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Neurogenin3 promotes early retinal neurogenesis

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Abstract

The transcriptional regulatory network governing the establishment of retinal neuron diversity is not well delineated. We report experimental results suggesting proneural gene *neurogenin3 (ngn3)* participating in this regulatory network. Retinal expression of chick *ngn3* was confined to early neurogenesis. Overexpression of *ngn3* in chick retina reduced cell proliferation and expanded the population of ganglion cells into the territory normally occupied by amacrine cells. *Ngn3* overexpression altered the expression of a number of regulatory genes, including *ash1*, *ath3*, *ath5*, *chx10*, *neuroD*, *ngn1*, *ngn2*, and *NSCL1*. Early gene *ngn1* was induced, but *ash1*, *ngn2*, *ath3*, and *chx10*, whose expressions persist through later phases of neurogenesis, were down-regulated. Expression of *ath5* was up-regulated at the locale corresponding to young ganglion cells, but was down-regulated at the locale corresponding to progenitor cells. These results suggest that *ngn3* regulates retinal neurogenesis by inducing regulatory genes for early-born neurons and repressing those for later-born cells.

Keywords

Transcriptional regulation; bHLH proneural gene; Retinal neurogenesis; Retinal ganglion cells

Introduction

It is unclear how the vertebrate retina achieves a balanced production of its diverse cell types, although the genesis of the different types of cells is known to progress in a stereotyped temporal and spatial order that is conserved among vertebrates (Adler, 2000). Retinal ganglion cells are born first, followed by horizontal cells, cone photoreceptors, amacrine cells, rod photoreceptors, bipolar neurons, and finally Müller glia (Rapaport et al., 2004). Spatially, newborn cells accumulate at their prospective locations, and as the retina matures, they organize into a well defined laminar structure and establish a complex neural circuitry communicating the visual signals initiated by photoreceptors to the brain through the axons of ganglion cells. Studies show that proliferating progenitor cells have some flexibility in adopting a final fate. For example, selective ablation of one neuronal cell type causes more progenitors to adopt that particular fate (Raymond, 1991; Reh, 1991). In addition, early born neurons influence future cell production (Waid and McLoon, 1998; Zhang and Yang, 2001), possibly by affecting progenitor cell proliferation (Mu et al., 2005). At the same time, experiments demonstrate that retinal progenitors show intrinsic variations in their fate potentials (Braisted et al., 1994; Belliveau et al., 2000; Cayouette et al., 2003). Furthermore, lineage-bias (Huang and Moody, 1997; Alexiades and Cepko, 1997) and lineage-dependence (Otteson and Hitchcock, 2003) have been demonstrated. The transcriptional regulatory mechanisms that

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direct the seemingly rigid yet flexible genesis of neural diversity, and thus ensure a balanced production of the different types of retinal cells, are largely unknown.

Genes encoding the basic helix–loop–helix (bHLH) family of transcriptional factors homologous to the *Drosophila* proneural genes *achaete-scute* and *atonal* play important roles in the generation of neuronal diversity in both the central and the peripheral nervous systems (CNS and PNS). Retinal neurogenesis employs several proneural genes, such as *achaete-scute* homologue 1 (*ash1*), atonal homologue 3 *(ath3), ath5, neuroD, neurogenin2 (ngn2), NSCL1*, and *NSCL2* (for reviews, see Vetter and Brown, 2001; Yan et al., 2005).

The mammalian *ngn* subfamily currently has three members; all are expressed in the developing CNS and PNS (Sommer et al., 1996). *Ngn1* and *ngn2* are well studied in neural development. *Ngn3*, on the other hand, is mostly known as a proendocrine factor that determines which precursor cells will become insulin-producing endocrine cells of the islets of Langerhans in the developing pancreas. Reports on *ngn3*'s role in the development of the nervous system are limited. In rodents, *ngn3* is expressed in glial precursors in the developing spinal cord (Liu et al., 2002) and regulates glial differentiation (Lee et al., 2003).

We have identified a chick bHLH gene homologous to mammalian *ngn3*. We found that retinal expression of chick *ngn3* was transient and restricted to early neurogenesis. Overexpression of *ngn3* affected the expression of a number of regulatory genes: those for early born neurons were up-regulated and those for later born cells were down-regulated. Furthermore, *ngn3* altered the expression of regulatory genes in a complex manner, not only activating or repressing specific genes, but also activating and repressing the same gene depending upon the anatomical location. Our study indicates that *ngn3* regulates retinal neurogenesis at an early point of an intricate transcriptional network that links diverse cell types in the developing retina.

Results

Ngn3 was transiently expressed in early retinal development

When mouse *ngn3* was used as a query to BLAST search the chick genome, the highest sequence identity (85%) was found to a single locus. This locus contained a bHLH domain that was 91% identical to mouse and human Ngn3 (Fig. 1). The full-length polypeptide deduced from the corresponding open reading frame consisted of 186 amino acids and shared 52% overall identity with mouse and human Ngn3 proteins. Because a Blastp search in NCBI's non-redundant database with the chick polypeptide showed Ngn3s from different species as the most homologous sequences (with next in line being Ngn2s from various species), the chick sequence was referred to as chick *ngn3*.

In situ hybridization was carried out to examine *ngn3* expression in the developing chick retina. Considering that retinal neurogenesis is mostly active between embryonic day 4 (E4) and E7, we first examined *ngn3* expression during these stages. At E4, *ngn3* expression was detected in cells localized to the outermost portion of the neuroepithelium (Figs. 2C–E), with a few found in the prospective location of ganglion cells (Fig. 2E, arrow). This pattern of expression was observed in the peripheral region (Fig. 2D) and the central region (Fig. 2E) and in E5 retina (Fig. 2F). By E6, *ngn3* expression became undetectable (Fig. 2G), except at the periphery, where development lags behind. In the peripheral retina at E10, only a few cells at the tip of the retinal neuroepithelium showed detectable *ngn3* expression, and these cells, like those expressed in E4 and E5 retinas, localized to the RPE side (Fig. 2H). To determine whether the spatially defined expression also occurred in retina younger than E4, we subsequently examined *ngn3* expression in retinas of E2 and E3. Expression of *ngn3* was detected in E2 retina, in cells confined to the apical side of the retinal neuroepithelium (Fig. 2A, arrow). At E3, some *ngn3*-expressing cells localized to the vitreal side where prospective ganglion cells

reside, while others remained on the RPE side (Fig. 2B, arrows). Expression of *ngn3* was also detected in the RPE (Figs. 2B, D, E, arrowheads).

The location of *ngn3*-exprssing cells on the apical side of the retinal neuroepithelium coincided with where M-phase cells reside. To examine whether cells undergoing mitosis (M-phase) expressed *ngn3*, double-labeling for *ngn3* mRNA and for phosphorylated histone H3 was carried out. Double-labeled cells were detected at E4 (Figs. 3A–C, arrowheads) and in E5 retina (Figs. 3D–F, arrowheads), when cell proliferation activity increases.

Double-labeling for *ngn3* expression and BrdU incorporation was carried out to determine whether *ngn3*-expressing cells were mostly postmitotic. Double-labeled cells were observed (Figs. 3G–I, arrowheads). The majority of the double-labeled cells localized to the outer most portion, where it accounted for more than 50% of *ngn3*-expressing cells, indicating that at least half of the *ngn3*-expressing cells at this location were still in the cell cycle. On the vitreal side, fewer than 10% of *ngn3*-expressing cells were $BrdU^+$.

The retinal neurogenesis involves several bHLH genes, such as *ath5*, *ngn2*, and *ash1* (Matter-Sadzinski et al., 2005; Vetter and Brown, 2001; Yan et al., 2005). To facilitate an understanding of how *ngn3* related to these bHLH genes, we compared the spatial pattern of *ngn3* expression with that of *ath5*, *ngn2*, and *ash1* using serial sections of E2 and E3 chick heads. While retinal expression of *ngn3* was detected in cells mostly localized to the apical side at E2 (Fig. 4A), expression of *ath5* at this early stage was detected only in a few cells in the central region within the retina (Fig. 4B, arrow). Expressions of *ngn2* (Fig. 4C) and *ash1* (Fig. 4D) at E2 were not detected in the E2 retina, even though their expression was evident in discrete domains of the developing brain (Figs. 4C, D, arrows). In E3 retina, while *ngn3* expression was visible in cells either at the RPE side or on the vitreal side in both central and periphery regions (Fig. 4E), the expression of *ath5*, *ngn2*, and *ash1* was detected in cells of the central region (Figs. 4F–H) within the retinal neuroepithelium. The difference in the spatial patterns of expression indicated that ngn3-exprssing cells belonged to a sub-population different from the *ath5*-, *ngn2*-, or *ash1*-expressing cells. The evident expression of *ngn3* in E2 retina and at the peripheral region of E3 retina suggested an earlier expression of *ngn3* than that of *ath5*, *ngn2*, and *ash1*.

Ngn3 overexpression altered retinal neurogenesis

Replication-competent retrovirus RCAS was used to drive overexpression and ectopic expression of *ngn3* in chick embryos. Concentrated virus was microinjected into the subretinal space, which is still connected with the neural tube at E2. This procedure produces widespread infection within and outside the nervous tissues of the embryo (Yan and Wang, 2001), as a result of both primary infections and subsequent infections by virus released from infected cells. The widespread RCAS viral infection and transduction do not affect retinal development; we have observed no abnormalities either at the gross level or at the microscopic level from hundreds of embryos infected with RCAS or RCAS-GFP (Yan and Wang, 2001; Li et al., 2002; our unpublished data). Embryos receiving RCAS-ngn3 survived to E6, but nearly all died between E7 and E8. Out of more than 100 embryos, one survived to E8, and this surviving embryo was found to have limited RCAS-ngn3 infection.

Overexpression of *ngn3* resulted in small eyes (Fig. 5A). Small eyes were observed in >95% of the embryos receiving RCAS-ngn3 ($n \geq 100$), including dead or dying ones. On average, the total number of cells in an E7.5 retina from RCAS-ngn3 infected embryo was reduced to ~50% of that in the control infected with RCAS-GFP. No small eye was observed with similar overexpression of bHLH genes *neuroD* (Yan and Wang, 1998) and NSCL2 (Li et al., 2001) or with similar overexpression of more than 10 homeodomain genes (Li et al., 2002; our unpublished data). To determine whether the small eye from *ngn3* overexpression was due to

enhanced cell death, or reduced cell proliferation, or both, BrdU incorporation and TUNEL assay were carried out. To minimize ambiguities arising from comparing retinas of varied cell proliferation activities due to difference in developmental stages, we compared the number of $BrdU^+$ cells in regions infected by RCAS-ngn3 with adjacent, uninfected regions on the same retinal section. We found that the infected regions contained fewer $BrdU^+$ cells in comparison with the internal control (the adjacent, uninfected region) in E7.5 retina (Figs. 5D, E). On average (10 areas of similar size), the number of $BrdU^+$ cells was reduced by more than 60% in infected region. Reduction of $BrdU^+$ cells was also evident at an early stage, E5 (Figs. 5B, C).

In normal retina, few cells are $TUNEL^+$ before E10, when developmental ganglion cell death commences (Cook et al., 1998). In E7.5 retinas infected with either RCAS-GFP (Figs. 5F, G) or RCAS-ngn3 (Figs. 5H, I), the number of $TUNEL^+$ cells was very low. From a total of 7 retinal cross-sections, 5 cells were found $TUNEL^+$ in retina infected with RCAS-ngn3, while 4 were $TUNEL^+$ in the control infected with RCAS-GFP.

Analyses of the cellular composition with specific markers showed altered retinal neurogenesis. The domain of cells expressing ganglion cell markers Islet-1 and Brn3A was expanded, and the expansion was observed with all embryos examined that showed nearly complete viral infections ($n \geq 5$). To avoid ambiguities from comparing different retinal sections that potentially differed in sectioning angle as well as in developmental stage, we used adjacent, uninfected regions in the same retinal section as internal controls. Within the regions infected with RCAS-ngn3, $Islet\text{-}1^+$ domain was expanded in comparison with the adjacent, uninfected region, and the expansion was evident at E5 (Figs. 6A, B) and E7.5 (Figs. 6C, D). The extent of expansion varied, from discernible thickening of this particular cell layer to its occupying nearly 1/3 the thickness of the retina (Fig. 6D). The variation was attributed to varied amounts of Ngn3 protein accumulated in the infected cells as a function of variations in the time of viral infection and in the number of viral insertions into host chromosomes. Expansion of $Brn3A^+$ domain was also observed in infection regions (Figs. 6E, F). Additionally, the domain of developing ganglion cells that transiently expressed *ath5* and *NSCL1* was also expanded (Fig. 7). In contrast to these expansions, domains of cells expressing amacrine cell markers Ap2α (Figs. 6G, H) and the vesicular inhibitory amino acid transporter (VIAAT, data not shown) were reduced in comparison to the control (Figs. 6I, J).

For a quantitative analysis, retinas from 3 infected embryos were pooled and their cells dissociated. After seeding for 4 h in a cell culture incubator, cells were fixed and stained with markers that identify different retinal cell populations. Scoring cell numbers revealed a 3-fold reduction in the number of $AP2\alpha^+$ cells, from 13.6% of total cell present in the control to 4.0% in the experimental retinas (Fig. 6K), while the number of $Brn3A^+$ cells was doubled, from 2.9% of total cells in the control to 6.1% in samples infected with RCAS-ngn3 (Fig. 6K). Note that because percentage of total cells is used, changes here reflect both a net increase in the number of a particular type of cells and the reduction of total retinal cell population from *ngn3* promoting cell cycle exit.

Cone genesis trails that of ganglion cells and is the dominant photoreceptor cell type in the chick. Cone photoreceptor cells were identified by specific antibodies against visinin, a calcium-binding protein (Yamagata et al., 1990). No significant changes in the number of photoreceptor cells were observed with either retinal cryosections or dissociated retinal cells, even though the territory of *neuroD* expression was markedly expanded (Figs. 10E, F) and the number of *neuroD*-expressing cells tripled (Fig. 6K). The death of embryos before E8 precluded in ovo analyses of bipolar and Müller glia populations.

Overexpression of ngn3 altered the expression of a number of regulatory genes

The confined *ngn3* expression to early retinal development suggested the possibility that *ngn3* is an early bHLH gene at an initial point of a transcriptional network regulating neuron diversity in the retina. To examine this possibility, we analyzed retinas infected with RCAS-ngn3 for alterations in the expression of genes encoding transcription factors known or implicated to participate in the development of different cell populations. We found alterations in the expression of a number of regulatory genes. Overall, genes associated with early retinal neurogenesis, including ganglion cell production, were up-regulated, and genes involved in progenitor cells and/or later born cells were suppressed or down-regulated. Because the developing retina is a pseudo-stratified structure within which young neurons accumulate at their prospective locations, alterations in gene expression conspicuously exhibited distinctive topographical patterns: (i) up- or down-regulation that occurred more or less evenly across the retina, (ii) up- or down-regulation that was spatially confined, and (iii) simultaneous up-regulation at one topographic location and down-regulation at another. These alterations were observed in all retinas examined that had near complete viral infection ($n \geq 5$). Among the embryos, the extent of alterations varied, and the variation was attributed to varied amounts of Ngn3 protein accumulated in the infected cells due to the nature of the viral vector.

Up-regulation of genes for ganglion cell development

A large number of studies have shown that ganglion cell development requires *ath5*, a bHLH gene predominantly expressed in the retina (for reviews see Vetter and Brown, 2001; Mu and Klein, 2004). *Ath5* also participates in the genesis of other retinal cells (Yang et al., 2003; Ma et al., 2004). In a normal retina, *ath5* expression spans more or less the entire process of neurogenesis and mostly in two zones of cells, with the outer zone adjacent to the prospective photoreceptor layer and the inner zone coinciding with young ganglion cells or their precursors (Liu et al., 2001; Brown et al., 2001; Ma et al., 2004) (Fig. 7A). Infection with RCAS-GFP did not alter the two zones of *ath5* expression (Figs. 7C, E). In retinas infected with RCAS-ngn3, the inner zone of *ath5* expression, which corresponded with ganglion cell development, was expanded (Figs. 7B, D, F). On the other hand, the outer zone of expression, presumably in multipotent progenitor cells (Yang et al., 2003), was diminished (Figs. 7B, D, F). The increase in the inner zone and the repression in the outer zone of *ath5* expression were apparent at both the peripheral retina (Figs. 7C, D) and the central retina (Figs. 7E, F), and also in E5 retina (Figs. 7G, H). Thus, *ngn3* enhanced ganglion cell-related *ath5* expression and at the same time suppressed progenitor cell-related *ath5* expression.

Another bHLH gene involved in ganglion cell development is *NSCL1*, which is distantly related to *atonal* and is expressed transiently in young ganglion cells during retinal neurogenesis and later in Műller cells (Li et al., 1999). *Ngn3* overexpression increased the *NSCL1*-expressing domain corresponding to young ganglion cells (Figs. 7I, J). Scoring cell numbers after dissociation of retinal cells showed a doubling in the number of *NSCL1*-expressing cells (Fig. 6K).

Induction of early proneural gene ngn1

In other regions of the CNS, *ngn1* plays a determinative role in generating neural diversity (Bertrand et al., 2002; Ross et al., 2003). In the chick retina, expression of *ngn1* was spatially confined to cells localized to the outermost portion of the neuroepithelium (Fig. 8A). Temporally, *ngn1* expression was detected in early phases of retinal neurogenesis and became undetectable by E7 (Fig. 8B), even though neurogenesis is still active at this time. This spatial and temporal pattern overlapped with *ngn3* expression. In E7.5 retinas infected with RCAS-ngn3, *ngn1* mRNA was readily detected, except in a few small regions that appeared to lack viral infection (Fig. 8D). A comparison of the histological locations using serial sections of the same eye showed that the *ngn1*-expressing domains corresponded well with regions

infected with the virus, whereas regions remaining negative for *ngn1* expression corresponded with uninfected regions (Figs. 8C, D). The level of ectopic *ngn1* expression appeared to display a spatial gradient across the thickness of the retinal neuroepithelium, with the highest level detected on the vitreal side and lowest on the RPE side (Fig. 8D), diametrically different from the normal spatial location of *ngn1* expression (Fig. 8A). This induction of *ngn1* at ectopic locations and outside the time window of its normal expression indicates that *ngn3* played a positive role on *ngn1* expression. Induction of *ngn1* was also detected in the cortex (data not shown), in some RPE cells at the periphery, and in a subset of head mesenchymal cells (Figs. 8E, F). No induction of *ngn1* was detected in head epidermal cells (Figs. 8E, F).

Down-regulation of genes for progenitor cells

Ash1 plays a determinative role in generating neural diversity in other regions of the CNS (Bertrand et al., 2002; Ross et al., 2003). In the retina, *ash1* expression is detected during both early and late phases of neurogenesis in a subpopulation of retinal progenitor cells. Mouse *ash1* is thought to participate in the generation of later-born neurons, such as rod photoreceptors and bipolar cells (Tomita et al., 2000), and chick *ash1* was believed to also participate in the formation of amacrine cells (Jasoni et al., 1994; Mao and Wang, submitted). In E7.5 retina, expression of *ash1* is abundantly detected in cells within the proliferating zone at both the peripheral (Fig. 9A) and the central (Fig. 9B) retina. In retinas infected with RCAS-ngn3, *ash1* expression was absent, except at the places corresponding to regions lacking viral infection (Figs. 9C, D). Thus, *ash1* expression was specifically inhibited in regions infected with RCAS-ngn3. Inhibition of *ash1* expression by *ngn3* was also evident at E5 (Figs. 9E, F).

Ngn2 is the third proneural bHLH gene playing a determinative role in generating neural diversity in other regions of the CNS (Bertrand et al., 2002; Ross et al., 2003). In the retina, *ngn2* participates in the development of progenitor cells that eventually differentiate into all major types of retinal neurons (Marquardt et al., 2001; Yan et al., 2001; Akagi et al., 2004; Ma and Wang, 2006). Like *ath5* and *ash1*, *ngn2* expression spans early and late phases of retinal neurogenesis. In the control E7.5 retina infected with RCAS-GFP, *ngn2* was expressed in a large number of progenitor cells distributed more or less across the entire thickness of the retinal neuroepithelium (Fig. 9G). In RCAS-ngn3 infected retinas of the same age, however, *ngn2* was down-regulated. The down-regulation appeared location-specific and was confined to the inner portion, leaving a narrow zone at the outer portion where *ngn2* expression remained (Fig. 9H). This location-dependent resistance to *ngn3*'s suppression reflects the pseudo-stratified nature of the developing retina and could be due to either *ngn3* alone being insufficient to suppress *ngn2* expression or other genes/factors promoting *ngn2* at the particular location, or both.

Down-regulation of genes for progenitors and bipolar cells

Proneural gene *ath3* and homeodomain gene *chx10* play important roles in the development of progenitor cells and bipolar cells (Hatakeyama et al., 2001; Burmeister et al., 1996). Expression of *ath3* at E7.5 in control retina infected with RCAS-GFP was detected mostly in cells localized to the prospective location of bipolar cells (Fig. 10A). At this location in retinas infected with RCAS-ngn3, *ath3* expression was detected in far fewer cells (Fig. 10B). At another cellular layer, where prospective amacrine cells would reside, *ath3* was expressed in more cells than in normal retina (Figs.10A, B). Similarly, the number of cells expressing *chx10* was reduced at the outer retina, the normal locale of *chx10* expression at this stage, but increased at the inner retina (Figs. 10C, D).

Up-regulation of neuroD

Location-dependent regulation by *ngn3* was also observed with the expression of proneural gene *neuroD*. *NeuroD* plays an important role in photoreceptor production, development, and

survival (Yan and Wang, 1998, 2004; Pennesi et al., 2003) and in the differentiation of a specific cone subtype (Liu et al., 2008). In the chick retina, *neuroD* is predominantly expressed in young photoreceptor cells and their precursors residing on the RPE side (Yan and Wang, 1998, 2004). Infection with RCAS-GFP did not alter this pattern of expression (Fig. 10E). *Ngn3* overexpression expanded *neuroD* expression to territories normally occupied by progenitors or prospective inner neurons (Fig. 10F). Notably, no ectopic *neuroD* expression was induced by *ngn3* at the location of young ganglion cells, where ectopic induction of *ngn1* was conspicuous (Fig. 8D). This further indicates the intricacy of the regulatory network governing a balanced production of diverse cell types in the developing retina. When the retinas from 3 infected embryos were pooled, and their cells were dissociated and scored, the number of *neuroD*-expressing cells was found to be more than tripled, from 15.5% of total cells in the control infected with RCAS-GFP to 52.0% in samples infected with RCAS-ngn3 (Fig. 6K).

Discussion

From NCBI's data base, we identified a chick gene encoding a bHLH protein, whose closest homologues were Ngn3s from different species. Expression of chick *ngn3* was detected in the developing retina and in some cells of the non-neural RPE. Mammalian *ngn3* is well known for its critical role in endocrine development, where *ngn3* determines which precursor cells will become insulin-producing endocrine cells of the islets of Langerhans. In the developing chick retina, expression of *ngn3* was detected as early as E2, persisted through E5, and became undetectable at E6 (except at the periphery). Spatially, cells expressing *ngn3* were mostly localized to the apical side of retinal neuroepithelium, where M-phase cells reside, with some at the prospective ganglion cell layer. The presence *ngn3* mRNA$^+$/BrdU$^+$ and *ngn3* mRNA$^+$/phosphoH3$^+$ cells in the developing retina suggests that *ngn3* is expressed in a subset of proliferating and dividing progenitors. The spatial and temporal pattern indicates that *ngn3* is transiently expressed in early phases of retinal neurogenesis, because at E6, retinal neurogenesis in the chick is still active and the expression bHLH regulatory genes *ath5*, *ngn2*, and *ash1* remains high. The onset of *ngn3* expression appeared to precede that of *ath5*, *ngn2*, and *ash1*, as our comparative analysis using serial sections showed that cells expressing *ngn3* were present in E2 retina, which has only a few cells expressing *ath5* and no cells expressing *ngn2* or *ash1*. The differences in temporal and spatial patterns of expression of these bHLH genes are consistent with the possibility of *ngn3* playing a role in regulating the other bHLH genes.

The early expression of *ngn3* suggested a role in the genesis of early-born neurons. Indeed, overexpression of *ngn3* expanded the domain of cells expressing ganglion cell markers (Islet-1, Brn3A, *ath5*, and *NSCL1*). The expansion of Islet-1$^+$ domain and Brn3A$^+$ domain were detected in internally controlled retinal sections and, thus, may reflect a net increase in the number of cells expressing these markers. A reduction in total cell population due to *ngn3* promoting cell cycle exit is expected to affect the ratio of ganglion cells to the total number of retinal cells, not the infection region-specific expansion of domains observed in internally control retinal sections. An increase in the number of cells expressing ganglion cell markers could result from *ngn3* promoting premature cell cycle exit, thus increasing the population of cells competent to become ganglion cells. It could also be due to overexpression of *ngn3* promoting migration of *ath5*-expressing cells in the progenitor zone to the prospective ganglion cell zone and/or preventing the proper turnover of *ath5*, which is known to promote ganglion cell development. The expansion of ganglion cell property appeared to occur at the expense of progenitor cells and of amacrine cells. The reduction of amacrine properties might be related to diminished *ash1* expression. While most published studies show *ash1* participating in the production of later-born neurons, such as rod photoreceptors and bipolar cells, Jasoni et al. (1994) proposed a role of *ash1* in amacrine cell genesis in the chick retina, and our unpublished results support *ash1* promoting amacrine cell production in the chick. Notably, the

photoreceptor population remained unchanged in retinas overexpressing *ngn3*, even though the expression of *neuroD*, a key regulator of photoreceptor development, was expanded into the inner retina. This indicates that the *neuroD* expression induced at the ectopic location was insufficient to switch cell identities at these places. Other factors required in photoreceptor production may be lacking at these locations or, alternatively, *ngn3* also induced genes antagonistic to *neuroD* or inhibitory to photoreceptor genesis.

In the retina, *ngn3* altered the expression of a number of regulatory genes, including *ash1*, *ath3*, *ath5*, *chx10*, *NSCL1*, *neuroD*, *ngn1*, and *ngn2*. These genes are associated with a wide range of cell types, including progenitor cells, photoreceptor cells, bipolar cells, amacrine cells, and ganglion cells. Alteration in their expression implies a link between *ngn3* and the genetic programs of the major cell populations present in the developing retina. Overall, *ngn3* increased the expression of those regulatory genes that are involved in the production of early born cells and suppressed others that are for later born cells. Admittedly, some of the alterations in expression could be indirect. Notably, overexpression of *ngn3* altered the expression of all 3 proneural genes (*ash1*, *ngn1*, and *ngn2*) known in other parts of the nervous system to play determinative roles in generating neuronal diversities.

Complexity in the effect of *ngn3* on regulatory genes was indicated by the changes in regulation as a function of variations in histological locations within the pseudostratified retinal neuroepithelium. Overexpression of *ngn3* both induced and suppressed the expression of *ath3*, *ath5*, and *chx10*, depending upon the histological location. While the location-specific alteration in *ath5* expression can be linked to promoting ganglion cell properties, no such link is obvious for the alterations in *ath3* and *chx10* expression. The location-dependency indicates the importance of cellular context in affecting the manner in which *ngn3* regulated gene expression and suggest collaborations between *ngn3* and cellular context-defined factors in generating retinal neural diversity. Such a location- or context-dependency was also manifested in *ngn3*-induced *ngn1* induction: not all tissues were competent to *ngn3*'s induction, and not all cells in a “competent” tissue responded to *ngn3*'s induction of *ngn1*. The competency could reflect the presence of co-factors, an absence of inhibitory factors, or cross-regulation of bHLH genes.

The properties of *ngn3*, including its expression pattern and its remarkably intricate regulation of a large number of regulatory genes, indicate that *ngn3* may reside at an early point of a regulatory network that links diverse cell populations in the developing retina for a balanced production of various cell types, perhaps by promoting early born neurons at the expense of later born ones. The complex manner in which *ngn3* altered the expression of other transcriptional factors offers a glimpse into the intricate regulatory network operating during retinal neurogenesis.

Experimental methods

Chick embryos

Fertilized, pathogen-free White Leghorn chicken eggs were purchased from Spafas and incubated in a Petersime incubator. All use of animals adhered to the procedures and policies set by the Institutional Animal Use and Care Committee at the University of Alabama at Birmingham.

Identification of chick ngn3

The mouse *ngn3* sequence (Sommer et al., 1996) was used to search for a chicken homologue in GenBank. The highest identity (85%) was found in a region of 157 nucleotides encoding a bHLH domain in *Gallus gallus* chromosome UNK clone CH261-5M18. The deduced amino

acid sequence of the corresponding open reading frame was then compared to that of mouse and human Ngn3.

Generation of RCAS-ngn3 retrovirus

The coding region of chick *ngn3* was PCR amplified from E5 chick retina cDNA using primers ccatggccccgcagagcgac and gtcgactcagaggaaggcggggaag. The PCR product was cloned and its sequence verified. From shuttle vector Cla12Nco, a Cla I fragment containing the chick sequence plus the 5 untranslated sequence of the *src* oncogene was then inserted into proviral vector RCAS (Hughes et al., 1987). Virus particles were produced by transfecting chick embryonic fibroblast cells with the recombinant proviral DNA. Concentrated viral stocks (~8×10^8 pfu/ml) were prepared as described (Yan and Wang, 1998).

Microinjection of retrovirus into chick embryos

Concentrated virus RCAS-ngn3, or control virus RCAS-GFP (Yan and Wang, 1998), was microinjected into the neural tube and the “subretinal” space (between the two layers of the optic cup) of day 2 chick embryos (E2, stage 15–17), as previously described (Yan and Wang, 1998). At E5 and E7.5, infected eyes were enucleated and fixed with ice-cold 4% paraformaldehyde, cryoprotected with OCT:sucrose (2:1), and frozen with liquid nitrogen. Infection by RCAS viruses (RCAS-ngn3 and RCAS-GFP) was identified by immunostaining with a specific antibody against a viral protein p27.

Dissociated retinal cells

E7.5 retinas were dissected and pooled from 3 embryos receiving RCAS-ngn3 virus at E2, with retinas infected by RCAS-GFP as control. Retinal cellswere dissociated with trypsin-EDTA. Dissociate cells were resuspended in culture medium and seeded onto the bottom of polyornithine-treated 35-mm culture dishes. Because on the average the number of total retinal cells in an E7.5 retina infected with RCAS-ngn3 was reduced to ~50% of that in the control infected with RCAS-GFP, dissociate cells for experimental retinas were resuspended in half the volume of what was used for the control, to achieve equivalent final cell densities at seeding. After 4 h in a cell culture incubator with Medium 199 supplemented with 10% fetal calf serum, cells were fixed with ice-cold 4% paraformaldehyde, before being subjected to immunostaining or in situ hybridization. The number of the positive cells and the number of total cells were scored from 10 view areas, each with 200–470 cells, under a 20× objective. The percentage of positive cells was calculated for one dish, and the means and SDs from 3 dishes were calculated using the computer program Origin 7.0 (OriginLab Corp.).

In situ hybridization

The coding regions of *ngn3, ngn1* (Perez et al., 1999), *ash1* (Jasoni et al., 1994), and *VIAAT* (GenBank accession # BI393349) were PCR amplified, based on published sequence information, from cDNA prepared from embryonic chick retina. PCR products were cloned and their sequences verified. Linearized plasmids harboring the sequences were used to synthesize digoxigenin (dig)-labeled antisense RNA probes using the Genius kit (Roche Molecular Biochemicals) following the manufacturer's instructions. Dig-labeled antisense RNA probes against chick *ath3, ath5, chx10, neuroD, ngn2*, and *NSCL1*, were prepared as previously described (Yan and Wang, 1998; Li et al., 1999; Yan et al., 2001, Ma et al., 2004). Retinal cryosections of 8–10 μm thick were used for in situ hybridization following procedures described previously (Li et al., 1999).

Immunocytochemistry

Monoclonal antibody RA4 (used at a 1:1000 dilution) was a gift from Dr. Steven McLoon (University of Minnesota). The following monoclonal antibodies were obtained from the

Developmental Studies Hybridoma Bank (University of Iowa): anti-bromodeoxyuridine (BrdU, clone G3G4, 1:100; developed by Dr. Stephen J. Kaufman), anti-islet-1 (clone 39.4D5, 1:100; developed by Dr. Thomas Jessell), anti-LIM (clone 4F2, 1:50; developed by Dr. Thomas Jessell), anti-AP2α (3B5, 1:50; developed by Dr. Trevor Williams), and anti-visinin (clone 7G4, 1:500; developed by Dr. Constance Cepko). Antibodies obtained from a commercial source included: monoclonal antibody against Brn3a (1:200; Chemicon); polyclonal antibody against red opsin (1:200; Chemicon); anti-phospho-Histone H3 (1:200; Biotin conjugated; Upstate Biotechnology), and polyclonal antibody against an RCAS viral protein, p27 (1:500; Spafas). Standard immunocytochemistry was performed using the ABC kit from Vector Laboratories with secondary antibodies conjugated with peroxidase, alkaline phosphatase (Vector Laboratories), or fluorophore (Molecular Probes).

Pulse-labeling with BrdU and double staining

BrdU (50 μg in 50 μl of HBSS) was dropped through an opening in the shell onto the vitelline membrane of E5 and E7.5 chick embryos. The embryos were incubated for 4 h before the eyes were harvested and fixed with 4% paraformaldehyde. For double labeling, cryosections on glass slides were first subjected to in situ hybridization with diglabeled anti-*ngn3* RNA probes and then to BrdU detection using a specific antibody as previously described (Li et al., 1999).

Acknowledgments

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cNgn3  MAPQSDRAPAAGQPYLGGPDAAASPAAGGSAGAGSRGASPARSALPPREVA-------RRK
hNgn3  .T..PSG..TV-.VTRETERSFPRASEDEVTCPT.APP..T.TRGNCA.AEEGGCRGAP..
mNgn3  .A.H.LD.LTI-.VSPETQQPFPGASDHEVLSSN.TPP..TLIPRDCS.AEVGDCRGTS..

cNgn3  GLGKARRG-LGKARGEGPLSKQKRSRRMKANDRERNRMHHLNSALDALRSVLPTFPDDAKL
hNgn3  -.-R....GRSRPKS.LA....R....K...........N.........G...........
mNgn3  -.-R....GRNRPKS.LA....R....K...........N.........G...........

cNgn3  TKIETLRFAHNYIWALTQSLLLAEQGLP--EPPPPPPPAAVAAAASPGPWGSP-CP--AA
hNgn3  ..................T.RI.DHS.YAL...A.HCGELGSPGG...D...LYS.VSQ.
mNgn3  ..................T.RI.DHSFYGP...V.CGELGSPGGG.N.D...IYS.VSQ.

cNgn3  P--PPAAP-RRGP---------------AAFPAFL
hNgn3  GSLS...SLEER.GLLGATFSACLSPGSL..SD..
mNgn3  GNLS.T.SLEEF.GLQVPSSPSYLLPGALV.SD..
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Fig. 1.

The deduced amino acid sequence of chick *ngn3* (cNgn3) and its alignment with human Ngn3 (hNgn3) and mouse Ngn3 (mNgn3). Gaps, indicated by hyphens, are introduced for optimal sequence identities. Dots indicate identical residues in hNgn3 and mNgn3 to that in cNgn3, and the bHLH domain is underlined.

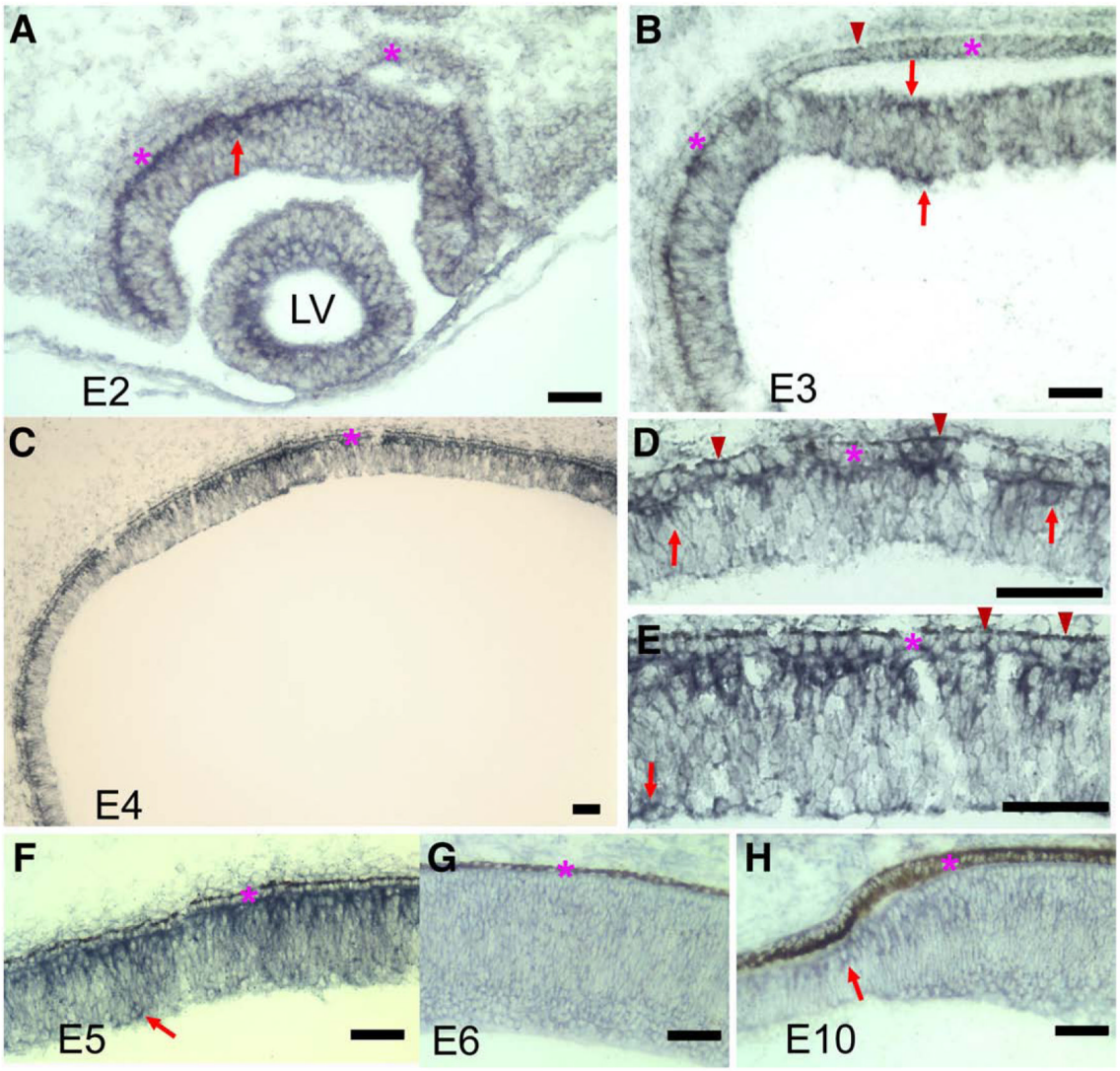

Fig. 2.

The expression of *ngn3* in the developing chick retina detected with in situ hybridization. (A, B) Cross-sections of an E2 eye (A) and an E3 eye (B). Arrows points to *ngn3*-expressing cells in the retina. Arrowhead points to a *ngn3*-expressing cell in the RPE. (C) E4 retinal neuroepithelium. (D) Higher magnification of a peripheral region of E4. Arrows point to *ngn3*-expressing cells in the retinal neuroepithelium. Arrowheads point to *ngn3*-expressing cells in the RPE. (E) Higher magnification of central region of E4. Arrow points to a *ngn3*-expressing cell in prospective ganglion cell layer. Arrowheads point to ngn3-expressing cells in the RPE. (F) E5 retinal neuroepithelium. Arrow points to a *ngn3*-expressing cell in the prospective ganglion cell layer. (G) E6 central retina. (H) the peripheral retina at E10. Arrow points to a *ngn3*-expressing cell at the tip of peripheral retina. The RPE layer is indicated by an asterisk. LV: the lens vesicle. Scale bars: 50 μm.

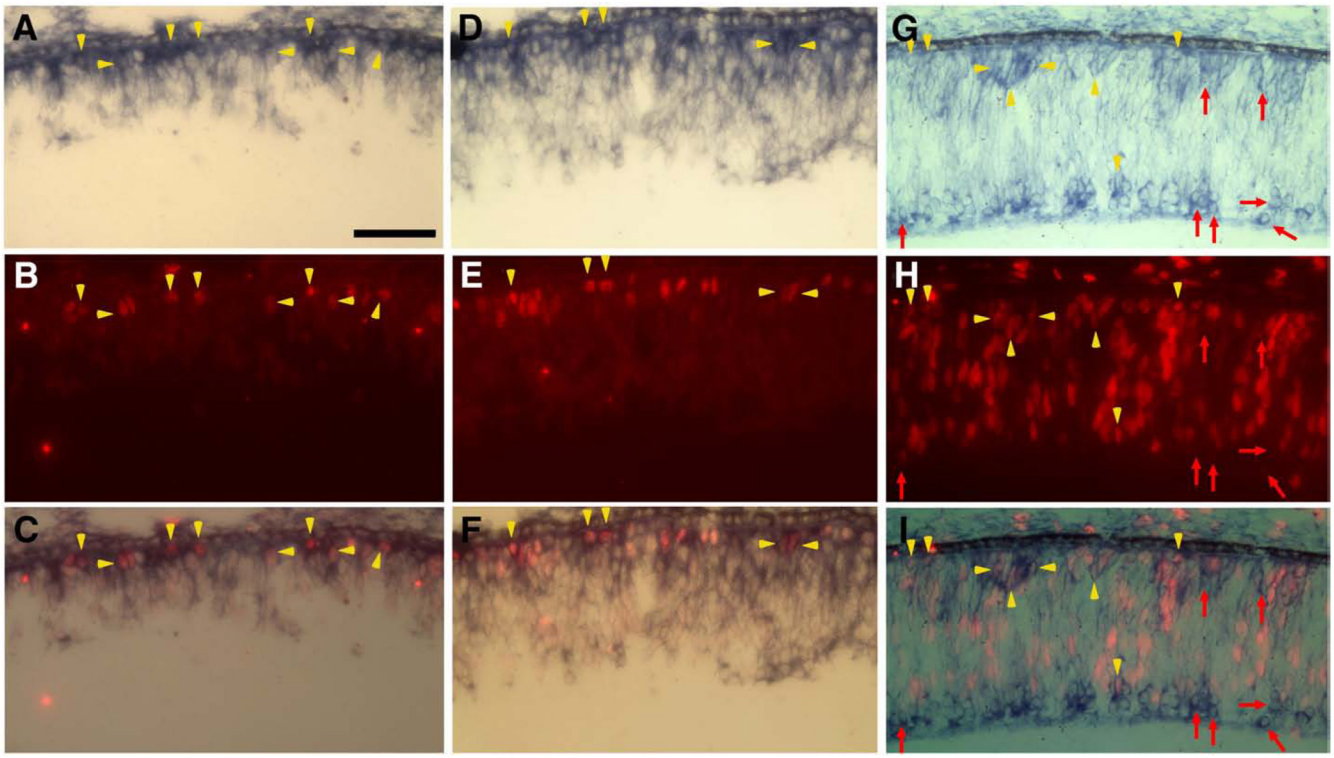

Fig. 3.

Double-labeling for *ngn3* expression and cell proliferation. (A–F) Double-labeling for *ngn3*mRNA (blue) and phosphoH3 (red) in E4 retina (A–C) and E5 retina (D–F). (A, D) In situ hybridization for *ngn3* mRNA. (B, E) Immunostaining for phosphoH3. (C, F) Simultaneous view of both. Yellow arrowheads point to double-labeled cells (*ngn3* mRNA$^+$/ phospoH3$^+$). (G–I) Double-labeling for *ngn3*mRNA (blue) and BrdU incorporation (red) in E5 retina. (G) In situ hybridization for *ngn3*mRNA. (H) immunostaining for BrdU incorporation. (I) Simultaneous view of both. Red arrows point to *ngn3*-expressing cells that lacked BrdU incorporation. Yellow arrowheads point to double-labeled cells (*ngn3* mRNA$^+$/ BrdU$^+$). Scale bars: 50 μm.

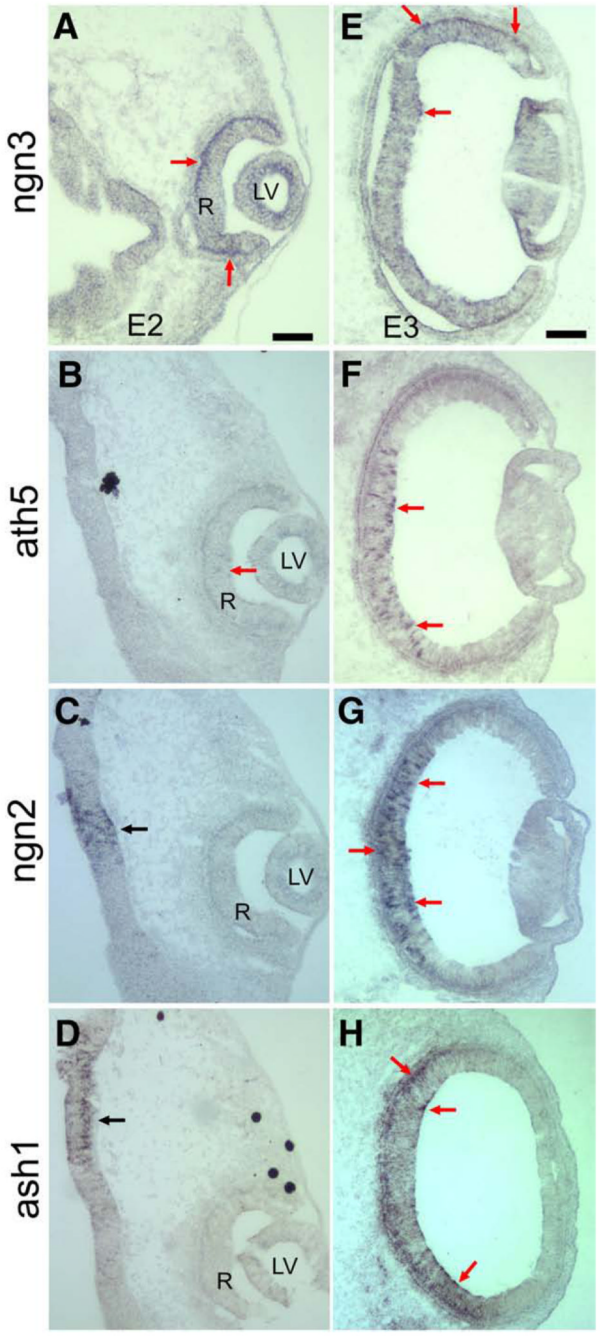

Fig. 4.

A comparison of the expression of *ngn3* with the expression of *ath5*, *ngn2*, and *ash1*. (A–D) Serial sections of an E2 embryo showing the expression of *ngn3* (A), *ath5* (B), *ngn2* (C), and *ash1* (D). Red arrows point to cells in the retinal neuroepithelium expressing the genes. (E–G) Expression of *ngn3* (E), *ath5* (F), *ngn2* (G), and *ash1* (H) in cross-sections of E3 eye. Arrows point to cells expressing the genes. LV: lens vesicle. R: retinal neuroepithelium. Scale bars: 100 μm.

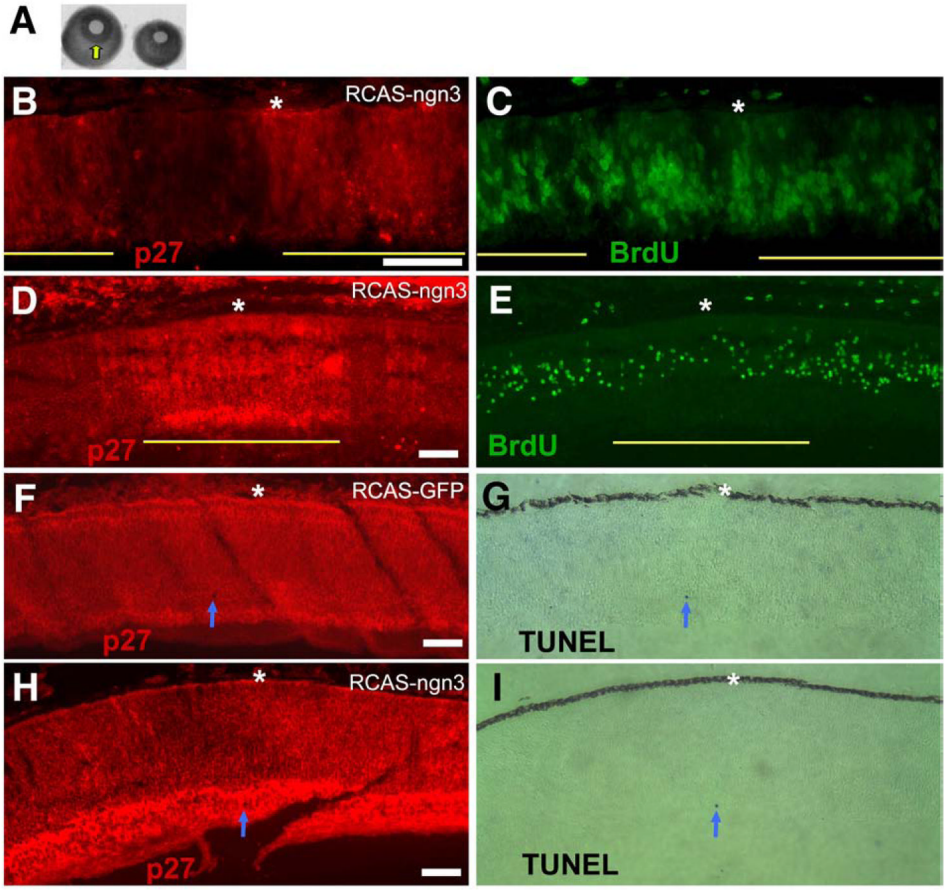

Fig. 5.

Analyses of cell proliferation with BrdU incorporation and of apoptosis with TUNEL assay of retinas infected with RCSA-ngn3. (A) Comparison of the eye size at E7.5 between a normal (left) and an experimental eye (right). Arrow points to the well-formed iris, which is missing in the experimental eye. (B, C) Double-labeling for an RCAS viral protein, p27 (B) and for BrdU incorporation (C) of an E5 retina with partial infection by RCAS-ngn3. The anti-p27 staining was used to identify regions, indicated by yellow lines, which were infected by the virus. (D, E) Double-labeling for the viral protein p27 (D) and for BrdU incorporation (E) of an E7.5 retina with partial infection by RCAS-ngn3. Yellow line indicates the infected region. (F, G) Double-labeling for the viral protein p27 (F) and for $TUNEL^+$ cells (G) of an E7.5 retina infected with RCAS-GFP. Arrow points to a $TUNEL^+$ cell. (H, I) Double-labeling for the viral protein p27 (H) and for $TUNEL^+$ cells (I) of an E7.5 retina infected with RCAS-ngn3. Arrow points to a $TUNEL^+$ cell. Scale bars: 50 μm.

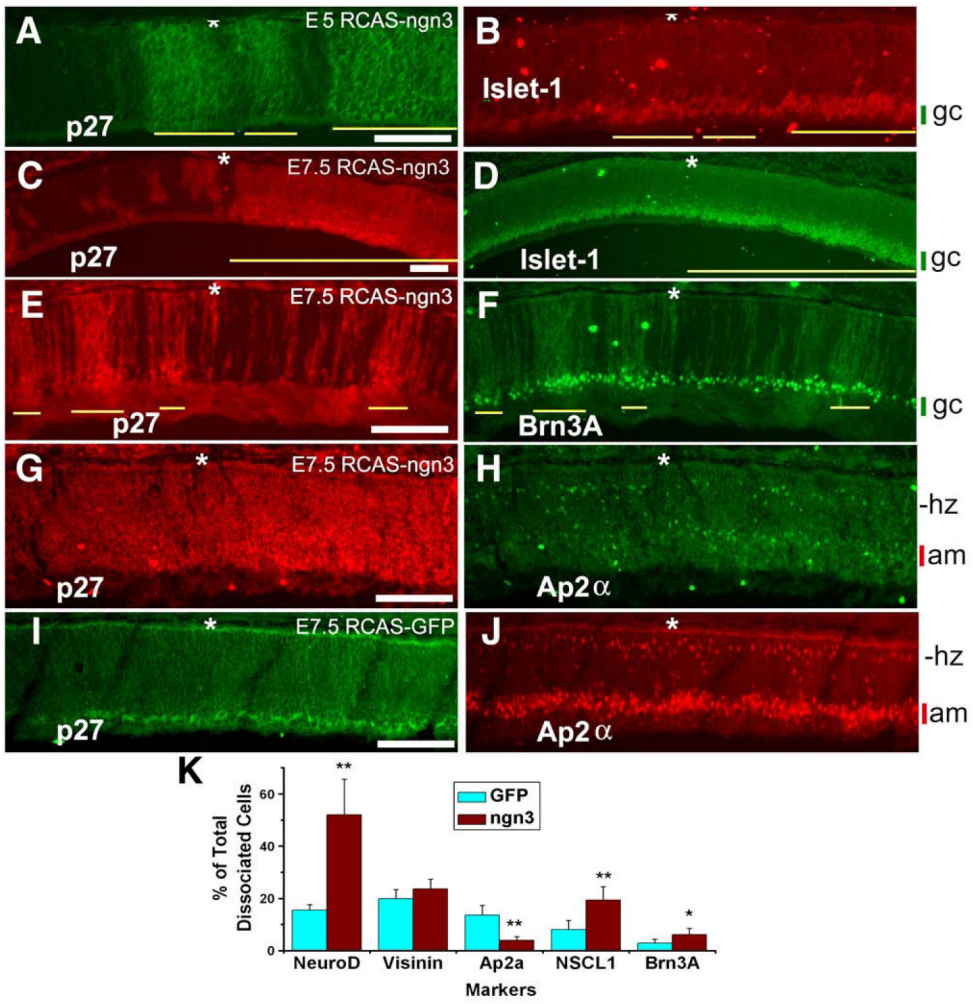

Fig. 6.

Alterations in retinal neurogenesis from *ngn3* overexpression. (A, B) Double-labeling for RCAS viral protein p27 to identify infected regions (yellow line) by RCAS-ngn3 (A) and for Islet-1 (B) of E5 retina partially infected by the virus. (C, D) Double-labeling for viral protein p27 (C) and Islet-1 (D) of E7.5 retina partially infected with RCAS-ngn3. Yellow line indicates the infected region. (E, F) Double-labeling for viral protein p27 (E) and Brn3A (F) of E7.5 retina partially infected with RCAS-ngn3. Yellow line indicates the infected region. (G, H) Double-labeling for viral protein p27 (G) and Ap2α (H) of E7.5 retina infected with RCAS-ngn3. (I, J) Double-labeling for viral protein p27 (I) and Ap2α (J) of control E7.5 retina infected with RCAS-GFP. The cellular (presumably ganglion cells, gc) layer that increased in thickness upon *ngn3* overexpression is marked by a green vertical bar, whereas the layer (presumably amacrine cells, am) with reduced thickness is marked by a red bar. The prospective location of horizontal cells (hz) is marked for convenience. The RPE layer is indicated by an asterisk. (K) A quantitative analysis of different cell populations using dissociated retinal cells from retinas infected with RCAS-GFP (GFP) or RCAS-ngn3 (ngn3). Shown are the means ± SDs of the calculated percentage of cells identified with each marker among dissociated E7.5 retinal cells. * and ** indicate statistical significance at 0.05 and 0.01 levels, respectively. Scale bars: 100 μm.

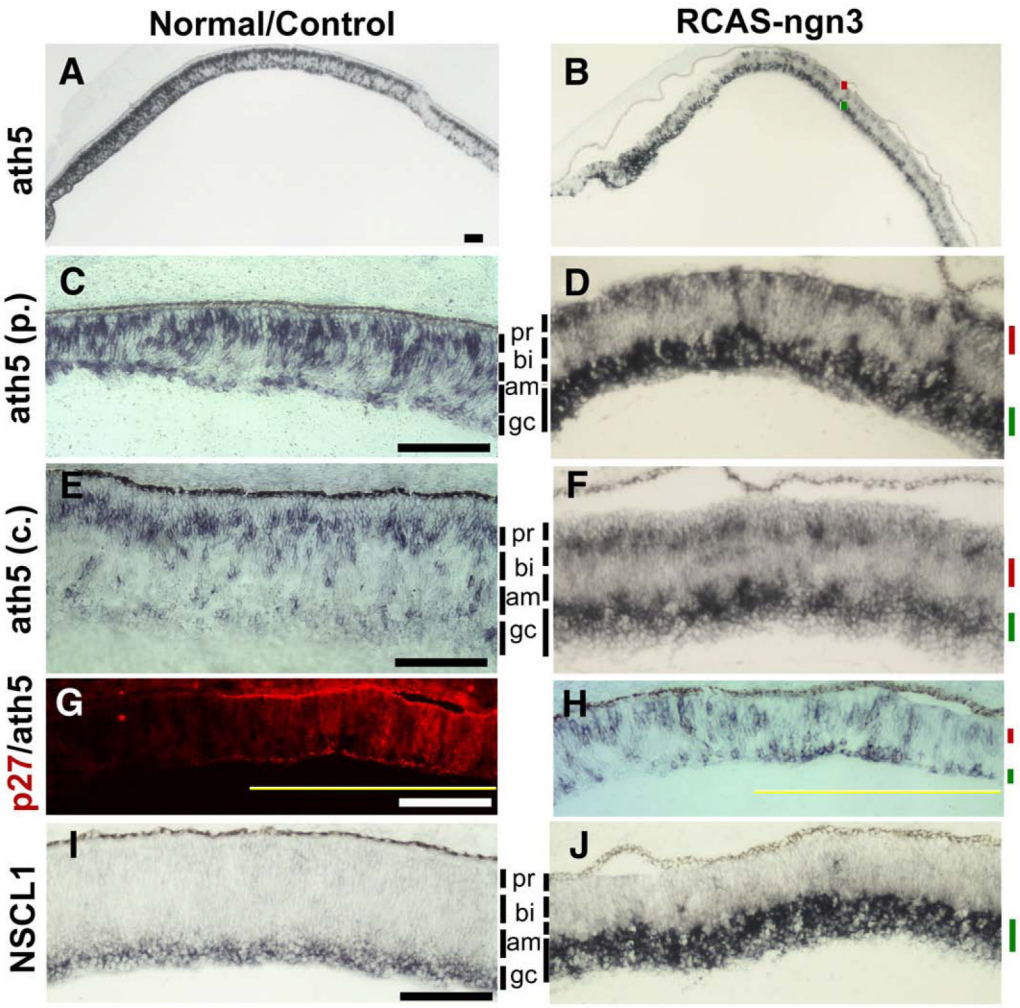

Fig. 7.

Alterations in the spatial patterns of *ath5* and *NSCL1* expression in retinas infected by RCAS-ngn3. (A, B) Low magnification views of in situ hybridization for *ath5* mRNA in normal retina (A) and experimental retina at E7.5 (B). (C, D) *ath5* expression in a peripheral region of the control retina infected with RCAS-GFP (C) and in the experimental retina at E7.5 (D). (E, F) *ath5* expression in a central region of the control retina infected with RCAS-GFP (E) or experimental retina (F). (G, H) Double-labeling for RCAS viral protein p27 (G) and *ath5* expression (H) of E5 retina partially infected with RCAS-ngn3. Yellow line indicates the infected region. (I, J) The spatial expression patterns of *NSCL1* in normal (I) and experimental (J) retinas. The cellular layer with diminished gene expression is marked by a red bar. The cellular layer with increased gene expression is marked by a green bar. To facilitate visualization of the pseudo-stratification of the E7.5 retina, the prospective cell layers are indicated by vertical lines in gray and marked for prospective positions of photoreceptors (pr), bipolar (bi), amacrine (am), and ganglion cells (gc). Scale bars: 100 μm.

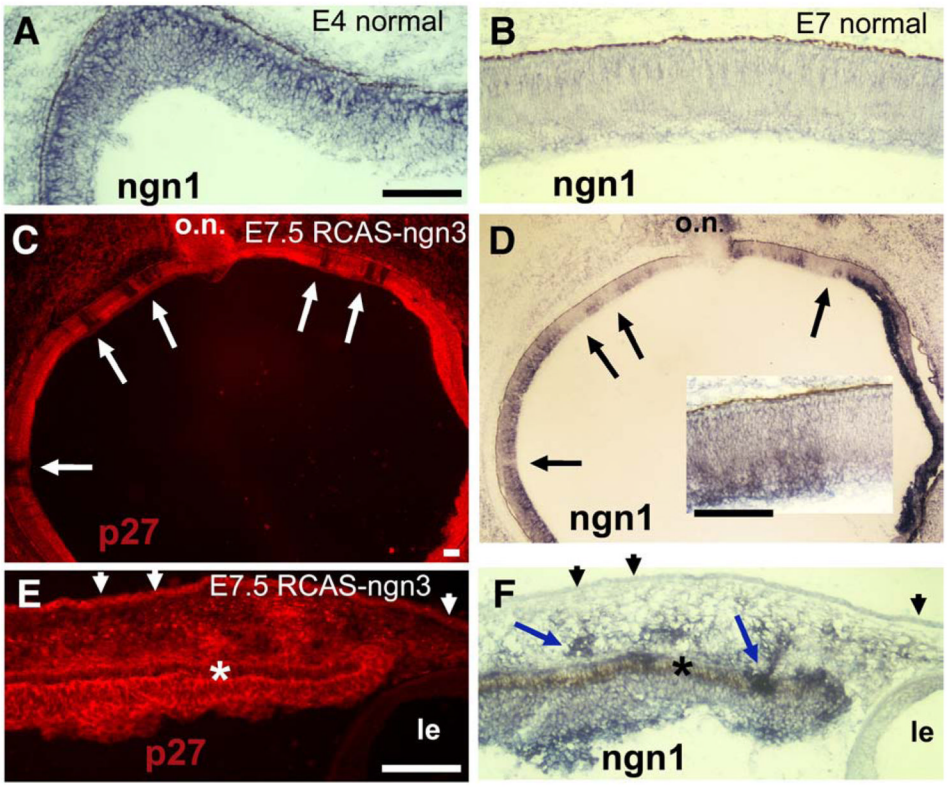

Fig. 8.

Induction of *ngn1* in eyes infected with RCAS-ngn3. (A, B) In situ hybridization detection for *ngn1* expression in normal retinas at E4 (A) and E7 (B). (C, D) Serial sections of an E7.5 eye infected with RCAS-ngn3 and subjected to immunostaining for RCAS viral protein p27 to identify regions infected with the virus (C) or to in situ hybridization to detect *ngn1* expression (D). Inset in D: higher magnification. Arrows point to corresponding regions lacking viral infection (C) and *ngn1* expression (D). (E, F) Serial sections of peripheral regions of an E7.5 eye infected with RCAS-ngn3 and stained for viral protein p27 (E) or *ngn1* mRNA by in situ hybridization (F). Arrowheads point to head epidermis infected with the virus (E) and lacking *ngn1* expression (F). Arrows in F point to *ngn1* expression in some mesenchymal cells and RPE cells. RPE is indicated by an asterisk. o.n., optic nerve. Le, lens. Scale bars: 100 μm.

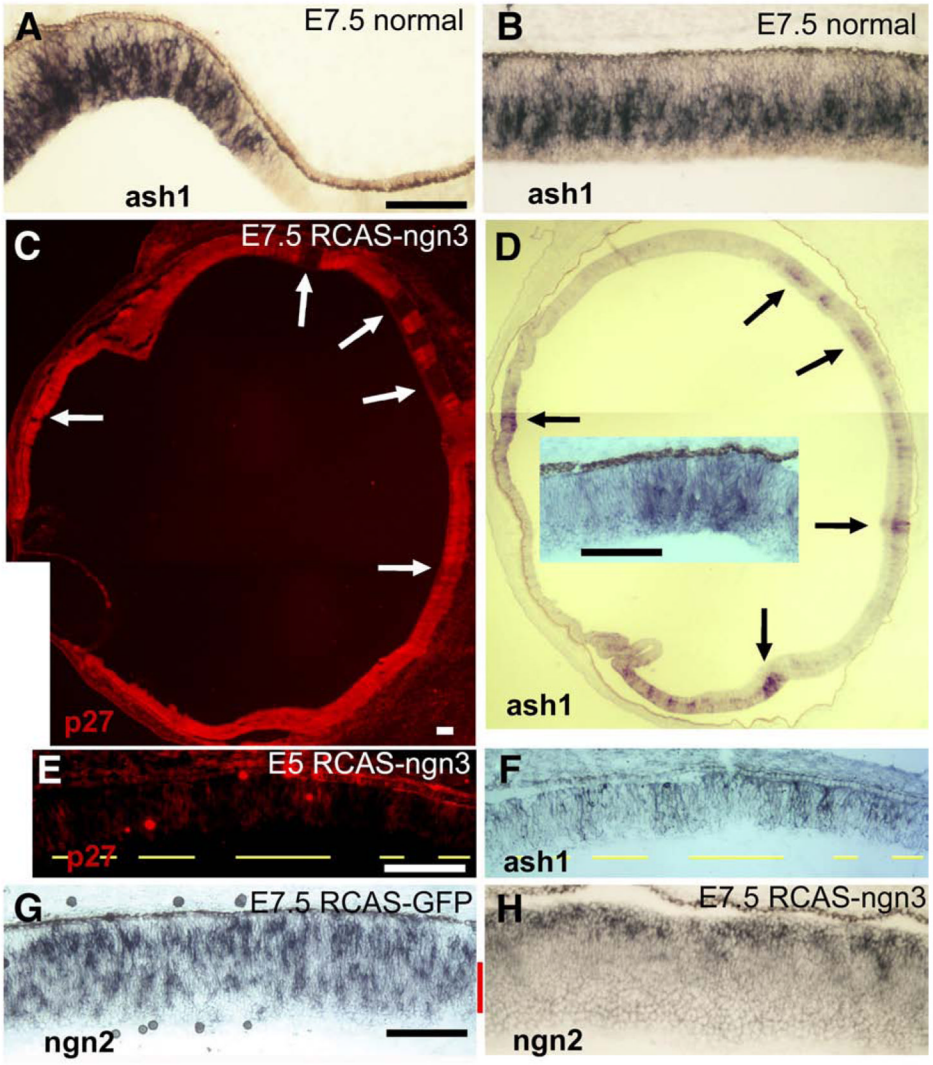

Fig. 9.

Down-regulation of *ash1* and *ngn2* expression in retinas infected by RCAS-ngn3. (A, B) Expression of *ash1* in normal E7.5 retina at the periphery (A) and the central region (B). (C, D) Serial sections of an E7.5 eye infected with RCAS-ngn3 and stained for viral protein p27 to identify regions infected by the virus (C) or stained for *ash1* mRNA by in situ hybridization (D). Arrows point to corresponding regions lacking viral infection (C) and retaining *ash1* expression (D). (E, F) Double-labeling for viral protein p27 (E) and *ash1* expression (F) of E5 retina partially infected with RCAS-ngn3. Yellow lines indicate infected regions. (G, H) The spatial expression patterns of *ngn2* in the control infected with RCASGFP (G) and experimental (H) retina. The cellular layer with diminished gene expression is marked by a red bar. Scale bars: 100 μm.

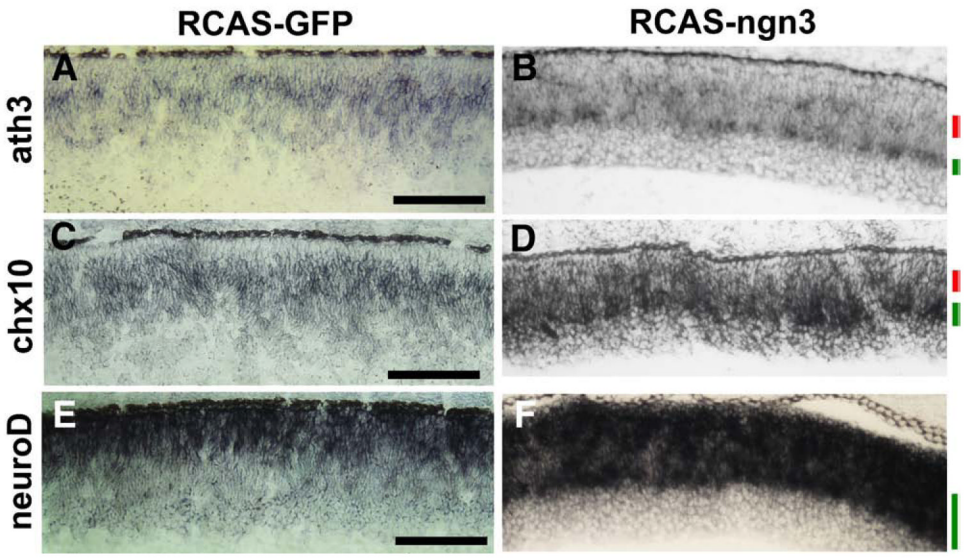

Fig. 10.

Alterations in the spatial locales of *ath3*, *chx10*, and *neuroD* expression in E7.5 retina infected by RCAS-ngn3. In situ hybridizationwas used to detect spatial expression patterns of *ath3* in the control infected with RCAS-GFP (A) and the experimental (B) retinas; of *chx10* in the control (C) and the experimental (D) retinas; of *neuroD* in the control (E) and the experimental (F) retinas. The cellular layer with diminished gene expression is marked by a red bar. The cellular layer with increased gene expression is marked by a green bar. Scale bars: 100 μm.