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Math3 and *NeuroD* regulate amacrine cell fate specification in the retina

Tomoyuki Inoue[1,2], Masato Hojo[1,*], Yasumasa Bessho[1], Yasuo Tano[2], Jacqueline E. Lee[3] and Ryoichiro Kageyama[1,†]

[1]Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan
[2]Department of Ophthalmology, Osaka University Medical School, Suita 565-0871, Japan
[3]Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO 80309, USA
*Present address: Department of Neurosurgery, Kurashiki Central Hospital, Okayama 710-8602, Japan
†Author for correspondence (e-mail: rkageyam@virus.kyoto-u.ac.jp)

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SUMMARY

The basic helix-loop-helix genes *Math3* and *NeuroD* are expressed by differentiating amacrine cells, retinal interneurons. Previous studies have demonstrated that a normal number of amacrine cells is generated in mice lacking either *Math3* or *NeuroD*. We have found that, in *Math3-NeuroD* double-mutant retina, amacrine cells are completely missing, while ganglion and Müller glial cells are increased in number. In the double-mutant retina, the cells that would normally differentiate into amacrine cells did not die but adopted the ganglion and glial cell fates. Misexpression studies using the developing retinal explant cultures showed that, although *Math3* and *NeuroD* alone only promoted rod genesis, they significantly increased the population of amacrine cells when the homeobox gene *Pax6* or *Six3* was co-expressed. These results indicate that *Math3* and *NeuroD* are essential, but not sufficient, for amacrine cell genesis, and that co-expression of the basic helix-loop-helix and homeobox genes is required for specification of the correct neuronal subtype.

Key words: Amacrine cell, bHLH, Homeobox, Pax6, Retina, Mouse

INTRODUCTION

A wide variety of neurons as well as glia differentiate from common precursors. The molecular mechanisms involved in generation of such diverse cell types in the developing nervous system are still obscure. Neural retina is an ideal model system with which to investigate the mechanisms for generation of multiple cell types (Harris, 1997; Cepko, 1999), as it has a relatively simple structure, mimics normal development in isolated explant cultures and is therefore easy to analyze (Caffé et al., 1989; Sparrow et al., 1990; Tomita et al., 1996; Morrow et al., 1999). In the neural retina, seven types of cells (six types of neuron and one type of glia) form three cellular layers: the outer nuclear layer (ONL), which contains rod and cone photoreceptors; the inner nuclear layer (INL), which contains bipolar, horizontal and amacrine interneurons, and Müller glia; and the ganglion cell layer (GCL), which contains ganglion and displaced amacrine cells. It has been shown that these seven types of cells differentiate from common precursors under the control of intrinsic cues, such as transcription factors, and extrinsic signals, such as neurotrophic factors (Harris, 1997; Cepko, 1999).

Recent studies have demonstrated that basic helix-loop-helix (bHLH)- and homeobox-type transcription factors contribute to the intrinsic properties of retinal precursors. (Kageyama and Nakanishi, 1997; Lee, 1997; Cepko, 1999; Livesey and Cepko, 2001). These transcription factors regulate determination and differentiation of multiple cell types. For example, the bHLH gene *NeuroD* (*Neurod1* – Mouse Genome Informatics) and the homeobox gene *Crx* regulate generation of photoreceptors (Furukawa et al., 1997; Furukawa et al., 1999; Freund et al., 1997; Chen et al., 1997; Morrow et al., 1999). Similarly, the bHLH genes *Mash1* and *Math3* (*Ascl1* and *Atoh3*, respectively – Mouse Genome Informatics) and the homeobox gene *Chx10* are required for specification of bipolar cells (Burmeister et al., 1996; Tomita et al., 2000; Hatakeyama et al., 2001). Interestingly, co-expression of *Mash1* or *Math3* with *Chx10* efficiently induces bipolar cell genesis, whereas misexpression of either *Mash1*/*Math3* or *Chx10* alone cannot (Hatakeyama et al., 2001). These results suggest that combinations of bHLH and homeobox genes may be important for specification of cell types. These transcription factor codes for cell fate specification are, however, only beginning to be elucidated.

Amacrine cells, interneurons present in the INL and GCL, make synapses onto bipolar cell terminals and ganglion cell dendrites, and modulate the synaptic connection between bipolar and ganglion cells (Kolb, 1997). There are multiple morphologically and functionally distinct subtypes of amacrine cells. Some of them are located in the GCL (displaced amacrine cells), while others are in the inner region of the INL. It has been shown that the bHLH gene *NeuroD* and the paired-type homeobox gene *Pax6* are expressed by differentiating amacrine cells (Jones et al., 1998; Morrow et al., 1999; Nishina et al., 1999). However, mutation for *NeuroD* or *Pax6* does not reduce amacrine cell genesis (Morrow et al., 1999; Marquardt et al., 2001) and, therefore, their functions are not yet known.

We have found that the bHLH gene *Math3* is also transiently

expressed by differentiating amacrine cells and that mice deficient for both *Math3* and *NeuroD* completely lack amacrine cells. Strikingly, the cells that fail to differentiate into amacrine cells do not die, but adopt the ganglion and Müller glial cell fates in the double-mutant retina. In addition, we found that, although *Math3* or *NeuroD* alone could not induce amacrine cell genesis, they could do so when *Pax6* or *Six3* is co-expressed. These data provide evidence that the bHLH genes *Math3* and *NeuroD* are essential but not sufficient for amacrine cell genesis and that combinations of bHLH and homeobox genes are important for cell type specification.

MATERIALS AND METHODS

Math3-NeuroD mutant mice

Both *Math3*- (Tomita et al., 2000) and *NeuroD*-mutant mice (Miyata et al., 1999) were crossed to ICR mice. *Math3/NeuroD* double-mutant mice were obtained by crossing *Math3*$^{-/-}$/*NeuroD*$^{+/-}$ male and *Math3*$^{+/-}$/*NeuroD*$^{+/-}$ female mice.

Construction of retroviruses

For construction of CLIG-Math3, CLIG-NeuroD, CLIG-Pax6 and CLIG-Six3, cDNAs for bHLH and homeobox factors were cloned into the *Eco*RI site of pCLIG, which directs expression of the cloned genes together with enhanced green fluorescent protein (GFP) from the upstream long terminal repeat (LTR) promoter (Hojo et al., 2000). For construction of CLIG-Pax6-Math3, CLIG-Pax6-NeuroD, CLIG-Pax6-Mash1, CLIG-Six3-Math3 and CLIG-Six3-NeuroD, the bHLH genes were cloned into the *Bsr*GI and *Cla*I sites of pCLIG-Pax6 and pCLIG-Six3, which are located in the 3′ region of GFP gene, so that GFP gene is fused in frame with each bHLH gene. Retroviral DNAs were transfected with LipofectAMINE (Gibco-BRL) into ψ2mp34, an ecotropic packaging cell line (Yoshimatsu et al., 1998). The supernatant was collected 2 days later and concentrated with Centricon Plus-20 (Millipore), as described previously (Ishibashi et al., 1994; Tsuda et al., 1998; Ohtsuka et al., 1999).

Retinal explant culture

The retinal explant culture was performed, as described previously (Tomita et al., 1996). Briefly, the neural retina without pigment epithelium was placed on a Millicell chamber filter (Millipore: diameter 30 mm, pore size 0.4 μm) with the ganglion cell layer upwards. The chamber was transferred to a six-well culture plate. Each well contained 1 ml of culture medium (50% MEM with Hepes, 25% Hank's solution, 25% heat-inactivated horse serum, 200 μM L-glutamine and 5.75 mg/ml glucose). Explants were cultured at 34°C in 5% CO_2, and the medium was changed every other day.

Immunochemical analysis

For immunohistochemistry, retinal explants were fixed with 4% paraformaldehyde for 10 minutes on ice, treated with 25% sucrose for 30 minutes, embedded in OCT compound (Miles) and sectioned (16 μm thickness). For immunocytochemistry, explants were dissociated, as previously described (Morrow et al., 1998; Hatakeyama et al., 2001). The samples were then preincubated with a blocking solution [5% normal goat serum and 0.1% Triton X-100 in phosphate-buffered saline (PBS)] for 1 hour and then incubated overnight at room temperature or for 2 days at 4°C in 1% goat serum and 0.1% Triton X-100 with the following antibodies: rabbit anti-GFP (Medical and Biological Laboratories), mouse anti-Myc (Invitrogen), rabbit anti-Myc (Medical and Biological Laboratories), rabbit anti-calbindin (Chemicon), mouse anti-HPC1/syntaxin (Sigma), rat anti-Thy1.2 (Pharmingen), mouse anti-p75 (Promega), mouse anti-protein kinase C (PKC) (Amersham), rabbit anti-neurofilament (NF) (Chemicon), rabbit anti-rhodopsin (LSL), mouse anti-glutamine synthetase (GS) (Chemicon), mouse anti-vimentin (Histofine), mouse anti-Ki67 (Pharmingen) and rabbit anti-β-galactosidase (Cortex Biochem). Retinal cell types were determined by the morphology, location and by using the following antibodies: anti-Thy1.2 and anti-p75 (ganglion cells), anti-calbindin and anti-HPC1 (amacrine cells), anti-PKC (bipolar cells), anti-calbindin and anti-NF (horizontal cells), anti-GS, anti-vimentin (Müller glia), and anti-rhodopsin (rods). To detect cell death, TUNEL assay was performed with a detection kit (Boehringer-Mannheim). Fluorescently labeled preparations were imaged using a Carl Zeiss confocal microscope.

X-gal staining

Retinal explants cultured for 2 weeks were fixed with 0.5% glutaraldehyde in PBS at 4°C for 1 hour and stained with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 35 mM potassium ferricyanide, 35 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.02% Nonidet P-40 and 0.01% deoxycholate in PBS. Frozen sections were prepared at 16 μm thickness.

In situ hybridization

In situ hybridization was performed as previously described (Hojo et al., 2000). Probes for Math3 (Takebayashi et al., 1997), NeuroD (Lee et al., 1995) and Math5 (Atoh7 – Mouse Genome Informatics) (Brown et al., 1998) were labeled with digoxigenin.

RESULTS

Math3 expression in the developing retina

Previous studies demonstrated that *Math3* is expressed in the INL and promotes bipolar cell genesis while inhibiting gliogenesis (Takebayashi et al., 1997; Tsuda et al., 1998; Tomita et al., 2000). To determine whether *Math3* is expressed by other cell types in the INL, *Math3* expression was examined by in situ hybridization. At postnatal day (P) 1, the retina consists of two layers, the ganglion cell layer and the ventricular zone, which contains common precursors for neurons and glia. At P1, *Math3* was expressed in the ventricular zone (Fig. 1A). During P4-P7, the ventricular cells differentiate into neurons and glia, which form the INL and ONL. At this stage, *Math3* was expressed only in the INL (Fig. 1B,C). The expression in the INL was broad during P4-P7, suggesting that *Math3* is expressed by amacrine and horizontal cells as well as by bipolar cells at this stage.

To show clearly that *Math3* is expressed by amacrine and horizontal cells, we examined *Math3* expression with specific markers: calbindin (amacrine and horizontal cells), HPC1 (amacrine cells) and neurofilament (NF, horizontal cells). At P7, *Math3* was co-expressed with amacrine and horizontal cell markers (Fig. 1G-O), suggesting that *Math3* may be involved in differentiation of these neurons. However, at P14, *Math3* expression became restricted to the outer region of the INL (Fig. 1D) and was observed with NF (Fig. 1S-U) but not with HPC1 (Fig. 1P-R), indicating that *Math3* expression disappears in amacrine cells by P14. This restricted expression was maintained until adulthood (Fig. 1E). A similar expression pattern was also reported for the chick ortholog (Roztocil et al., 1997).

Loss of amacrine cells and concomitant increase of ganglion and Müller glial cells in *Math3-NeuroD* double-mutant retina

Although *Math3* is transiently expressed by differentiating amacrine cells, our previous data have shown that retinal development proceeds normally in the absence of *Math3*

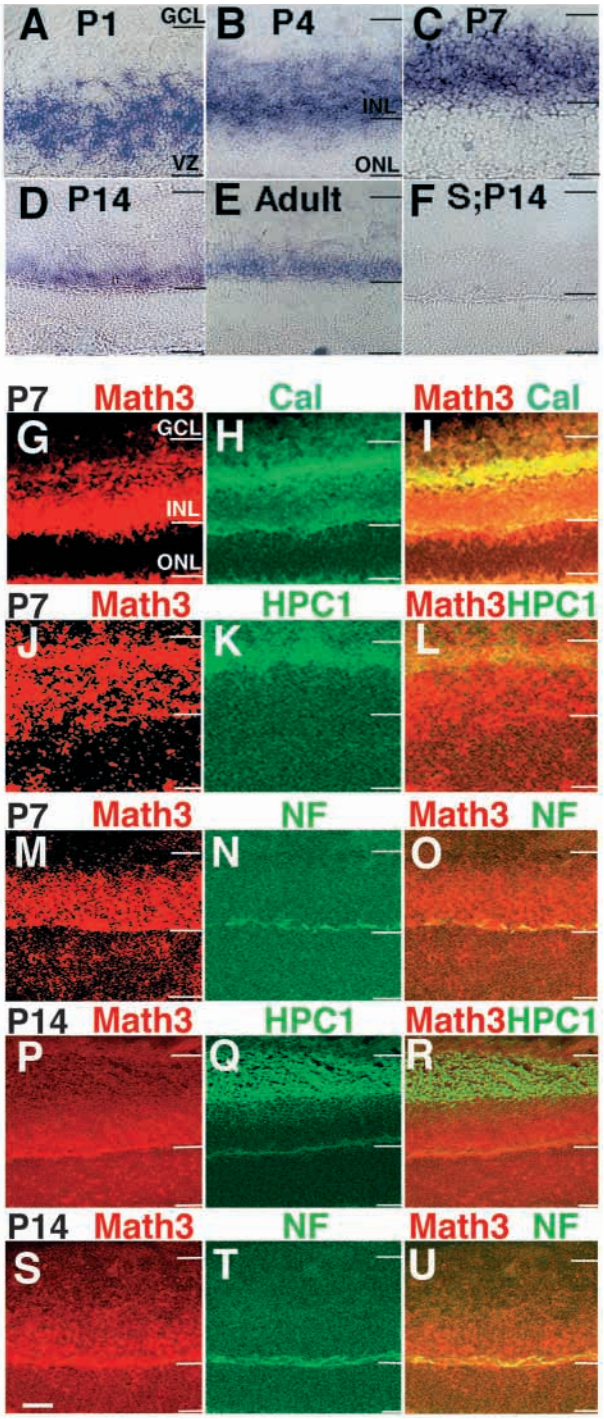


Fig. 1. Expression of *Math3* in the developing retina. In situ hybridization of *Math3* in mouse retina. (A) At P1, the retina consists of the ganglion cell layer (GCL) and ventricular zone (VZ). *Math3* is expressed in the ventricular zone. (B,C) At P4 and P7, the retina consists of three cellular layers. *Math3* is expressed broadly in the inner nuclear layer (INL). (D,E) At P14 and adult, *Math3* is expressed in the outer region of the INL. (F) The sense strand was used as a probe. No signal is observed. (G-I) At P7, *Math3* is expressed by calbindin$^+$ amacrine and horizontal cells. (J-L) At P7, *Math3* is expressed by HPC1$^+$ amacrine cells. (M-O) At P7, *Math3* is expressed by NF$^+$ horizontal cells. (P-R) At P14, *Math3* is not expressed by HPC1$^+$ amacrine cells. (S-U) At P14, *Math3* is still expressed by NF$^+$ horizontal cells. Scale bar: 25 μm.

(Tomita et al., 2000). It has previously been reported that amacrine cell differentiation is delayed in *NeuroD*-deficient retina although the final number is not affected (Morrow et al., 1999). These results raise the possibility that *Math3* and *NeuroD* might be functionally redundant for amacrine cell genesis. To investigate this possibility, mice that lack both *Math3* and *NeuroD* were generated. Because most of the double-mutant mice died soon after birth, retinal explants were prepared from E17.5 embryos and cultured for 2 weeks to examine the postnatal development of the mutant retina. Retinal explants were also prepared from wild-type, *Math3*$^{-/-}$ and *NeuroD*$^{-/-}$ embryos for comparison. After 2 weeks of culture, the three cellular layers, the GCL, INL and ONL, were formed in *Math3*$^{-/-}$-*NeuroD*$^{-/-}$ retinal explants as in the wild type (Fig. 2A, parts a-d). Whereas the absolute cell number of the ONL was slightly reduced in *NeuroD*$^{-/-}$ and *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina (Fig. 2A, parts c,d, Fig. 2B, parts c,d and Fig. 3A, green bars), that of the other layers was not significantly affected in the mutant retina (Fig. 3A). As rod genesis is known to be decreased in the absence of *NeuroD* (Morrow et al., 1999), the decrease of the ONL cell number in the double-mutant retina may be due to *NeuroD* deficiency. In the INL of wild-type, *Math3*$^{-/-}$ and *NeuroD*$^{-/-}$ retina, amacrine cells (calbindin$^+$, HPC1$^+$) were normally generated (Fig. 2D, parts a-c, black arrowheads; Fig. 2E, parts a-c; Fig. 3B,C, red bars). By contrast, in the double-mutant retina, amacrine cells (including displaced amacrine cells in the GCL) were completely missing (Fig. 2D, part d, asterisks, Fig. 2E, part d and Fig. 3B,C, asterisks), although the total number of the INL and GCL cells was not affected (Fig. 3A). Strikingly, ganglion cell number (Thy1.2$^+$, p75$^+$) was significantly increased not only in the GCL but also ectopically in the INL of the double-mutant retina (Fig. 2F, part d, Fig. 2G, part d and Fig. 3B,C, black bars). Furthermore, Müller glial cells (vimentin$^+$, glutamine synthetase$^+$) were slightly increased in the double-mutant retina (Fig. 2H, part d, Fig. 2I, part d and Fig. 3C, blue bar), whereas bipolar and horizontal cells were normally generated (Fig. 2C, part d, Fig. 2D, part d, white arrowhead, and Fig. 3C). Thus, the double-mutant retina displayed the lack of amacrine cells and concomitant increase of ganglion and Müller glial cells.

It was previously reported that *NeuroD* mutation exhibits three- to fourfold increase of Müller glia and two to threefold increase of bipolar cells (Morrow et al., 1999). However, we only observed small increase of Müller glia and no increase of bipolar cells in *NeuroD*$^{-/-}$ retina (Fig. 3C). This discrepancy may be due to the different genetic backgrounds, C57BL/6 (Morrow et al., 1999) and ICR (this study).

Because the absolute number of ganglion cells was significantly increased in *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina, we next examined whether the optic nerves, which contain the axons of ganglion cells, were thickened in the double-mutant embryos. The area of the optic nerve section was increased about 1.7-fold in the double mutant embryos (Fig. 4B) compared with the wild type (Fig. 4A). In addition, the NF-positive axon bundles of ganglion cells were increased in the double mutant optic nerve (Fig. 4C-E). These results are consistent with the increase of the ganglion cell number in *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina.

Fate switch from amacrine cells to ganglion and Müller glial cells in the double-mutant retina

The lack of amacrine cells in the double-mutant retina could

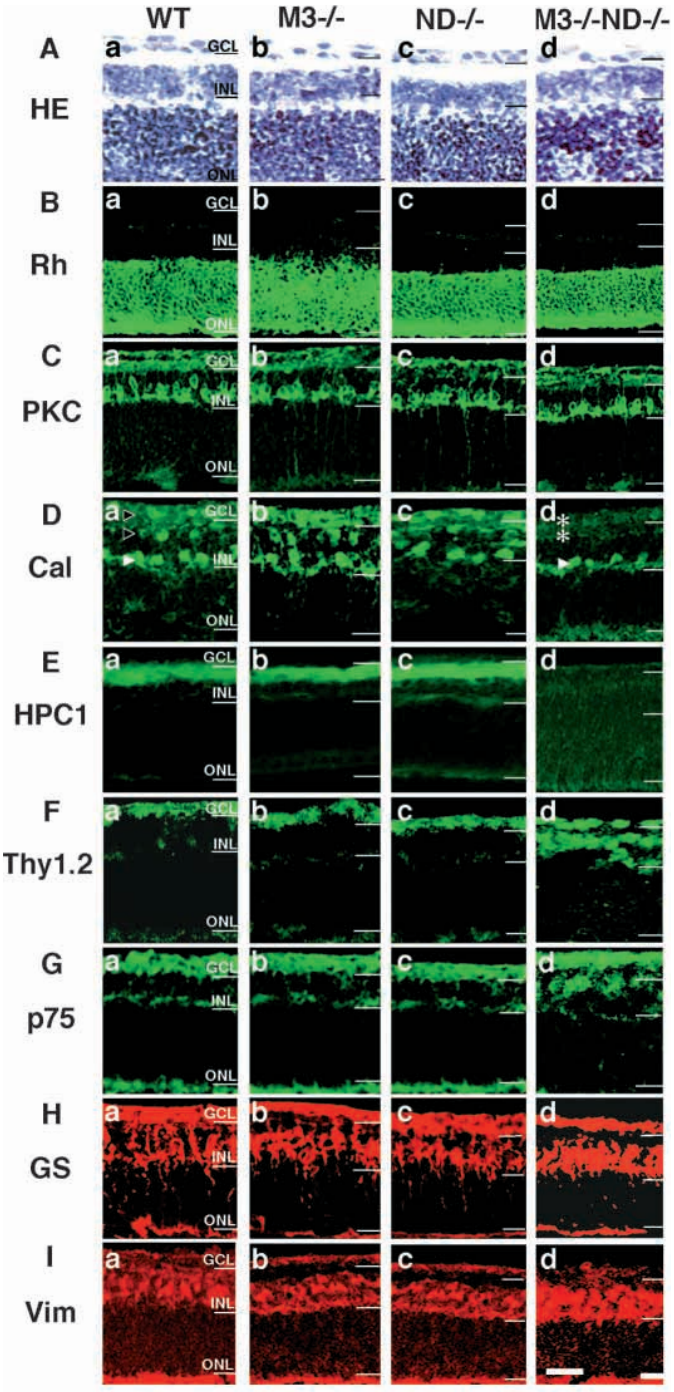


Fig. 2. The lack of amacrine cells and concomitant increase of ganglion and Müller glial cells in *Math3*/*NeuroD* double-mutant retina. The retinal explants were prepared from wild-type (a), *Math3*$^{-/-}$ (b), *NeuroD*$^{-/-}$ (c) and *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ embryos (d) at E17.5 and cultured for 2 weeks. (A) HE staining. The GCL, INL and ONL are formed in all retinal explants. (B) Immunohistochemistry for rhodopsin. There are fewer rods (rhodopsin^{+}) in *NeuroD*$^{-/-}$ (c) and *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina (d). (C) PKC-positive cells (bipolar cells) are normally generated in all retinal explants. (D) Calbindin-positive amacrine (black arrowheads) and horizontal cells (white arrowhead) are normally generated in wild-type (a), *Math3*$^{-/-}$ (b) and *NeuroD*$^{-/-}$ retina (c). By contrast, in *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina (d), amacrine cells are completely missing (asterisks), whereas calbindin-positive horizontal cells are normally generated (white arrowhead). (E) Amacrine cells (HPC1^{+}) are missing in *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina (d). (F,G) Ganglion cells (Thy1.2^{+}, p75^{+}) are significantly increased in *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina (d). There are ectopic ganglion cells in the inner region of the INL of *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina (d). (H,I) The number of Müller glial cells (glutamine synthetase^{+}, vimentin^{+}) is slightly increased in *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina (d). Scale bar: 25 μm.

be due to cell death. To investigate this possibility, a TUNEL assay was performed at days 1, 3, 5, 7 and 14 of culture. However, no significant increase in the number of TUNEL-positive cells was observed in the double-mutant retina (data not shown), suggesting that the cells that would normally differentiate into amacrine cells did not die but remained in the retina. As ganglion and Müller glial cells were increased in the double-mutant retina, it is most likely that the cells that failed to differentiate into amacrine cells became ganglion and Müller glial cells instead. To test this idea, we monitored the fates of the mutant cells. As *lacZ* was knocked into the *NeuroD* locus (Miyata et al., 1999), the cells that would normally differentiate into amacrine cells (NeuroD^{+}) could be monitored by X-gal staining in the double-mutant retina. In *NeuroD*$^{+/-}$ retina, rods and amacrine cells were labeled (Fig. 5A, part a), as described previously (Morrow et al., 1999). By contrast, in *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina, many labeled cells were present in the GCL and the inner region of the INL, the majority being small in size (Fig. 5A, part b) and others displaying a Müller glia-like morphology (Fig. 5A, part b, arrowhead).

To further characterize the nature of the *lacZ*$^{+}$ cells, retinal explants that were cultured for seven days from E17.5 were dissociated and subjected to immunocytochemistry. Many of the *lacZ*$^{+}$ cells from *NeuroD*$^{+/-}$ retina expressed the amacrine cell marker HPC1 (Fig. 5B, parts a-d, arrows) but not ganglion (Fig. 5B, parts i-l) and Müller glial cell markers (Fig. 5B, parts q-t). By contrast, those from the double-mutant retina expressed the ganglion (Fig. 5B, parts m-p, arrows) and Müller glial cell markers (Fig. 5B, parts u-x, arrow) but not the amacrine cell marker (Fig. 5B, parts e-h). These results strongly indicate that the cells that failed to differentiate into amacrine cells adopted the ganglion and Müller glial cell fates in the double-mutant retina.

Math5 expression is increased in *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina

It has previously been shown that the bHLH gene *Math5* is essential for ganglion cell genesis (Brown et al., 2001; Wang et al., 2001). As the ganglion cell number was significantly increased in *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina, *Math5* expression was next compared between the wild-type and double-mutant retina. At E17.5, *Math5* was expressed weakly by subsets of cells in the ventricular zone of the wild-type retina (Fig. 6E). By contrast, in the double-mutant retina, *Math5* expression was significantly upregulated (Fig. 6F), suggesting that this upregulation may lead to the increase of the ganglion cell number. These results also suggest that, in the wild-type retina, *Math5* expression is downregulated by *Math3* and *NeuroD*.

We also examined whether *Math3*/*NeuroD* expression was changed in single-mutant retinas. In *NeuroD*$^{-/-}$ retina, *Math3* expression was not changed (Fig. 6A,B), and, similarly, in

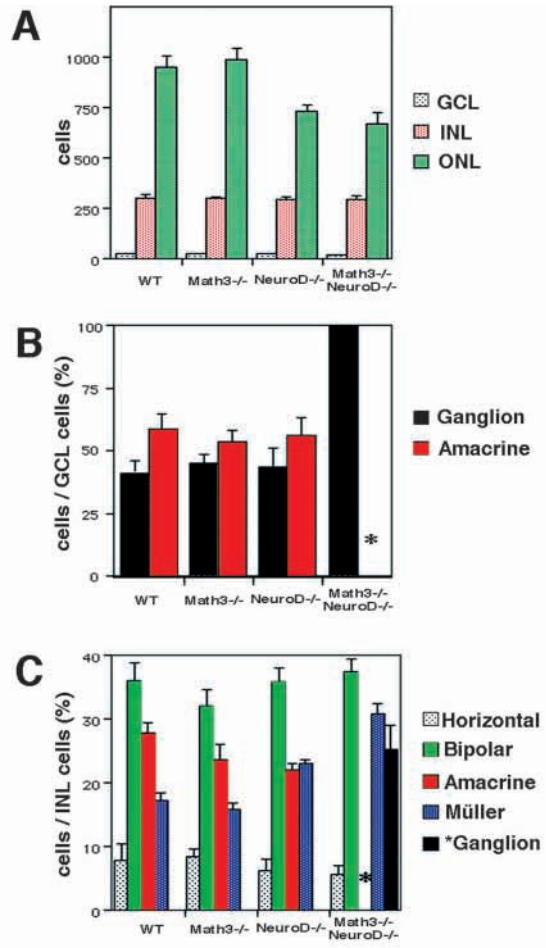


Fig. 3. Quantification of retinal cells of the mutant retina. (A) The cell number was counted in a section (16 μm thick, 500 μm wide) of the central region of at least three independent samples from each genotype. The number of the ONL cells is reduced in *NeuroD*$^{-/-}$ and *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina. The absolute numbers of the GCL and INL cells are not significantly affected by mutations. (B) Ratios of ganglion and amacrine cells in the GCL. The percentage of calbindin-positive and Thy1.2-positive cells per the total GCL cells was calculated. About a half of the GCL cells are amacrine cells in wild-type, *Math3*$^{-/-}$ and *NeuroD*$^{-/-}$ retina. By contrast, there are no amacrine cells (asterisk) in the GCL of *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina. As the absolute number of the GCL cells is not affected (A), these data indicate that the absolute number of ganglion cells is increased in *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina. (C) Ratios of cells in the INL. The cell number was counted as above, and the percentage of each retinal cell type per the total INL cells was calculated. There are ectopic ganglion cells but no amacrine cells (asterisk) in *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ INL.

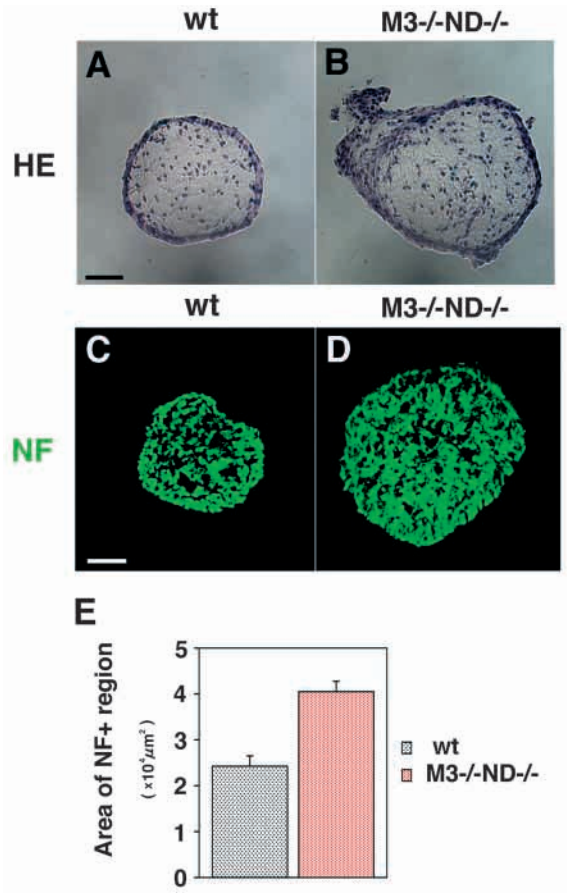


Fig. 4. The optic nerves are thickened in the double-mutant mice. (A,B) Section of the wild-type (A) and *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ optic nerve (B) at E17.5. (C,D) NF staining of the wild-type (C) and *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ optic nerve (D) at E17.5. Scale bar: 50 μm. (E) The NF^{+} region of the optic nerve sections was quantified (*n*=3). The section of the double-mutant optic nerves is about 1.7-fold wider than that of the wild type.

Math3$^{-/-}$ retina, *NeuroD* expression was not changed (Fig. 6C,D). Thus, *Math3* and *NeuroD* do not seem to cross-regulate each other, unlike *Math3*/*NeuroD* and *Math5*.

Misexpression of *Math3* or *NeuroD* alone does not induce amacrine cell genesis

The above observation, that the normal number of amacrine cells were generated in the mouse retina lacking either *Math3* or *NeuroD* whereas no amacrine cells were generated in the double-mutant retina, indicates that these two bHLH genes are functionally redundant for amacrine cell genesis. In order to determine whether either *Math3* or *NeuroD* is sufficient for amacrine cell genesis, each gene was misexpressed with retrovirus in the retinal explant cultures. We used a replication-incompetent retrovirus, CLIG, which directs expression of green fluorescent protein (GFP) as a marker from the upstream LTR promoter (Fig. 7A) (Hojo et al., 2000). Math3 or NeuroD cDNA was inserted into the upstream of the internal ribosomal entry site (IRES) so that both the bHLH and GFP genes were expressed bicistronically (Fig. 7A). Virus was applied to retinal explants, which were prepared from mouse embryos at E17.5. After 2 weeks of culture, by which time most retinal cells finished differentiation, the fates of the virus-infected cells were determined by monitoring GFP-positive cells. When the control virus CLIG was applied, approximately 81±0.6% of the virus-infected cells became rods in the ONL while the other cells differentiated mostly into bipolar and Müller glial cells in the INL (Fig. 7B, part a, Fig. 7C), as previously described (Turner and Cepko, 1987). By contrast, when CLIG-Math3 or CLIG-NeuroD was applied, almost all virus-infected cells became rods, the most preferred cell fate during this culture

period (Fig. 7B, parts b,c, Fig. 7C). However, *Math3* or *NeuroD* alone did not promote amacrine cell genesis under this condition (Fig. 7B, part b,c, Fig. 7C), although *NeuroD* has previously been shown to increase it slightly when misexpressed in the postnatal rat retina (Morrow et al., 1999). These results indicate that *Math3* or *NeuroD* alone is not sufficient for amacrine cell genesis. Interestingly, misexpression of these bHLH genes almost completely inhibited gliogenesis (Fig. 7C), as previously described (Brown et al., 1998; Morrow et al., 1999; Bae et al., 2000; Cai et al., 2000; Hatakeyama et al., 2001).

Co-expression of *Math3* or *NeuroD* with *Pax6* promotes amacrine cell genesis

It has been shown that *Pax6* is expressed by amacrine and horizontal cells (Jones et al., 1998; Nishina et al., 1999). However, misexpression of *Pax6* alone was not sufficient for amacrine cell genesis (Fig. 8B, parts b,c, Fig. 9A), as previously described (Hatakeyama et al., 2001). Because the combination of homeobox and bHLH genes is important for retinal cell type specification (Hatakeyama et al., 2001), *Pax6* was co-expressed with *Math3* or *NeuroD* in the retinal explant cultures. For co-expression of the two factors, the bHLH factors were fused with GFP and each of them was co-expressed with Pax6 (Fig. 8A). More than 99% of the cells infected with these retroviruses successfully co-expressed both bHLH and homeobox genes (data not shown). Co-expression of *Math3* and *Pax6* significantly increased generation of both amacrine (calbindin$^+$, HPC1$^+$) and horizontal cells (calbindin$^+$) (Fig. 8C, parts b,c, arrowheads; Fig. 9A,C, blue bars). However, these genes did not promote generation of bipolar (PKC$^+$) or Müller glial cells (GS$^+$) (Fig. 8C, parts d,e; Fig. 9B,E), suggesting that a combination of *Math3* and *Pax6* prefers amacrine and horizontal cells to the other cell types. Co-expression of *NeuroD* and *Pax6* predominantly increased amacrine cell genesis (Fig. 8D, parts b,c, arrowheads; Fig. 9A, red bar) and no other cell types were promoted (Fig. 8D, parts

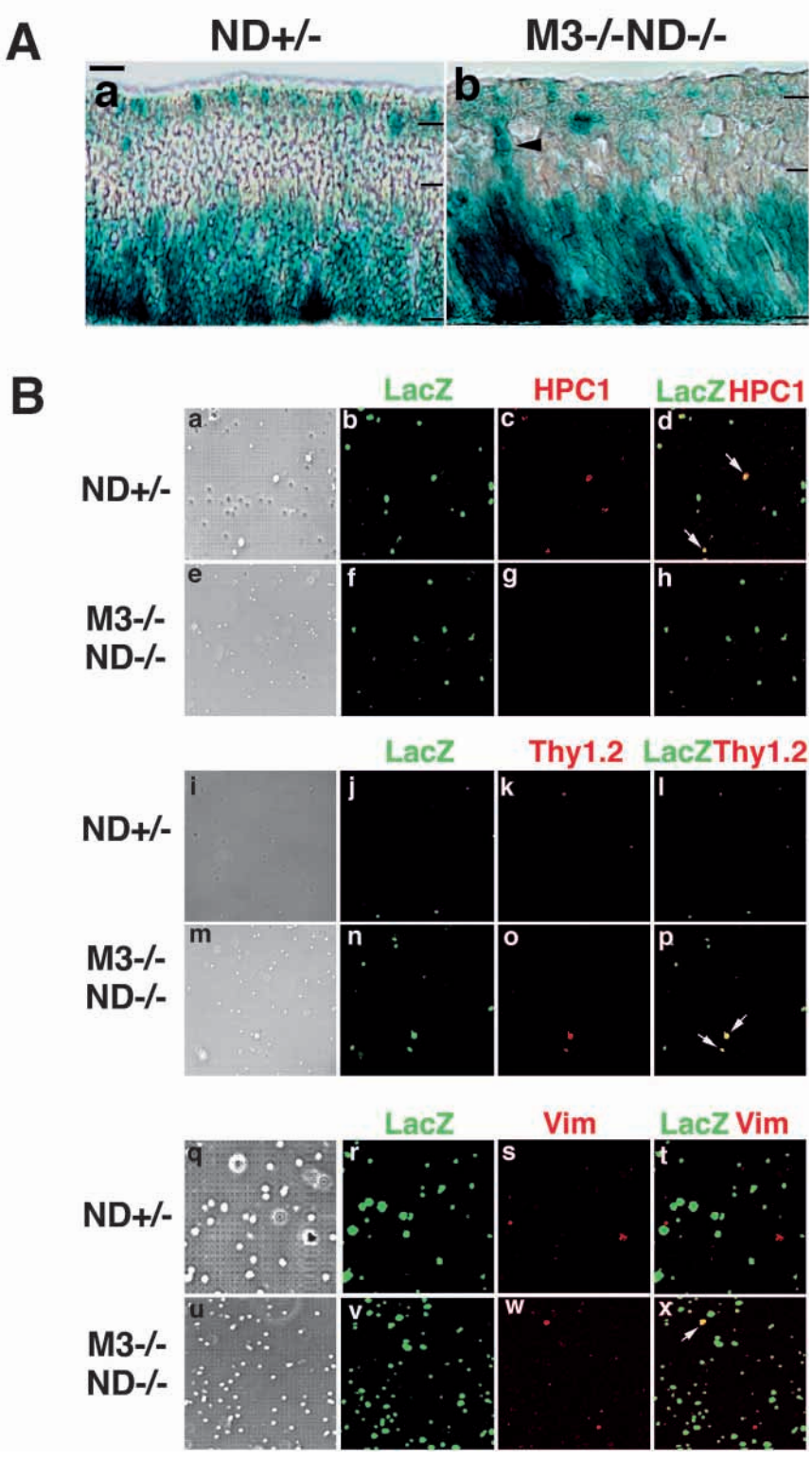


Fig. 5. The fate switch from amacrine cells to ganglion and Müller glial cells in *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina. (A) X-gal staining of the retinal explants that were prepared at E17.5 and cultured for 14 days. (a) In *NeuroD*$^{+/-}$ retina, X-gal staining is observed in *NeuroD*-expressing cells: rods in the ONL and amacrine cells in the GCL and the inner region of the INL. (b) In *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina, X-gal-positive cells are not missing but present in the GCL and INL as well as in the ONL. The majority of them are small in size, while others display a Müller glia-like morphology (arrowhead). Scale bar: 25 μm. (B) Retinal explants were prepared at E17.5, cultured for seven days, dissociated and subjected to immunocytochemistry (n=3). (a-d) In *NeuroD*$^{+/-}$ retina, some (2.7±0.3%) of the *lacZ*$^+$ cells express the amacrine cell marker HPC1 (arrows). (e-h) In *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina, no *lacZ*$^+$ cells express HPC1. (i-l) In *NeuroD*$^{+/-}$ retina, no *lacZ*$^+$ cells express the ganglion cell marker Thy1.2. (m-p) In *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina, some (4.3±0.6%) of the *lacZ*$^+$ cells express Thy1.2 (arrows). (q-t) In *NeuroD*$^{+/-}$ retina, no *lacZ*$^+$ cells express the Müller glial marker vimentin. (u-x) In *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina, some (0.9±0.2%) of the *lacZ*$^+$ cells express vimentin (arrow).

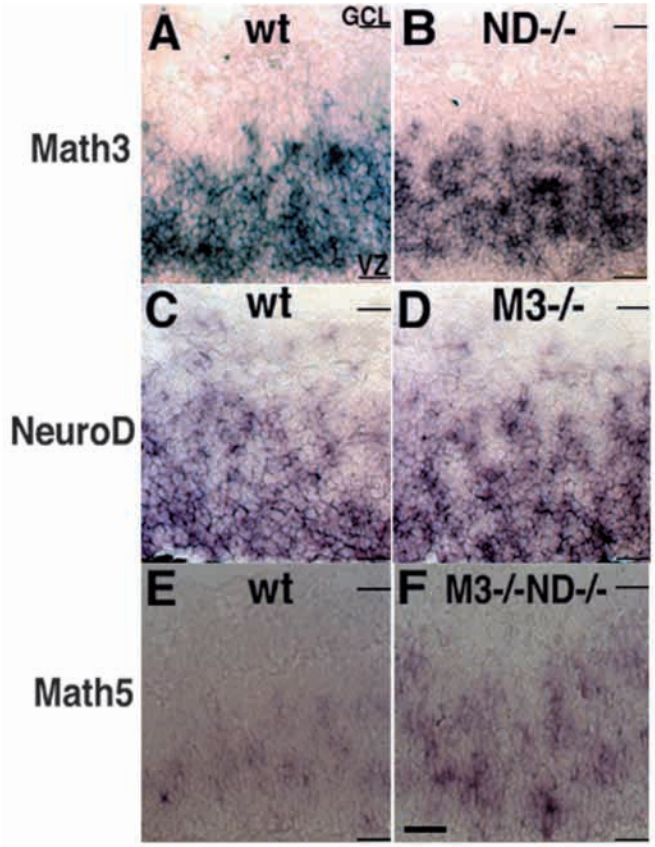


Fig. 6. bHLH gene expression in the mutant retina. In situ hybridization was performed with E17.5 retina. (A,B) *Math3* expression is not changed in *NeuroD*$^{-/-}$ retina. (C,D) *NeuroD* expression is not changed in *Math3*$^{-/-}$ retina. (E,F) *Math5* expression is upregulated in *Math3*$^{-/-}$/*NeuroD*$^{-/-}$ retina, suggesting that this upregulation leads to the increase of ganglion cells in the double-mutant retina. Scale bar: 15 μm.

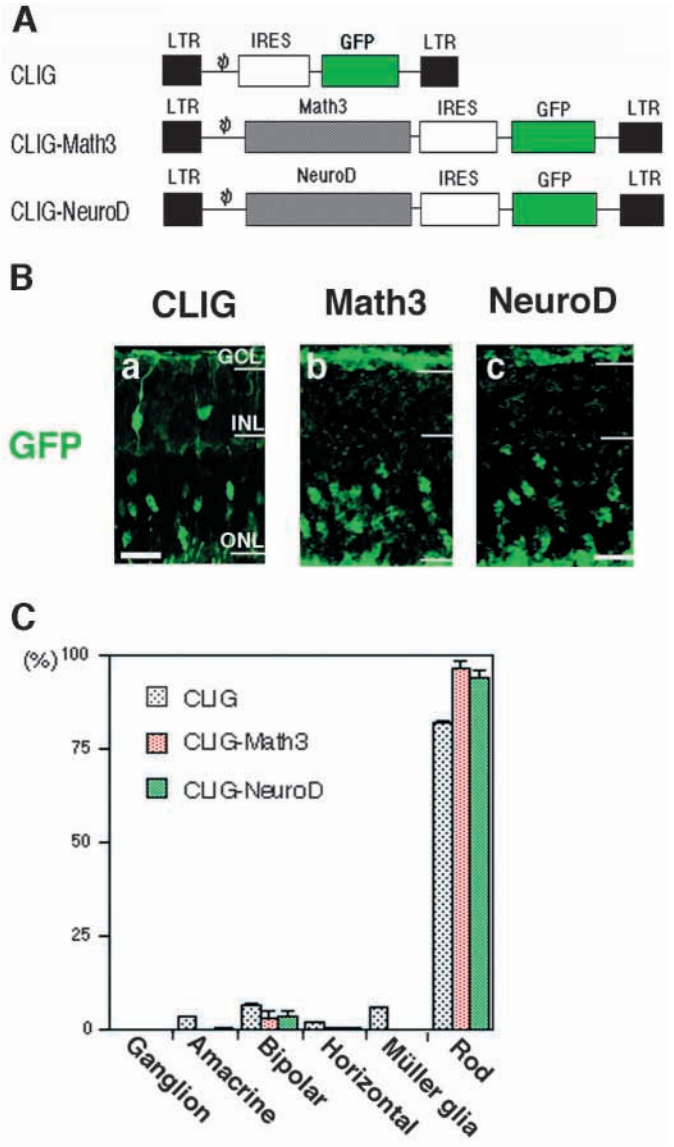


Fig. 7. Misexpression of *Math3* and *NeuroD* with retrovirus. (A) Retroviral vectors. GFP, green fluorescent protein; IRES, internal ribosomal entry site; LTR, long terminal repeat. (B) Retinal explants were prepared from E17.5 mouse embryos and infected with CLIG, CLIG-Math3, and CLIG-NeuroD. After 2 weeks, the explants were subjected to immunohistochemistry using anti-GFP antibody. (C) Ratios of retinal cell types infected with CLIG, CLIG-Math3 and CLIG-NeuroD. Rod genesis is increased while gliogenesis is inhibited by *Math3* and *NeuroD*.

d,e; Fig. 9B-E), suggesting that a combination of *NeuroD* and *Pax6* is more specific to amacrine cell genesis. These results demonstrated that, although *Math3* and *NeuroD* alone cannot specify the amacrine cell fate, they can do so with *Pax6*.

We next examined whether another neuronal bHLH gene *Mash1* is also able to induce amacrine cell genesis when *Pax6* is co-expressed. *Mash1* (*Ascl1* – Mouse Genome Informatics) is expressed by bipolar cells in the INL (Jasoni and Reh, 1996) and co-expression of *Mash1* and *Chx10* is able to induce bipolar cell genesis (Hatakeyama et al., 2001). However, co-expression of *Mash1* and *Pax6* did not promote generation of amacrine or horizontal neurons (Fig. 8E, parts b,c; Fig. 9A,C). These results suggest that the amacrine cell-inducing activity is rather unique to *Math3* and *NeuroD* and not compensated by *Mash1*. Interestingly, co-expression of *Mash1* and *Pax6* did not promote bipolar cell genesis either (Fig. 8E, part d; Fig. 9B), suggesting that proper combinations between bHLH and homeobox genes are important for retinal cell type specification.

The increase in amacrine cell number by *Pax6* and *Math3*/*NeuroD* could be the result of proliferation of amacrine cells and apoptosis of other cell types rather than conversion of precursors to the amacrine cell fate at the expense of other cell types. To distinguish between these possibilities, proliferation and death of virus-infected cells were analyzed at days 3, 7 and 14 of the cultures. Cell proliferation was examined by Ki67, a nuclear antigen expressed by proliferating cells. The majority of the cells infected with CLIG, CLIG-Pax6-Math3 or CLIG-Pax6-NeuroD were negative for Ki67 at all the time points (data not shown), indicating that *Pax6* and *Math3*/*NeuroD* did not promote cell proliferation. To determine the extent of cell death, the retinal explants were subjected to a TUNEL assay. The majority of the virus-infected cells were TUNEL negative at all the time points (data not shown). These results suggest that the amacrine cell genesis

induced by *Pax6* and *Math3*/*NeuroD* was not the result of proliferation of amacrine cells or apoptosis of other cell types, but most probably of conversion of precursors towards the amacrine cell fate at the expense of other cell types.

Co-expression of *Math3* or *NeuroD* with *Six3* also promotes amacrine cell genesis

It was recently reported that *Pax6* mutation lost the multipotentiality of retinal progenitor cells, resulting in production of only amacrine cells (Marquardt et al., 2001), suggesting that *Pax6* is not essential for amacrine cell genesis. In this mutant retina, the homeobox gene *Six3* as well as *NeuroD* are expressed (Marquardt et al., 2001), raising the possibility that *Six3* is also involved in amacrine cell genesis. To test this possibility, *Six3* was misexpressed with or without *Math3*/*NeuroD* (Fig. 10A). *Six3* alone was not sufficient for amacrine cell genesis (Fig. 10B, part a, Fig. 10C, part a). However, co-expression of *Six3* with *Math3* or *NeuroD* generated amacrine cells (Fig. 10B, parts b,c, arrowheads; Fig. 10C, part a). These results suggest that *Pax6* and *Six3* are redundant for amacrine cell fate specification. Interestingly, *Math3* induced horizontal cell genesis more efficiently than amacrine cell genesis, while *NeuroD* predominantly induced amacrine cell genesis (Fig. 10C), suggesting that *Math3* and *NeuroD* have distinct activities in retinal cell fate specification.

DISCUSSION

Math3 and *NeuroD* are essential for amacrine cell genesis

We have found that, in *Math3/NeuroD* double-mutant retina, amacrine cells are completely missing without significant cell death. The cells that would normally differentiate into amacrine cells, which can be monitored by X-gal staining, did not die but remained in the GCL and INL of the double-mutant retina. The majority of the *lacZ*$^+$ cells in the GCL and INL were small in size and showed a ganglion cell phenotype, while others displayed a Müller glia phenotype. In accordance with this observation, ganglion and Müller glial cells were increased in number in the double-mutant retina. Normally, nearly 60% of the GCL cells are displaced amacrine cells and the others are ganglion cells (Jeon et al., 1998). By contrast, in the double-mutant retina, all of the GCL cells were ganglion cells. Furthermore, there were many ectopic ganglion cells and extra Müller glial cells in the INL of the double-mutant retina. These results indicate that there is a fate switch from amacrine cells to ganglion and Müller glial cells in the absence of *Math3* and *NeuroD*. As amacrine cell genesis overlaps with ganglion and Müller glial cell genesis, it is likely that the double-mutant cells may adopt the alternatively available cell fates.

Neuronal versus glial fate determination and neuronal subtype specification by bHLH genes

The phenotype similar but opposite to the *Math3*/*NeuroD* double mutation was observed in the retina lacking the bHLH gene *Math5* in mouse and its ortholog in zebrafish, which shows a fate switch from ganglion cells to amacrine cells (Kay et al., 2001; Wang et al., 2001). Thus, *Math5* regulates ganglion versus amacrine cell fate, suggesting that this bHLH gene is involved

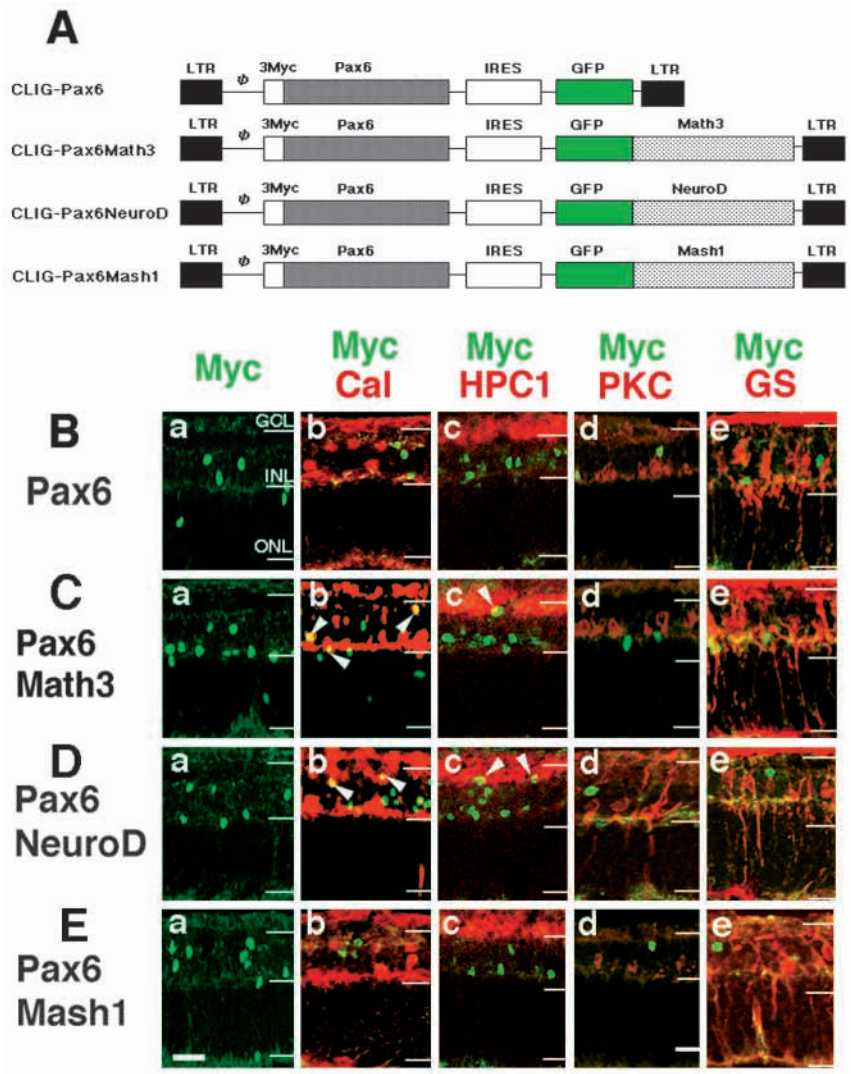


Fig. 8. Co-expression of *Pax6* and bHLH genes. (A) Retroviral vectors. The bHLH genes (*Math3*, *NeuroD* and *Mash1*) were fused with *GFP*, and Pax6 has three repeats of Myc tag. (B-E) Retinal explants were infected with CLIG-Pax6 (B), CLIG-Pax6-Math3 (C), CLIG-Pax6-NeuroD (D) and CLIG-Pax6-Mash1 (E). After 2 weeks of culture, the explants were sectioned and subjected to immunohistochemistry for Myc only (a) or Myc and either calbindin (b), HPC1 (c), PKC (d) or GS (e). (B) Misexpression of *Pax6* alone generates INL cells, but they are negative for the markers. (C) Co-expression of *Math3* and *Pax6* significantly increases the population of amacrine and horizontal cells (b,c, arrowheads) but not bipolar (d) or Müller glial cells (e). (D) Co-expression of *NeuroD* and *Pax6* significantly increases the population of amacrine cells (b,c, arrowheads) but not horizontal (b), bipolar (d) or Müller glial cells (e). (E) Co-expression of *Mash1* and *Pax6* does not induce mature INL cells. Scale bar: 25 μm.

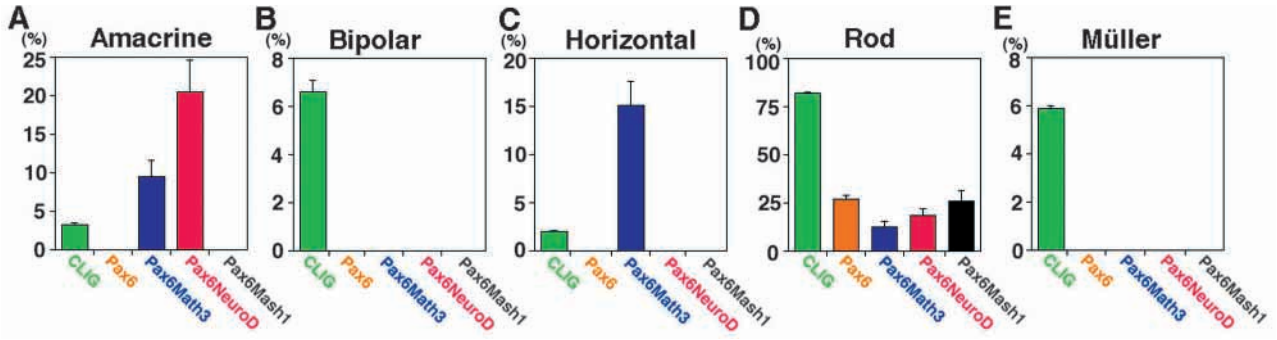


Fig. 9. Ratios of retinal cell types induced by misexpression of bHLH genes and *Pax6*. Ratios with s.e. of amacrine cells (A, calbindin$^+$), bipolar cells (B, PKC$^+$), horizontal cells (C, calbindin$^+$), rods (D, rhodopsin$^+$) and Müller glial cells (E, GS$^+$) are the average of at least three independent experiments. Co-expression of *Pax6* and *Math3* significantly increases the population of amacrine and horizontal cells while that of *Pax6* and *NeuroD* only increases the population of amacrine cells. By contrast, co-expression of *Pax6* and *Mash1* does not increase the number of any mature cells.

in the neuronal subtype specification rather than the neuronal versus glial cell fate decision. By contrast, the retina lacking *Mash1* and *Math3* exhibits a fate switch from bipolar cells to Müller glial cells, indicating that *Mash1* and *Math3* regulate neuronal versus glial fate determination in the retina (Tomita et al., 2000). Our present data show that *Math3-NeuroD* double mutation leads to increase of both ganglion cells and Müller glia at the expense of amacrine cells, suggesting that *Math3* and *NeuroD* regulate both neuronal subtype specification and neuronal versus glial fate determination. Although these bHLH genes seem to have distinct activities, we speculate that the two types of fate switches, neurons to glia and neuronal subtype changes, may simply reflect the different competence of retinal precursors. Because ganglion cell genesis overlaps with amacrine cell genesis but not with Müller glial cell genesis (Young, 1985), it is likely that the cells that would differentiate into ganglion cells have a potential to become amacrine cells but not others (Fig. 11A). Thus, in the absence of *Math5*, the cells that fail to differentiate into ganglion cells may predominantly become amacrine cells. Likewise, as bipolar and Müller glial cells are the last cell types to be generated, the cells that fail to differentiate into bipolar cells may have the only choice to become Müller glia in the absence of *Mash1* and *Math3* (Fig. 11A). By contrast, as amacrine cell genesis overlaps with both ganglion and Müller glial cell genesis (Young, 1985),

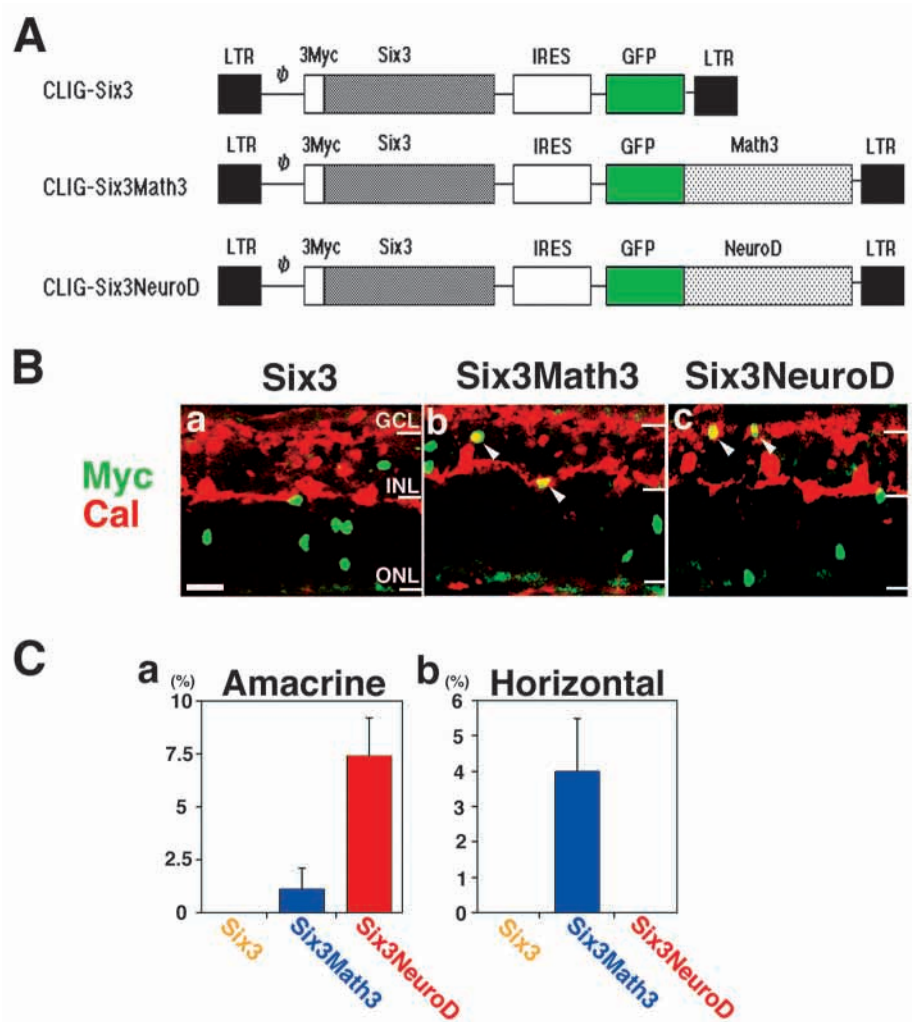
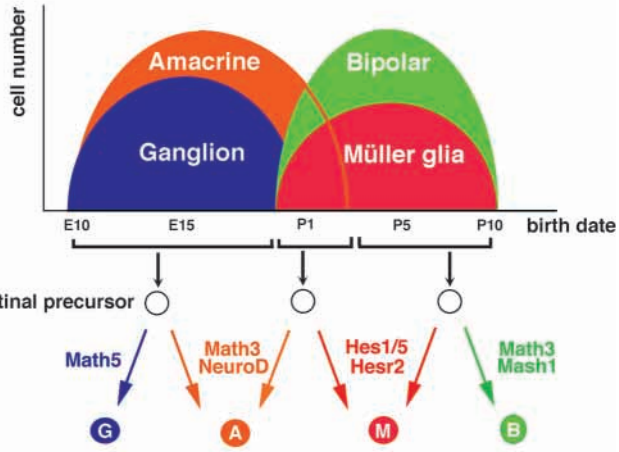


Fig. 10. Co-expression of *Six3* and bHLH genes. (A) Schematic structure of retroviral vectors. The bHLH genes (*Math3*, *NeuroD*) were fused with GFP, and Six3 has three repeats of Myc tag. (B) Retinal explants were infected with CLIG-Six3 (a), CLIG-Six3-Math3 (b) and CLIG-Six3-NeuroD (c). After 2 weeks of culture, the explants were sectioned and subjected to immunohistochemistry for Myc and calbindin. Misexpression of *Six3* alone generates some INL cells, but they are negative for calbindin expression (a). Co-expression of *Six3* and *Math3*/*NeuroD* increases the population of calbindin-positive cells (b,c, arrowheads). Scale bar: 25 μm. (C) Ratios of amacrine (a) and horizontal cells (b). Co-expression of *Six3* and *NeuroD* efficiently generates amacrine cells while co-expression of *Six3* and *Math3* efficiently generates horizontal cells.

A



B

Cell type	bHLH	Homeobox
Ganglion	Math5	
Amacrine	Math3 / NeuroD	Pax6 / Six3
Bipolar	Math3 / Mash1	Chx10
Horizontal	Math3	Pax6 / Six3
Rod / Cone	NeuroD	Crx
Müller glia	Hes1 / Hes5 / Hesr2	Rax / Chx10

Fig. 11. Transcription factors for retinal cell fate specification. (A) Model for fate switches. The time course of ganglion, amacrine, bipolar and Müller glial cell genesis (Young, 1985) and the bHLH genes that regulate generation of these cells are shown. Because ganglion cell genesis is overlapped with amacrine cell genesis, the cells that are blocked from differentiation into ganglion cells may adopt the amacrine cell fate. By contrast, as amacrine cell genesis is overlapped with ganglion and Müller glial cell genesis, the cells that are blocked from differentiation into amacrine cells may adopt the ganglion and Müller glial cell fates. Because bipolar and Müller glial cells are the last cell types to be generated, the cells that are blocked from differentiation into bipolar cells may adopt Müller glial cell fate. (B) The transcription factor code for retinal cell fate specification. Proper combinations of bHLH and homeobox genes are important for retinal cell fate specification.

the cells that fail to differentiate into amacrine cells may have a potential to become both ganglion and Müller glial cells and thereby adopt these two fates in the absence of *Math3* and *NeuroD* (Fig. 11A). Thus, the two types of fate switches, neurons to glia and neuronal subtype changes, might mostly reflect the competence of retinal precursors, and it is likely that the cells that are blocked from differentiation to a particular cell type may simply adopt alternatively available cell fates.

The exact mechanism for the fate switch in the absence of bHLH genes is not known. However, we found that *Math5*, which is essential for ganglion cell genesis, is upregulated in the retina that lack both *Math3* and *NeuroD*. Thus, it is likely that this upregulation leads to the increase of ganglion cells in the double-mutant retina. These results also suggest that *Math3* and *NeuroD* may normally restrict the ganglion cell number by repressing *Math5* expression. Similarly, it is possible that *Math3*/*NeuroD* expression is upregulated in the retina that lack *Math5*, where the cells that fail to differentiate into ganglion cells adopt the amacrine cell fate. If this is the case, cross-inhibitory regulation between *Math3*/*NeuroD* and *Math5* might determine the normal numbers of ganglion and amacrine cells. The similar type of regulation has been reported in other regions. *Mash1* and neurogenins (Ngns) display complementary expression patterns in the telencephalon and, in *Ngn1*/*Ngn2* double-mutant telencephalon, *Mash1* expression is ectopically upregulated in the regions where only Ngns are normally expressed, indicating that Ngns repress *Mash1* expression (Fode et al., 2000). Similarly, in the spinal cord, cross-inhibitory regulation between *Math1* and *Ngn1* controls the development of distinct types of interneurons (Gowan et al., 2001). Thus, it is likely that the antagonistic regulation between neuronal bHLH genes is a general mechanism for generating the correct number of distinct types of neurons.

Previous studies have demonstrated that *NeuroD* regulates only the neuronal differentiation/maturation step but not the determination step of most of the nervous system (Lee et al., 1995; Miyata et al., 1999; Liu et al., 2000a; Liu et al., 2000b; Schwab et al., 2000; Kim et al., 2001). However, our data indicate that *NeuroD* also regulates the neuronal versus glial fate choice in the retina like other bHLH-type neuronal determination genes such as *Mash1* and *Math3* and the genes for Ngns (Tomita et al., 2000; Nieto et al., 2001; Sun et al., 2001). This finding is consistent with the previous report by Morrow et al. (Morrow et al., 1999). Thus, *NeuroD* has both differentiation and determination activities, depending on the regions of the nervous system.

Neuronal subtype specification by bHLH genes requires proper homeobox genes

Although *Math3* and *NeuroD* are essential for amacrine cell genesis, misexpression of these bHLH genes alone only induced rod genesis. However, previous analysis has demonstrated that misexpression of *NeuroD* alone is able to induce an approx. twofold increase in amacrine cell number (Morrow et al., 1999). This discrepancy could be due to different experimental conditions: in vivo retina of postnatal rats (Morrow et al., 1999) and in vitro retinal explants prepared from mouse embryos (this study). As retinal precursors are known to display different competence as development proceeds, further analysis with retinas of various developmental stages would be necessary to determine to what extent bHLH genes alone can specify the retinal neuronal subtypes.

Even though *Math3* and *NeuroD* alone can induce amacrine cell genesis under some conditions, their effect is still rather small (Morrow et al., 1999). However, they can more efficiently specify the correct neuronal subtypes when the homeobox gene *Pax6* or *Six3* is co-expressed. Interestingly, *Math3* exhibits preference of the horizontal cell fate over the amacrine cell fate, whereas *NeuroD* exhibits the opposite. *Math3* expression is observed only transiently in amacrine cells, but permanently in horizontal cells, while *NeuroD* is expressed by amacrine cells but not by horizontal cells. Thus, the preference of cell types by these bHLH genes well reflects their expression patterns, and these data clearly demonstrate that bHLH genes are involved in neuronal subtype specification in addition to simply making the neuronal versus glial fate choice. The involvement of bHLH genes in neuronal subtype specification has been shown in both invertebrates and vertebrates. In *Drosophila*, the bHLH genes *scute* and *atonal* promote external sensory and chordotonal organs, respectively, and *atonal* mutation is not rescued by *scute* (Chien et al., 1996). Domain swapping experiments indicate

that the basic region is responsible for the specificity of the neuronal subtypes (Chien et al., 1996). Similarly, in mice that have *Mash1* gene at the *Ngn2* locus, *Mash1* partially rescues *Ngn2* mutation but confers a different identity on rescued neurons (Fode et al., 2000).

Although combinations of *Math3*/*NeuroD* and *Pax6*/*Six3* efficiently induced amacrine cell genesis, still many cells failed to express mature neuronal markers. At E17.5, when retrovirus was applied, rods are the most preferred cell type, and it is possible that retrovirus-mediated misexpression of bHLH and homeobox genes is not sufficient to override the rod fate specification. Such cells could halt during terminal differentiation and fail to express mature neuronal markers. It is also possible that additional factors may be necessary to convert the cell fates more efficiently.

Interestingly, *Mash1* does not induce any cell types when *Pax6* is co-expressed. As *Mash1* can induce bipolar cell genesis when *Chx10* is co-expressed (Hatakeyama et al., 2001), it is likely that proper combinations with homeobox genes are essential for bHLH genes to specify the correct neuronal subtypes. The current model for the transcription factor codes for the retinal cell type specification is as follows: rods and cones, *NeuroD* and *Crx* (Furukawa et al., 1997; Freund et al., 1997; Chen et al., 1997; Morrow et al., 1999); horizontal cells, *Math3* and *Pax6*/*Six3* (this study); bipolar cells, *Math3*/*Mash1* and *Chx10* (Burmeister et al., 1996; Tomita et al., 2000; Hatakeyama et al., 2001); amacrine cells, *Math3*/*NeuroD* and *Pax6*/*Six3* (Morrow et al., 1999) (this study); ganglion cells, *Math5* (Brown et al., 1998; Wang et al., 2001); and Müller glia, *Hes1*/*Hes5*/*Hesr2* and *Rax*/*Chx10* (Fig. 11B) (Furukawa et al., 2000; Hojo et al., 2000; Hatakeyama et al., 2001; Satow et al., 2001). However, this list is still incomplete. For example, in *Math3*$^{-/-}$ retina, horizontal cells develop normally, and it remains to be determined which bHLH gene compensates for *Math3* in horizontal cell development. One of the candidate genes is *Ngn2*, which is expressed in the developing retina (Gradwohl et al., 1996; Sommer et al., 1996). Homeobox genes are also functionally redundant. For example, *Crx* and *Pax6* are not essential for neuronal subtype specification. In *Crx*-deficient retina, rods are born but only their outer segments are not formed, suggesting that *Crx* is essential for maturation of rods (Furukawa et al., 1999). It is possible that a related homeobox gene *Otx2* may compensate *Crx* for the earlier stages of rod genesis. Similarly, in *Pax6*-deficient retina, amacrine cells are generated and it is likely that *Six3* compensates *Pax6* for amacrine cell genesis (Marquardt et al., 2001) (this study). Thus, double-mutation analysis of homeobox genes is necessary to clarify their functions in neuronal subtype specification.

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