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The final fates of *neurogenin2*-expressing cells include all major neuron types in the mouse retina

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Abstract

The molecular mechanism underlying vertebrate retinal development is not well understood. To examine whether *neurogenin2* (*ngn2*) expression determines cell fate in the retina, we mapped the final fates of cells that once expressed *ngn2*, using the conditional, binary CreERTM-LacZ system. We found LacZ⁺ cells in all 3 nuclear layers of the mouse retina and including all major types of neurons: photoreceptors, horizontal, bipolar, amacrine, and ganglion cells. The distribution of LacZ⁺ cells among the 3 nuclear layers closely resembled a theoretical distribution of total retinal cells. The temporal window in which each cell type was marked appeared nonrandom, and was similar to its birthdate and birth sequence. These data indicate that cells expressing *ngn2* at some point in their life histories may later differentiate into a variety of cell types.

Keywords

Retina; Development; Progenitor cells; Cell fate; Tracing; CreER

Introduction

The vertebrate retina contains six major types of cells: photoreceptors, horizontal cells, bipolar cells, amacrine cells, ganglion cells, and Müller glia. The cells are organized into 3 nuclear layers: the outer nuclear layer (ONL), the inner nuclear layer (INL), and the ganglion cell layer (GCL). Degeneration of retinal neurons leads to irreversible vision loss, and future therapies may include stem cell-based replacement. Imperative to such an approach is an understanding of the molecular control over retinal cell type specification.

The majority of the retinal cells are generated from a pool of multipotent progenitors (Cepko, 1999; Adler, 2000), and the rest may derive from lineage-restricted progenitors (Huang and Moody, 1997; Alexiades and Cepko, 1997). During development, precursors of each type accumulate and differentiate in stereotypical spatial orders and temporal gradients. Ganglion cells are believed to be born first, followed by cone photoreceptors, horizontal, and amacrine cells. Rod photoreceptors and bipolar cells are born later. The maturation of Müller glia marks the termination of retinal neurogenesis. The molecular mechanisms underlying the generation of the diverse types of retina cells are not fully understood. Recent studies show that transcription factors of the homeodomain family and the basic helix–loop–helix (bHLH) family play key roles in the process of retinal neurogenesis (for recent reviews, see Mu and Klein, 2004; Yan et al., 2005).

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Neurogenin2 (*ngn2*) is one of 3 currently known members of the *neurogenin* subfamily of bHLH proteins homologous to *Drosophila* proneural gene *atonal* (Sommer et al., 1996). Studies show that *ngn2* specifies neural cell types in the peripheral nervous systems (PNS; Ma et al., 1999; Fode et al., 1998), in the spinal cord (Mizuguchi et al., 2001; Novitsch et al., 2001; Lee et al., 2005), and in the brain (Fode et al., 2000; Schuurmans et al., 2004). The developing vertebrate retina also transiently expresses *ngn2*, but whether *ngn2* expression determines the fate of a particular type (or types) of retinal neurons remains to be determined. Experimental observations indicate that *ngn2* participates in a genetic pathway that eventually leads to the genesis of photoreceptor cells. In the mouse retina, regions where *pax6* is inactivated lack *ngn2* expression and contain no photoreceptor cells (Marquardt et al., 2001). Gain-of-function studies show that *ngn2* induces cultured, nonneural RPE cells to transdifferentiate along the photoreceptor pathway (Yan et al., 2001). These findings raise a critical question of whether *ngn2* specifies the photoreceptor fate. However, *ngn2* also induces RPE cells to transdifferentiate into cells that closely resemble retinal ganglion cells. This suggests that *ngn2* may induce not just one, but multiple neural differentiation programs. The establishment of this notion, however, needs direct evidence from experiments in vivo.

In this study, we used a conditional, bipartite genetic system based on a LacZ reporter (Seibt et al., 2003) and a Cre-estrogen receptor (Metzger et al., 1995) to permanently label cells expressing *ngn2* at defined developmental stages in mice (Zirlinger et al., 2002). We report that LacZ⁺ cells were detected in all 3 layers of the retina and included all major cell types, with a temporal order of LacZ labeling closely resembling cell birth sequence. Thus, in the retina, cells transiently expressing *ngn2* were not confined to one specific final fate.

Results

Spatial/temporal patterns of *ngn2* expression in the mouse retina

To gain insight into how *ngn2* might participate in retinal neurogenesis, we examined its expression with in situ hybridization. The retinal neuroepithelium is a pseudostratified structure composed mainly of proliferating progenitor cells. As development proceeds, an increasing number of cells withdraw from the cell cycle, become specified, and accumulate at their prospective anatomic locations. Mouse retinal neurogenesis commences around E10 and is essentially complete near P10 (Young, 1985). At E10, *ngn2* expression was detected in a few cells (Fig. 1A). The number of *ngn2*-expressing cells increased dramatically in E12 retina (Fig. 1B), remained high in E16 retina (Fig. 1C), decreased in E18 retina (Fig. 1D), and remained low at E19 (Fig. 1E). Expression of *ngn2* became undetectable at P2 (Fig. 1F) and remained undetectable at P5 (Fig. 1G). The temporal pattern indicated a developmental down-regulation of *ngn2* expression at late developmental stages to a level undetectable with in situ hybridization. Spatially, cells expressing *ngn2* were more or less scattered across the neuroepithelium, except that the vitreal side (occupied by young ganglion cells) had few or no such cells (Figs. 1B-E).

The final fates of cells that had transiently expressed *ngn2*

The down-regulation of *ngn2* and the lack of distinguishable characteristics in those cells that transiently expressed *ngn2* confounded the effort to identify the developmental potential of those *ngn2*-expressing cells. We therefore deployed the binary, conditional CreERTM-LacZ reporter system (Metzger et al., 1995; Soriano, 1999) to map the fate of those *ngn2*-expressing cells in *Ngn2*-CreERTM mice (Zirlinger et al., 2002). In these mice, the coding sequence of *ngn2* was replaced by CreERTM. Homozygotes were not viable. Heterozygous *Ngn2*-CreERTM^{+/-} mice, verified with PCR (Fig. 2), were crossed with homozygous ROSA26-LacZ reporter mice. Administration of 4-OHT results in functional Cre recombinase, which irreversibly activates the LacZ gene by removing a floxed cassette. Because the activated LacZ

expression persists through the life of the cell and because the half-life of 4-OHT is 0.5–2 h (Danielian et al., 1998), this system offers the opportunity to track the final fates of cells that expressed *ngn2* during the brief presence of 4-OHT administered during retinal development.

LacZ⁺ cells were detected in all 3 nuclear layers of the retina. Within the ONL, where photoreceptor cells reside, cells were marked by 4-OHT administration at both early (E15.5) and late developmental stages (P4). Those marked during an early stage localized at the outer edge of the ONL (Fig. 3A), indicative of cones. Those marked during a late stage distributed across essentially the entire thickness of the ONL (Fig. 3B), indicative of rods. In the INL, LacZ⁺ cells included calbindin⁺ horizontal cells (Fig. 3C), which reside at the outermost edge of the INL, cells in a zone of G0α⁺ (Vardi, 1998) cone bipolar cells (Fig. 3D), PKC⁺ (Negishi et al., 1988; Milam et al., 1993) rod bipolar cells (Fig. 3E), and Chx10⁺ bipolar cells (Fig. 3F). LacZ⁺ cells also included AP2⁺ amacrine cells (Fig. 3G), which typically reside in the lower half of the INL. In the GCL, LacZ⁺ cells were Brn3A⁺ (Fig. 3H), a marker for ganglion cells (Xiang et al., 1995). Overall, LacZ⁺ cells included all major types of retinal neurons.

Distribution of LacZ⁺ cells among the 3 nuclear layers

The observation of LacZ⁺ cells containing all major cell types indicated that *ngn2* expression did not favor certain retinal cell type(s). To examine the issue further, we compared the overall distribution of LacZ⁺ cells among the 3 nuclear layers with the published information on total retinal cells (Jeon et al., 1998). The stereotyped organization of the retina into 3 well-defined, clearly laminated nuclear layers makes it possible to have an unambiguous analysis. From a total of 3617 LacZ⁺ cells scored from 242 retinal sections with 4-OHT administered between E12 and P6, 2140 (59%) were in the ONL, 1371 (38%) in the INL, and 106 (3%) in the GCL (Fig. 4). Remarkably, this distribution was essentially the same as the retinal cell distribution among the 3 layers extrapolated from a study of the major cell populations of the mouse retina by Jeon et al. (1998).

The developmental window and temporal order of LacZ labeling

To examine the temporal order of LacZ labeling (i.e., *ngn2* expression), we identified and scored the number of LacZ⁺ cells with 4-OHT administered at different time points, from E10 to P10. We detected no LacZ⁺ cells with 4-OHT administered at E10, likely because few cells expressed *ngn2* at this time as shown with in situ hybridization (Fig. 1A). When 4-OHT was administered at E12 or E13, the only LacZ⁺ cells were horizontal cells (Fig. 5A), and no ganglion cells were labeled at these stages. This was unexpected, since it is well established that ganglion cells are the first to be born (Polley et al., 1989; Rapaport et al., 2004).

Ganglion cells began to be marked at E14 and peaked at E20. Photoreceptor cells began to become labeled with 4-OHT injection at E15. Amacrine cells were marked during a wide window, from E15 through P5. Most bipolar cells were labeled between P3 and P5 (Fig. 5A). No LacZ⁺ cells were found with 4-OHT administration at P7 and P10.

A comparison of the temporal order LacZ labeling with the timing and topography of cell genesis in the mouse retina (Young, 1985) indicated a rather striking similarity between the 2 events (Fig. 5B). The developmental window at which each cell type expressed *ngn2* was in substantial agreement with their birth dates, albeit cell birth occurs in a wider temporal window than *ngn2* expression marked by LacZ (Fig. 5B).

Expression of *ngn2* in proliferating cells

The diverse types of LacZ⁺ cells, together with the spatial location of cells expressing *ngn2* within the retinal neuroepithelium, suggested that *ngn2* might be expressed in proliferating,

multipotent progenitor cells. We tested this possibility by examining whether *ngn2*-expressing cells incorporated BrdU. Two sets of experiments were carried out. In the first set, E14 embryos were pulsed with BrdU for 2 h and subsequently analyzed for *ngn2* expression (in situ hybridization) and BrdU incorporation. In the second set, BrdU was administered at the same time as 4-OHT to Ngn2-CreERTM to day 18 embryos, which were analyzed at P7 by double-labeling for LacZ and BrdU incorporation. Double-labeled cells were detected in both cases (Figs. 6A and B). The presence of *ngn2*⁺/BrdU⁺ cells indicated that some S-phase cells expressed *ngn2*. The presence of LacZ⁺/BrdU⁺ cells indicated that cells expressing *ngn2* underwent S-phase of the cell cycle at the time of *ngn2* expression or thereafter.

Discussion

To examine whether *ngn2* was expressed in cells that would differentiate into a specific type of cells in the retina, we used the conditional, binary CreERTM-LacZ system to map the final fates of cells that expressed *ngn2* during retinal neurogenesis in the mouse retina. Similar fate mapping systems have recently been employed to track down the final fate of cells expressing various genes in the developing retina (Yang et al., 2003; Zhang et al., 2003; Badea et al., 2003; Rowan and Cepko, 2004). In the study by Yang et al. (2003), cells expressing *ath5* are found to have broad developmental potentials; these cells include ganglion cells, photoreceptor cells, and amacrine cells. We found LacZ⁺ cells in all 3 nuclear layers, and they included all major types of neurons. Furthermore, the quantitative distribution of LacZ⁺ cells was essentially identical to that of the overall retinal cells among the 3 nuclear layers. These findings suggest that transient expression of *ngn2* per se may not be the deterministic factor in retinal cell fate specification. This is consistent with the spatial/temporal pattern of *ngn2* expression. In situ hybridization showed *ngn2* expression in cells within the neuroepithelium and the expression became undetectable in those cells that had migrated to their final anatomical destination. Similar spatial expression patterns are reported by Brown et al. (1998) and Akagi et al. (2004). Furthermore, two sets of double-labeling experiments showed *ngn2* expression in cells still in the cell cycle.

We noted that ganglion cells were not marked by 4-OHT administration at E12 nor at E13, the time points at which horizontal cells became marked. Although Young (1985) also noted that the peak of horizontal cell birth preceded that of ganglion cells, it is well established that ganglion cells are born first (Polley et al., 1989; Rapaport et al., 2004). The lacking of LacZ⁺ cells is perhaps due to that another gene, such as *ath5*, is at work during the initial phase of retinal neurogenesis. *Ath5* has been shown to be expressed earlier than *ngn2* in the mouse retina (Brown et al., 1998) and to play an important role in early retinal development in zebrafish (Masai et al., 2000; Stenkamp and Frey, 2003). Additionally, our recent study indicates that *ath5* and *ngn2* may represent different transcriptional hierarchies (Ma et al., 2004). We noted that only a few cells were positive in our in situ hybridization for *ngn2* expression at E10 and the number dramatically increased at E12. Thus, it is possible *ath5* is functionally equivalent to *ngn2* at early time points in retinal neurogenesis. If this scenario is correct, it raises an intriguing question of whether the progression of a retinoblast through the various development stages is reflected by the expression of different proneural bHLH genes that bring about the observed intrinsic differences in their developmental potentials at different stages (Cayouette et al., 2003). Clearly, rigorous studies are needed to test this possibility.

The essentially identical quantitative distribution of LacZ⁺ cells and of the overall retinal cells among the three nuclear layers is rather striking and provides additional support to the notion that transient expression of *ngn2* is not a determinative factor in retinal cell specification. The similarity, nonetheless, should not be simply taken to rule out the possibility that *ngn2* biases one, or a subset, of cell types. *ngn2* could promote the production of early-born neurons (or

photoreceptor cells in particular), even though it was also expressed in progenitors that later developed as later-born cells. This could be brought about through a number of mechanisms. First, the function of *ngn2* could be cell-context dependent. Certain interacting proteins, particularly those synergizing with Ngn2 protein, might be present only during an early phase of development, and the combinatorial cue would cause a bias for early-born neurons. Conversely, there might be antagonistic proteins present during later phases of retinal neurogenesis to neutralize Ngn2's activity, thereby allowing progenitors to develop into later-born cells. It is well-known that bHLH proteins may form heterodimers with other bHLH proteins or cooperate with homeodomain proteins (Lee and Pfaff, 2003) in regulating gene expression. Second, *ngn2*'s function may be modulated by environmental cues, whose prevalence may vary during the course of retinal development. For example, *ngn2* expression during a later phase of retinal neurogenesis could be null in terms of its biological function. Third, the level and duration of *ngn2* expression might differ during the course of retinal neurogenesis, and this varied expression may dictate whether or not the expression is biologically significant. The CreERTM-LacZ system has been mostly used to map the fate of cells expressing a gene at a qualitative, rather than quantitative, level. To this end, it is difficult to conclude that all LacZ⁺ cells had the same level and/or duration of *ngn2* expression. Notably, however, in situ hybridization did, in fact, show high levels of *ngn2* expression at E12 and E16, suggesting that the level of *ngn2* expression during later phases of retinal neurogenesis was substantially lower than at the early phase. This raises the question of whether the low level of *ngn2* expression during the late phase of retinal neurogenesis merely reflects the tailing off of its expression or, alternatively, was programmed to be at a basal level that could be readily increased in case retinal neurogenesis encounters pro-grammatic problems, such as the genetic alterations observed in experimental knockouts, which have been shown to enhance the expression of some bHLH genes (Akagi et al., 2004).

The diversity of LacZ⁺ cells suggests that *ngn2* expression may provide a proliferating retinal progenitor the competence to differentiate into the various cell types comprising the retina. Given its ability to guide RPE cells to transdifferentiate into cells that molecularly and morphologically resemble retinal neurons (Yan et al., 2001), *ngn2* seems capable of bringing forth retinal neural differentiation programs in even nonneural (RPE) cells.

Experimental methods

Mouse manipulations

All experimental procedures involving the use of animals were approved by the institutional review board at the University of Alabama at Birmingham. The Ngn2-CreERTM mice (Zirlinger et al., 2002) were a generous gift from Dr. David Anderson (Caltech). Rosa26-loxP-LacZ reporter mice (Soriano, 1999) were obtained from the Jackson Laboratory [Stock #003310, 129s-Gt(ROSA)26Sor]. The 129s-Gt(ROSA)26Sor male mice were crossed with 129S1/SvImJ (Stock #002448, Jackson Laboratory) female mice to generate heterozygous Rosa26-loxP-lacZ mice. The heterozygous male and female mice were then used to generate homozygous Rosa26-loxP-lacZ reporter mice. Heterozygous Ngn2-CreERTM male mice were crossed to Rosa26-loxP-lacZ homologous female mice to generate embryos for analysis. Timed pregnancies were determined by vaginal plug observation, with midday time of plug observation counted as E0.5.

E14 embryos were pulsed for 2 h with BrdU injected intraperitoneally with 50 μ g body weight into normal C57 pregnancy mice.

Genotype detection

The genotypes of the mice were verified with PCR using genomic DNA isolated from tail tissue. The primers and PCR conditions to verify the Rosa26-loxP-LacZ reporter genotypes were as instructed by the Jackson Laboratory. To verify the Ngn2-CreERTM genotype, primers for Cre (atccgaaaagaaaacgttga and atccaggttacggatatagt) and for *ngn2* (ctgagactctggagttgaag and ctgatacagtccttgccg) were used. Also included were primers for glyceraldehyde-3-phosphate dehydrogenase (catgaccacagtcctcatccat and cctctctgtcctagtcctt) as an internal control.

Injection of 4-hydroxy tamoxifen

An estrogen analog, 4-hydroxy tamoxifen (4-OHT), was used to activate CreERTM. A single injection of 4-OHT (500 µg per mouse, i.p.) was carried out at the different developmental stages, from E10 to P10, which covers essentially the entire period of retinal neurogenesis in the mouse (Young, 1985). At each stage, multiple (2–5) mice were used. Mice were sacrificed at P13 to P17, when the retinal histogenesis has finished, and those carrying the Ngn2-CreERTM and 129s-Gt(ROSA)26Sor were identified by PCR. For BrdU labeling, the same mice were injected with BrdU (20 µg/g body weight, i.p.) immediately after 4-OHT injection.

X-gal staining

Eyes were enucleated and fixed with 4% formaldehyde on ice for 30 min. After rinsing with PBS, the sample was washed twice each for 10 min in lacZ rinse buffer (0.2 M sodium phosphate, pH 7.3, 2 mM magnesium chloride, 0.02% NP-40, 0.01% sodium deoxycholate) and was then incubated overnight in the dark with lacZ staining buffer (lacZ rinse buffer plus 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml xgal). After 3 washes with PBS, the samples were postfixed 30 min with 4% formaldehyde, embedded with OCT:sucrose (2:1), and frozen with liquid nitrogen. Retinal cryosections of 5–12 µm were collected onto Superfrost/Plus glass slides and examined for LacZ⁺ cells under a light microscopy. Immunohistochemistry with specific antibodies was carried out to facilitate the identification of LacZ⁺ cells as a particular type of retinal neurons.

Immunohistochemistry

Primary antibodies used in this study included polyclonal anti-AP2 antibodies (used at a 1:500 dilution; Santa Cruz Biotechnology), polyclonal anti-Chx10 (1:100, Exalpha Biologicals), and monoclonal antibodies against Brn3a (1:200, Chemicon), G0α (1:500; Chemicon), calbindin (Clone DL-300; 1:200, Sigma), PKC (clone MC5; 1:500; Sigma), and BrdU (clone G3G4; 1:100; developed by Dr. Stephen J. Kaufman and obtained from the Developmental Studies Hybridoma Bank at Iowa University). Standard immunohistochemistry was carried out with 5–12 µm cryosections of the retina that had been stained with x-gal prior to tissue freezing (see previous paragraph).

In situ hybridization

A 779-base pair fragment within the *ngn2* coding sequence was PCR amplified with primers (ctgagactctggagttgaag and ctgatacagtccttgccg) based on the published sequence (Seibt et al., 2003). The PCR product was cloned into pGEM-T and its sequence verified. Digoxigenin-labeled antisense RNA probes were synthesized from cloned DNA using the Genius Kit (Roche). In situ hybridization was carried out on retinal sections as previously described (Li et al., 1999).

Cell counting

LacZ⁺ cells were counted and categorized according to their anatomical localizations (the ONL and the GCL) and double-labeling with a marker that identifies a particular cell type (within the INL). The numbers of LacZ⁺ cells in each of the three nuclear layers of the retina were counted according to their localizations in the ONL, the INL, and the GCL in the same slides. The total numbers of LacZ⁺ cells and that of each of the three nuclear layers were the sum of over 250 retinal sections representing 4-OHT administration at different developmental stages, from E10 to P10.

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Fig 1.

Spatial/temporal expression pattern of *ngn2* in the developing mouse retina examined with in situ hybridization using digoxigenin-labeled probes. (A) E10 retina. Only a few cells (arrows) expressed detectable levels of *ngn2*. (B) E12 retina. More cells expressed *ngn2* at this stage. (C) E16 retina. The number of *ngn2*-expressing cells remained high. (D) E18 retina. The level of *ngn2* expression appeared to decrease. (E) E19 retina. The level of *ngn2* expression continued to decline. (F) P2 retina. *Ngn2* expression was undetectable. (G) P5 retina. *Ngn2* expression remained undetectable. Magnifications: $\times 10$.

Fig 2. PCR genotyping of Ngn2-CreERTM mice. Shown are PCR products for *cre* recombinase, *ngn2*, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Fig 3.

The spatial distribution of LacZ⁺ cells in the mouse retina examined by histology and immunohistology. (A) P17 retina with 4-OHT administered at E15.5. (B) P18 retina, 4-OHT at P4. (C) P15 retina double-labeled with anti-calbindin antibody to identify horizontal cells, 4-OHT at E12. (D) P18 retina double-labeled with anti-G0 α antibody to mark bipolar cells, 4-OHT at P4. (E) P18 retina double-labeled with antibodies against PKC to mark rod bipolar cells, 4-OHT at P4. (F) P14 retina double-labeled with antibodies against Chx10 to identify bipolar cells, 4-OHT at P5. (G) P16 retina double-labeled with anti-AP2 antibody to identify amacrine cells, 4-OHT at P3. (H) P17 retina double-labeled with anti-Brn3A antibody to identify ganglion cells, 4-OHT at E15.5. Blue arrow: LacZ⁺ cells; black arrow: double-labeled cells. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Magnifications: $\times 40$.

Fig 4.

Distributions of LacZ⁺ cells and theoretical distribution of total retinal cells among the three nuclear layers of the mouse retina. A total of 3617 LacZ⁺ cells from 242 retinal sections were scored. Among them, 2140 (59%) were in the ONL, 1371 (38%) in the INL, and 106 (3%) in the GCL. The theoretical numbers were extrapolated from the study of Jeon et al. (1998).

Fig 5.

The temporal order and the developmental windows in which the major cell types became marked by LacZ expression. (A) The means and SD (graphed in log 10) of the number of LacZ⁺ cells per retinal section calculated from 4 to 50 sections at each of the developmental time points at which 4-OHT was administered. (B) A comparison of the temporal order and the developmental windows of each major cell type between *ngn2* expression (i.e., becoming marked by LacZ expression in the study; colored solid lines) and birth dates of mouse retinal cells (dashed lines) extrapolated from the study of Young (1985).

Fig 6. Detection of *ngn2*⁺/BrdU⁺ and LacZ⁺/BrdU⁺ cells. (A) Double-labeling for *ngn2* expression (in situ hybridization staining in darker blue of the cytoplasm) and BrdU incorporation (immunostaining in red of the nucleus) E14 retina. (B) Double-labeling for LacZ and BrdU in P7 retina with 4-OHT and BrdU administered at E18. Black arrows: double-labeled cells; red arrow: BrdU⁺ cells; blue arrow: LacZ⁺ cells. Magnifications: $\times 40$.