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Role of the *Barhl2* homeobox gene in the specification of glycinergic amacrine cells

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Summary

The mammalian retina contains numerous morphological and physiological subtypes of amacrine cells necessary for integrating and modulating visual signals presented to the output neurons. Among subtypes of amacrine cells grouped by neurotransmitter phenotypes, the glycinergic and γ-aminobutyric acid (GABA)ergic amacrine cells constitute two major subpopulations. To date, the molecular mechanisms governing the specification of subtype identity of amacrine cells remain elusive. We report here that during mouse development, the *Barhl2* homeobox gene displays an expression pattern in the nervous system that is distinct from that of its homologue *Barhl1*. In the developing retina, *Barhl2* expression is found in postmitotic amacrine, horizontal and ganglion cells, while *Barhl1* expression is absent. Forced expression of Barhl2 in retinal progenitors promotes the differentiation of glycinergic amacrine cells, whereas a dominant-negative form of Barhl2 has the opposite effect. By contrast, they exert no effect on the formation of GABAergic neurons. Moreover, misexpressed Barhl2 inhibits the formation of bipolar and Müller glial cells, indicating that Barhl2 is able to function both as a positive and negative regulator, depending on different types of cells. Taken together, our data suggest that Barhl2 may function to specify the identity of glycinergic amacrine cells from competent progenitors during retinogenesis.

Key words: Barhl2, Homeobox gene, Retina, Retinogenesis, Glycinergic amacrine cell

Introduction

The vertebrate retina is a highly organized laminar structure composed of seven classes of neuronal and glial cells. These include the rod and cone photoreceptor cells in the outer nuclear layer (ONL); the horizontal, bipolar and amacrine interneurons, plus the Müller glial cells in the inner nuclear layer (INL); and the ganglion and displaced amacrine cells in the ganglion cell layer (GCL). In addition, a small number of displaced amacrine and ganglion cells are located in the inner plexiform layer. Apart from these seven major cell classes, some classes of retinal neurons contain many diverse subtypes that differ in morphologies, physiological properties and functions (Vaney, 2002; Wassle and Boycott, 1991).

The amacrine cells are important interneurons that serve to integrate and modulate visual signals presented to ganglion cells, the output neurons of the retina. In the mouse retina, at least 26 morphological types of amacrine cells have been identified (MacNeil and Masland, 1998). The majority of them contain either glycine or GABA inhibitory neurotransmitters and hence amacrine cells can be divided into two major groups – glycinergic and GABAergic (Vaney, 1990). Glycinergic amacrine cells, which in the mouse comprise ~35% of the amacrine cell population (Marquardt et al., 2001), are usually small-field amacrine cells with diffuse dendritic trees (Menger et al., 1998; Pourcho and Goebel, 1985). AII cells are thus far the best characterized glycinergic amacrine cells with a bistratified morphology. They are the major amacrine cells that participate in the rod pathway circuitry (Famiglietti and Kolb, 1975; Kolb and Famiglietti, 1974; Strettoi et al., 1992; Vaney, 1985). GABAergic amacrine cells generally have wider dendritic fields than those of glycinergic cells and they comprise ~40% of all amacrine cells in the mouse retina (Marquardt et al., 2001; Pourcho and Goebel, 1983; Vaney, 1990). Many GABAergic amacrine cells contain other neurotransmitters such as acetylcholine and dopamine in addition to GABA (Vaney, 1990; Wassle and Boycott, 1991), as exemplified by the direction-selective starburst amacrine cells which are not only GABAergic but cholinergic as well (Brecha et al., 1988; Fried et al., 2002; Kosaka et al., 1988; O'Malley and Masland, 1989; O'Malley et al., 1992; Vaney and Young, 1988).

During retinogenesis, the seven classes of retinal cells are produced from multipotent progenitor cells following a loose temporal order, with amacrine cells being generated in a period spanning from embryonic day 11 (E11) to postnatal day 4 (P4) (Young, 1985). It has been postulated that, in response to changes of intrinsic and extrinsic cues, retinal progenitors undergo a series of changes in competence to give rise to the various retinal cell types (Cepko, 1999; Harris, 1997; Livesey and Cepko, 2001). Recent advances by molecular genetic approaches have begun to unravel the molecular bases

underlying the determination and differentiation of different retinal cell types, including amacrine cells. It has been shown by gene targeting that the homeobox gene *Pax6* is required to maintain the multipotency of retinal progenitors to generate all retinal cell types but amacrine cells (Marquardt et al., 2001; Marquardt and Gruss, 2002). Overexpression experiments have demonstrated that the basic helix-loop-helix (bHLH) factor Neurod1 alone or in combination with Pax6 is capable of promoting amacrine cell differentiation (Inoue et al., 2002; Morrow et al., 1999). Similarly, another bHLH factor Math3 (Neurod4 – Mouse Genome Informatics) together with Pax6 can promote amacrine cell differentiation but Math3 alone lacks this activity (Inoue et al., 2002). In compound knockout mice deficient for both *Neurod1* and *Math3*, no amacrine cells are produced in the retina (Inoue et al., 2002). However, the formation of amacrine cells is essentially normal in single mutants null for either *Neurod1* or *Math3* (Inoue et al., 2002; Morrow et al., 1999), suggesting that *Neurod1* and *Math3* are redundantly required for fate determination of amacrine cells. The absence of *Pax6* does not alter *Neurod1* expression (Marquardt et al., 2001), thereby allowing the differentiation of amacrine cells to occur.

Despite our current knowledge of amacrine cell development, it remains unclear what factors are involved in conferring retinal progenitors with the potential to generate amacrine cells, or in specifying their subtype identity. *Barhl1* and *Barhl2/MBH1*, two mammalian homeobox genes, represent homologues of the *Drosophila BarH* genes that are required for normal development of the compound eye and external sensory organs (Bulfone et al., 2000; Higashijima et al., 1992a; Higashijima et al., 1992b; Kojima et al., 1991; Li et al., 2002; Saito et al., 1998). In the mouse, *Barhl1* is expressed in the developing inner ear hair cells and central nervous system (CNS), and has been shown by gene-targeting to be essential for the long-term maintenance of cochlear hair cells (Li et al., 2002). The expression pattern of *Barhl2* has not yet been well characterized during mouse development. One area of *Barhl2* expression is in the interneurons of the spinal cord and ectopic *Barhl2* expression has been reported to promote the differentiation and migration of these neurons (Saba et al., 2003). *Barhl2* expression has also been found in the GCL of the developing retina (Saito et al., 1998); however, it is not yet known whether *Barhl2* has a role during retinal development. In this study, we aimed to: (1) systemically examine the spatial and temporal expression pattern of *Barhl2* during mouse development; (2) identify the types of retinal cells that express *Barhl2*; and (3) investigate whether *Barhl2* plays a role during retinogenesis. We find that *Barhl2* displays an expression pattern in the CNS that is distinct from that of *Barhl1*. In the retina, *Barhl2* is expressed by developing amacrine, horizontal and ganglion cells. Forced *Barhl2* expression promotes the formation of glycinergic amacrine cells but has no effect on GABAergic neurons, suggesting that *Barhl2* is involved in the specification of subtype identity of amacrine cells.

Materials and methods

Isolation of *Barhl2* cDNA and RNA in situ hybridization

The full-length *Barhl2* open reading frame was assembled by PCR from two partial *Barhl2* cDNA clones isolated from a mouse E14.5 cDNA library (Stratagene) using a human EST (GenBank accession no. AI367090) as screening probe. The *Barhl1* cDNA was described previously (Li et al., 2002). RNA in situ hybridization was carried out as previously described (Sciavolino et al., 1997) using digoxigenin-labeled riboprobe prepared following the manufacturer's protocol (Roche Diagnostics).

Generation of an anti-Barhl2 antibody

DNA fragment corresponding to amino acids 3-132 of the mouse Barhl2 protein was amplified by PCR and inserted into pGEMEX (Promega) and pMAL-cR1 (New England Biolabs) vectors to express fusion proteins with the bacteriophage T7 gene 10 protein and bacterial maltose-binding protein, respectively. Antibody production and affinity purification were performed as described previously by Xiang et al. (Xiang et al., 1995; Xiang et al., 1993).

Plasmid construction and virus preparation and injection

The control-GFP plasmid used is pLZRSΔ-IRES-EGFP (Kim et al., 2002), a replication-incompetent murine retroviral vector derived from LZRSpBMN-Z (Kinsella and Nolan, 1996). To construct the Barhl2-GFP plasmid, the above-mentioned cDNA fragment containing the entire Barhl2-coding region was subcloned into the *Xho*I site of the control-GFP vector. For construction of the Barhl2-EnR-GFP plasmid, DNA segment corresponding to amino acids 208-314 of Barhl2 containing the homeodomain was PCR amplified and inserted into the *Nco*I and *Eco*RI sites of the EnR-Slax13 shuttle vector (Morgan and Fekete, 1996). The fused sequence was then released, blunt-ended and transferred into the control-GFP vector. Control-GFP, Barhl2-GFP and Barhl2-EnR-GFP viruses were produced in Phoenix Eco retroviral packaging cells (ATCC) by transfection of relevant plasmids using the Lipofectamine reagent (Gibco-BRL). Ten to 12 days after transfection, viruses were collected and concentrated as described (Morgan and Fekete, 1996).

In vivo infection of P0 mouse retinas by retroviruses was performed as described (Turner et al., 1990). Infected retinas were harvested from P30 mice for analysis. Retinal explant culture and viral infection were carried out also as previously described (Tomita et al., 1996).

Immunostaining and antibodies

To prepare retinal sections, eyeballs were enucleated and immersion-fixed with 4% paraformaldehyde in PBS for 1 hour at 4°C. Following fixation the retinas were dissected out, treated with 30% sucrose in PBS for 1-2 hours at 4°C with gentle shaking, embedded in OCT compound and cryosectioned at 12-16 μm. Immunoperoxidase staining of mouse retinal sections was performed as described (Xiang et al., 1995; Xiang et al., 1993). For double immunofluorescence, retinal sections were treated with methanol for 2 minutes at 4°C, rinsed with PBS and then stained with 0.02 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in PBS for 5 minutes at room temperature. After washes in PBS, they were blocked with 5% normal serum, incubated overnight with a mixture of primary antibodies at 4°C, rinsed with PBS and then incubated with a mixture of fluorophore-conjugated secondary antibodies for 1 hour at room tempeature. Images were captured by a digital camera mounted on a Nikon Eclipse E800 microscope.

The following primary antibodies were used: anti-syntaxin (Sigma); anti-protein kinase Cα (PKCα) (Amersham); anti-glutamine synthase (GS) (Chemicon); anti-Pax6 (Developmental Studies Hybridoma Bank); anti-Brn3a (Chemicon); anti-choline acetyltransferase (ChAT) (Chemicon); anti-green fluorescent protein (GFP) (mouse monoclonal, Chemicon; rabbit polyclonal, MBL International Corporation); anti-calbindin-D28K (Sigma); anti-calretinin (Chemicon); anti-$GABA_B$ (Sigma); anti-GABA (Sigma); anti-GABA transporter-1 (GAT-1) (Chemicon); anti-tyrosine hydroxylase (TH) (Chemicon); anti-recoverin (Dizhoor et al., 1991); anti-Barhl2 (this work); and anti-glycine transporter 1 (GLYT1) (Chemicon).

Quantification

To quantify GFP$^+$ cells and GFP$^+$ cells colocalized with cell type-specific markers on sections of retinas infected with control-GFP, Barhl2-GFP or Barhl2-EnR-GFP viruses, 500-3500 GFP$^+$ cells were usually scored for each retina and at least three retinas were analyzed for each type. For retinas labeled with the anti-TH antibody; however, more than 10000 GFP$^+$ cells were counted in each retina owing to the paucity of TH-immunoreactive cells. All data were tested for significance using two sample Student's *t*-test with unequal variances.

Results

Patterns of *Barhl2* expression during CNS development

To understand the role of the *Barhl2* gene in the mouse, we systemically analyzed by RNA in situ hybridization its spatial and temporal expression patterns during embryonic and postnatal development. We found that the expression of *Barhl2* was restricted only to select areas of the CNS and was absent from non-neural tissues. During embryogenesis, *Barhl2* is first expressed in two symmetric stripes within the diencephalon at E9.5 (Fig. 1A). By E11.5, its expression extends to the mesencephalon and the entire length of the dorsal neural tube (Fig. 1B). In the neural tube, *Barhl2* appears to be expressed in the D1 dorsal sensory interneurons (Fig. 1C). These cells migrate ventrally following a superficial pathway, eventually localizing into two small lateral columns between the alar and basal plates of the spinal cord by E14.5 (Fig. 1C,D).

From E14.5 to P1, *Barhl2* expression is found in neurons of the pyriform cortex and diagonal band within the telencephalon (Fig. 1E,F,J,K). In the diencephalon, *Barhl2* is prominently expressed in the epithalamic and intermediate thalamic neuroepithelia, ventral thalamus, preoptic area, paraventricular nucleus, as well as in the mammillary body (Fig. 1E-G,K,L,P). *Barhl2* expression in the mesencephalon is detected in the superior and inferior colliculi, periaqueductal gray and tegmentum (Fig. 1G,H,M,N). In the developing cerebellum, no signal is observed at E14.5 (Fig. 1H,I). Only by P1 is *Barhl2*

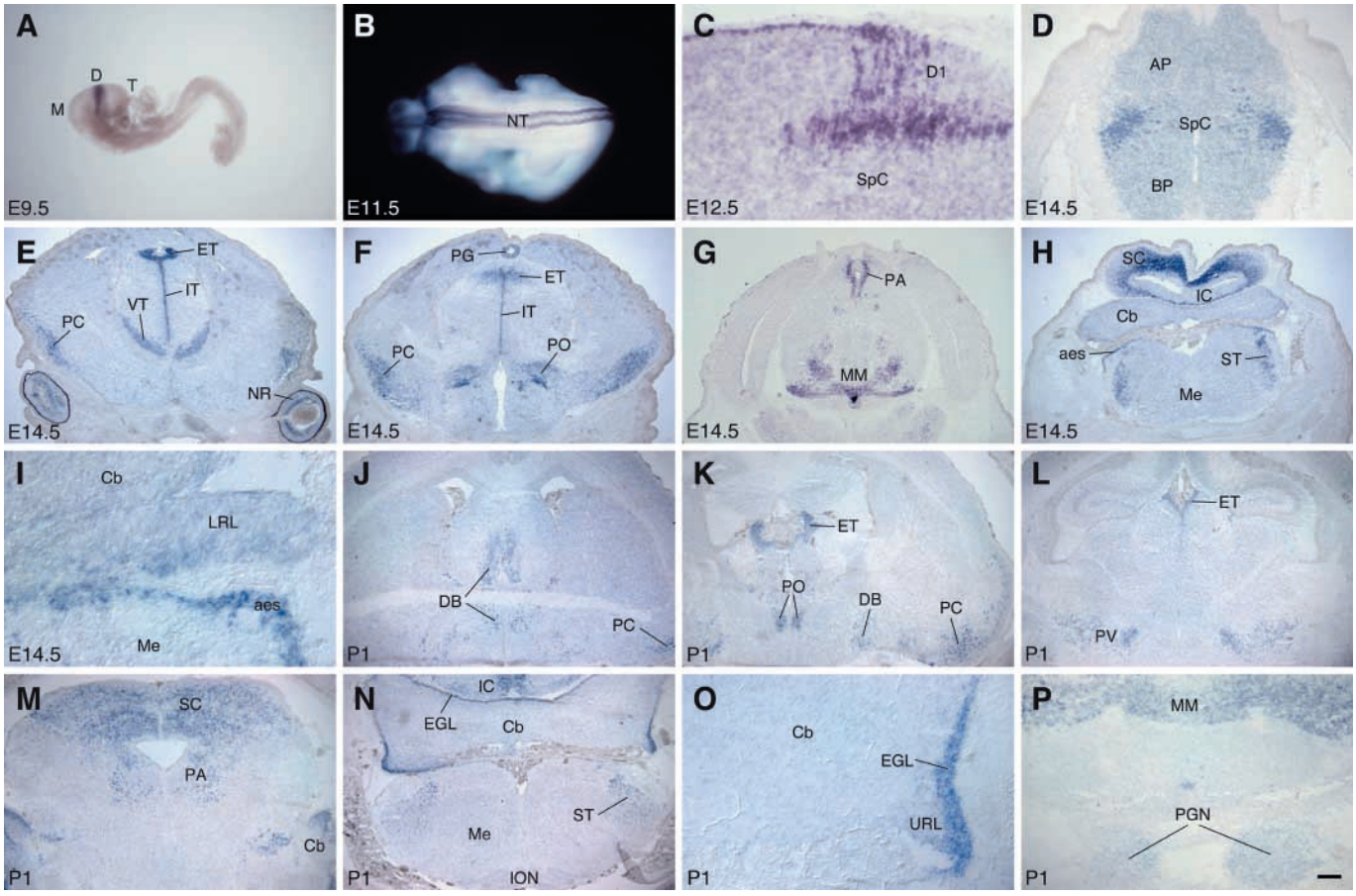

Fig. 1. Expression of *Barhl2* during CNS development. (A-P) The *Barhl2* transcript was detected by in situ hybridization in whole-mount embryos (A,B) and in sagittal (C) and coronal (D-P) sections from embryonic and postnatal mice at the indicated developmental stages. *Barhl2* expression is initiated at E9.5 in two symmetric stripes within the diencephalon (A). At later stages, its expression is found in select regions of the telencephalon, diencephalon, mesencephalon, cerebellum, medulla and spinal cord (B-P), as well as in the retina and pineal gland (E,F). aes, anterior extramural migratory stream; AP, alar plate; BP, basal plate; Cb, cerebellum; D, diencephalon; D1, D1 interneuron; DB, diagonal band; EGL, external granule layer; ET, epithalamic neuroepithelium; IC, inferior colliculus; ION, inferior olivary nucleus; IT, intermediate thalamic neuroepithelium; LRL, lower rhombic lip; M, mesencephalon; Me, medulla; MM, mammillary region; NR, neural retina; NT, neural tube; PA, periaqueductal gray; PC, pyriform cortex; PG, pineal gland; PGN, pontine gray nucleus; PO, preoptic area; PV, paraventricular nucleus; SC, superior colliculus; SpC, spinal cord; ST, spinal trigeminal nucleus; T, telencephalon; URL, upper rhombic lip; VT, ventral thalamus. Scale bar: 25 μm in I; 50 μm in C,O; 100 μm in D,P; 250 μm in A,E-N; 333 μm in B.

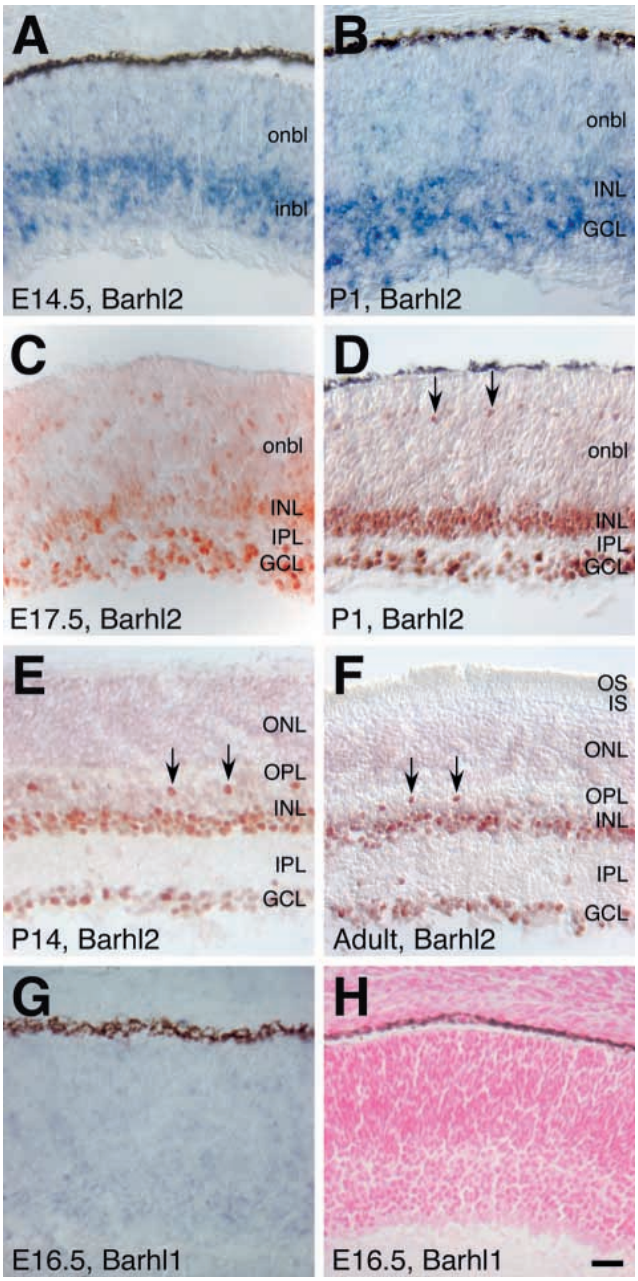

Fig. 2. Spatial and temporal expression patterns of *Barhl* genes during mouse retinogenesis. (A,B) In situ hybridization analysis of E14.5 and P1 retinal sections using a *Barhl2* antisense probe showed prominent signals in the inner retina. (C-F) Retinal sections from the indicated developmental stages were immunostained with an anti-Barhl2 antibody. In embryonic, postnatal and adult retinas, the anti-Barhl2 antibody labeled many cells in the inner nuclear and ganglion cell layers (C-F), as well as some migratory cells within the outer neuroblastic layer (C,D). Arrows indicate representative differentiating and mature horizontal cells. (G) In situ hybridization analysis of E16.5 retinal sections using a *Barhl1* antisense probe detected no signals in the retina. (H) X-gal staining of retinal sections from E16.5 *Barhl1*$^{lacZ/+}$ embryos counterstained with Fast Red. No β-galactosidase activity was detected in the retina. GCL, ganglion cell layer; inbl, inner neuroblastic layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS, inner segment; onbl, outer neuroblastic layer; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment. Scale bar: 25 μm.

expression seen in the upper rhombic lip and external granule layer (Fig. 1N,O) In the developing precerebellar system, however, there is *Barhl2* expression in the anterior extramural migratory stream at E14.5, even though it is absent from any of the precerebellar nuclei, including the pontine gray and reticulotegmental nuclei derived from the anterior extramural migratory stream (Fig. 1H,I,P). In the medulla, *Barhl2* is expressed in neurons of developing principal and spinal trigeminal nuclei (Fig. 1H,N). In addition to these brain structures, *Barhl2* expression is also found in developing neural retina and pineal gland (Fig. 1E,F).

Expression of *Barhl2* in amacrine, horizontal and ganglion cells but not in mitotic progenitors during retinogenesis

As revealed by RNA in situ hybridization, during retinogenesis, *Barhl2* transcripts were found primarily in the inner neuroblastic layer at E14.5 and then in the INL and GCL at late embryonic and postnatal stages (Fig. 2A,B). *Barhl1* transcripts, by contrast, were not detectable in embryonic and postnatal retinas (Fig. 2G). We further made use of a *lacZ* reporter knocked in the *Barhl1* locus to examine whether *Barhl1* was expressed in the retina (Li et al., 2002). No β-galactosidase activity was ever seen in retinas of *Barhl1*$^{lacZ/+}$ mice at any developmental stages (Fig. 2H), demonstrating that of the two *Barhl* genes, *Barhl2* is uniquely expressed in the developing retina.

As several cell classes, including amacrine, bipolar, horizontal, Müller and ganglion cells, reside within the INL and GCL, it was not possible to identify the types of cells that express *Barhl2* by in situ hybridization. Thus, we raised a specific polyclonal anti-Barhl2 antibody (see Materials and methods) to determine the cellular and subcellular distribution of Barhl2 protein during retinal development. This antibody was shown to be specific based on the following observations: (1) immunostaining with anti-Barhl2 gave the same labeling pattern in the mouse embryo as revealed by in situ hybridization; and (2) anti-Barhl2 did not stain inner ear hair cells where only *Barhl1* is expressed (data not shown).

By immunostaining retinal sections from various developmental stages, we used the anti-Barhl2 antibody to examine retinal expression patterns of the protein. Consistent with it being a homeodomain transcription factor, Barhl2 is nuclear (Fig. 2C-F). Similar to its transcripts detected by in situ hybridization, Barhl2 is first seen in cells within the inner neuroblastic layer at E13.5. By E17.5, it is distributed largely in cells of the INL and GCL (Fig. 2C). Interestingly, however, anti-Barhl2 immunolabeled additional cells scattered within the outer neuroblastic layer at E13.5-17.5 (Fig. 2C). At P1, anti-Barhl2 also additionally labeled a layer of evenly spaced cells in the outer neuroblastic layer (Fig. 2D), which at P14 became a layer of cells lining at the border between the outer plexiform layer and INL (Fig. 2E), a position where horizontal cells are normally situated. At P14 and in the adult, the large majority of Barhl2-immunoreactive cells are located within the inner half of the INL as well as in the GCL, where amacrine and ganglion cells reside (Fig. 2E,F).

The cells expressing Barhl2 in the outer neuroblastic layer of embryonic retinas could be either mitotic progenitors or newly generated cells en route to the INL and GCL or both. To distinguish these possibilities, S-phase cells within E17.5

retinas were pulse labeled by the thymidine analog BrdU, followed by double-immunostaining using anti-Barhl2 and anti-BrdU antibodies (Fig. 3A). In the outer neuroblastic layer, we found that none of the small number of scattered cells immunoreactive for Barhl2 colocalized with any of the large number of proliferative cells labeled by anti-BrdU (Fig. 3A). Thus, Barhl2 appears to be expressed exclusively by postmitotic cells during retinal development.

To determine the types of cells that express Barhl2, we double-immunostained adult retinal sections with the anti-Barhl2 antibody and antibodies against markers for different cell types. As shown in Fig. 3B, there appears to be a complete colocalization between Barhl2 and syntaxin, a marker for all amacrine cells, in cells within the inner half of the INL, indicating that Barhl2 may be expressed by all amacrine cells. In agreement with this notion, Barhl2 is expressed by all amacrine cells that are immunoreactive for GLYT1, the GABA receptor $GABA_B$, calretinin or calbindin D-28k (Fig. 3C-F). Similarly, all horizontal cells positive for calbindin are immunoreactive for Barhl2 as well (Fig. 3F). Double-immunostaining with an antibody against Brn3a, a marker for ~70% of ganglion cells (Xiang et al., 1995), showed that only 33% of Brn3a-immunoreactive cells were labeled by anti-Barhl2 (Fig. 3G), implicating that many cells in the GCL which are positive for Barhl2 but negative for Brn3a must be displaced amacrine cells (Fig. 3G). In support of this explanation, within the GCL, the large majority of cells immunoreactive for $GABA_B$ or calretinin, two proteins expressed in displaced amacrine cells and some ganglion cells, are colocalized with Barhl2 (Fig. 3D,E). However, we observed no colocalization in retinal cells between Barhl2 and PKCα or GS (Fig. 3H,I), which respectively are markers for rod bipolar and Müller cells. Thus, our results suggest that during retinal development Barhl2 is expressed only in developing and mature amacrine and horizontal cells plus a subpopulation of ganglion cells.

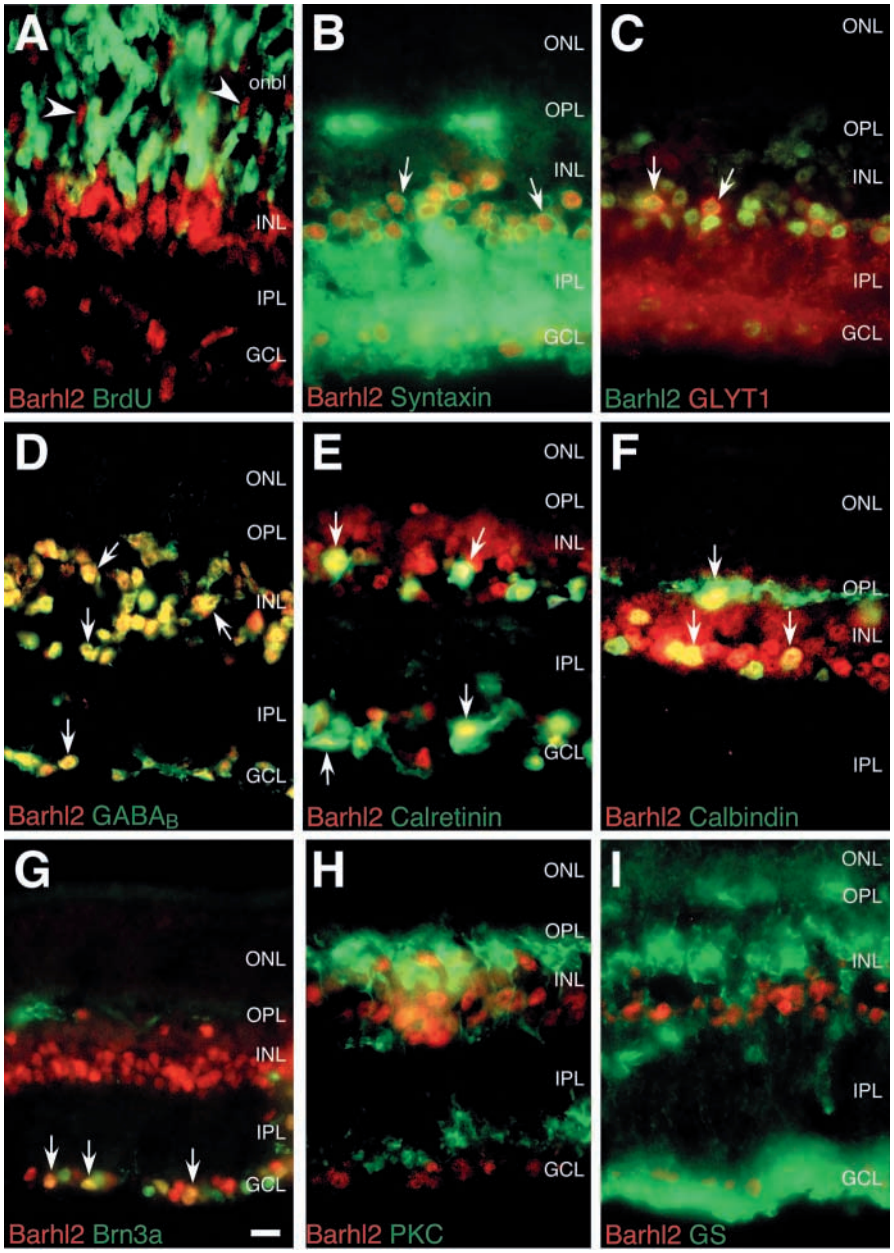

Fig. 3. Localization of Barhl2 protein in postmitotic amacrine, horizontal and ganglion cells. (A) Retinal sections from BrdU-labeled E17.5 embryos were double-immunostained with antibodies against Barhl2 (red) and BrdU (green). Barhl2-immunoreactive cells, even those within the outer neuroblastic layer (indicated by arrowheads), do not overlap the S-phase cells immunoreactive for BrdU. (B-I) Double-immunolabeling of adult retinal sections using an anti-Barhl2 antibody and antibodies against several indicated cell type-specific marker proteins. Barhl2 appears to be expressed in all amacrine cells immunoreactive for syntaxin (B), glycine transporter 1 (GLYT1) (C), GABA receptor $GABA_B$ (D), calretinin (E) and calbindin D-28k (F); and in all horizontal cells immunoreactive for calbindin (F), but only in a subset of ganglion cells immunoreactive for Brn3a (G). By contrast, Barhl2 is absent from rod bipolar and Müller cells immunoreactive for protein kinase Cα (PKC) and glutamine synthase (GS), respectively (H,I). Arrows indicate representative colocalized cells. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; onbl, outer neuroblastic layer; ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bar: 10 μm in A-F,H,I; 16.7 μm in G.

Forced Barhl2 expression promotes the formation of glycinergic amacrine cells

As a first step to understand the role of Barhl2 during retinal development, we investigated the effect of forced Barhl2 expression in retinal progenitors. Overexpression of Barhl2 in the mouse retina was achieved by a replication-incompetent murine retroviral vector derived

from LZRSpBMN-Z (Fig. 4A) (Kim et al., 2002; Kinsella and Nolan, 1996). Retinas were infected at P0 by subretinal injection of Barhl2-GFP or control-GFP viruses and analyzed at P30. The laminar position and morphology of GFP-positive cells were monitored in retinal sections. Although most virus-infected GFP$^+$ cells became rod cells located within the ONL in retinas infected with either control-GFP or Barhl2-GFP viruses, there appeared to be a significant increase in the number of GFP$^+$ cells residing in the inner region of the INL in retinas infected with Barhl2-GFP viruses (Fig. 4B,C). To more accurately assess cell distribution, we quantified the number of GFP$^+$ cells located in different retinal layers. In retinas infected with Barhl2-GFP viruses, we found that the percentage of GFP$^+$ cells located within the inner half of the INL increased from 8.6% in the control to 16.9% (Fig. 4D). By contrast, the proportion of GFP$^+$ cells distributed within the outer half of the INL dropped sharply from 9.4% in the control to only 1.5% in retinas infected with Barhl2-GFP viruses (Fig. 4D). There was no difference in the number of GFP$^+$ cells distributed in the ONL and GCL in retinas infected with either control-GFP or Barhl2-GFP viruses (Fig. 4D). Thus, forced Barhl2 expression significantly increases the formation of cells located within the inner half of the INL at the expense of cells located within the outer half of the INL.

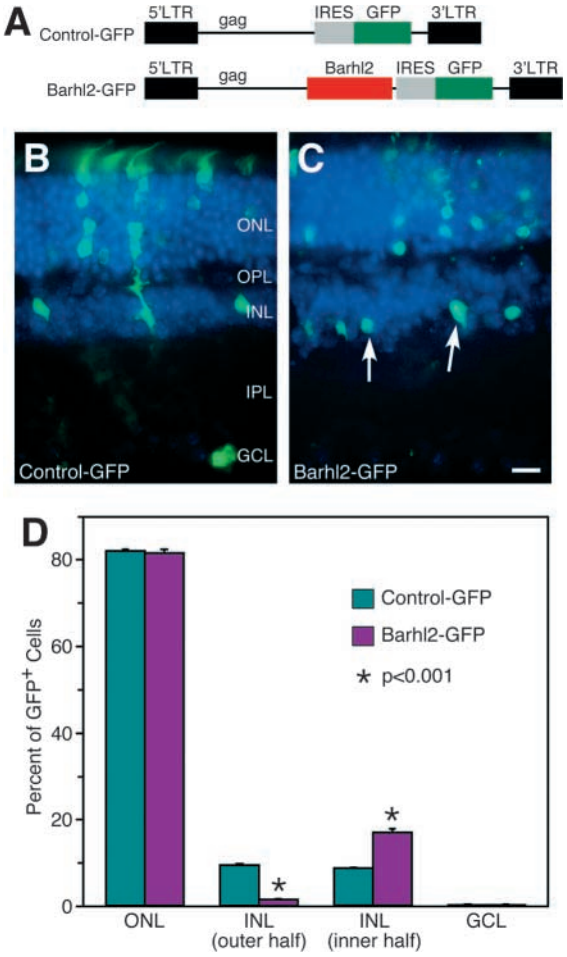

Fig. 4. Effect of forced Barhl2 expression on retinal inner nuclear cells. (A) Schematic of control-GFP and Barhl2-GFP retroviral constructs. In Barhl2-GFP, a bicistronic transcript containing an internal ribosomal entry site (IRES) is produced from the viral LTR promoter, which allows efficient expression of both Barhl2 and the GFP marker protein. (B,C) Virus-transduced GFP$^+$ (green) cells were visualized in P30 retinal sections that were weakly counterstained with nuclear DAPI. Infection with Barhl2-GFP viruses caused a significant increase of cells situated within the inner region of the inner nuclear layer. Arrows in C indicate two such cells.
(D) Percentages of virus-transduced cells located in different cellular layers of the retina (means±s.d.). More than 600 GFP$^+$ cells in three independent retinas were scored for either control-GFP or Barhl2-GFP virus. The inner nuclear layer was divided into the outer and inner halves for quantitation. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bar: 10 μm.

Given the location of amacrine cells within the inner half of the INL we speculated that amacrine cell differentiation might be promoted by overexpression of Barhl2. To confirm this effect, we utilized a variety of cell-type specific antibodies to determine the types of GFP$^+$ cells. By double-immunofluorescence, we found that ectopically expressed Barhl2 appeared to significantly increase the number of GFP$^+$ cells immunoreactive for syntaxin, Pax6 or GLYT1, all proteins expressed in amacrine cells (Fig. 5A-F). As well, it increased the number of GFP$^+$ cells that were weakly immunoreactive for calbindin (Fig. 5G,H). As shown in Fig. 6A, quantitation of colocalized cells revealed that forced Barhl2 expression increased the percentage of syntaxin$^+$ cells from 3.8% to 14.7%, Pax6$^+$ cells from 2.1% to 12.7%, GLYT1$^+$ cells from 0.7% to 4.5%, and calbindin$^+$ cells from 0.7% to 2.4%. By contrast, no change was observed in the number of GFP$^+$ cells immunoreactive for GABA, GAT-1, ChAT or TH in retinas infected with Barhl2-GFP viruses (Fig. 5I,J, Fig. 6A). As cholinergic (ChAT$^+$) and dopaminergic (TH$^+$) amacrine cells are GABAergic as well, it appears that Barhl2 is able to promote the differentiation of amacrine cells and glycinergic amacrine cells in particular, but has no effect on the formation of GABAergic amacrine cells. In agreement with this notion, only weak calbindin-immunoreactive cells were increased by Barhl2 overexpression, whereas the number of strong calbindin-immunoreactive amacrine cells, which have been shown to represent cholinergic neurons (Haverkamp and Wassle, 2000), was not altered (Fig. 5G,H).

Forced Barhl2 expression suppresses the formation of bipolar and Müller cells

Given the substantial decrease of GFP$^+$ cells within the outer half of the INL in retinas infected with Barhl2-GFP viruses (Fig. 4), there might be potential reduction of bipolar, horizontal and/or Müller cells in these retinas. We investigated this possibility by immunofluorescence using antibodies specific to these and other cell types. Interestingly, essentially no GFP$^+$ cells became PKCα-immunoreactive rod bipolar cells in retinas infected with Barhl2-GFP viruses, whereas in control retinas, 4.2% of GFP$^+$ cells were immunoreactive for PKCα (Fig. 5K,L, Fig. 6A). Similarly, the fraction of GFP$^+$ cells that were immunoreactive for GS, was diminished from 8.4% in control retinas to only 3% in retinas infected with Barhl2-GFP viruses (Fig. 5M,N, Fig. 6A). By contrast, as revealed by immunostaining with antibodies against calbindin and Brn3a, no GFP$^+$ cells became calbindin$^+$ horizontal cells or Brn3a$^+$

ganglion cells in either control retinas or retinas infected with Barhl2-GFP viruses (Fig. 5G,H,O,P, Fig. 6A). Moreover, immunostaining with an anti-recoverin antibody showed that ~82% of all GFP$^+$ cells differentiated into photoreceptors in both control retinas and retinas infected with Barhl2-GFP viruses (Fig. 6A). Thus, forced Barhl2 expression in retinal progenitors at P0 completely blocks the formation of rod bipolar cells as well as strongly suppresses the formation of Müller cells, whereas it does not affect the formation of photoreceptor, horizontal or ganglion cells.

The effect of forced Barhl2 expression on bipolar and Müller cells could result from increased death of virus-transduced cells. To test this possibility, we measured the number of apoptotic GFP$^+$ cells by assaying for the active caspase 3 immunoreactivity in P4 and P10 retinas infected with control-GFP or Barhl2-GFP viruses. No significant difference was observed in the percentage of apoptotic GFP$^+$ cells between control retinas and retinas infected with Barhl2-GFP viruses at P4 (Barhl2 mean±s.d., 0.29±0.10%, n=4; control, 0.22±0.06%, n=3) or P10 (Barhl2, 0.12±0.06%, n=3; control, 0.15±0.03%, n=3). As Barhl2 is expressed only by postmitotic cells and hence is unlikely to affect cell proliferation, it most probably exerts its retinal function by influencing specification and/or differentiation of retinal cell types.

As retinal progenitors change their competent states during development, we investigated whether overexpressed Barhl2 had different effects at embryonic stages, in particular, on the earlier-born horizontal and ganglion cells. In these experiments, retinal explants from E13.5, E17.5 or P0 mice were infected with Barhl2-GFP or control-GFP viruses and cultured in vitro for 14 days before analysis. Compared with P0 retinas infected in vivo, we found that very similar results could be obtained from these explants (Fig. 6). For example, with E17.5 retinal explants, overexpressed Barhl2 increased amacrine cells positive for syntaxin, Pax6, GLYT1 or calbindin, reduced cells immunoreactive for PKCα or GS, but did not alter the number of cells positive for GAT-1 (Fig. 6B). Similarly, it had no effect on calbindin$^+$ horizontal cells or Brn3a$^+$ ganglion cells (Fig. 6B).

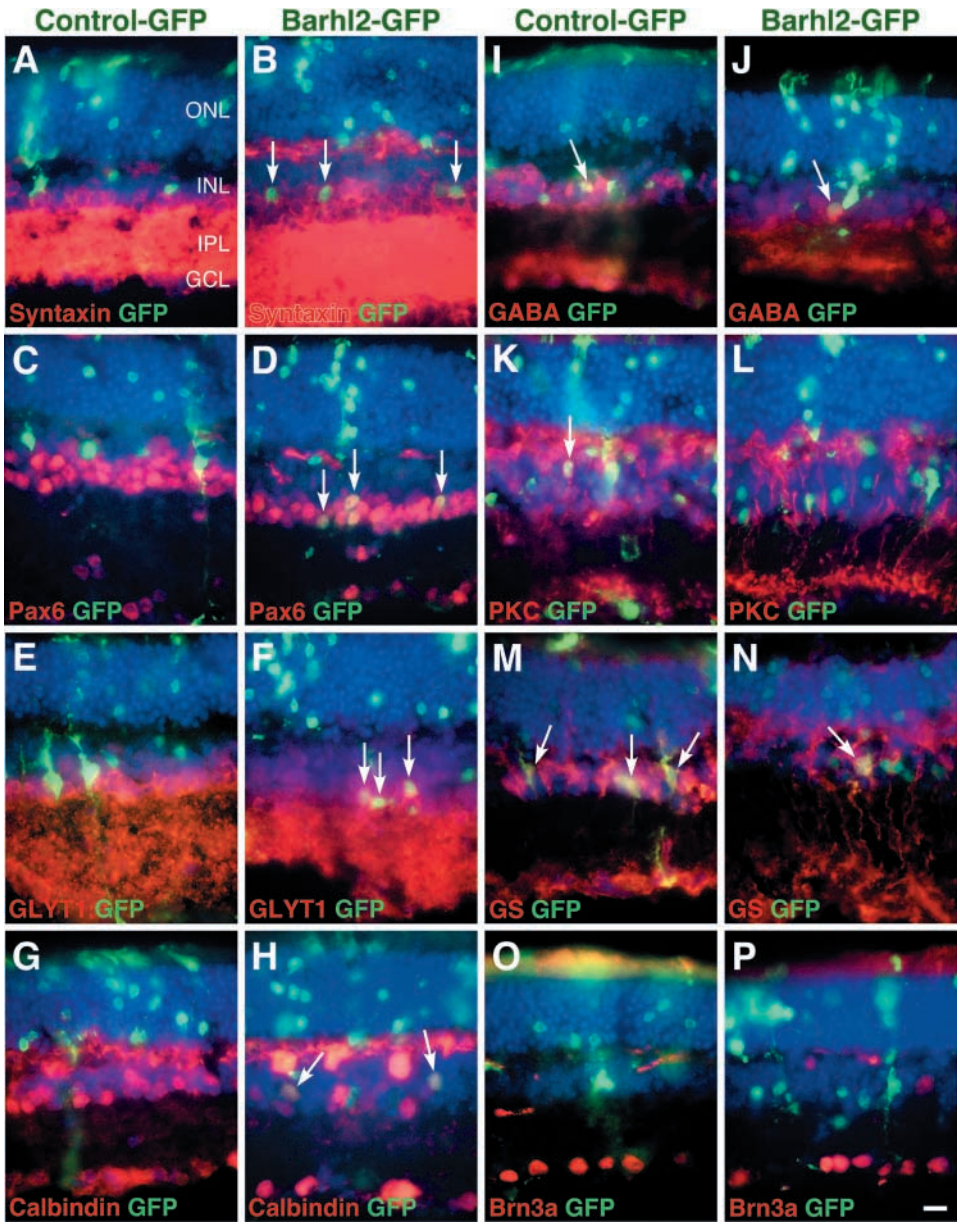

Fig. 5. Effect of misexpressed Barhl2 on the formation of different retinal cell types. (A-P) Sections from P30 retinas infected with control-GFP (A,C,E,G,I,K,M,O) or Barhl2-GFP (B,D,F,H,J,L,N,P) viruses were double-immunostained with an anti-GFP antibody and antibodies against syntaxin (A,B), Pax6 (C,D), glycine transporter 1 (GLYT1) (E,F), calbindin (G,H), GABA (I,J), PKCα (K,L), glutamine synthase (GS) (M,N) or Brn3a (O,P). All sections were weakly counterstained with nuclear DAPI. Forced Barhl2 expression resulted in a significant increase in the number of amacrine cells immunoreactive for syntaxin (A,B), Pax6 (C,D), GLYT1 (C,D) and calbindin (E,F), whereas it had no effect on amacrine cells immunoreactive for GABA (I,J) and ganglion cells immunoreactive for Brn3a (O,P). Moreover, it greatly suppressed the formation of rod bipolar cells immunoreactive for PKCα (K,L) and Müller cells immunoreactive for GS (M,N). Arrows indicate representative colocalized cells. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer. Scale bar: 25 μm.

Overexpression of a dominant-negative form of Barhl2 inhibits the formation of glycinergic amacrine cells

To test whether Barhl2 acts as a transcriptional activator for the specification of glycinergic amacrine cells, we constructed a dominant-negative viral plasmid, Barhl2-EnR-GFP, by fusing the repressor domain of the *Drosophila* engrailed protein to the Barhl2 homeodomain region (Fig. 7A). Compared with retinas infected with control-GFP viruses, we found that the fraction of GFP$^+$ cells distributed in the INL was reduced from 19.7% to 10.4% in retinas infected with Barhl2-EnR-GFP viruses (Fig. 7D,E). This decrease manifested a proportional reduction of GFP$^+$ cells within both the inner and outer halves of the INL and was accompanied by an ~10% increase in the number of GFP$^+$ cells located in the ONL (Fig. 7B).

To determine the types of GFP$^+$ cells diminished in the INL of retinas infected with Barhl2-EnR-GFP viruses, we measured the number of GFP$^+$ cells immunoreactive for various cell type-specific markers. First, for GFP$^+$ amacrine cells immunoreactive for Pax6, calbindin or GLYT1, overexpressed Barhl2-EnR decreased their proportions from 2.5% in the control to 1.5%, 0.9 to 0.4% or 0.9 to 0.3%, respectively (Fig. 7C,F-I), consistent with Barhl2 being a positive regulator for glycinergic amacrine cell formation. By contrast, it notably did not alter the percentage of GFP$^+$ amacrine cells immunoreactive for GABA (Fig. 7C,J,K). Second, in agreement with the notion that Barhl2 acts as a negative regulator for the formation of bipolar and Müller cells, overexpressed Barhl2-EnR markedly reduced the fractions of GFP$^+$ cells that became immunoreactive for PKCα or GS (Fig. 7C). Third, accompanying the reduced number of amacrine, bipolar and Müller cells in retinas infected with Barhl2-EnR-GFP viruses, there was a corresponding 9% increase in the number of GFP$^+$ photoreceptor cells immunoreactive for recoverin (Fig. 7C). Finally, overexpressed Barhl2-EnR resulted in no changes in the number of GFP$^+$ horizontal cells immunoreactive for calbindin, or GFP$^+$ ganglion cells immunoreactive for Brn3a (Fig. 7C).

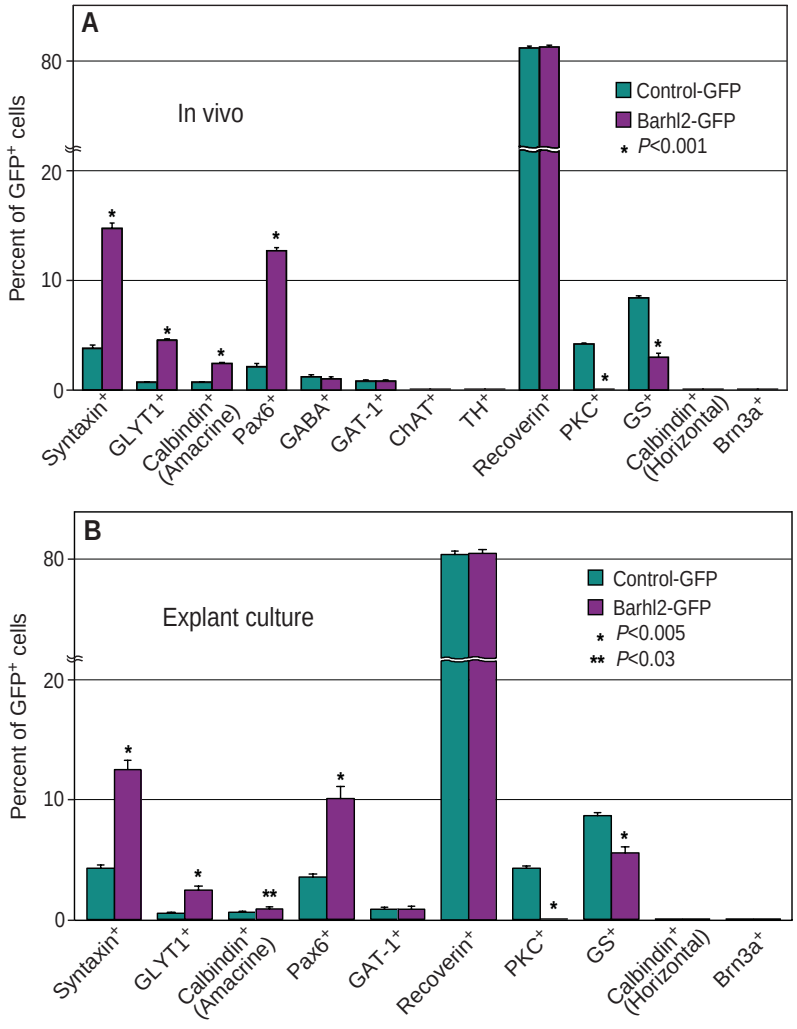

Fig. 6. Quantitation of virus-transduced retinal cells that became immunoreactive for a series of cell type-specific markers. (A) P0 retinas infected in vivo. (B) E17.5 retinal explants infected in vitro. Each histogram represents the mean±s.d. for three or four retinas. More than 530 GFP$^+$ cells were scored in each retina. ChAT, choline acetyltransferase; GAT-1, GABA transporter 1; GLYT1, glycine transporter 1; GS, glutamine synthase; TH, tyrosine hydroxylase.

Discussion

Distinct expression patterns of *Barhl* genes in the nervous system

Two homologous *Barhl* genes, *Barhl1* and *Barhl2*, have been identified in mammals (Bulfone et al., 2000; Saito et al., 1998). A rather complete analysis of the *Barhl1* expression profile in the mouse has been reported (Li et al., 2002; Bulfone et al., 2000), whereas the expression pattern of *Barhl2* has not been thoroughly investigated (Saito et al., 1998). The systematic analysis of *Barhl2* expression pattern in this work allows us to compare the similarities and differences of these two genes in their expression patterns during mouse development. We find that *Barhl1* and *Barhl2* display distinct expression patterns in the nervous system while sharing some overlapping expression profiles in the CNS. First, in sensorineural tissues, *Barhl2* is uniquely expressed in the retina, while *Barhl1* is uniquely expressed in the inner ear hair cells (Li et al., 2002), suggesting that each may play a unique, nonredundant role in the respective tissue. This is indeed the case as demonstrated by this and our previous work (Li et al., 2002). Second, in the brain, *Barhl2* is uniquely expressed in the telencephalon, thalamus, preoptic area, paraventricular nucleus, sensory trigeminal nuclei and pineal gland but absent from the posterior extramural migratory stream and all mossy fiber precerebellar nuclei (Fig. 1). The opposite is true for *Barhl1* (Bulfone et al., 2000). Third, *Barhl1* and *Barhl2* have overlapping expression patterns within the mammillary body, midbrain, cerebellum and spinal cord (Bulfone et al., 2000), suggesting a possible functional redundancy between these two genes in these CNS regions.

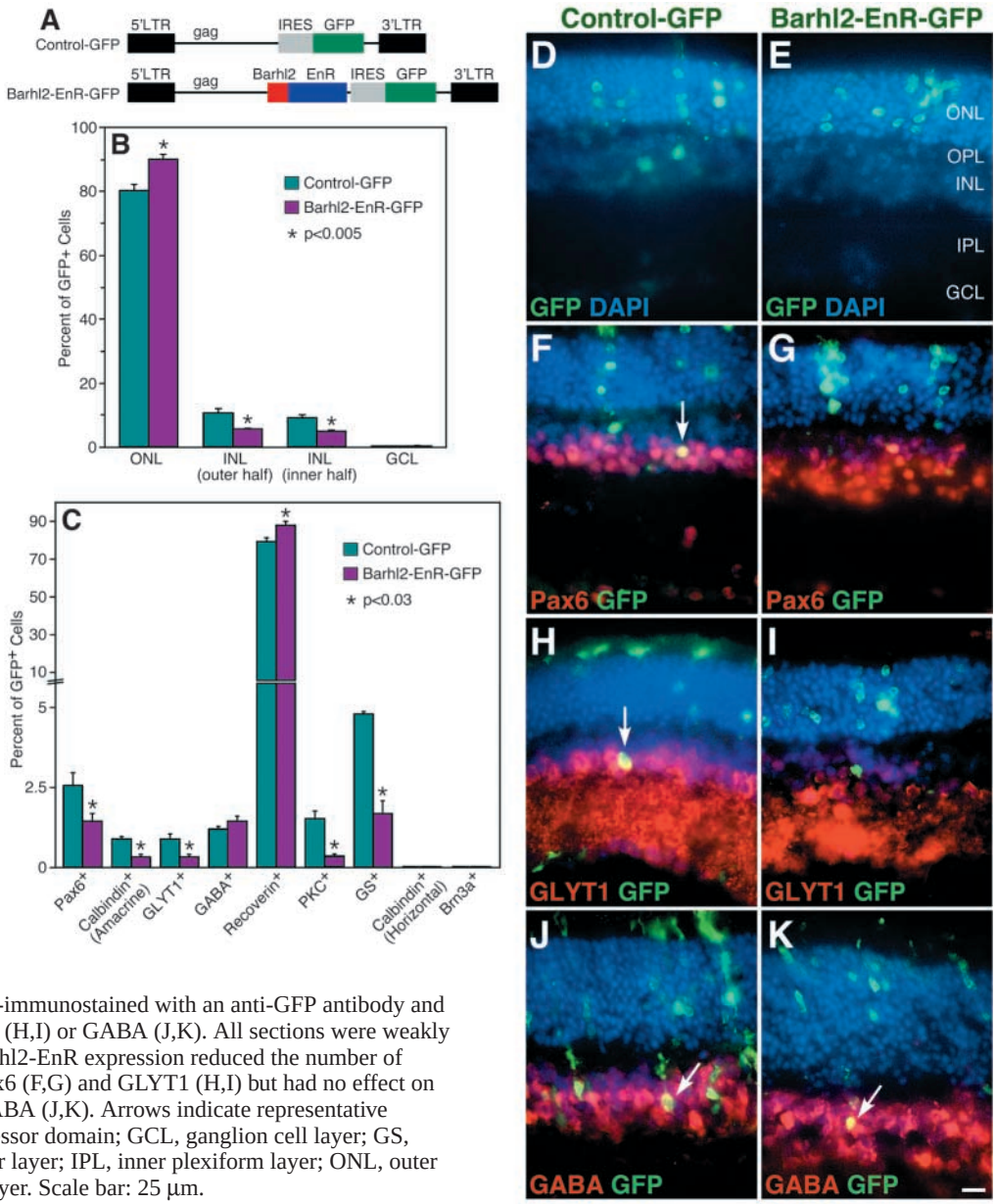

Fig. 7. Effect of a dominant-negative form of Barhl2 on the formation of different retinal cell types. (A) Schematic of control-GFP and Barhl2-EnR-GFP retroviral constructs. (B) Percentages of virus-transduced cells located in different cellular layers of the retina (means±s.d.). More than 500 GFP$^+$ cells in three or four independent retinas were scored for either control-GFP or Barhl2-EnR-GFP virus. (C) Percentages of virus-transduced cells that became immunoreactive for a series of cell type-specific markers. Each histogram represents the mean±s.d. for three to five retinas. More than 500 GFP$^+$ cells were scored in each retina. (D,E) Virus-transduced GFP$^+$ cells were visualized in P30 retinal sections that were weakly counterstained with DAPI. Infection with Barhl2-EnR-GFP viruses resulted in a significant decrease of INL cells. (F-K) Sections from P30 retinas infected with control-GFP or Barhl2-EnR-GFP viruses were double-immunostained with an anti-GFP antibody and antibodies against Pax6 (F,G), GLYT1 (H,I) or GABA (J,K). All sections were weakly counterstained with DAPI. Forced Barhl2-EnR expression reduced the number of amacrine cells immunoreactive for Pax6 (F,G) and GLYT1 (H,I) but had no effect on amacrine cells immunoreactive for GABA (J,K). Arrows indicate representative colocalized cells. EnR, engrailed repressor domain; GCL, ganglion cell layer; GS, glutamine synthase; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bar: 25 μm.

Barhl2 is expressed in postmitotic amacrine, horizontal and ganglion cells during retinogenesis

The expression of *Barhl2* in the retina has provided an excellent opportunity to study its function in the CNS using retina as a model system because of its easy accessibility and well-characterized cell classes. We therefore focused our efforts to investigate the cellular localization of Barhl2 factor and its function during retinogenesis. RNA in situ hybridization reveals the presence of *Barhl2* transcripts within the inner neuroblastic layer at E14.5 and in the INL and GCL in postnatal retinas. Owing to the presence of several cell classes in these layers, however, it is impossible to unequivocally identify the types of cells that express *Barhl2* by in situ hybridization. We thus produced a specific anti-Barhl2 antibody and unambiguously identified by immunostaining all classes of cells that express Barhl2, which include amacrine, horizontal and ganglion cells (Fig. 3). In addition, BrdU labeling experiment indicated that Barhl2 was expressed only by postmitotic cells.

During mouse retinogenesis, birthdating studies have demonstrated that amacrine, horizontal and ganglion cells begin to exit the cell cycle at E11 (Young, 1985). Our analysis has revealed the onset of *Barhl2* expression at E13.5, indicating that *Barhl2* is unlikely to be involved in the fate commitment and initial generation of amacrine, horizontal and ganglion cells. The scattered cells immunoreactive for Barhl2 in the outer neuroblastic layer resemble those of Brn3b$^+$ migrating ganglion cells or Lim1$^+$ migrating horizontal cells (Liu et al., 2000; Xiang, 1998). They most probably represent newly generated neurons that are differentiating and migrating toward the inner retina to become amacrine, horizontal and ganglion cells. Thus, given its expression only in differentiating and mature neurons, *Barhl2* may play a role in the differentiation and maintenance of amacrine, horizontal and ganglion cells.

Barhl2 promotes the differentiation of glycinergic amacrine cells at the expense of bipolar and Müller cells

The amacrine cells come as many as more than 26 morphological types to fine tune the physiological output of the retina (MacNeil and Masland, 1998). For example, the starburst amacrine cells play a central role in the establishment of retinal direction-selectivity (Fried et al., 2002; Vaney and Taylor, 2002). Amacrine cells can also be divided into two major nonoverlapping groups according to the types of neurotransmitters they contain. These are glycinergic and GABAergic amacrine cells. To date, it remains essentially unknown what factors are involved in the differentiation of these different amacrine cell subtypes. Our work identifies Barhl2 as a key regulator that confers the identity of glycinergic amacrine cells. This effect appears to be specific as overexpressed Barhl2 does not cause even a slight change in the number of GABAergic cells (Fig. 6). Similarly, the Barhl2 homeodomain, when fused with the engrailed repressor, suppresses glycinergic amacrine cells but has no effect on GABAergic neurons. Interestingly, *Pax6*, apart from its essential role in early retinal development, is expressed in differentiating amacrine cells and has been shown to positively regulate the formation of glycinergic amacrine cells as well (Marquardt et al., 2001).

Despite the requirement of Pax6 for retinal progenitors to acquire multipotency, the absence of *Pax6* in mice permits the formation of amacrine cells (Marquardt et al., 2001), implicating that another unknown factor(s) must be required to make the progenitors competent for the generation of amacrine cells. Furthermore, gene targeting has demonstrated that *Math3* and *Neurod1* are redundantly required to specify amacrine cells from progenitors (Inoue et al., 2002; Morrow et al., 1999). These data and our present study have prompted us to propose a genetic pathway governing the determination and differentiation of amacrine cells (Fig. 8). In this model, Math3 and Neurod1 together specify amacrine cells from retinal progenitors rendered competent by an unknown regulator(s). Barhl2, probably together with Pax6, then promotes the differentiation of glycinergic amacrine cells from fate-restricted amacrine progenitors. Such fate-restricted amacrine progenitors have been identified by fate tracing experiments in early rat and *Xenopus* retinas (Alexiades and Cepko, 1997; Huang and Moody, 1997; Moody et al., 2000). However, it remains to be determined what factors are involved in the differentiation of GABAergic amacrine cells. Apart from the absolute requirement of Pax6 for retinal progenitors to produce all retinal cells other than amacrine neurons (Marquardt et al., 2001), the possibility cannot be ruled out that Pax6 also plays a redundant role to confer progenitors with the capacity of amacrine cell generation. Consistent with this notion, forced Pax6 expression can promote an amacrine cell fate in combination with Math3 or Neurod1 (Inoue et al., 2002). Thus, there is a possibility that $Pax6^+$ progenitor cells may also be specified into amacrine cells by Math3 and Neurod1 (Fig. 8).

We do not know how Barhl2 specifies the identity of only glycinergic amacrine cells while it appears to be expressed by all amacrine cell population. Similarly, it is not clear why the lack of Pax6 activity leads to the loss of only glycinergic amacrine cells while Pax6 is apparently expressed by all differentiating amacrine cells as well (Davis and Reed, 1996; Marquardt et al., 2001). One explanation is that the differentiation of a specific amacrine subtype requires a particular code of transcription factors. Thus, even though Barhl2 is a pro-glycinergic amacrine cell factor, it may promote the differentiation of this subtype only from amacrine cell precursors that express a certain combination of transcription factors. In the spinal cord, it has been demonstrated that the various subtypes of interneurons and motor neurons are specified by different transcriptional codes (Jessell, 2000; Shirasaki and Pfaff, 2002). Our overexpression experiments did not reveal a role for Barhl2 to promote the differentiation of horizontal and ganglion cells in which Barhl2 is also expressed. As viral infections were performed in neonatal retinas in this study, one likely possibility is that postnatal retinal progenitors may lose their competence to give rise to these two cell types because nearly all horizontal and ganglion cells are produced prior to birth (Young, 1985). However, the finding that the Prox1 homeoprotein can promote the formation of horizontal cells from postnatal progenitors appears to dispute this possibility (Dyer et al., 2003). In addition, overexpressed Barhl2 in cultured E13.5 and E17.5 retinas did not affect horizontal cell formation. Another possibility is that Barhl2 is adventitiously expressed in horizontal and ganglion cells without any functional consequence. Efforts are under way to produce *Barhl2* knockout mice to distinguish these possibilities.

Many of the transcription factors involved in retinal development have been shown to act as both positive and negative

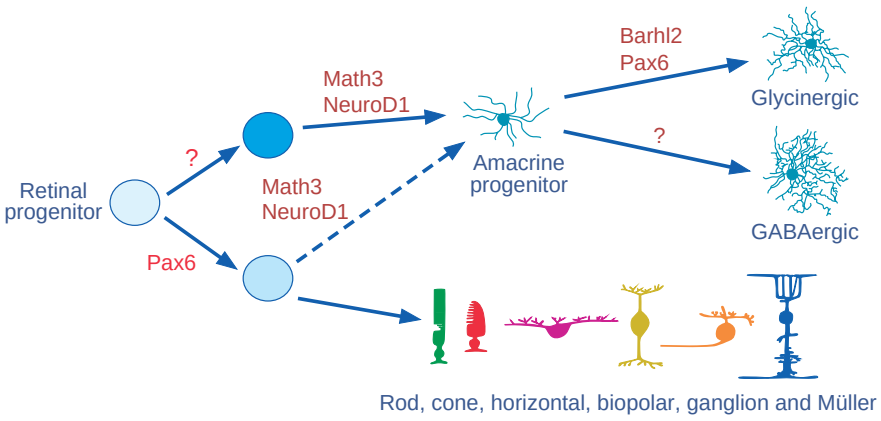

Fig. 8. Schematic depicting genetic relationships between Barhl2 and other transcription factors involved in the development of amacrine cells. Retinal progenitors may acquire the competence to produce amacrine cells by the activation of an unknown factor(s). Competent progenitor cells are then specified by Math3 and Neurod1 to become amacrine cells. Some of the newly generated amacrine cells may differentiate into glycinergic cells by the action of Barhl2 and Pax6. Alternatively, Math3 and Neurod1 may also be able to specify $Pax6^+$ progenitors into amacrine cells (broken arrow). It is known that $Pax6^+$ progenitor cells have the potential to differentiate into rod, cone, horizontal, bipolar, ganglion and Müller cells.

regulators depending on different cell types. For example, Rax1 and Hes1 are able to promote a Müller glial cell fate as well as inhibit neuron differentiation (Furukawa et al., 2000). By contrast, Xath5 promotes a ganglion cell fate but blocks the formation of Müller and bipolar cells (Kanekar et al., 1997). Similarly, we show here that Barhl2 can function as a positive factor to specify glycinergic amacrine cells while negatively regulating the formation of bipolar and Müller cells. As Barhl2 is not expressed in mitotic retinal progenitors, conceivably, it may prevent newly selected amacrine cells from differentiating into bipolar or Müller cells. This bifunctional property of retinal developmental regulators may serve to control the production of proper numbers of different cell types and to minimize developmental errors during retinogenesis.

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