchased from The Jackson Laboratory (Stock number: 003920), and genotyping was performed according to protocols provided by The Jackson Laboratory. Embryos were identified as E0.5 at noon on the day at which vaginal plugs were first observed. All animal procedures in this study were approved by University Committee of Animal Resources at University of Rochester.

Immunohistochemistry, *in situ* hybridization, and X-Gal staining. Staged mouse embryos and enucleated eyes of postnatal mice were fixed with 4% paraformaldehyde in PBS for 1–2 h at 4°C. After the fixation, samples were cryopreserved with 20% sucrose, embedded in OCT compound, and cryosectioned at a thickness of 14 μ m. Immunostaining, *in situ* hybridization, and X-Gal staining were performed as described previously (Pan et al., 2005). For whole-mount immunostaining, animals were anesthetized with halothane and perfused with 4% paraformaldehyde in PBS. Eyecups were isolated and blocked in 10% horse serum with 0.5% Triton X-100 for 4 h and incubated with primary antibody for 2–3 d at 4°C. Then, eyecups were washed in PBS and incubated with fluorescently labeled secondary antibody for 1 d at 4°C. After three rinses of 15 min each, retinas were dissected out and mounted in PBS on slides. Retinal images were captured with a Zeiss LSM 510 META confocal microscope. To quantify the numbers of immunolabeled cells, five or more retinas for each genotype were analyzed with Image J software (National Institutes of Health).

The following antibodies and dilutions were used: rabbit anti- β -galactosidase (lacZ) (1:500; Millipore Bioscience Research Reagents), goat anti-POU4F2 (1:200; Santa Cruz), mouse anti-calbindin (1:2000; Sigma), mouse anti-POU4F1 (1:200; Millipore Bioscience Research Reagents), mouse anti-syntaxin (1:5000; Santa Cruz), mouse anti-PAX6 [1:200; Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Isl1/2 (1:200; DSHB), goat anti-ChAT (1:100; Millipore Bioscience Research Reagents), sheep anti-Chx10 (Exalpha), rabbit anti-PKC α

(1:5000; Sigma), mouse anti-GAD65 (1:200; BD Bioscience), rabbit anti-activated caspase3 (1:200; BD PharMingen), goat anti-GLYT1 (1:5000; Millipore Bioscience Research Reagents), rabbit anti-opsin (1:200), chicken anti-GFP (1:500; Abcam). Alexa-conjugated secondary antibodies (Invitrogen) were used at a concentration of 1:500.

Multielectrode array recording. The procedures of retina preparation, action potential recording, and data analysis have been described previously in detail (Tian and Copenhagen, 2003). Briefly, mice were dark adapted for 30 min before being killed. Retinas were isolated under infrared illumination in oxygenated extracellular solution, which contained (in mM) 124 NaCl, 2.5 KCl, 2 CaCl₂, 2 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and glucose 22 (pH 7.35 with 95% O₂ and 5% CO₂). Isolated retina was mounted in the multielectrode array (MEA-60) chamber with the ganglion cell layer facing the recording electrodes and continuously perfused with oxygenated extracellular solution at 34°C. For spontaneous retinal waves, data were collected for 30 min. For light-evoked action potentials, a white rectangular moving bar (600 $\times$ 4000 μ m) was used to stimulate the retinas, while moving on a black background and perpendicular to its long axis at 1000 mm/s and in 12 directions at 30° intervals with a pseudorandom sequence. The stimulations were repeated 30 $\times$ with random sequences. Data were collected using a PC-based interface card and software (Multi Channel System). The signals were filtered between 100 Hz (low cutoff) and 200 or 250 kHz (high cutoff). Off-line data analysis was performed on a PC computer using off-line sorter (Plexon).

MEA data analysis. For spontaneous retinal waves, the cell with highest firing rate was selected for each electrode for the calculation of correlation index. The correlation index for a given pair of neurons was calculated based on the following equation (Wong et al., 1993; Torborg et al., 2005):

$$\text{Correlation index} = \frac{N_{AB(-0.1s, 0.1s)} \times T}{N_{A(0,T)} \times N_{B(0,T)} \times (0.2s)},$$

where N_{AB} is the number of spike pairs from cells A and B for which cell B fires within ± 100 ms of cell A, T is the total recording time, $N_{A(0,T)}$ is the total number of spikes in cell A, and $N_{B(0,T)}$ is the total number of spikes in cell B. The frequencies of action potentials of all sorted cells were averaged to calculate the average firing rate. The interwave interval is the time between the two subsequent waves determined manually based on the spike trains.

For light-evoked directional selective responses, self-programmed software was used to calculate the peak spike frequency of leading and trailing edge (ON and OFF) responses for each cell. The responses were averaged from 30 recording trials. The directional selectivity index (DSI) was calculated as follows:

$$\text{DSI} = \frac{\text{Preferred peak frequency} - \text{Null peak frequency}}{\text{Preferred peak frequency} + \text{Null peak frequency}}.$$

Results

Targeted deletion of *Barhl2* and retinal expression of *lacZ* knock-in reporter gene

To investigate the role of *Barhl2* in retinal development, we generated a targeted deletion of *Barhl2* by homologous recombination (supplemental Fig. S1 A, B, available at www.jneurosci.org as supplemental material). The *Barhl2*^{lacZ} knock-in allele was created by replacing 88 bp in exon 1 with the *lacZ* reporter gene and SV40 polyadenylation sequences to prevent the transcription of the remaining *Barhl2*-coding sequences. *In situ* hybridization, using *Barhl2* antisense probe, showed that the expression of *Barhl2* was abolished in *Barhl2*^{lacZ/lacZ} (*Barhl2* null) retinas and anti-lacZ immunostaining revealed the expression of lacZ in the embryonic retinas (supplemental Fig. S1C, available at www.jneurosci.org as supplemental material). The heterozygous *Barhl2*^{lacZ/+} mice were indistinguishable from the WT and, therefore, included as controls. Offspring from the *Barhl2*^{lacZ/+} intercross were born in a standard Mendelian ratio. Out of the first 289

mice weaned, 22.5% were WT, 52.9% were *Barhl2*^{lacZ/+}, and 24.6% were *Barhl2*^{lacZ/lacZ}. *Barhl2*-nulls (*Barhl2*^{lacZ/lacZ}) mice were indistinguishable from littermate controls at birth. However, they did not gain weight at the same rate as controls and gradually displayed signs of impaired motor coordination. *Barhl2*-null mice died before postnatal day 24 (P24).

Previous studies have shown that the earliest expression of *Barhl2* in mouse retina is at E13.5 in the inner neuroblastic layer (NBL). In the adult retina, *Barhl2* is expressed in amacrine, horizontal, and ganglion cells (Mo et al., 2004). To examine the retinal cell types that *Barhl2*-*lacZ* is expressed in and to determine whether the expression pattern of *lacZ* reflects that of endogenous *Barhl2*, we double-immunolabeled retinas from *Barhl2*^{lacZ/+} mice with anti-*lacZ* antibody and specific cell type-specific markers. *LacZ*⁺ cells were in three distinct locations in the retina, in the GCL and the inner and outer portions of the INL. In the GCL, *LacZ*⁺ cells colabeled with an RGC marker, POU4F1, in some RGCs (supplemental Fig. S1D, available at www.jneurosci.org as supplemental material). All *Barhl2*-expressing cells in the GCL and the inner portions of the INL colabeled with PAX6, a marker for all RGCs and ACs (supplemental Fig. S1E, available at www.jneurosci.org as supplemental material). Double staining with amacrine subtype-specific markers revealed that *lacZ* was expressed in all cholinergic ACs (ISL1⁺), selective glycinergic amacrine (GLYT1⁺), and GABAergic (GAD65⁺) ACs (supplemental Fig. S1F–H, available at www.jneurosci.org as supplemental material). At the outer border of the INL, all horizontal cells (calbindin⁺) expressed *lacZ* (supplemental Fig. S1I, available at www.jneurosci.org as supplemental material). The absence of colabeling of *lacZ* with CHX10, opsin, and GFAP excluded the possibility that *Barhl2* is expressed in bipolar, photoreceptor, and glial cells, respectively (data not shown). These results are consistent with the expression pattern of *Barhl2* reported previously (Mo et al., 2004) and indicated that the knock-in *lacZ* allele recapitulated endogenous *Barhl2* expression.

Retinal defects in *Barhl2*-null mice

To investigate the role of *Barhl2* in retinal development, we first assessed the retinal histology of *Barhl2*-null retinas at different developmental stages. Before E15.5, no alterations in retinal laminar organization and thickness were apparent in *Barhl2* nulls (Fig. 1A). Starting at E16.5, *Barhl2*-null retinas had a noticeable reduction in GCL thickness, and by P7, the INL thickness was also reduced (Fig.

Figure 1. Developmental abnormality of *Barhl2*-null retinas. Retinas from control and *Barhl2*-null mice were analyzed by hematoxylin and eosin staining at different developmental stages. **A**, Compared with the controls, no overt change in retinal thickness and laminar structures is observed in *Barhl2*-null retinas before E15.5. **B–F**, From E16.5, the GCL of *Barhl2*-null retina is thinner, and the number of cells in the GCL is significantly reduced. This hypoplasia is maintained till P21. From P7, the INL also exhibits a marked reduction in cell numbers; at P21, the cell number in the INL is reduced 35.6%. The ONL thickness is normal. **G**, Quantitation of cells in the GCL and INL per 250 μm length of retinal section at P0–P21. Cell counting in NBL in **C** and ONL in **E** and **F** covers 62.5 μm linear length. **t* test $p < 0.01$; ***t* test $p < 0.001$. Scale bar, 50 μm.

1B–D). Although the overall laminar structure of *Barhl2*-null retinas resembled those of the controls at the completion of retinogenesis (at P21), *Barhl2*-null retinas had $16.3 \pm 2\%$ fewer cells in the GCL and $35.6 \pm 6\%$ less cells in the INL (Fig. 1E–G).

Figure 2. Effect of *Barhl2*-null mutation on RGCs and ACs in P21 retinas. Retinas were immunolabeled with indicated antibodies (green) and nuclear counterstained with propidium iodide (red). **A**, Loss of *Barhl2* leads to a severe loss of RGCs immunoreactive for POU4F1 (average number of POU4F1+ RGCs $\pm$ SD: controls, 33 ± 5 , $n = 4$; *Barhl2* nulls, 22 ± 3 , $n = 4$; $p < 0.01$, Student's *t* test). **B**, Loss of amacrine cells immunoreactive for PAX6 (average number of PAX6+ ACs $\pm$ SD: controls, 217 ± 11 , $n = 4$; *Barhl2* nulls, 142 ± 13 , $n = 4$; $p < 0.01$, Student's *t* test). **C–E**, No significant alteration in horizontal cells immunoreactive for calbindin (**C**, average number of calbindin+ horizontal cells $\pm$ standard SD: controls, 8 ± 2 , $n = 4$; *Barhl2* nulls, 7 ± 2 , $n = 4$; $p = 0.63$, Student's *t* test), bipolar cells immunoreactive for CHX10 (**D**, average number of CHX10+ bipolar cells $\pm$ SD: controls, 155 ± 11 , $n = 4$; *Barhl2* nulls, 161 ± 17 , $n = 4$; $p = 0.75$, Student's *t* test), and photoreceptors immunoreactive for Opsin (**E**). **F**, Quantitation of immunoreactive cells per 250 μ m length of retinal section at P21. **t* test $p < 0.01$. Scale bar, 100 μ m.

Figure 3. Analysis of RGCs and displaced ACs in the GCL of *Barhl2*-null retina. **A–D**, Retinas from control and *Barhl2*-null mice at P21 were coimmunolabeled with anti-PAX6 (red) and anti-POU4F2 (green) (**A**, **B**) or with anti-ISL1 (red) and anti-POU4F2 (green) (**C**, **D**). **E**, Quantitation of immunolabeling results. In the GCL of *Barhl2*-null retina, the total number of ACs and RGCs labeled by anti-PAX6 and anti-ISL1 are reduced by 13.8 and 28.3%, respectively. Specifically, the POU4F2-immunoreactive RGCs are decreased to 53%, whereas the POU4F2-negative and ISL1-positive cells are increased more than twofold. Cell numbers were counted in the central regions of at least three independent samples from each genotype. **t* test $p < 0.01$. Scale bar, 50 μ m.

The expression of *Barhl2* in amacrine, horizontal, and ganglion cells, and the marked loss of cells in the GCL and INL in *Barhl2*-null retinas, suggested that deletion of *Barhl2* could impair the differentiation and/or survival of neurons in the GCL and INL. To test this possibility, cell type-specific markers were used to analyze the development of retinal cell types and subtypes. Immunostaining for POU4F1, which labels ~70% of RGCs (Xiang et al., 1993), revealed a 35% decrease of these cells in the GCL (Fig. 2A). In the INL, anti-PAX6 labeling revealed a 39% loss of ACs (Fig. 2B). No significant changes in horizontal (calbindin+), bipolar (CHX10+), and photoreceptor (Opsin+) cells (Fig. 2C–F). In summary, the targeted deletion of *Barhl2* selectively affects the development of RGCs and ACs but not horizontal cells that also express *Barhl2*.

Developmental loss of RGCs in *Barhl2*-null retinas

The loss of RGCs in *Barhl2*-null retinas prompted us to analyze further the changes in cell composition in the GCL. Whole-mount immunostaining of the adult retinas revealed an ~47% decrease of POU4F2+ RGCs (Fig. 3A–E) and a 13.8 $\pm$ 4% decrease in PAX6+ cells (Fig. 3A,B,E) in *Barhl2* nulls. Although ISL1 immunostaining for RGCs and cholinergic ACs revealed a 28.3 $\pm$ 3.0% loss of all ISL1+ cells in the GCL, the POU4F2- and ISL1+ cells (presumably the displaced cholinergic ACs) increased by more than twofold (Fig. 3C–E), suggesting that the loss of RGCs in *Barhl2* nulls is accompanied by an increase in the displaced ACs in the GCL, particularly the cholinergic amacrine subtypes.

To investigate whether the loss of RGCs results from a dys-

regulation of neurogenesis or cell death, we next tested the generation of RGCs in the absence of *Barhl2*. POU4F2 expression marks the majority of RGCs during embryogenesis (Young, 1985; Gan et al., 1999). Immunostaining of POU4F2 revealed no change in the number of RGCs in *Barhl2*-null retinas before E15.5 (Fig. 4*A,B,M*), suggesting that the genesis of RGCs was not affected in *Barhl2* nulls. However, a 19.4% decrease of POU4F2+ RGCs was observed starting at E16.5 in *Barhl2*-null retinas compared with controls (Fig. 4*C,D,M*). The number of POU4F2+ RGCs continued to decrease with age (Fig. 4*E–H,M*). Consistently, the reduction of RGCs was confirmed by a 33.7% reduction in the cross-section areas of the *Barhl2*-null optic nerves (Fig. 4*I–L*). To determine whether the loss of RGCs resulted from apoptosis in *Barhl2*-null retinas, we immunolabeled retinas with anti-activated CASP3, one of the main effectors in developmental cell death (Kuida et al., 1996). *Barhl2*-null retinas showed a gradual increase of apoptotic cells in the GCL from E16.5 to P0 compared with controls (supplemental Fig. S2, available at www.jneurosci.org as supplemental material), suggesting that the newly generated RGCs undergo apoptosis in *Barhl2*-null retinas.

Previous studies have shown that BARHL2 is expressed in ~33% of RGCs (Mo et al., 2004). The loss of RGCs in *Barhl2* nulls is of comparable number, suggesting a cell-autonomous role of BARHL2 in RGCs. Coimmunolabeling *Barhl2*-null retinas using anti-POU4F2 and anti-lacZ demonstrated that 90–95% of RGCs immunoreactive for lacZ and POU4F2 were absent in *Barhl2*-null retinas (Fig. 4*N–O*, arrows). In contrast, the number of RGCs immunoreactive for POU4F2 alone (green) remained the same, consistent with the cell-autonomous role for BARHL2 in development and survival of a selective set of RGCs.

Although BARHL2 expression is restricted to a subset of RGCs in the adult, it may also be expressed transiently in other RGCs during development. To investigate the fate of all *Barhl2* lineage RGCs, we used a lineage tracing strategy to permanently mark cells that ever expressed *Barhl2*. *Barhl2*-Cre knock-in mice were generated and expressed Cre recombinase under *Barhl2* regulatory sequences. *Barhl2*-Cre mice were bred with the Z/EG conditional GFP reporter mice, which constitutively expresses an enhanced GFP after the Cre-mediated recombination (Novak et al., 2000), to generate *Barhl2*^{Cre/+};Z/EG/+ mice for the analysis of *Barhl2*-lineage cells in control retinas (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). Similarly, *Barhl2*-null (*Barhl2*^{Cre/lacZ};Z/EG/+) mice were generated from *Barhl2*^{Cre/+};Z/EG/+ and *Barhl2*^{lacZ/+} heterozygotes crossing to determine *Barhl2*-lineage cells in the absence of *Barhl2*. Colabeling of GFP and POU4F2 demonstrated

Figure 4. Loss of RGCs in *Barhl2*-null retina. *A, B*, No overt change in the number of POU4F2 immunoreactive RGCs is detected in *Barhl2*-null retinas at and before E15.5. *C–H*, Starting at E16.5, there is a progressive reduction in RGCs in *Barhl2*-null retinas. Insets show the enlarged views of boxed regions in the central retina. *I, J*, Ventral views of brains reveal optic nerves (arrowheads) and optic chiasm (arrow). *K, L*, Hematoxylin and eosin staining of optic nerve cross sections cut at the level indicated by the dashed lines in *I* and *J*. The cross-section areas (arrowheads) of the *Barhl2*-null optic nerves are reduced by ~33.7% compared with controls ($n = 3$, $p < 0.001$, Student's *t* test). *M*, Quantitation of RGCs immunoreactive for POU4F2 in late embryonic stages and early postnatal days. * t test $p < 0.01$. *N, O*, Immunostaining of whole-mount retinas with anti-POU4F2 (green) and anti-lacZ (red) reveals a severe loss of lacZ+ RGCs (arrowhead; yellow) in *Barhl2* null. *P, Q*, Lineage tracing analysis by immunostaining of whole-mount retinas with anti-GFP (green) and anti-POU4F2 (red) shows a severe reduction of *Barhl2*-lineage RGCs (arrowhead; yellow) in *Barhl2* null. OC, Optic chiasm; OT, optic tract. Scale bars: (in *B, D, F*, and *H*) *A–H*, 50 μ m; (in *I–L*), 300 μ m; (in *Q*) *N–Q*, 25 μ m.

that compared with heterozygous retina at P21, *Barhl2*-null retinas had a loss of ~95% of *Barhl2*-lineage RGCs (Fig. 4*P, Q*), thus confirming the cell-autonomous role of *Barhl2* in the development of RGCs.

Altered amacrine subtype composition in *Barhl2*-null retinas

The partial loss of ACs in the INL (Fig. 2*B*) and the twofold increase in POU4F2+ and ISL1+ cells (presumably the displaced cholinergic ACs) in the GCL (Fig. 3*C–E*) prompted us to examine the subtype-specific changes of ACs in *Barhl2*-null retinas. Immunostaining of GAD65 for GABAergic ACs showed that the number of GABAergic ACs was significantly reduced in *Barhl2*-null retinas by 34% compared with the controls (Fig. 5*A*). Immunolabeling of BHLHB5, a bHLH TF expressed in a group of GABAergic ACs (Feng et al., 2006), revealed a similar loss of

Figure 5. Subtype-specific effects on ACs *Barhl2*-null retina. Sections from P21 mouse retinas were immunolabeled with AC subtype-specific markers (green) and nuclear counterstained with PI (red). **A–C**, Loss of *Barhl2* leads to a severe loss of ACs immunoreactive for GAD65 (**A**, average number of GAD65 + ACs $\pm$ SD: controls, 62 ± 6 , $n = 4$; *Barhl2* nulls, 39 ± 5 , $n = 4$; $p < 0.01$, Student's *t* test), Bhlhb5 (**B**), and GLYT1 (**C**, average number of GLYT1 + ACs $\pm$ SD: controls, 89 ± 6 , $n = 4$; *Barhl2* nulls, 50 ± 3 , $n = 4$; $p < 0.001$, Student's *t* test). **D–F**, There is also an overt increase of amacrine cells immunoreactive for Isl1 (**D**, average number of ISL1 + ACs $\pm$ SD: controls, 10 ± 2 , $n = 4$; *Barhl2* nulls, 18 ± 3 , $n = 4$; $p < 0.01$, Student's *t* test), ChAT (**E**), and calretinin (**F**). Scale bar, 100 μ m.

GABAergic ACs (Fig. 5B). Likewise, anti-GLYT1 labeling demonstrated a 40% reduction in glycinergic ACs in *Barhl2*-null retinas compared with the controls (Fig. 5C). In contrast, the cholinergic ACs labeled by anti-ISL1, ChAT, and calretinin in the ACL were increased (Fig. 5D–F). Whole-mount immunolabeling showed that in the ACL, ISL1 expression completely overlapped that of ChAT and that there was a threefold increase in the cholinergic amacrine cell density in *Barhl2*-null retinas (Fig. 6A,B). Similarly, ChAT- and ISL1-labeled displaced cholinergic ACs were increased by twofold (Fig. 6C,D).

ISL1 is expressed in developing and mature cholinergic ACs. Retina-specific deletion of *Isl1* abolishes all cholinergic ACs (Elsathory et al., 2007). To elucidate the mechanism underlining the increase of cholinergic ACs in *Barhl2*-null retinas, we assessed ISL1 expression during early postnatal retinal development. We found that there were approximately twofold increases in the

number of cells expressing ISL1 at the inner edge of the NBL (presumably the developing ACL) in *Barhl2*-null retinas at P0–P5 (Fig. 6E,F, brackets). In contrast, there was a reduction of 40% differentiating PAX6+ ACs in *Barhl2*-null retinas at P5 (Fig. 7A,B). Anti-BHLHB5 immunolabeling detected a similar reduction of a subset of GABAergic ACs at this stage (Fig. 7C,D). Moreover, using anti-activated CASP3 immunostaining, we detected an increase of apoptotic cells in the NBL where the nascent ACs were localized (Fig. 7E,F,J). Colabeling with anti-PAX6 and anti-CASP3 confirmed some of these apoptotic cells as ACs (Fig. 7G–I, arrows). Thus, loss of *Barhl2* affects the amacrine subtype composition by differentially changing the genesis and survival of different amacrine subtypes.

Roles of *Barhl2* in the genetic regulatory networks of ACs and RGCs

Neurod1 and *Neurod4* are redundantly required for AC genesis (Inoue et al., 2002). To define the role of *Barhl2* in the genetic regulatory network of AC cell differentiation, we analyzed the expression of *Neurod4* and *Neurod1* in control and *Barhl2*-null retinas by *in situ* hybridization and observed no detectable alteration in their expression from E14.5 to P0 (Fig. 8A,B) (data not shown), suggesting that *Barhl2* unlikely functions upstream of *Neurod4* and *Neurod1* during AC differentiation. Rather, *Barhl2* could function downstream of *Neurod4* and *Neurod1* in the AC differentiation pathway to negatively regulate the development of cholinergic amacrine subtype by suppressing *Isl1*.

In *Drosophila*, the proneural gene *ato* is required for the specification of the first born retinal neurons, R8 photoreceptors (Brunet and Ghysen, 1999). During retinogenesis, *ato* expression is sequentially restricted from the most anterior region of morphogenetic furrow to R8 founder cells. This restricted expression of *ato* is partially controlled by *BarH1* and *BarH2*. Loss of *BarH* induces cell-autonomous ectopic expression of *ato* (Lim and Choi, 2004). The murine orthologue of *ato*, *Atoh7*, is transiently expressed in ganglion-competent retinal precursors and is essential for RGC differentiation (Wang et al., 2001; Yang et al., 2003). Thus, we asked whether *Barhl2* could negatively regulate the expression of *Atoh7*. As shown by *in situ* hybridization, loss of *Barhl2* had no effect on the expression of *Atoh7* (Fig. 8C). Conversely, the expression of *Barhl2* was significantly downregulated in both the GCL and the inner NBL of *Atoh7*-null retinas at E13 (Fig. 8D). Furthermore, *Barhl2* expression was greatly downregulated in *Pou4f2*-null retinas at E13.5 (Fig. 8E). Therefore, *Barhl2* functions downstream of *Atoh7*–*Pou4f2* pathway to regulate the development of a subset of RGCs.

Figure 6. Cholinergic ACs are increased in *Barhl2*-null retinas. **A, B**, Double immunolabeling of control and *Barhl2*-null retinas with anti-ISL1 (green) and anti-ChAT (red) reveals a threefold increase in cholinergic ACs in the absence of *Barhl2* at P21. **C, D**, In the GCL, there is a twofold increase in the displaced cholinergic ACs in *Barhl2*-null retinas. **E, F**, Upregulation of ISL1 expression in the ACL of *Barhl2*-null retina during the development of cholinergic amacrine cells (brackets). Scale bars, 25 μ m.

Functional impairments in *Barhl2*-null mice

Finally, we determined the functional impacts of *Barhl2* deletion on retinal cholinergic ACs by recording spontaneous and light-evoked synaptic activity of RGCs. We first examined how genetic deletion of *Barhl2* affects cholinergic synaptic activity in the developing retina. We used a multielectrode array (MEA) recording to examine spontaneous RGC activity in *Barhl2*-null mice at P3 and P13 (Fig. 9A). The spontaneous retinal waves at P3 are mediated by nicotinic acetylcholine receptors (nAChRs) and the retinal waves at P13 are mediated by glutamate receptors (GluRs) (Bansal et al., 2000). The frequency of nAChR-mediated retinal waves, measured as the interwave interval, in *Barhl2*-null mice at P3 was $\sim$ 50% lower than that of age-matched controls, whereas the interwave interval of GluR-mediated retinal waves in *Barhl2*-null mice at P13 was not different from age-matched controls (Fig. 9B–D), demonstrating that genetic deletion of *Barhl2* preferentially affects nAChRs-mediated spontaneous synaptic transmission in developing retina.

We then examined whether genetic deletion of *Barhl2* affects the light-evoked synaptic transmission specific to cholinergic ACs by examining the light-evoked responses of directional selective (DS) RGCs. DSRGCs exhibit vigorous spiking activity when a visual object moves in their preferred direction across the receptive field but minimum response when the same object sweeps across in the opposite (null) direction (compare Fig. 10A,B) because of spatially offset GABAergic inhibition from cholinergic starburst ACs (Demb, 2007). In mouse retina, most of the ON-OFF responsive RGCs are DSRGCs. We specifically examined the strength of the directional selectivity, as a measure of the specific asymmetric inhibitory synaptic transmission between cholinergic starburst ACs and DSRGCs, and the peak frequency of light-evoked responses, as a measure of the strength of overall inhibitory synaptic inputs, of ON-OFF DSRGCs in *Barhl2*-null mice. Consistent with an increase of GABAergic synaptic inputs from cholinergic starburst ACs, the average peak frequency of light-evoked responses of ON-OFF DSRGCs in

Figure 7. *Barhl2*-null retinas exhibit an elevated cell apoptosis in ACs. **A, B**, Immunostaining of retina sections with PAX6 reveals a significant loss of PAX6+ amacrine cells (brackets) in *Barhl2*-null retina at P5. **C, D**, Anti-BHLHB5 immunostaining shows a decrease in a selective group of GABAergic amacrine cells in *Barhl2*-null retina (yellow arrowhead). **E, F**, Anti-activated CASP3 immunostaining shows an increase of apoptotic cells in the INL of *Barhl2*-null retina at P3. **G–I**, Expression of PAX6 (red) in apoptotic cells shown by anti-CASP3 (green) in *Barhl2*-null retina (yellow arrowhead) at P3. **J**, Quantification of apoptotic cells in the INL of the developing retina by averaging activated caspase3-positive cells in 10 sections per retina. The differences in apoptotic cell numbers in control and *Barhl2*-null retina are significant from P0 to P5. * t test $p < 0.01$. Scale bars, 100 μ m.

Barhl2-null mice is significantly lower than that of controls (Fig. 10D,E). In contrast, the average peak frequency of light-evoked responses of ON RGCs in *Barhl2*-null mice, which are dominated by non-DSRGCs, is not different from that of controls (Fig. 10C). Interestingly, the peak frequency of light-evoked responses of the

null direction of ON-OFF DSRGCs is more significantly suppressed in *Barhl2*-null mice, which resulted in a slight increase of the strength of directional selectivity (Fig. 10F). This is consistent with an asymmetric GABAergic inhibition from cholinergic starburst ACs to ON-OFF DSRGCs with more effective inhibition on the null direction. Together, these results strongly support the notion that genetic deletion of *Barhl2* specifically impaired GABAergic synaptic transmission mediated by cholinergic starburst ACs in mouse retina.

Discussion

In this report, we have generated *Barhl2*-null mice and demonstrated *Barhl2*'s essential role in the development of multiple retinal cell types. Loss of *Barhl2* results in a marked decrease of RGCs. During the development of ACs, *Barhl2*-null mutation affects the development of distinct AC subtypes. Loss of *Barhl2* leads to the partial loss of glycinergic and GABAergic ACs, a significant increase in cholinergic ACs, and altered synaptic inputs of cholinergic ACs to RGCs in both immature and mature retinas. Gene expression studies show that *Barhl2*-null mutation leads to the upregulation of ISL1, the key regulator of cholinergic ACs in the INL and the GCL. Furthermore, we have demonstrated that *Barhl2* functions downstream of *Atoh7*–*Pou4f2* pathway in RGC development and likely downstream of *Neurod1* and *Neurod4* in differentiating ACs. The differential effect of *Barhl2*-null mutation on each retinal neuronal subtype implies a mechanism that BARHL2 and other TFs form a unique combinatorial code in each subtype and regulate the acquisition of subtype identities.

Essential roles of *Barhl2* in the development of RGCs

Previous studies have demonstrated that ATOH7 and POU4F2 constitute the essential genetic cascade of RGC development. ATOH7 determines the RGC competence of retinal precursors (Brown et al., 2001; Wang et al., 2001; Yang et al., 2003), whereas POU4F2 is required for the terminal differentiation of RGCs including axon growth and pathfinding as well as survival (Gan et al., 1996, 1999). The downregulation of *Barhl2* expression in *Atoh7*-null and *Pou4f2*-null retinas (Fig. 8D,E) and the partial loss of RGCs in *Barhl2*-null retinas suggest that *Barhl2* functions downstream of *Atoh7* and *Pou4f2* to control the differentiation of a subset of RGCs. Similarly, in *Xenopus*, Xbh1 (orthologous to BARHL2) is reported to interact with Xath5 and Xath3 and act as a late transcriptional repressor downstream of *Xath5* in the RGCs development (Poggi et al., 2004). Although *Barhl2* is a direct downstream target of MATH1 in the spinal cord (Saba et al., 2005), it remains to be determined whether ATOH7 directly regulates *Barhl2* in retinas. However, the late onset of *Barhl2* expression at E13.5 and the downregulation of *Barhl2* in *Pou4f2*-null retinas suggest that *Barhl2* is an indirect downstream target of *Atoh7* during RGC development.

In *Barhl2*-null mice, there is no change in the initial formation and migration of the nascent RGCs (POU4F2+) to the GCL, but there is a progressive reduction of ~35% RGCs accompanied by the reduced thickness of optic nerves (Figs. 2–4), suggesting that *Barhl2* is dispensable for RGC genesis but necessary for RGC maturation and survival. Targeted deletion of *Barhl2* could result in the RGC defects through two possible mechanisms. First, BARHL2 could function cell autonomously to control the maturation and survival of the BARHL2-expressing RGCs. Without BARHL2, these RGCs fail to develop properly and undergo apoptosis. Such cell-autonomous effects were previously observed during retinal development in mice null for several TFs. For ex-

Figure 8. Analysis of potential upstream and downstream genes of *Barhl2*. **A–C**, The expression profiles of retinogenic bHLH genes, *Atoh7*, *Neurod4*, and *Neurod1*, are unaffected in *Barhl2*-null retinas by *in situ* hybridization at E16.5. **D, E**, *Barhl2* expression is downregulated in *Atoh7*-null and *Pou4f2*-null retinas. *In situ* hybridization reveals a reduced expression of *Barhl2* in *Atoh7*-null (**D**) and *Pou4f2*-null (**E**) retinas at E13.5. L, Lens; NBL, neuroblast layer retina. Scale bars, 100 μ m.

ample, BHLHB4 is specifically expressed in rod-bipolar cells, and *Bhlhb4* knockout abolishes the rod-bipolar cell population (Bramblett et al., 2004). Similarly, ISL1 is required cell autonomously for the development of bipolar cells and cholinergic ACs, and POU4F2 and SHH act cell autonomously in RGCs (Elshatory and Gan, 2008; Pan et al., 2008; Sanchez-Camacho and Bovolenta, 2008). Alternatively, BARHL2 could function in a non-cell-autonomous manner. Loss of *Barhl2* may not affect the RGCs of BARHL2+ subset. Rather, it indirectly influences the maturation and survival of the RGCs that never express BARHL2. Non-cell-autonomous effect was previously seen in retinas transfected with SMO-M2, a constitutive active allele of Smoothened, that SMO-M2 expression inhibits rhodopsin expression in uninfected photoreceptor cells (Yu et al., 2006). Interestingly, the extent of RGC loss is in proportion to the number of RGCs expressing *Barhl2* as shown by the colocalization of POU4F2 and lacZ in *Barhl2*^{lacZ/+}, suggesting a cell-autonomous role of BARHL2 in the maturation and survival of some RGCs. We performed a lineage analysis using *Barhl2-Cre* knock-in allele (Fig. 4N–Q) and demonstrated the loss of RGCs that are mostly from *Barhl2*-cell lineage, thus confirming the cell-autonomous role of BARHL2 in RGCs.

The roles of *Barhl2* in the subtype specification of ACs

ACs form synapses with bipolar cells and RGCs and modulate the signal processing by excitatory and inhibitory effects. The development of ACs depends on several TFs. FOXN4 regulates amacrine genesis by activating the expression of amacrine differentiation factors, *Neurod4* and *Neurod1* (Li et al., 2004). PTF1, a bHLH TF, is essential for the specification of amacrine and horizontal cells, acting as a primary target of FOXN4. These above TFs do not regulate the subtype specification of ACs, and little is known about how the mammalian retina generates the numerous different subtypes of ACs. PAX6, a key regulator in the early retinal development, is expressed in differentiating ACs and positively regulates the formation of glycinergic ACs (Marquardt et al., 2001). Recently, we show that BHLHB5 and ISL1 are uniquely expressed and required for the differentiation of GABAergic (Feng et al., 2006) and cholinergic (Elshatory et al., 2007) amacrine subtypes, respectively. Although *Barhl2* is expressed in all amacrine subtypes, loss of *Barhl2* does not affect the initial formation of ACs expressing *Neurod1* and *Neurod4*. Rather, it has subtype-specific effects on ACs, resulting in a 40% loss of glycin-

ergic and GABAergic ACs and in a significant increase of cholinergic ACs. These differential effects imply that BARHL2's role in amacrine cell differentiation depends on the unique cellular environment of each amacrine subtypes. Therefore, our data indicate that the subtype specification of ACs is determined after the acquisition of pan-amacrine properties and is governed by the unique combinatorial activities of overlapping transcriptional activators and repressors.

The combinatorial codes of TFs play many roles in embryonic development. It is first revealed in *Drosophila* that the overlapping expression of transcriptional activators and repressors in early embryos generates the combinatorial TF codes essential for the formation of segmentation stripes (Stanojevic et al., 1991). During vertebrate neurogenesis, the combinatorial actions of HD, bHLH, and LIM-HD proteins regulate the specification of neural cell types in the spinal cord (Jessell, 2000; Bertrand et al., 2002). In ventral spinal cord, for example, the partial overlapping expression of PAX6, NKX6.1, and NKX2.2, and their mutual repressive activities, define a progenitor domain from which motor neurons are generated (Tanabe et al., 1998). The function of the combinatorial activities of partially overlapping transcriptional activators and repressors is similarly proposed to regulate the neurogenesis during cortical development (O'Leary and Nakagawa, 2002). Recently, we show that *Bhlhb5* is expressed in neurons in layers II–V of somatosensory and visual cortex and that targeted deletion of *Bhlhb5* leads to altered gene expression patterns in area- and layer-specific manners. For example, the expression of ROR β in layer IV of *Bhlhb5* nulls is downregulated in somatosensory but not visual cortex where *Bhlhb5* is also highly expressed and is required for the expression of Eph7A. Additionally, BHLHB5 regulates the expression of Ephrin-A5 in layers IV–V but not layers II–III of somatosensory cortex despite BHLHB5's expression in layers II–V. These results provide direct evidence for this combinatorial code model within the developing cortex (Joshi et al., 2008).

Ectopic expression of bHLH and HD TFs in different combinations in retina are capable to uniquely enhance or suppress the differentiation of the six major retinal cell types (Wang and Harris, 2005). Here, we show that although *Barhl2* is expressed in horizontal, amacrine, and ganglion cells, targeted disruption of *Barhl2* does not appear to affect horizontal cells but leads to the altered subtype composition of ACs and a partial loss of RGCs. Despite *Barhl2*'s expression in the three AC subtypes, loss of

Figure 9. The retinal waves mediated by cholinergic synaptic transmission were affected in *Barhl2*-null mice. Spontaneous retinal waves were recorded from P3 and P13 *Barhl2*-null mice and age-matched WT controls. **A**, Example of retinal waves recorded from a P3 WT retina. Left column, Spike trains of 20 neurons. Center column, A diagram of the array showing a propagating retinal wave. Each frame shows the mean firing rate of each cell (averaged >0.5 s). The small black dots represent the position of electrodes. Each black circle represents one cell, with the radius of the circle proportional to its firing rate. Right column, Spike trains of 10 neurons selected from the time window shown in the left column and the position of each neuron is represented with the number in the center column. **B**, Normalized correlation index as a function of distance between the recording electrodes from both WT and *Barhl2*-null mice at the ages of P3 and P13, respectively. **C**, Average interwave interval of the spontaneous retinal waves of both *Barhl2*-null mice and age-matched WT controls, showing that waves are much less frequent in *Barhl2*-null mice in the first postnatal week. **D**, Average firing rate of RGCs of *Barhl2*-null mice and age-matched WT controls at the ages of P3 and P13, respectively.

Barhl2 results in a significant reduction of glycinergic and GABAergic ACs accompanied by a substantial increase in cholinergic ACs (Fig. 5–7). Our results indicate that BARHL2 and other yet to be identified TFs form unique combinatorial codes in each AC subtype, a mechanism comparable with that of BHLHB5 in cortex, and thus provide an example of combinatorial TF codes that specify AC subtypes. Future identification of additional regulators of AC subtype differentiation will increasingly elucidate the precise mechanism by which combinatorial TF codes determine the AC subtypes. In RGCs, the expression of BARHL2 in combination with other RGC-specific factors, such as POU4F2 and ISL1, defines a particular group of RGCs. Future morphological, physiological, and functional analyses of the *Barhl2*-lineage

RGCs will shed light on their subtype identity and difference from other RGCs expressing POU4F2 and ISL1 but not BARHL2.

Published studies have shown that forced expression of *Barhl2* by viral infection in mouse retinas promotes the differentiation of glycinergic ACs but has no effect on the differentiation of GABAergic ACs, RGCs, and horizontal cells (Mo et al., 2004). The reported nonessential role of BARHL2 in horizontal cells is consistent with our targeted disruption study and with our model that retinal cell types are regulated by unique combinatorial TF codes. The increase in glycinergic ACs in *Barhl2* over-expression retina also agrees with the reduction in glycinergic ACs in *Barhl2*-null retinas, demonstrating the essential role of *Barhl2* in these ACs. However, the effect of ectopic *Barhl2* expression on

GABAergic ACs and RGCs appears to differ from the loss-of-function data presented here showing that *Barhl2*-null mutation results in a decrease in RGCs, GABAergic ACs, and an increase in cholinergic ACs. One likely explanation is that the viral infection is performed in neonatal retinas at P0 after the differentiation of most ACs and RGCs and likely after BARHL2 executes its roles. The reduction of RGCs in *Barhl2*-null retinas occurs mostly during late embryogenesis (Fig. 4), when BARHL2 is critically required and functions in a proper combination with other TFs in RGCs. In the BARHL2 gain-of-function experiments, BARHL2 is expressed in postnatal retinas. Possibly, attributable to the lack of necessary combinatorial TF codes, the ectopic, postnatal expression of BARHL2 has no detectable effect on RGCs and the GABAergic ACs. Further experiments with the BARHL2 gain of function in embryonic retinas will help to address these seemingly different observations in GABAergic ACs.

References

- Auerbach W, Dunmore JH, Fairchild-Huntress V, Fang Q, Auerbach AB, Huszar D, Joyner AL (2000) Establishment and chimera analysis of 129/SvEv- and C57BL/6-derived mouse embryonic stem cell lines. *Biotechniques* 29: 1024–1028, 1030, 1032.
- Bansal A, Singer JH, Hwang BJ, Xu W, Beaudet A, Feller MB (2000) Mice lacking specific nicotinic acetylcholine receptor subunits exhibit dramatically altered spontaneous activity patterns and reveal a limited role for retinal waves in forming ON and OFF circuits in the inner retina. *J Neurosci* 20:7672–7681.
- Bertrand N, Castro DS, Guillemot F (2002) Pro-neural genes and the specification of neural cell types. *Nat Rev Neurosci* 3:517–530.
- Bramblett DE, Pennesi ME, Wu SM, Tsai MJ (2004) The transcription factor Bhlhb4 is required for rod bipolar cell maturation. *Neuron* 43:779–793.
- Brown NL, Patel S, Brzezinski J, Glaser T (2001) Math5 is required for retinal ganglion cell and optic nerve formation. *Development* 128:2497–2508.
- Brunet JF, Ghysen A (1999) Deconstructing cell determination: proneural genes and neuronal identity. *Bioessays* 21:313–318.
- Demb JB (2007) Cellular mechanisms for direction selectivity in the retina. *Neuron* 55:179–186.
- Elshatory Y, Gan L (2008) The LIM-homeobox gene *Islet-1* is required for the development of restricted forebrain cholinergic neurons. *J Neurosci* 28:3291–3297.
- Elshatory Y, Everhart D, Deng M, Xie X, Barlow RB, Gan L (2007) *Islet-1* controls the differentiation of retinal bipolar and cholinergic amacrine cells. *J Neurosci* 27:12707–12720.
- Feng L, Xie X, Joshi PS, Yang Z, Shibasaki K, Chow RL, Gan L (2006) Requirement for Bhlhb5 in the specification of amacrine and cone bipolar subtypes in mouse retina. *Development* 133:4815–4825.
- Gan L, Xiang M, Zhou L, Wagner DS, Klein WH, Nathans J (1996) POU domain factor Brn-3b is required for the development of a large set of retinal ganglion cells. *Proc Natl Acad Sci U S A* 93:3920–3925.
- Gan L, Wang SW, Huang Z, Klein WH (1999) POU domain factor Brn-3b is

Figure 10. Light-evoked responses of ON-OFF DSRGCs are impaired in *Barhl2*-null mice. Responses to the moving bars of 12 different directions were recorded from the cells located in the RGC layer of P15 *Barhl2*-null mice and age-matched WT controls. The cells were identified as ON, OFF, and ON-OFF cells based on the patterns of light responses, and the DSI and peak frequency of their light responses were calculated from the responses of each group of the cells. **A**, Representative polar plot turning curve (center) and peristimulus response histograms to the moving bars at 12 different directions of a non-DS ON cell from a P15 WT retina. This cell showed a weak directional selectivity (DSI = 0.022). **B**, Representative polar plot turning curve and peristimulus response histograms of a DS ON-OFF cell from a P15 WT retina. This cell had a stronger ON directional selectivity (DSI = 0.6 at 210°) but a weaker OFF directional selectivity (DSI = 0.333). **C**, Cumulative distribution curves of peak frequency of ON cells of *Barhl2*-null mice and WT controls. Inset, Average peak frequencies. **D**, Cumulative distribution curves of peak frequency of the preferred and null directions of ON responses of ON-OFF DSRGCs of *Barhl2*-null mice and WT controls. Inset, Average peak frequencies. **E**, Cumulative distribution curves of peak frequency of the preferred and null directions of OFF responses of ON-OFF DSRGCs of *Barhl2*-null mice and WT controls. Inset, Average peak frequencies. **F**, Average DSI of ON and OFF responses of ON-OFF DSRGCs of *Barhl2*-null mice and WT controls.

essential for retinal ganglion cell differentiation and survival but not for initial cell fate specification. *Dev Biol* 210:469–480.

Higashijima S, Kojima T, Michiue T, Ishimaru S, Emori Y, Saigo K (1992) Dual Bar homeo box genes of *Drosophila* required in two photoreceptor cells, R1 and R6, and primary pigment cells for normal eye development. *Genes Dev* 6:50–60.

Inoue T, Hojo M, Bessho Y, Tano Y, Lee JE, Kageyama R (2002) Math3 and NeuroD regulate amacrine cell fate specification in the retina. *Development* 129:831–842.

Jessell TM (2000) Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat Rev Genet* 1:20–29.

Joshi PS, Molyneaux BJ, Feng L, Xie X, Macklis JD, Gan L (2008) Bhlhb5 regulates the postmitotic acquisition of area identities in layers II–V of the developing neocortex. *Neuron* 60:258–272.

- Kong JH, Fish DR, Rockhill RL, Masland RH (2005) Diversity of ganglion cells in the mouse retina: unsupervised morphological classification and its limits. *J Comp Neurol* 489:293–310.
- Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Rakic P, Flavell RA (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* 384:368–372.
- Li S, Mo Z, Yang X, Price SM, Shen MM, Xiang M (2004) Foxn4 controls the genesis of amacrine and horizontal cells by retinal progenitors. *Neuron* 43:795–807.
- Lim J, Choi KW (2004) Induction and autoregulation of the anti-proneural gene *Bar* during retinal neurogenesis in *Drosophila*. *Development* 131:5573–5580.
- Lin B, Wang SW, Masland RH (2004) Retinal ganglion cell type, size, and spacing can be specified independent of homotypic dendritic contacts. *Neuron* 43:475–485.
- Marquardt T, Ashery-Padan R, Andrejewski N, Scardigli R, Guillemot F, Gruss P (2001) Pax6 is required for the multipotent state of retinal progenitor cells. *Cell* 105:43–55.
- Masland RH (2001a) The fundamental plan of the retina. *Nat Neurosci* 4:877–886.
- Masland RH (2001b) Neuronal diversity in the retina. *Curr Opin Neurobiol* 11:431–436.
- Mo Z, Li S, Yang X, Xiang M (2004) Role of the *Barhl2* homeobox gene in the specification of glycinergic amacrine cells. *Development* 131:1607–1618.
- Novak A, Guo C, Yang W, Nagy A, Lobe CG (2000) Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* 28:147–155.
- O'Leary DD, Nakagawa Y (2002) Patterning centers, regulatory genes and extrinsic mechanisms controlling arealization of the neocortex. *Curr Opin Neurobiol* 12:14–25.
- Pan L, Yang Z, Feng L, Gan L (2005) Functional equivalence of Brn3 POU-domain transcription factors in mouse retinal neurogenesis. *Development* 132:703–712.
- Pan L, Deng M, Xie X, Gan L (2008) ISL1 and BRN3B co-regulate the differentiation of murine retinal ganglion cells. *Development* 135:1981–1990.
- Patterson KD, Cleaver O, Gerber WV, White FG, Krieg PA (2000) Distinct expression patterns for two *Xenopus* *Bar* homeobox genes. *Dev Genes Evol* 210:140–144.
- Poggi L, Vottari T, Barsacchi G, Wittbrodt J, Vignali R (2004) The homeobox gene *Xbh1* cooperates with proneural genes to specify ganglion cell fate within the *Xenopus* neural retina. *Development* 131:2305–2315.
- Saba R, Johnson JE, Saito T (2005) Commissural neuron identity is specified by a homeodomain protein, *Mbh1*, that is directly downstream of *Math1*. *Development* 132:2147–2155.
- Sanchez-Camacho C, Bovolenta P (2008) Autonomous and non-autonomous Shh signalling mediate the in vivo growth and guidance of mouse retinal ganglion cell axons. *Development* 135:3531–3541.
- Stanojevic D, Small S, Levine M (1991) Regulation of a segmentation stripe by overlapping activators and repressors in the *Drosophila* embryo. *Science* 254:1385–1387.
- Tanabe Y, William C, Jessell TM (1998) Specification of motor neuron identity by the MNR2 homeodomain protein. *Cell* 95:67–80.
- Tian N, Copenhagen DR (2003) Visual stimulation is required for refinement of ON and OFF pathways in postnatal retina. *Neuron* 39:85–96.
- Torborg CL, Hansen KA, Feller MB (2005) High frequency, synchronized bursting drives eye-specific segregation of retinogeniculate projections. *Nat Neurosci* 8:72–78.
- Wang JC, Harris WA (2005) The role of combinatorial coding by homeodomain and bHLH transcription factors in retinal cell fate specification. *Dev Biol* 285:101–115.
- Wang SW, Kim BS, Ding K, Wang H, Sun D, Johnson RL, Klein WH, Gan L (2001) Requirement for *math5* in the development of retinal ganglion cells. *Genes Dev* 15:24–29.
- Wong RO, Meister M, Shatz CJ (1993) Transient period of correlated bursting activity during development of the mammalian retina. *Neuron* 11:923–938.
- Xiang M, Zhou L, Peng YW, Eddy RL, Shows TB, Nathans J (1993) *Brn-3b*: a POU domain gene expressed in a subset of retinal ganglion cells. *Neuron* 11:689–701.
- Yang Z, Ding K, Pan L, Deng M, Gan L (2003) *Math5* determines the competence state of retinal ganglion cell progenitors. *Dev Biol* 264:240–254.
- Young RW (1985) Cell differentiation in the retina of the mouse. *Anat Rec* 212:199–205.
- Yu C, Mazerolle CJ, Thuring S, Wang Y, Pacal M, Bremner R, Wallace VA (2006) Direct and indirect effects of hedgehog pathway activation in the mammalian retina. *Mol Cell Neurosci* 32:274–282.