Requirement of Multiple Basic Helix-Loop-Helix Genes for Retinal Neuronal Subtype Specification*

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Retinal precursor cells give rise to six types of neurons and one type of glial cell during development, and this process is controlled by multiple basic helix-loop-helix (bHLH) genes. However, the precise mechanism for specification of retinal neuronal subtypes, particularly horizontal neurons and photoreceptors, remains to be determined. Here, we examined retinas with three different combinations of triple bHLH gene mutations. In retinas lacking the bHLH genes Ngn2, Math3, and NeuroD, horizontal neurons as well as other neurons such as bipolar cells were severely decreased in number. In the retina lacking the bHLH genes Mash1, Ngn2, and Math3, horizontal and other neurons were severely decreased, whereas ganglion cells were increased. In the retina lacking the bHLH genes Mash1, Math3, and NeuroD, photoreceptors were severely decreased, whereas ganglion cells were increased. In all cases, glial cells were increased. The increase and decrease of these cells were the result of cell fate changes and cell death and seem to be partly attributable to the remaining bHLH gene expression, which also changes because of triple bHLH gene mutations. These results indicate that multiple bHLH genes cross-regulate each other, cooperatively specify neuronal subtypes, and regulate neuronal survival in the developing retina.

In the neural retina, there are six types of neurons and one type of glial cell forming three cellular layers as follows: (i) rod and cone photoreceptors in the outer nuclear layer (ONL); (ii) horizontal, bipolar, and amacrine interneurons and Müller glia in the inner nuclear layer (INL); and (iii) ganglion and displaced amacrine cells in the ganglion cell layer (GCL). During retinal development, these seven types of cells are generated from common precursors in sequences relatively conserved among species (1, 2); ganglion cells are generated first, followed in overlapping phases by horizontal cells, cones, amacrine cells, rods, bipolar cells and, finally, Müller glial cells. The retina is a good model system for investigating the mechanism of neural development because it has a relatively simple structure and mimics normal development in isolated explant cultures.

It has been shown that retinal cell fate determination and differentiation are controlled by intrinsic cues, such as transcription factors, and extrinsic signals, such as neurotrophic factors (3–6). The basic helix-loop-helix (bHLH) genes are good candidates for intrinsic regulators of retinal cell fate decision (7–9). In the developing mammalian eye, neurogenic bHLH genes such as Mash1, Math3, Math5, Ngn2, and NeuroD are expressed by retinal precursor cells (10–16). Loss of function studies indicate that these bHLH genes play an essential role in specification of many retinal cell types. For example, in the absence of Math5, ganglion cells are severely decreased, whereas amacrine cells are increased, indicating that Math5 regulates ganglion versus amacrine cell fate decision (17–19). In contrast, in the absence of Math3 and NeuroD amacrine cells are severely decreased, whereas ganglion cells are increased (20). Interestingly, Math3 is up-regulated in retinas lacking both Math3 and NeuroD (20), suggesting that antagonistic regulation between Math3 and Math3/NeuroD regulates ganglion/amacrine cell fate choice. For specification of bipolar cells, either Mash1 or Math3 is required (21, 22). In the absence of Mash1 and Math3 bipolar cells are missing, and those that should become bipolar cells adopt the Müller glial cell fate, indicating that Mash1 and Math3 regulate bipolar versus Müller glial cell fate decision (21, 22). Interestingly, Hes1 and Hes5, which can functionally antagonize neurogenic bHLH genes such as Mash1 and Math3 (23–25), induce Müller glial cell fate choice (26, 27). Thus, bHLH genes play an essential role in neuron/glia cell fate decision as well as in neuronal subtype specification. It is likely that, when compensatory neurogenic bHLH gene expression does not occur, the mutant cells adopt the glial fate while becoming different subtypes of neurons when compensatory neurogenic bHLH gene expression occurs.

Despite these extensive studies, the precise mechanism for retinal cell type specification remains to be determined. In particular, the bHLH genes that regulate horizontal and photoreceptor cell genesis remains to be determined. It is likely that, in single or double mutations of bHLH genes, other neurogenic bHLH genes may compensate for horizontal and photoreceptor cell development. In the present study, we analyzed the retina with three different combinations of triple compound mutations for neurogenic bHLH genes. We found that rod genesis is strongly reduced in the Mash1;Math3;NeuroD triple-mutant retina, whereas horizontal cell genesis is significantly...
impaired in Mash1, Ngn2, Math3 and Ngn2, Math3, NeuroD triple-mutant retina. These results indicate that retinal cell fate specification depends on the compensatory expression of multiple bHLH genes.

EXPERIMENTAL PROCEDURES

Generation of Double and Triple Mutant Mice—Mash3 (21), Mash1 (28), NeuroD (29), and Ngn2 (30) mutant mice were generated previously. Mash1, NeuroD, Ngn2, NeuroD and Ngn2, Math3 double-mutant mice were obtained by crossing each double heterozygous mouse. Mash1, Math3, NeuroD, Ngn2, Math3, and Ngn2, Math3, NeuroD triple-mutant mice were obtained by crossing each triple heterozygous mouse or triple-heterozygous female and (Mash1+/–: NeuroD+/–, Mash1+/–: Ngn2+/–, or Ngn2+/–: NeuroD+/–): Math3+/– male mouse. Their genetic background was mostly ICR. The mutant mice were obtained from embryonic day 17.5 (E17.5) because most of the double or triple mutant mice died around birth. Retinal explants were prepared from E17.5 embryos and cultured for 4, 8, or 14 days to examine the postnatal development of the mutant retinas.

Retinal Explant Culture—The retinal explant culture was performed as described previously (31, 32). Briefly, eyes were isolated from E17.5 mouse embryos and transferred to a phosphate-buffered solution. The neural retina without pigment epithelium was placed on a Millicell chamber filter (Millipore; diameter 30 mm, pore size 0.4 mm) 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% minimum Eagle’s medium with Heps, 25% Hanks’ solution, 25% heat-inactivated horse serum, 200 mm L-glutamine, and 5.75 mg/ml glucose). Explants were cultured at 34 °C in 5% CO2, and the medium was changed every other day.

Immunohistochemistry—The retinal explants were first fixed with 4% paraformaldehyde in a phosphate-buffered solution for 10 min and incubated in 25% sucrose in a phosphate-buffered solution for 30 min on ice, embedded in optimal cutting temperature compound, and sectioned (16-mm thickness). For immunohistochemistry, the samples were preincubated with a blocking solution (5% normal goat serum with or without 0.1% Triton X-100 in a phosphate-buffered solution) for 1 h and then incubated overnight or for 2 days at 4 °C in 1% goat serum with or without 0.1% Triton X-100 with the following antibodies: mouse anti-RETPI/rodopsin (Sigma); mouse anti-protein kinase C (Amersham Biosciences); mouse anti-HPCL/syntaxin (Sigma); rabbit anti-recoverin (kind gift of J. F. McGinnis and R. J. Elias) (33); rabbit anti-calbindin (Chemicon); mouse anti-Inlet1 (Developmental Studies Hybridoma Bank); mouse anti-glutamine synthetase (GS, Chemicon); rabbit anti-p75 (Promega); mouse anti-cyclooxygenase 2 (Santa Cruz) and mouse anti-Ki67 (Pharmentigen). Cell nuclei were counterstained with propidium iodide. To detect cell death, terminal deoxynucleotide transferase-mediated digoxigenin-labeled RNA probes for Mash1, Ngn2, Math3, NeuroD (J–L). The wild type and mutant retinas were prepared from E17.5 embryos. A higher magnification is shown in the insets. A–C, Mash1 expression is up-regulated in the Ngn2+/–: (B) and Math3+/– (C) retinas. D–F, Ngn2 expression is up-regulated in the Mash1+/– (E) and Math3+/– (F) retinas. G–I, Math3 expression is weakly up-regulated in the Mash1+/– (B) and Ngn2+/– (D) retinas. J–L, NeuroD expression is slightly up-regulated in the Ngn2+/– retina (K) but not significantly changed in the Math3+/– retina (L). Scale bar, 100 μm. RPE, retinal pigment epithelium; VZ, ventricular zone; VT, wild type.

RESULTS

Retinal bHLH Gene Expression Is Affected by the Lack of Other bHLH Genes—It has been shown that retinal neuronal subtype specification is controlled by multiple bHLH genes such as Mash1, Ngn2, Math3, NeuroD, and Math5 (7–9, 16–22, 35). Among them, Math5 induces ganglion cell fate specification, whereas the other bHLH genes regulate other cell types. To determine which genes regulate horizontal and photoreceptor cells, we focused on the neurogenic bHLH genes: Mash1, Ngn2, Math3, NeuroD, and Math5. We first examined the regulatory interaction of bHLH genes in retinal development. In the absence of either Ngn2 or Math3, Mash1 expression is up-regulated in the retina (Fig. 1, A–C). Similarly, in the absence of either Mash1 or Math3, Ngn2 expression is up-regulated (Fig. 1, D–F). Math3 expression is also weakly up-regulated in Mash1+/– or Ngn2+/– retina (Fig. 1, G–I). These results indicate that Mash1, Ngn2, and Math3 antagonistically cross-regulate expression in the retina. It is likely that, in the absence of one or two bHLH genes, the remaining genes may be up-regulated and compensate for the deficiency. In the absence of NeuroD, however, expression of other bHLH genes is not changed (data not shown). NeuroD expression is slightly up-regulated in the absence of Ngn2 but not in the absence of Math3 (Fig. 1, J–L).

To identify the bHLH genes that regulate horizontal and photoreceptor cell development, we examined the retina with double mutations of Mash1+/–: Ngn2+/–, Mash1+/–: Math3+/–, Mash1+/–: NeuroD+/–, Ngn2+/–: Math3+/–, Ngn2+/–: NeuroD+/–, and Math3+/–: NeuroD+/–. These mutations have a normal number of horizontal cells (20–22, data not shown). Although some of them have a reduced number of photoreceptors, none of them has fewer photoreceptors than does the NeuroD+/– retina (16, data not shown). Thus, it is likely that, in the double mutations, the other genes may compensate horizontal and photoreceptor cell development. We therefore examined three sets of triple mutations, Ngn2+/–: NeuroD+/–; Math3+/–: NeuroD+/–; and Mash1+/–: NeuroD+/–, to identify the bHLH genes that regulate horizontal and photoreceptor cell development. Because these triple mutant mice die around birth, we prepared retinal explants from E17.5 embryos and performed a two-week culture to examine the postnatal development of the mutant retina.

Defects of Ngn2+/–: Math3+/–: NeuroD+/– Retina—We first examined the defects of the Ngn2+/–: Math3+/–: NeuroD+/–.
roD−/− retina. This triple mutant retina consisted of two cellular layers (Fig. 2, G–L), unlike the three cellular layers of the wild type retina (Fig. 2, A–F). The majority of the outer layer cells were found to be rod photoreceptors (rhodopsin “recoverin”) (Fig. 2, G and H). The inner boundary of the photoreceptor layer was irregular, indicating that the arrangement of photoreceptors was disorganized in this mutant retina (Fig. 2, G and H, arrowheads). This layer also contained a small number of amacrine cells (calbindin) (Fig. 2F). However, there were virtually no horizontal (calbindin) and bipolar cells (protein kinase C “Islet-1”) (Fig. 2, I–K). Horizontal and bipolar cells form a synapse with photoreceptors in the outer plexiform layer that separates the ONL and the INL. Thus, it is likely that the lack of horizontal and bipolar cells leads to the fusion of the INL and ONL in this triple mutant retina. The GCL contains ganglion cells (p75 “Islet-1”), but their number was also significantly reduced (Fig. 2, K and L). In contrast, Müller glial cells (GS “cyclinD3”) were increased in number (Fig. 3, C and D) as compared with the wild type (Fig. 3, A and B). Interestingly, the cell bodies of Müller glia, which are normally located in the INL (Fig. 3, A and B), were scattered throughout the outer layer (Fig. 3, C and D), indicating that the migration of Müller glial cell bodies is impaired.

Quantification of the cell numbers showed that only Müller glial cells were increased, whereas the other cell types, including photoreceptors, were decreased in number in the Ngn2−/−;Math3−/−;NeuroD−/− retina (Fig. 4). In particular, bipolar and horizontal cells were virtually missing, indicating that Ngn2, Math3, and NeuroD are essential for bipolar and horizontal cell genesis. Because these cells are generated in the double mutations of Ngn2−/−;Math3−/−; Ngn2−/−; NeuroD−/− and Math3−/−; NeuroD−/− (20, data not shown), these bHLH genes compensate each other for bipolar and horizontal cell development.

Defects of Mash1−/−;Ngn2−/−;Math3−/− retina—We next examined the defects of the Mash1−/−;Ngn2−/−; Math3−/− retina. Like the wild type retina, this triple mutant retina consisted of three cellular layers (Fig. 2, M–R). The ONL contained a normal number of photoreceptors (rhodopsin “recoverin”; Fig. 2, M and N, and Fig. 4). However, the INL contained fewer amacrine and horizontal cells (calbindin, Figs. 2O and 4) and even fewer bipolar cells (protein kinase C “Islet1”, Figs. 2, N, P, Q, and F, and Fig. 4). Instead, there were more Müller glial cells in the mutant INL (GS “cyclinD3”; Fig. 3, E and F, and Fig. 4). In addition, ganglion cells were significantly increased in the GCL (p75 “Islet-1”, Fig. 2, Q and R, and Fig. 4). These results indicate that Mash1, Ngn2, and Math3 are essential for generation of the correct numbers of the INL neurons and for suppression of the overproduction of ganglion cells.

Defects of Mash1−/−;Math3−/−;NeuroD−/− retina—We next examined the defects of the Mash1−/−;Math3−/−; NeuroD−/− retina. This mutant retina also consisted of three cellular layers (Fig. 2, S–X). However, the number of photoreceptors in the ONL was significantly reduced as compared with the wild type (rhodopsin “recoverin”, Fig. 2, S and T, and Fig. 4). The number of photoreceptors in this triple mutant retina was about one-third of the wild type (Fig. 4), indicating that Mash1, Math3, and NeuroD play an important role in the generation of photoreceptors. In addition, there were very few bipolar cells in the INL (recoverin “protein kinase C “Islet1”;
Fig. 2, T, V, and W, and Fig. 4), as observed in the Mash1−/−; Math3−/− double-mutant retina (21, 22). Surprisingly, there were almost normal numbers of amacrine and horizontal cells in the INL (calbindin−, Figs. 2U and 4), raising the possibility that the Mash1 mutation, in addition to the Math3 and NeuroD double mutations, may revive amacrine cells, because these cells are mostly missing in Math3 and NeuroD double mutations (20). Interestingly, there were more ganglion cells in the GCL (p75+S+Ilet−; Figs. 2, W and X, and Fig. 4) and more Müller glial cells in the INL (GS+cyclinD3−; Fig. 3, G and H, and Fig. 4).

Taken together, in the retina with triple mutations of neurogenic bHLH genes, many subtypes of neurons were missing or decreased whereas Müller glial cells were increased, a finding that is consistent with the idea that neurogenic bHLH genes promote neuronal versus glial formation. However, in some cases ganglion cells were increased, suggesting that the remaining neurogenic bHLH gene expression is important for the compensated neuronal subtypes.

Increased Cell Death and Fate Switch in the Triple Mutant Retina—The decrease or the absence of subsets of neurons and the concomitant increase of Müller glia could be involved in either neuronal death and glial proliferation or the cell fate switch from neurons to glia. To differentiate between these possibilities, we next examined cell proliferation and death of the retina at several time points, namely days 4, 8, and 14 of the explant cultures. Cell proliferation was analyzed by the immunostaining of Ki67, an antigen expressed in all phases of cell cycles (36). During the time course examined, cell proliferation was not altered in the mutant retina (data not shown), suggesting that cell proliferation is not involved in increase of glial cells.

Cell death was next analyzed by TUNEL. In the wild type retina, subsets of cells underwent apoptosis at day 4 (Fig. 5A), but their number was decreased at later time points (Fig. 5, B and C). In Ngn2−/−;Math3−/−;NeuroD−/− retina, cell death was not significantly increased at day 4 of culture (Fig. 5D). However, at later stages, TUNEL+ cells were increased in the photoreceptor layer (Fig. 5, E and F). Because photoreceptors were significantly decreased in this triple mutant retina, it is likely that this decrease is due to cell death. Cell death was not observed in the region that contained ganglion and amacrine cells (Fig. 5, E and F). In addition, cell death was not increased at day 4 (Fig. 5D), by which time the fate of most cell types is determined. Thus, the decrease or absence of ganglion and the INL-specific neurons is not due to cell death but is likely the result of the fate switch from ganglion to INL neurons to Müller glial cells in the absence of the neurogenic bHLH genes Ngn2, Math3, and NeuroD. In contrast, cell death was not increased in the Mash1−/−;Ngn2−/−;Math3−/−; NeuroD−/− retina (Fig. 5G), suggesting that the decrease or lack of amacrine, bipolar, and horizontal cells and the increase of ganglion and Müller glial cells are due to fate switches of retinal precursors.

In the Mash1−/−;Math3−/−;NeuroD−/− retina, cell death was increased slightly in the ONL (Fig. 5H) as compared with the wild type (Fig. 5C). However, the cell death was almost the same level as that of the NeuroD−/− retina (Fig. 5I) where photoreceptors underwent apoptosis (16). Because in this triple mutant retina there were fewer photoreceptors than in the NeuroD−/− retina, the reduction of photoreceptors in the triple mutations cannot be explained solely by cell death. It is likely that the fate switch from photoreceptors to ganglion and Müller glial cells may occur in the absence of Mash1, Math3, and NeuroD.
Expression of the Remaining bHLH Genes in the Triple Mutant Retina—The expression of the remaining bHLH genes in the triple mutant retina was next examined by in situ hybridization. Mash1 expression was up-regulated in the Math3+/− retina (Fig. 6B), the double mutations Math3−/−NeuroD−/− and Ngn2−/−NeoD−/−, and the triple mutation Ngn2−/−Math3−/−NeuroD−/− (Fig. 6C–E). Ngn2 expression was also up-regulated in the Math3−/−, Mash1−/−Math3−/−, Math3−/−NeuroD−/−, and the Mash1−/−Math3−/−NeuroD−/− retinas (Fig. 6, G–J). In contrast, NeuroD expression was not significantly changed in the Math3−/−, Ngn2−/−, Math3−/−, Mash1−/−Math3−/−, and Mash1−/−Ngn2−/− retinas (Fig. 6, L–O).

Math5 expression was not changed in the Math3−/− retina (Fig. 6Q), which has a normal number of ganglion cells (21). In contrast, Math5 expression was greatly up-regulated in the Mash1−/−Math3−/−NeuroD−/− and Mash1−/−Ngn2−/− retinas (Fig. 6, R and S), a finding that is consistent with the observation that ganglion cells are increased in these triple mutants (Fig. 4). Strikingly, Math5 expression was significantly down-regulated in the Ngn2−/−Math3−/−NeuroD−/− retina at both E14 and E17.5 (Fig. 6, T and V; compare with Fig. 6, P and U, respectively), which is consistent with the observation that ganglion cells are decreased in this triple mutant retina (Fig. 4). These results indicate that Math5 expression is regulated differently by the other bHLH genes.

DISCUSSION

Specification of Retinal Neuronal Subtypes by Multiple Neurogenic bHLH Genes—Previous studies demonstrated that the generation of retinal neurons is controlled by multiple neurogenic bHLH genes (7–9). However, the bHLH genes that regulate the formation of horizontal and photoreceptor cells remain to be determined. Here, we found that in the triple mutations Ngn2−/−Math3−/−NeuroD−/− and Mash1−/−Ngn2−/−Math3−/− the generation of horizontal cells as well as other neurons is severely impaired (summarized in Fig. 7). Furthermore, photoreceptor cell genesis is also reduced in the triple mutant Mash1−/−Math3−/−NeuroD−/− (Fig. 7). However, in any of the single and double mutations that we tested, horizontal and photoreceptor cell genesis is not affected so significantly. These results indicate that more than two bHLH genes cooperatively regulate horizontal and photoreceptor cell development.

Horizontal cell genesis is affected only in Ngn2−/−Math3−/−NeuroD−/− and Mash1−/−Ngn2−/−Math3−/− retinas but not in the Mash1−/−Math3−/−NeuroD−/− retina, suggesting that Ngn2 may be most important for horizontal cell genesis (Fig. 7). However, horizontal cell development normally occurs in the double mutations Mash1−/−Ngn2−/−,
Ngn2\(\sim\)Math3\(\sim\), and Ngn2\(\sim\)NeuroD\(\sim\), indicating that Ngn2 is not essential for horizontal cell genesis. Thus, all four bHLH genes, Mash1, Ngn2, Math3, and NeuroD, are involved in horizontal cell development.

It was shown previously that retinal neuronal subtypes are specified by combinations of bHLH and homeodomain genes (20, 22). For example, bHLH genes alone are not sufficient for bipolar cell development, but combinations of the bHLH genes Mash1/Math3 and the homeodomain gene Chx10 are required for bipolar cell formation (22). Chx10 determines the layer identity (the INL), whereas Mash1/Math3 specifies the INL-specific neuronal subtype. Similarly, the homeodomain gene Prox1 was shown to be essential for horizontal cell development (37). Thus, it is likely that combinations of Mash1/Ngn2/Math3/NeuroD and Prox1 are required for horizontal cell genesis.

Our results also demonstrated that the three bHLH genes Mash1, Math3, and NeuroD are important for photoreceptor cell formation. In the absence of these bHLH genes, many photoreceptors underwent apoptosis. However, the number of dying cells in the Mash1\(\sim\)/Math3\(\sim\)/NeuroD\(\sim\) retina is almost the same as that of the NeuroD\(\sim\) retina, suggesting that NeuroD is most important for the survival of photoreceptors. Importantly, there were even fewer photoreceptors in the Mash1\(\sim\)/Math3\(\sim\)/NeuroD\(\sim\) retina, indicating that cell death alone cannot explain the reduction of photoreceptors. It is likely that the cells that should normally differentiate into photoreceptors adopted other cell types in the triple mutations. These results indicate that Mash1, Math3, and NeuroD cooperatively regulate photoreceptor specification. It was shown that the homeodomain genes Crx and Otx2 are essential for photoreceptor development (38–43). These results suggest that combinations of Mash1/Math3/NeuroD and Crx/Otx2 are required for photoreceptor specification.

Cross-regulation of Neurogenic bHLH Genes—We showed that the three bHLH genes Mash1, Ngn2, and Math3 mutually regulate their expression in the retina. In the absence of one of the bHLH genes, expression of the other two is up-regulated, indicating that these bHLH genes antagonistically regulate their expression. In contrast, the expression of these bHLH genes is not significantly affected by NeuroD deficiency, and NeuroD expression is not significantly affected by mutations of other bHLH genes, suggesting that NeuroD expression could be regulated independently of most other bHLH genes in the retina.

Math5 expression is also controlled by other bHLH genes. Interestingly, Math5 expression is up-regulated in Mash1\(\sim\)/Math3\(\sim\)/NeuroD\(\sim\) and Mash1\(\sim\)/Ngn2\(\sim\)/Math3\(\sim\)/NeuroD\(\sim\) retinas but down-regulated in the Ngn2\(\sim\)/Math3\(\sim\)/NeuroD\(\sim\) retina (Fig. 7). The up-regulation and down-regulation of Math5 expression observed in the triple mutant retina correlated well with the increase and decrease of ganglion cell genesis (Fig. 7), a finding that is consistent with the previous report that Math5 is essential for ganglion cell development (17, 18). In Mash1\(\sim\)/Math3\(\sim\)/NeuroD\(\sim\) and Mash1\(\sim\)/Ngn2\(\sim\)/Math3\(\sim\)/NeuroD\(\sim\) retinas, Ngn2 and NeuroD, respectively, are expressed, suggesting that Ngn2 and NeuroD do not repress Math5 expression. In contrast, in the Ngn2\(\sim\)/Math3\(\sim\)/NeuroD\(\sim\) retina, Mash1 expression is up-regulated, and Mash1 expression is down-regulated, suggesting that Mash1 may repress Math5 expression (Fig. 7). Further studies will be required to determine whether Mash1 directly represses Math5 transcription by binding to its promoter.

The Difference in Remaining bHLH Gene Expression May Be Responsible for the Difference in Neuronal Subtypes Compen-sated in Triple Mutations—In the Ngn2\(\sim\)/Math3\(\sim\)/NeuroD\(\sim\) retina, the number of amacrine cells is severely reduced. These triple mutations increase the expression of Mash1 (Fig. 7), which is inhibitory to amacrine cell genesis (20). In contrast, a normal number of amacrine cells are generated in the Mash1\(\sim\)/Math3\(\sim\)/NeuroD\(\sim\) retina. These triple mutations increase Ngn2 expression, which could promote amacrine cell development (Fig. 7). We previously found that two bHLH genes Math3 and NeuroD are essential for amacrine cell genesis (20). However, because a normal number of amacrine cells are generated in the Mash1\(\sim\)/Math3\(\sim\)/NeuroD\(\sim\) retina, the loss of amacrine cells in the retina lacking Math3 and NeuroD (but not Mash1) is not simply the result of muta-tions of the amacrine cell genesis-promoting bHLH genes. It is likely that up-regulation of the inhibitory bHLH gene Mash1 is also important for the loss of amacrine cells in the retina lacking Math3 and NeuroD. These results suggest that the remaining bHLH gene expression may be responsible for determining which cell types are compensated.

In the retina lacking the three bHLH genes Ngn2, Math3, and NeuroD, a reduced number of amacrine cells were generated, although we found previously that, in the retina lacking two bHLH genes, Math3 and NeuroD, amacrine cells are mostly missing (20). We do not have definitive evidence to explain why amacrine cells were slightly increased in the triple mutations as compared with the double mutations, because Mash1 expression was up-regulated in both retina (Fig. 6, C–E). It was reported previously that NeuroD-mutant phenotypes depend upon the genetic backgrounds; NeuroD-null mice on a 129/SvEv genetic background die of severe diabetes shortly after birth, whereas those on different backgrounds survive to adulthood (44). Thus, the difference in amacrine cell genesis between Math3\(\sim\)/NeuroD\(\sim\) and Ngn2\(\sim\)/Math3\(\sim\)/NeuroD\(\sim\) retinas could be due to different genetic backgrounds, because ICR mice of Math3\(\sim\)/NeuroD\(\sim\) and of Ngn2\(\sim\) derive from different colonies.

It is interesting that bipolar cells are mostly missing in all of the triple mutations that we examined (Fig. 7). We found previously that either Mash1 or Math3 can induce generation of bipolar cells with the homeodomain gene Chx10, whereas in the absence of both Mash1 and Math3 all bipolar cells are missing (21, 22). Thus, it is reasonable that there are virtually no bipolar cells in the Ngn2\(\sim\)/Math3\(\sim\)/NeuroD\(\sim\) retina. However, it remains to be determined why bipolar cells are missing in the Ngn2\(\sim\)/Math3\(\sim\)/NeuroD\(\sim\) retina, because Mash1 is still expressed. Because cell death is increased in the Ngn2\(\sim\)/Math3\(\sim\)/NeuroD\(\sim\) retina, it is possible that bipolar cells may die during development.

In conclusion, our data indicate that multiple neurogenic bHLH genes cross-regulate each other, cooperatively specify retinal neuronal subtypes, and regulate the survival of retinal neurons.

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