Development/Plasticity/Repair

In Vitro Generation of Early-Born Neurons from Late Retinal Progenitors

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Evidence suggests that, as development ensues, the competence of neural progenitors is progressively altered, such that they become fated to give rise to neurons of a particular stage. Here, we demonstrate that late retinal progenitors can give rise to retinal ganglion cells (RGCs), an example of an early-born cell type in the retina. A subset of late retinal progenitors in vitro responds to cues that favor RGC differentiation by displaying markers characteristic of RGCs. In addition, mechanisms used during normal RGC differentiation are recruited by these cells toward their differentiation along RGC lineage. Our observations suggest that late neural progenitors may not be irreversibly fated but may appear as such under the constraints dictated by epigenetic cues.

Key words: retinal ganglion cells; Notch; stem cells/progenitors; differentiation; Ath5; Brn3b

Introduction

One of the central issues in neurobiology is the generation of cellular diversity in the CNS. The vertebrate retina is a simple and accessible model of the CNS suitable for investigating mechanisms underlying cellular diversity. The stereotypical laminar organization of retina consists of seven major cell types that include rod and cone photoreceptors, retinal ganglion cells (RGCs), horizontal cells, amacrine cells, bipolar cells, and Müller glia. Thymidine birth-dating studies have shown that the generation of these cells follows an evolutionarily conserved temporal sequence, in which RGCs, cone photoreceptors, horizontal cells, and the majority of amacrine cells are born during early histogenesis, whereas bipolar cells, Müller glia, and the majority of rod photoreceptors are born during late histogenesis (Sidman, 1961; Kahn, 1974; Young, 1985; LaVail et al., 1991; Prada et al., 1991). Despite overlaps in generation of certain cell types, the early and late stages of histogenesis are temporally segregated such that the majority of early and late-born neurons are generated approximately between embryonic day 10.5 (E10.5) and E16, and between E18 and postnatal day 6 (PN6), respectively, in the mouse retina (Sidman, 1961; Young, 1985; Robinson, 1991). Given the species difference in the gestation period, the majority of early- and late-born neurons are generated approximately between embryonic day 10.5 (E10.5) and E16, and between E18 and postnatal day 6 (PN6), respectively, in the mouse retina (Sidman, 1961; Young, 1985; Robinson, 1991). Given the species difference in the gestation period, the majority of early-born neurons are believed to be generated between E11.5 and E17 in the rat retina (Robinson, 1991). Evidence from a variety of experimental approaches including cell ablation studies (Negishi et al., 1982; Reh and Tully, 1986) and lineage analyses (Turner and Cepko, 1987; Holt et al., 1988; Wets and Fraser, 1988; Turner et al., 1990) suggest that the retinal progenitors are multipotent, and that the decision taken by a progenitor to differentiate along a particular path depends on local cell–cell interactions. In vitro coculture experiments, from different embryonic stages, offered additional proof that the differentiation of retinal progenitors can be influenced by epigenetic cues, and that these cues could be diffusible factors elaborated by previously differentiated cells and/or differentiating cells. The known diffusible factors that have been shown to affect differentiation of retinal progenitors include epidermal growth factor (EGF) (Anchan et al., 1991; Ahmad et al., 1998a), TGF-β (Anchan et al., 1991; Lillien and Cepko, 1992), basic FGF (Hicks and Courtois, 1992), acidic FGF (Lillien and Cepko, 1992), tauroine (Altshuler et al., 1993), CNTF (Kirsch et al., 1996; Ezzeddine et al., 1997), leukemia inhibitory factor (Neophytou et al., 1997), retinoic acid (Kelley et al., 1994), and sonic hedgehog (Levine et al., 1997; Zhang and Yang, 2001). There are yet-unidentified diffusible factors that have been shown to influence the differentiation of rods (Watanabe and Raff, 1990; Altshuler and Cepko, 1992; Harris and Messersmith, 1992; Reh, 1992; Watanabe and Raff, 1992; Belliveau et al., 2000), RGCs (Waid and McLoon, 1998), and amacrine cells (Belliveau and Cepko, 1999). Recent evidence suggests that, besides diffusible factors, cell–cell interactions mediated by membrane-bound receptors and ligand interactions, exemplified by Notch signaling, may play a critical role in retinal neurogenesis (Dorsky et al., 1995, 1997). Although it is still unclear how it achieves the specificity of cell fate determination, Notch signaling has been demonstrated to regulate both the temporal and spatial specification of retinal neurons, particularly RGCs (Ahmad, 1995; Austin et al., 1995; Ahmad et al., 1997).

Although these studies clearly demonstrate that the epigenetic cues play a critical role in differentiation of retinal progenitors, the conserved sequence of the generation of retinal cell types suggests that the progenitors that define the early and late stages of histogenesis have distinct competence to give rise to specific cell types. Additionally, evidence based on coculture studies suggests that the epigenetic cues work under the constraints of competence dictated by intrinsic factors. For example, early retinal progenitors, when cocultured with cells from the late stage of
suggest that a subset of late retinal progenitors is competent to
regulators of RGC differentiation, Ath5 and Brn3b. Our results
ceded by and/or accompanied with the expression of two known
by late retinal progenitors involved Notch signaling and was pre-
crease RGC phenotype of late retinal progenitor is competent to respond to cues that
sure of these progenitors to a condition that is conducive for RGC
differentiation condition, expressed RGC-specific markers, sug-
genearly-born neurons and mechanisms that are used in vivo are recruited by these cells to differentiate into RGCs in vitro.

Materials and Methods

Progenitor cell culture. Timed-pregnant (E18) Sprague Dawley rats were obtained from Sasco (Wilmington, MA). The gestation day was confirmed by the morphological examination of embryos (Christie, 1964). Fertilized hen eggs (SPAFAS, Wilmington, MA) were incubated in a humidified chamber at 38°C, and embryos were staged according to Hamburger and Hamilton (1951). Embryos were harvested at appropriate gestation periods, and eyes were enucleated. Retinas were dissected out and dissociated as previously described (Ahmad et al., 1999). Cells were plated in six-well culture plates at a density of 2 × 10⁶ cells/well in DMEM:F12, 1× N2 supplement (Invitrogen, San Diego, CA), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 ng/ml EGF for 5 d to generate clonal neurospheres. 5-Bromo-2′-deoxyuridine (BrdU) (10 μM) was added to the culture for the final 24 h. The next day, neurospheres were collected, washed extensively to remove BrdU, and cocultured on poly–d-lysine- and laminin-coated glass coverslips with E3 chick retina/PN1 rat cerebral cortex served as controls. For RT-PCR analysis of transcripts expressed in E18 rat retinal cells, coculture was performed across a 0.4 μm membrane (Millipore, Bedford, MA). The medium was changed every other day, and culture was continued for 5–6 d. Cells were either frozen for RNA extraction or fixed with 4% paraformaldehyde for 15 min at 4°C followed by immunofluorescence analysis.

**Table 1. List of primers and their respective sequences used for RT-PCR analysis**

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<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing temperature</th>
<th>Product size (bp)</th>
<th>GenBank accession number</th>
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<td>50°C</td>
<td>543</td>
<td>XM037235</td>
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<td>Thy1</td>
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Hoechst dye efflux assay. The progenitor and precursors in E18 neurospheres cultured in E3 conditioned medium were enriched using a Hoechst dye efflux assay (Bhattacharya et al., 2003). Briefly, dissociated cells were resuspended in Hoechst Iscove medium containing 2% FCS at 4°C overnight followed by an overnight incubation in antibodies against rhodopsin, PKC, Nestin, RPF1, Islet1, Brn3b, Thy1, and BrdU at 4°C. Cells were examined for epifluorescence after incubation in IgG conjugated to cyanin 3 (Cy3)/FITC. Images were captured using a cooled CCD camera (Princeton Instruments, Trenton, NJ) and Openlab software (Improvision, Lexington, MA).

RT-PCR analysis. RNA was isolated from frozen cells using a Qiazol kit (Valencia, CA) RNA isolation kit, and cDNA synthesis was performed as previously described (Bhattacharya et al., 2003). Specific primers were amplified with gene-specific forward and reverse primers on a Roche-480 (Stratagene, La Jolla, CA). The gene-specific primers used for RT-PCR are described in Table 1.

Transplantation of E18 retinal progenitors. E18 retinal cells were obtained from green fluorescent protein (GFP)-expressing transgenic mice (Okabe et al., 1997) and cultured to obtain GFP-positive neurospheres as described above. Transplantation of GFP-positive retinal progenitors was performed in PN10 P23H transgenic rats containing the proline-23-histidine (Pro23His) rhodopsin mutation [line TgN(P23H)3] as previously described (Lewin et al., 1998; Chacko et al., 2003). Animals were anesthetized with 20–25 µl of a 1:1 dilution of ketamine (60 mg/ml) and xylazine (8 mg/ml), injected intraperitoneally. A 30-gauge needle was inserted into the eye near the equator. The needle was retracted, and a glass micropipette connected to a 10 µl Hamilton syringe was inserted through the wound to deliver 50,000 cells in a 1 µl volume. Animals were injected with cyclosporin (5 mg · kg⁻¹ · d⁻¹) and killed at 1, 2, and 3 weeks after transplantation.

Immunofluorescence analysis. Detection of cell-specific markers and BrdU was performed as previously described (Ahmad et al., 1999). Briefly, paraformaldehyde-fixed cells were incubated in PBS containing 5% NGS and 0.2–0.4% Triton X-100 followed by an overnight incubation in antibodies against rhodopsin, PKC, Nestin, RPF1, Islet1, Brn3b, Thy1, and BrdU at 4°C. Cells were examined for epifluorescence after incubation in IgG conjugated to cyanin 3 (Cy3)/FITC. Images were captured using a cooled CCD camera (Princeton Instruments, Trenton, NJ) and Openlab software (Improvision, Lexington, MA).

Results

A subset of late retinal progenitors expresses RGC-specific markers in vitro

E18 retinal progenitors normally generate rod photoreceptors, bipolar cells, Müller glia, and a subset of amacrine cells. It has been observed that these progenitors have intrinsic limitations regarding the range of cell types that they can generate (Belliveau et al., 2000). To investigate the extent of plasticity of late retinal progenitors, E18 retinal dissociates were cultured in the presence of EGF. In this culture condition, progenitors are selectively amplified, giving rise to neurospheres that consist of Nestin-positive proliferating cells (Fig. 1A–D). E18 retinal progenitors, thus enriched and tagged with BrdU, were allowed to differentiate by substituting FBS for mitogens in the culture medium. As expected, BrdU-positive cells that expressed rhodopsin and PKC were observed, suggesting the differentiation of progenitors into late-born neurons, rod photoreceptors, and bipolar cells, respectively (Fig. 1E–L). To determine the plasticity of the retinal progenitors in terms of generating early-born neurons, expression of markers characteristic of differentiating RGCs was analyzed. These include RPF1, a member of VI POU family.
domain subfamily, which is one of the earliest RGC differentiation markers, detected as early as E11 in the mouse retina (Zhou et al., 1996). Islet1, a member of the homeodomain family, is another early RGC marker (Rachel et al., 2002). BrdU-positive cells were detected that expressed RPF1, suggesting that a subset of E18 retinal progenitors may possess the capacity to differentiate along RGC lineage (Fig. 1). Similar immunocytochemical results were obtained using antibody against Islet1 (data not shown).

Cells from an early stage of retinal histogenesis facilitate differentiation of late retinal progenitors into RGCs

Next, we wanted to know whether the potential of cultured E18 retinal progenitors can be influenced by epigenetic cues, because evidence has emerged that cell-cell interaction plays a critical role in the specification of retinal progenitors (Cepko, 1999). The differentiation of E18 retinal progenitors was analyzed in cultured conditions that are conducive for the differentiation of early-born neurons. BrdU-tagged E18 retinal progenitors were cocultured with an excess of E3 chick retinal cells. Several factors were considered for choosing E3 chick retinal cells for inducing RGC differentiation. First, stages E3–E5 in chick retina represent the peak of RGC genesis (Prada et al., 1991). Second, it is likely that the cues that influence RGC differentiation are evolutionarily conserved, because, regardless of species, RGCs are the first retinal cell types to differentiate. Therefore, rat retinal progenitors may be able to read out cues for RGC differentiation elaborated by E3 chick retinal cells. Third, more cells can be harvested with relative ease from E3 chick retina than from E13–E14 rat retina, the peak of RGC genesis. When E18 retinal progenitors were cultured with E3 chick retinal cells, the proportion of BrdU-positive cells expressing RGC markers increased compared with controls. In the presence of FBS, 18.70 ± 1.22 and 10.17 ± 1.00% of BrdU-positive cells expressed RPF1 and Islet1, respectively. In the presence of E3 chick cerebral cortex cells, 15.3 ± 1.90 and 6.5 ± 0.50% of BrdU-positive cells expressed RPF1 and Islet1, respectively (Fig. 2). In contrast, the proportion of BrdU-positive cells expressing RGF markers increased significantly when cocultured with E3 chick retinal cells; 32.53 ± 1.98 and 34.70 ± 1.90% of BrdU-positive cells expressed RPF1 and Islet1, respectively, suggesting a positive influence of E3 chick retinal cells on RGC differentiation (Fig. 2A–D, I). Three different approaches were taken to corroborate the plasticity of late retinal progenitors to generate RGCs. First, we investigated their ability to express Brn3b, which is a member of the pou domain subfamily and expressed in a large subpopulation (~70%) of differentiating and matured RGCs (Xiang et al., 1993; Gan et al., 1996, 1999). In the presence of FBS, 9.12 ± 1.52% of BrdU-positive cells expressed Brn3b. In contrast, 24.48 ± 1.88% of BrdU-positive cells expressed Brn3b when cocultured with E3 chick retinal cells, a significant increase similar to those observed for RFP1 and Islet1 in coculture conditions (Fig. 3). Second, we performed coculture across a 0.4 μm membrane to corroborate results by analyzing the expression of transcripts corresponding to RGC markers. RT-PCR analysis performed on late retinal progenitors identified the expression of transcripts corresponding to Islet1, RPF1, Brn3b, and Thy1 whose levels increased in the presence of E3 chick retinal cells, suggesting that RGC-specific genes are activated by epigenetic cues, and that these cues are likely to be diffusible (Fig. 4A). Third, we cultured late retinal progenitors in E3 chick-conditioned medium to further confirm their differentiation along RGC lineage by colocalization of different RGC differentiation markers. Double-immunocytochemical analysis revealed that a subset of cells coexpressed Islet1 and RPF1 (Fig. 4B–E)/Brn3b (F–I), suggesting that late retinal progenitors possess the ability to express multiple markers of RGC differentiation.
in coculture condition. To ascertain that cues promoting RGC differentiation are not species specific, BrdU-tagged late retinal progenitors were cocultured with E14 rat retinal cells. The proportion of BrdU-positive cells expressing RGC-specific markers increased significantly compared with controls, suggesting that factors that participate in RGC differentiation during early histogenesis are conserved across species (Fig. 5A–D).

Cells from an early stage of retinal histogenesis inhibit differentiation of late retinal progenitors into rod photoreceptors

To determine whether E3 chick retinal cells influence the differentiation of late retinal progenitors into late-born neurons such as rod photoreceptors, expression of rhodopsin was analyzed in BrdU-tagged late retinal progenitors after coculture. A significant decrease in the proportion of BrdU-positive cells expressing opsin was observed in the presence of E3 chick retinal cells compared with control (23.24 ± 1.92 vs 15.3 ± 2.52%; p < 0.01), suggesting that the presence of cells from early stages of retinal histogenesis compromises the differentiation of late-born neurons (Fig. 6A). A negative effect on rod photoreceptor differentiation was also induced by cells from the cerebral cortex, as ascertained by a relative decrease in the levels of opsin transcripts (Fig. 6B). However, to rule out an alternative possibility that the negative influence of E3 chick retinal cells on rod photoreceptor differentiation does not reflect an altered potential of E18 retinal progenitors in vitro, we cocultured these cells in the presence of postnatal retinal cells that have been observed to secrete factors that promote rod photoreceptor differentiation (Altshuler and Cepko, 1992; Watanabe and Raff, 1992; Ahmad et al., 1999). The presence of postnatal retinal cells significantly increased the proportion of opsin-positive cells and levels of opsin transcripts compared with controls, suggesting that E18 progenitors maintain their normal potential to differentiate into rod photoreceptors in response to conducive conditions (Fig. 6).

The RGC potential is inherent to a subset of late retinal progenitors and is constrained in vivo

Although the majority of RGCs are born between E11.5 and E17, a few of these cells are reported to be generated in the peripheral E18 retina (Reese and Colello, 1992). Therefore, it is probable that RGCs generated in vitro are derived from few residual early progenitors. We addressed this issue in two different ways. First, we determined whether, as in Long–Evans (pigmented) rats (Reese and Colello, 1992), RGCs are born in the E18 retina of Sprague Dawley (albino) rats. Pregnant rats at day 18 of gestation were injected with BrdU, and eyes were enucleated from PN1 pups for immunohistochemical analysis of the retina. Although BrdU-positive cells were readily observed, none of these cells expressed RGC-specific markers, either in the central or peripheral retina (Fig. 7A–P), suggesting that, in Sprague Dawley rats, RGC genesis is complete by E18; and therefore, our observation of RGC generation in vitro is unlikely to be attributable to residual early progenitors. The difference in the genesis of RGCs between Long–Evans and Sprague Dawley is likely attributable to albinism; it has been demonstrated recently that the temporal aspects of RGC genesis is different between albino and wild-type mice, being completed earlier in the former than in the latter (Rachel et al., 2002). Second, we performed RGC birth dating in PN1 rats and investigated the potential of PN1 retinal progenitors to differentiate into RGCs. This experiment was performed to address the concern that, regardless of species difference, we might have overlooked a small number of RGCs generated in the peripheral retina at E18 or staged the gestation day of pregnant rats incorrectly. Similar to our observations in the E18 retina, we did not detect proliferating cells in the PN1 retina that expressed RGC-specific markers (data not shown). As observed for late progenitors derived from the E18 retina, PN1 retinal progenitors differentiated into RGCs; the proportion of RGCs generated was higher in coculture condition than in the control (Islet1, 5.57 ± 0.99 vs 11.53 ± 2.74%; RPF1, 13.52 ± 1.91 vs 23.26 ± 1.66%). Similar results were obtained with PN3 retinal progenitors. However, there was a progressive decrease in the proportion of cells expressing RGC markers in coculture condition at each subsequent time point (Fig. 7Q). Together, these observations suggest that a subset of retinal progenitors derived from the postnatal retina possess the potential to give rise to early-born neurons, and that they are depleted as...
histogenesis progresses toward completion. Next, we wanted to know whether RGC potential of late retinal progenitors are inherent or acquired de novo because of prolonged exposure to mitogens during the enrichment process. We analyzed the potential of freshly dissociated late retinal progenitors to generate RGCs without being exposed to mitogens. Pregnant rats were injected with BrdU to tag the progenitors in vivo. E18 retinal cells were harvested, an aliquot of cells was processed directly for immunocytochemical analysis, and the rest were cocultured with E3 chick retinal cells for 5 d. BrdU-tagged neurospheres were used as control; cell dissociates from mitogen-exposed neurospheres were cultured in the presence of 1% FBS for 5 d before immunocytochemical analysis. In E18 retinal dissociates that were analyzed immediately after harvest, BrdU-positive cells expressing RGC markers were not observed, suggesting that late retinal progenitors do not give rise to RGCs in vivo (data not shown). However, when freshly dissociated E18 retinal progenitors were cocultured with E3 chick retinal cells, a small proportion of BrdU-positive cells was observed that expressed RPF1 (3.09 ± 0.30%) and Islet1 (5.2 ± 0.53%), suggesting that a subset of late retinal progenitors may possess the potential to give rise to RGCs but are constrained in vivo, and that the culture condition promotes this potential (Fig. 8A). In contrast, a higher proportion of cells obtained from neurospheres expressed RPF1 (18.70 ± 1.22%) and Islet1 (10.17 ± 1.00%), suggesting that the subset of late retinal progenitors that possess the potential to generate RGCs is amplified during the enrichment process involving mitogens. To investigate further, if the in vivo environment has a negative influence on the ability of late retinal progenitors to generate early-born neurons, enriched late retinal progenitors were transplanted intravitreally into a 10-d-old rat pup containing the P23H mutation in the opsin gene that leads to retinal degeneration. We showed that retinas containing genetic or traumatic injury promote incorporation of grafted cells in the laminar structure (Chacko et al., 2003). Grafted progenitors were observed in the retina that expressed RPF1, suggesting their differentiation along RGC lineage (Fig. 8B–F). However, the proportion of cells that expressed RPF1 was significantly less than those in vitro (0.13 vs 20%; p < 0.001), suggesting that late retinal progenitors are constrained from differentiating into RGC in vivo.

Differentiation of late retinal progenitors into RGCs involves Notch signaling

Several lines of evidence suggest that Notch signaling is involved in the specification of RGCs. First, both the Notch1 receptor and its ligand Delta1 are expressed during the genesis of RGCs (Ahmad et al., 1995, 1997), and second, functional analyses of Notch1 and Delta1 have demonstrated that the temporal and spatial specification of RGCs is regulated by Notch signaling (Austin et al., 1995; Ahmad et al., 1997). We were interested in knowing whether the differentiation of late retinal progenitors into RGCs in vitro involves Notch signaling. Levels of transcripts corresponding to Notch1 and Delta1 were analyzed in late retinal progenitors, cultured either in the presence of FBS or E3 chick retinal cells. In addition, we analyzed levels of Ath5 and Brn3b mRNA. Ath5, a bHLH transcription factor, is required for the genesis of RGCs (Brown et al., 2001) and regulates Brn3b, a POU domain transcription factor, expressed during early differentiation of RGCs (Xiang et al., 1993; Gan et al., 1996, 1999). The levels of Notch1 and Delta1 transcripts were observed to be increased in E18 retinal progenitors in coculture condition compared with those of the FBS control, suggesting that Notch signaling is enhanced during their differentiation into RGCs (Fig. 9A, B). There was a corresponding increase in the levels of Ath5 and Brn3b transcripts that, together with the observation of an increase in Notch signaling, suggests that mechanisms involved in the genesis of RGCs in vivo are recruited by late retinal progenitors during their differentiation into RGCs in vitro. Evidence suggests that, during RGC differentiation, there is a relative increase in Notch signaling in progenitors: first, to maintain their uncommitted state, and second, to accentuate their difference from differentiating precursors (Ahmad et al., 1997). To test this notion in the context of differentiation of late retinal progenitors into RGCs, progenitors and postmitotic precursor populations were isolated using the Hoechst dye efflux assay (Bhattacharya et al., 2003). This assay separates the two populations on the basis of their relative ability to exclude the Hoechst dye; retinal progenitors that exclude the dye constitute the SP, whereas postmitotic precursors and differentiated cells with relative inability to exclude the dye form the NSP (Fig. 9C). The levels of Notch signaling in these two populations were evaluated by measuring levels of transcripts corresponding to Notch1, Delta1, and Hes1, a transcriptional repressor activated in response to Notch signaling (Fig. 9D). Whereas transcripts corresponding to Notch1, Delta1, and Hes1 are expressed in the SP cells, those corresponding to Delta1

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**Figure 4.** Diffusible factors secreted by E3 chick retinal dissociate influence late retinal progenitors to express multiple RGC-specific transcripts. Immunocytochemical results were corroborated by RT-PCR analysis in late retinal progenitors (A). The levels of Islet1, RPF1, Thy1, and Brn3b) increased in late retinal progenitors cocultured with E3 chick retinal cells across a membrane (lane 2) compared with those cultured in the presence of 1% FBS (lane 1). Culture of late retinal progenitors with E3 chick-conditioned medium influenced late retinal progenitors to express multiple RGC markers such as Islet1 and RPF1 (B–E), arrow), and Islet1 and Brn3b (F–I, arrow). Scale bar, 20 μm.
assessed by Student’s test. Scale bar, 100 μm.

are detectable in the NSP cells, suggesting that Notch signaling is relatively enhanced in the progenitor population, presumably to maintain the progenitor population and promote gradual differentiation between progenitors and precursors that have decided to become RGCs.

**Discussion**

In the retina, neurons belonging to specific types are born in two distinct stages of histogenesis in a lineage-independent manner. The temporal segregation in the generation of different types of neurons indicates that progenitors that define early and late stages of histogenesis are intrinsically different (i.e., they have distinct competence to respond to stage-specific epigenetic cues). This notion presupposes that, as development proceeds, progenitors progressively express different combination of genes that allow them to read epigenetic cues of a particular stage, and it is this communication between cell-intrinsic and cell-extrinsic factors that ultimately specifies the fate of a progenitor at a given stage. Consequently, early retinal progenitors are incompetent to give rise to late-born neurons even when exposed to an environment that promotes generation of the latter (Morrow et al., 1998; Belliveau and Cepko, 1999; Rapaport et al., 2001) and vice versa (Belliveau et al., 2000). A similar developmental constraint on progenitors to give rise to specific neuronal types has been observed in the cerebral cortex, in which cells of different cortical layers are generated in an inside-out manner, in a strict temporal sequence (O’Rourke et al., 1995). As in the developing retina, the late cortical progenitors fated to become neurons of superficial layers were found to be incompetent to generate early-born deep-layer neurons when transplanted in younger ferret hosts (Frantz and McConnell, 1996).

The observations from the retina and cerebral cortex suggest that the competence of the retinal progenitor changes irreversibly as development ensues. Alternatively, the change in competence may not be irreversible, but progenitors are constrained by overwhelming epigenetic influence from giving rise to any other neuronal types than those born during that particular stage. To test this notion, we investigated the ability of enriched late retinal progenitors to give rise to early-born neurons, specifically RGCs, in vitro. We observed that a small subset of late retinal progenitors, enriched from E18, PN1, or PN3 retinas, possesses the capacity to generate cells that display RGC phenotypes. These progenitors, like their early retinal counterparts, respond favorably to cues present at early histogenesis. These cues, yet unknown, may play a critical role in promoting the differentiation of RGCs in vivo. This notion is supported by the fact that they are evolutionarily conserved, because cells removed from the early stage of retinal histogenesis, either from chick or rat, have a similar RGC-promoting effect on late retinal progenitors in vitro. Remarkably, cues emanating from these cells in the early stage of histogenesis have a potent inhibitory effect on the differentiation of late-born neurons. A similar rod-inhibitory influence of embryonic retina, partly mediated through the CNTF family of cytokines, has been reported recently (Belliveau et al., 2000). These observations suggest that, although the environment in the early stages of histogenesis is naturally geared toward promoting the specification of early-born neurons, it also ensures that premature differentiation of late-born neurons does not take place. Whereas cues present in the early stages of histogenesis promote RGC differentiation, those present in the late stages have a negative influence on the differentiation of progenitors along the RGC lineage. Waid and McLoon (1998) have identified a factor secreted by matured chick RGCs that actively prevents progenitors from choosing the RGC fate. It is therefore likely that the late retinal progenitors, fated to generate late-born neurons, are constrained from giving
rise to RGCs under the influence of such factors (see below). The presence of inhibitory factors may also explain why the differentiation of enriched late retinal progenitors into RGCs is severely compromised when transplanted into the retina.

The differentiation of late retinal progenitors into RGCs involves the recruitment of mechanisms that are used during the normal development of RGCs. This notion is supported by the following observations. First, Notch signaling, which has been shown to play a critical role in both temporal and spatial specification of RGCs (Ahmad et al., 1995, 1997; Austin et al., 1995), is activated in late retinal progenitors when cocultured with cells from an early stage of retinal histogenesis. The levels of components of Notch signaling, the \( \text{Notch1} \) receptor and the downstream effector \( \text{Hes1} \), are increased during RGC differentiation. However, like that observed in vivo, there was a relative difference in the levels of Notch signaling between progenitors and committed precursor populations, as suggested by the difference in the levels of transcripts corresponding to components of Notch signaling in progenitor population (SP cells) and the precursor population (NSP cells). This difference in Notch signaling between progenitors and RGC precursors may accentuate the phenotypic differences between the two (Ahmad et al., 1997). It is likely that proneural genes are upregulated in response to a decrease in Notch signaling in RGC precursors, one of the effects of which is an increase in the levels of Delta1 (Kunisch et al., 1994; Heitzler et al., 1996). These precursor cells that overexpress Delta1 may accentuate Notch signaling in the neighboring progenitors, thereby amplifying initial minor differences between the two cell populations; cells in which Notch signaling is relatively weak differentiate as RGCs, whereas cells in which Notch signaling is relatively strong remain uncommitted retinal progenitors (Tanabe and JesSELL, 1996; Ahmad et al., 1997; Bertrand et al., 2002). The increase in the levels of \( \text{Delta1} \) mRNA in SP cells, although it appears contradictory in the presence of enhanced Notch signaling, is
likely in response to a possible parallel signaling used by progenitors to ensure their proliferation and noncommitted state. One such signaling may involve the EGF receptor (Ahmad et al., 1998a). It has been shown recently that the activation of the Ras-MAPK (mitogen-activated protein kinase) pathway, a downstream component of signaling through the EGF receptor, leads to upregulation of Delta in the progenitor population in Drosophila (Carmena et al., 2002). It is likely that Delta1 expressed by the progenitor population further reinforces Notch signaling (Karanu et al., 2001). Second, the changes in the levels of Notch signaling during RGC differentiation were accompanied by an increase in the levels of a regulator of RGCs, Ath5, and its target, Bmr3b. Evidence has emerged from a variety of experimental approaches that Ath5 is a key regulator of RGC differentiation (Vetter and Brown, 2001). Expression of Ath5 coincides with RGC genesis (Kanekar et al., 1997; Brown et al., 1998; Matter-Sadzinski et al., 2001), and experiments on both the loss and gain of Ath5 function have demonstrated that Ath5 is required for the generation of RGCs (Kanekar et al., 1997; Brown et al., 2001; Wang et al., 2001). Evidence suggests that one of the targets of Ath5 in RGC precursors is Bmr3b, a known regulator of RGC differentiation and survival (Xiang et al., 1993; Gan et al., 1999). Therefore, our observations suggest that a subset of late retinal progenitors use both Notch signaling- and Ath5-related cascade reactions for differentiation along RGC lineage. Although the exact nature of cooperation between the two mechanisms during RGC differentiation is not known, a recent study of cell fate specification in the ciliary margin zone of Xenopus retina shed light on their interactions; activation of Notch signaling promotes cell cycle exit that potentiates the RGC determination activity of Ath5 (Ohnuma et al., 2002).

An interesting question that emerges from our study is how a small subset of late retinal progenitors maintains its competence to differentiate into early-born neurons. This can be explained on the presumption that the developmental change in the competency of retinal progenitors is not absolute. As development progresses, the progenitors acquire the ability to respond more favorably to cues that promote the generation of late-born neurons than to those conducive for giving rise to early-born neurons. This premise presupposes that the late retinal progenitors may have a residual ability to generate RGCs, but odds are heavily against such specification as they find themselves inundated with cues for the generation of late-born neurons and inhibitory factors for RGC differentiation, elaborated by early-born neurons such as the RGCs (Waid and McLoon, 1998). Enrichment of late retinal progenitors in vitro is likely to dilute these negative environmental influences, thus removing the impediments toward their differentiation into RGCs. A similar selection of alternate fates has been observed in the case of ectodermal stem cells/progenitors that decide to differentiate into RGCs. A similar selection of alternate fates has been observed in the case of ectodermal stem cells/progenitors that decide to differentiate into RGCs. A similar selection of alternate fates has been observed in the case of ectodermal stem cells/progenitors that decide to differentiate into RGCs.
Additional evidence that these cells belong to the late progenitor population and may not represent a special, more plastic subpopulation set aside during early histogenesis comes from the observation that their number decreases progressively as histogenesis proceeds toward completion. As late histogenesis continues, cells are likely to be drawn from this subpopulation to generate late-born neurons and Müller glia, thereby progressively depleting their number and, hence, a progressive decrease in the number of RGCs born under the influence of E3 chick retinal cells. Besides, even at the late stage of differentiation, precursors that are fated to become rods are not irreversibly committed to the photoreceptor lineage. This notion is supported by the observation that prospective rods, even when they become postmitotic, could be respecified by epigenetic factor CNTF along a completely different lineage to give rise to bipolar cells (Ezzeddine et al., 1997). Together, our observations suggest that late retinal progenitors may not be irreversibly committed to give rise to late-born neurons. The repertoire of genes expressed by them at that stage makes them partial toward cues that promote the specification of late-born neurons. Their residual potential to give rise to early-born neurons is likely to be counterbalanced by inhibitory factors elaborated by mature early-born neurons. Thus, late retinal progenitors may appear irreversibly fated under constraints dictated by epigenetic cues.

References


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