Math3 and NeuroD regulate amacrine cell fate specification in the retina

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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-/- 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 EcoRI 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 BsrGI and Clal 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 92mp34, an ecotropic packaging cell line (Yoshimatsu et al., 1998). The supernatant was collected 2 days later and concentrated with Centricron 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: 832 m thickness). For immunocytochemistry, explants were dissociated, as previously described (Ishibashi et al., 1994; 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.
Math3 and NeuroD in amacrine cell fate specification (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...
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 \textit{lacZ} was knocked into the \textit{NeuroD} locus (Miyata et al., 1999), the cells that would normally differentiate into amacrine cells (\textit{NeuroD}+) could be monitored by X-gal staining in the double-mutant retina. In \textit{NeuroD}+/– retina, rods and amacrine cells were labeled (Fig. 5A, part a), as described previously (Morrow et al., 1999). By contrast, in \textit{Math3}–/–/\textit{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, arrowhead).

To further characterize the nature of the \textit{lacZ}+ cells, retinal explants that were cultured for seven days from E17.5 were dissociated and subjected to immunocytochemistry. Many of the \textit{lacZ}+ cells from \textit{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 \textit{Math5} is essential for ganglion cell genesis (Brown et al., 2001; Wang et al., 2001). As the ganglion cell number was significantly increased in \textit{Math3}–/–/\textit{NeuroD}–/– retina, \textit{Math5} expression was next compared between the wild-type and double-mutant retina. At E17.5, \textit{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, \textit{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, \textit{Math5} expression is downregulated by \textit{Math3} and \textit{NeuroD}.

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

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.
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 T).

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**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).
Math3 and NeuroD in amacrine cell fate specification

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-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.

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.
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...
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),
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 Math1 gene at the Ngn2 locus, Math1 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, Math1 does not induce any cell types when Pax6 is co-expressed. As Math1 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 Rex1/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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