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Posttranslational Mechanisms Control the Timing of bHLH Function and Regulate Retinal Cell Fate

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Summary

During central nervous system development, neurons are often born in a precise temporal sequence. Basic helix-loop-helix (bHLH) transcription factors are required for the development of specific subpopulations of neurons, but how they contribute to their ordered genesis is unclear. We show that the ability of bHLH factors to regulate the development of distinct neuronal subtypes in the Xenopus retina depends upon the timing of their function. In addition, we find that the timing of bHLH function can be regulated posttranslationally, so that bHLH factors with overlapping expression can function independently. Specifically, XNeuroD function in the retina can be inhibited by glycogen synthase kinase 3β (GSK3β), while Xath5 function can be inhibited by Notch. Thus, the potential of bHLH factors to regulate the development of neuronal subtypes depends upon the context in which they function.

Introduction

The vertebrate central nervous system (CNS) contains a vast array of distinct neuronal cell types. In many regions of the nervous system, these cell types arise in precise ratios from a pool of multipotent progenitor cells, often in a particular order. Understanding the molecular mechanisms by which this diversity is achieved is a fundamental challenge of developmental neurobiology. The vertebrate retina is an attractive CNS structure in which to elucidate the molecular basis of neuronal differentiation, since it contains a limited number of cell types organized in a stereotypical laminar pattern (Altshuler et al., 1991). The seven classes of neurons and one glia cell type found in the mature vertebrate retina are generated from a common pool of multipotent progenitor cells in a roughly conserved histogenic order: ganglion cells and cones are born first, while rods and Müller glia are born last (reviewed in Cepko et al., 1996). This fidelity of birth order across divergent species suggests that control of retinal cell differentiation is a highly regulated process.

There are several transcription factor families that have been shown to be important intrinsic regulators of neuronal differentiation (Edlund and Jessell, 1999). The spatial and temporal control of such factors is likely to play a role in the commitment of undifferentiated retinal progenitor cells to specific phenotypic fates. Consistent with this idea, gene disruption studies have shown that a number of these transcription factors are required for retinal development (reviewed in Harris, 1997; Livesey and Cepko, 2001). In addition, there is evidence that the pattern of transcription factor expression changes during retinogenesis, suggesting that specific combinations of transcription factors may regulate the development of specific cell types (Perron et al., 1998). The question, then, is how these factors ultimately contribute to the full array of retinal cells found in the mature retina.

In many developmental systems, basic helix-loop-helix (bHLH) transcription factors are intimately involved in the determination and differentiation of cells. In the nervous system, proneural bHLH factors share a common role of regulating neuronal differentiation (Lee, 1997). It is less clear how these genes participate in the acquisition of specific neuronal phenotypes. During *Xenopus laevis* retinogenesis, there are several bHLH factors that are coexpressed both temporally and spatially but promote the formation of different retinal cell types when overexpressed (Perron et al., 1998). For example, *Xath5*, *Xath3*, and *XNeuroD* are coexpressed in retinal progenitors. However, overexpression of Xath5 or Xath3 during *Xenopus* retinal development promotes the formation of early cell types, such as ganglion cells (Kanekar et al., 1997; Perron et al., 1999), while overexpression of XNeuroD promotes the differentiation of later-born cell types (Kanekar et al., 1997).

Gene disruption studies have demonstrated that bHLH factors are required for the differentiation of specific subpopulations of retinal neurons. For example, mutation of the *ath5* gene results in a loss of retinal ganglion cells in mice (Brown et al., 2001; Wang et al., 2001) and zebrafish (Kay et al., 2001), while the differentiation of amacrine cells is delayed in *NeuroD* knockout mice (Morrow et al., 1999). Such results indicate that bHLH factors can influence neuronal differentiation but raise the question of whether they contribute cell-type information during retinal neurogenesis. bHLH factors might intrinsically specify neuronal subtypes, as has been suggested for atonal and achaete-scute genes in the fly peripheral nervous system and eye (Chien et al., 1996; Sun et al., 2000). Alternatively, the ability of bHLH factors to promote the differentiation of specific cell types may depend, in part, upon the cellular context in which they are functioning. Since retinal progenitors have been proposed to change over developmental time in their competence to generate subtypes of retinal neurons (Cepko et al., 1996; Livesey and Cepko, 2001), this model would suggest that the timing of bHLH factor expression or function would be an important component in determining the phenotype of the cell that differentiates in response to bHLH factor activity.

To distinguish between these possibilities, we have focused on two closely related members of the vertebrate atonal-related bHLH family: Xath5, which promotes retinal ganglion cell differentiation when overex-

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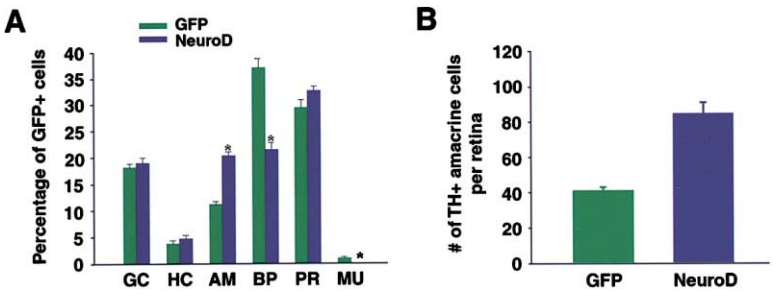

Figure 1. Overexpression of XNeuroD Promotes an Increase in Amacrine Cell Differentiation

(A) Overexpression of XNeuroD by 16-cell RNA injection caused a 2-fold increase in the representation of amacrine cells and a decrease in bipolar cells and Müller glia, as compared to GFP controls. The percent representation of each cell type was calculated as a weighted average. n = 3655 cells from 17 embryos for GFP and n = 2886 cells from 21 embryos for XNeuroD. Error represents SEM; asterisk, $p < 0.01$ by Student's t test. GC, ganglion cells; HC, horizontal cells; AM, amacrine cells; BP, bipolar cells; PR, photoreceptor cells; and MU, Müller glial cells.

(B) To confirm that mature amacrine cells had increased, retinal sections from injected embryos were stained with antibodies to tyrosine hydroxylase (TH), which labels dopaminergic amacrine cells in the *Xenopus* retina. There was a 2-fold increase in the number of TH-positive cells per retina from XNeuroD-injected embryos, compared to control GFP-injected embryos. n = 10 embryos for GFP and n = 12 embryos for XNeuroD.

pressed, and XNeuroD, which promotes differentiation of the later-born, amacrine cell type (Kanekar et al., 1997). We demonstrate that posttranslational mechanisms independently regulate the timing of Xath5 and XNeuroD function, so that they promote the differentiation of early and late-born cell types, respectively. Furthermore, we find that other bHLH factors from multiple species, including Xash1, Mash1, and scute, can also be regulated posttranslationally to provide control over the timing of their function. Our results suggest that during *Xenopus* retinogenesis, related bHLH factors have the ability to regulate the differentiation of overlapping subsets of retinal neuron types rather than a unique cell type. Thus, the actual role that an individual bHLH factor has in retinal cell fate specification in part depends upon the context in which it is active.

Results

Glycogen Synthase Kinase 3β Inhibits XNeuroD, but Not Xath5, Function in the Neural Plate

Xath5 and *XNeuroD* commence expression at the same stage of retinal neurogenesis, as the first populations of neurons are beginning to differentiate (Kanekar et al., 1997). However, when overexpressed in retinal precursor cells, Xath5 promotes the differentiation of retinal ganglion cells, the earliest-born retinal cell type, while XNeuroD promotes the differentiation of later-born cell types, such as amacrine cells (Kanekar et al., 1997; Figure 1). This raises the possibility that the function of XNeuroD, but not Xath5, is inhibited at early stages of retinal neurogenesis, thus preventing XNeuroD from promoting the differentiation of the earliest born cell types. To explore this possibility, we have considered the serine-threonine kinase GSK3β as a candidate regulator of XNeuroD function during retinal development.

During primary neurogenesis in *Xenopus*, GSK3β can inhibit the function of XNeuroD and prevent it from promoting neuronal differentiation (Marcus et al., 1998). GSK3β is also expressed in the developing retina (Marcus et al., 1998; data not shown). In addition, XNeuroD, but not Xath5, contains a consensus phosphorylation site for GSK3β (SPPLSV; Marcus et al., 1998) (Figure 2A). To confirm that GSK3β can inhibit XNeuroD function and determine whether it also inhibits Xath5 function, we injected mRNA encoding GSK3β, along with mRNA for either XNeuroD or Xath5, into one cell of two- to four-cell stage embryos. mRNA encoding β-galactosidase was included as a tracer. To assay effects on neuronal differentiation, we processed neural plate-staged embryos (stage 14–16) for whole-mount in situ hybridization, using a probe for the neural-specific N-tubulin. Ectopically expressed Xath5 or XNeuroD converted cells within the neural plate and ventral ectoderm into differentiated neurons (Kanekar et al., 1997; Lee et al., 1995; Figures 2D and 2F). As expected, the ability of XNeuroD to promote ectopic neurogenesis was inhibited by coexpression of GSK3β (33/36 embryos; Marcus et al., 1998) (Figure 2E), while Xath5 activity was not inhibited (0/51 embryos; Figure 2G). Xath5 retained the ability to promote ectopic neurogenesis, even in the presence of high doses of GSK3β mRNA, which alone inhibited primary neurogenesis (22/27 embryos; Figures 2C and 2G). We conclude that GSK3β can differentially inhibit the ability of related bHLH factors to promote neuronal differentiation at neural plate stages.

To provide evidence that GSK3β is directly modulating XNeuroD function rather than acting through intermediate factors, we mutated the putative GSK3β phosphorylation site in XNeuroD (Figure 2A) to create a GSK3β-insensitive version of XNeuroD ($XNeuroD_{[S\rightarrow A]}$). Ectopically expressed $XNeuroD_{(S\rightarrow A)}$ was capable of converting cells within the neural plate and ventral ectoderm into differentiated neurons (59/62 embryos; Figure 2H), and this was not inhibited by co-injection of GSK3β mRNA (0/48 embryos; Figure 2I). Thus, $XNeuroD_{(S\rightarrow A)}$ is both active in promoting neuronal differentiation and insensitive to the effects of GSK3β in the neural plate.

Inhibition of GSK3β Signaling in the Retina Causes XNeuroD to Promote the Earliest-Born Cell Fate

GSK3β is expressed in the neural plate, early eye fields, and throughout the eye primordia, as well as the mature retina (Marcus et al., 1998). If GSK3β is inhibiting XNeuroD function at early stages of retinal neurogenesis, then blocking GSK3β activity should relieve this inhibition and allow XNeuroD to promote early-born cell fates. To test this, we coexpressed XNeuroD with a dominant-

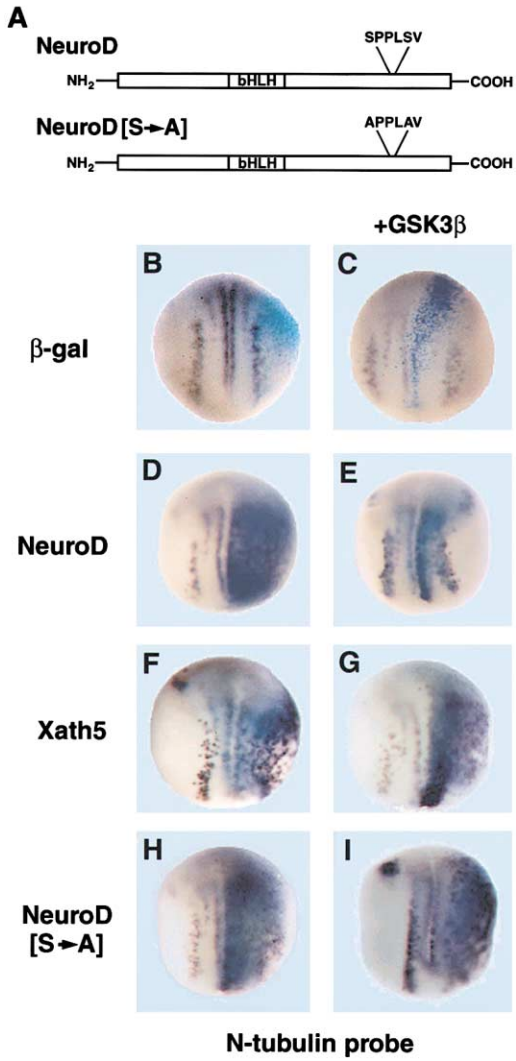

Figure 2. GSK3β Inhibits XNeuroD, but Not Xath5, Activity

(A) XNeuroD contains a consensus phosphorylation site for GSK3β (SPPLSV). This site was mutated to APPLAV in $XNeuroD_{(S\rightarrow A)}$.

(B–I) RNA was injected into one cell of two-cell stage embryos and assayed at stage 14 by whole-mount in situ hybridization for expression of N-tubulin (dark purple). The embryos are shown in a dorsal view with anterior at the top. The injected side is to the right and is marked by β-galactosidase expression (aqua). (B) A control embryo injected with RNA for β-galactosidase showing normal N-tubulin expression in the developing primary neurons. (C) Co-injection of RNA for GSK3β suppresses primary neurogenesis on the injected side. (D, F, and H) Injection of RNA for either XNeuroD, Xath5, or $XNeuroD_{(S\rightarrow A)}$ promotes ectopic N-tubulin expression on the injected side. (E, G, and I) Co-injection of RNA for GSK3β suppresses ectopic neurogenesis induced by expression of XNeuroD (E), but not Xath5 (G) or $XNeuroD_{(S\rightarrow A)}$ (I).

negative form of GSK3β (dnGSK3β; Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995) by injecting mRNA at the 16-cell stage. mRNA for green fluorescent protein (GFP) was co-injected, permitting identification of cells derived from the injected blastomere. 16-cell RNA injection was used to ensure efficient coexpression of multiple proteins and allow for control of levels of expression. For key experiments, results were confirmed by in vivo transfection of plasmids directly into retinal progenitors at optic vesicle stages, as previously described (Holt et al., 1990). To minimize the pleiotropic effects on head and eye development often seen with higher doses of dnGSK3β (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995), we used 10 pg of dnGSK3β mRNA, a dose that does not cause gross phenotypic abnormalities. Retinal neurons derived from blastomeres co-injected with dnGSK3β mRNA and XNeuroD mRNA showed a significant shift toward a retinal ganglion cell fate, compared to GFP controls or injections of 10 pg of dnGSK3β alone (Figure 3A; $p < 0.003$). 63% of labeled cells adopted ganglion cell fates at the expense of later-born cell types; this 3-fold increase was similar to that observed in retinal progenitors in Xath5-injected embryos (Kanekar et al., 1997). Co-injection of higher doses of dnGSK3β mRNA (50 pg) with XNeuroD (10pg) mRNA had an even more dramatic effect, causing 87.6% ± 4.3% of labeled cells within the retina to differentiate as retinal ganglion cells ($n = 687$ cells from 13 embryos). Thus, endogenous GSK3β in the developing retina appears to inhibit the ability of ectopically expressed XNeuroD to promote retinal ganglion cell differentiation. This effect is not observed with all bHLH factors, since dnGSK3β did not alter the ability of ectopically expressed Math5 to promote bipolar cell differentiation (data not shown; Brown et al., 1998). Furthermore, retinal cells derived from blastomeres co-injected with GSK3β and Xath5 mRNAs retained their bias toward ganglion fates (data not shown).

To determine whether GSK3β modulates the activity of endogenous bHLH factors during retinal development, we injected increasing amounts dnGSK3β mRNA and assayed for effects on retinal cell fate. Injection of low amounts of dnGSK3β mRNA (10 pg) caused a slight but significant increase in the representation of retinal ganglion cells (RGCs; 23.5% ± 1.5%), compared to GFP controls (18.2% ± 0.7%) (Figure 3B). With increasing doses of dnGSK3β mRNA, there was an increased shift toward the RGC fate (Figure 3B) (32.7% ± 3.8% RGCs with 30 pg mRNA and 44.4% ± 10.6% RGCs with 50 pg mRNA). Similarly, lipofection of dnGSK3β plasmid DNA into the optic vesicle resulted in significantly increased RGC differentiation (28.5% ± 2.2%, $n = 264$ cells from five embryos, $p < 0.01$), compared to GFP controls (20.3% ± 1.1%, $n = 1225$ cells from 12 embryos). These results suggest that GSK3β might function to inhibit the activity of endogenous bHLH factors, preventing them from promoting early retinal cell differentiation.

Targeted Overexpression of a GSK3β-Insensitive Form of XNeuroD Increases Differentiated Cells within the Ganglion Cell Layer

To directly demonstrate that XNeuroD can promote RGC differentiation when GSK3β inhibition is prevented, we tested the effects of overexpressing the GSK3β-insensitive version of XNeuroD ($XNeuroD_{[S\rightarrow A]}$) in the retina by 16-cell RNA injection. Retinal neurons derived from blastomeres injected with mRNA for GFP and $XNeuroD_{(S\rightarrow A)}$ showed a significant shift toward the retinal ganglion cell fate (Figures 3C and 3G). 40% of neurons derived from blastomeres injected with $XNeuroD_{(S\rightarrow A)}$ mRNA

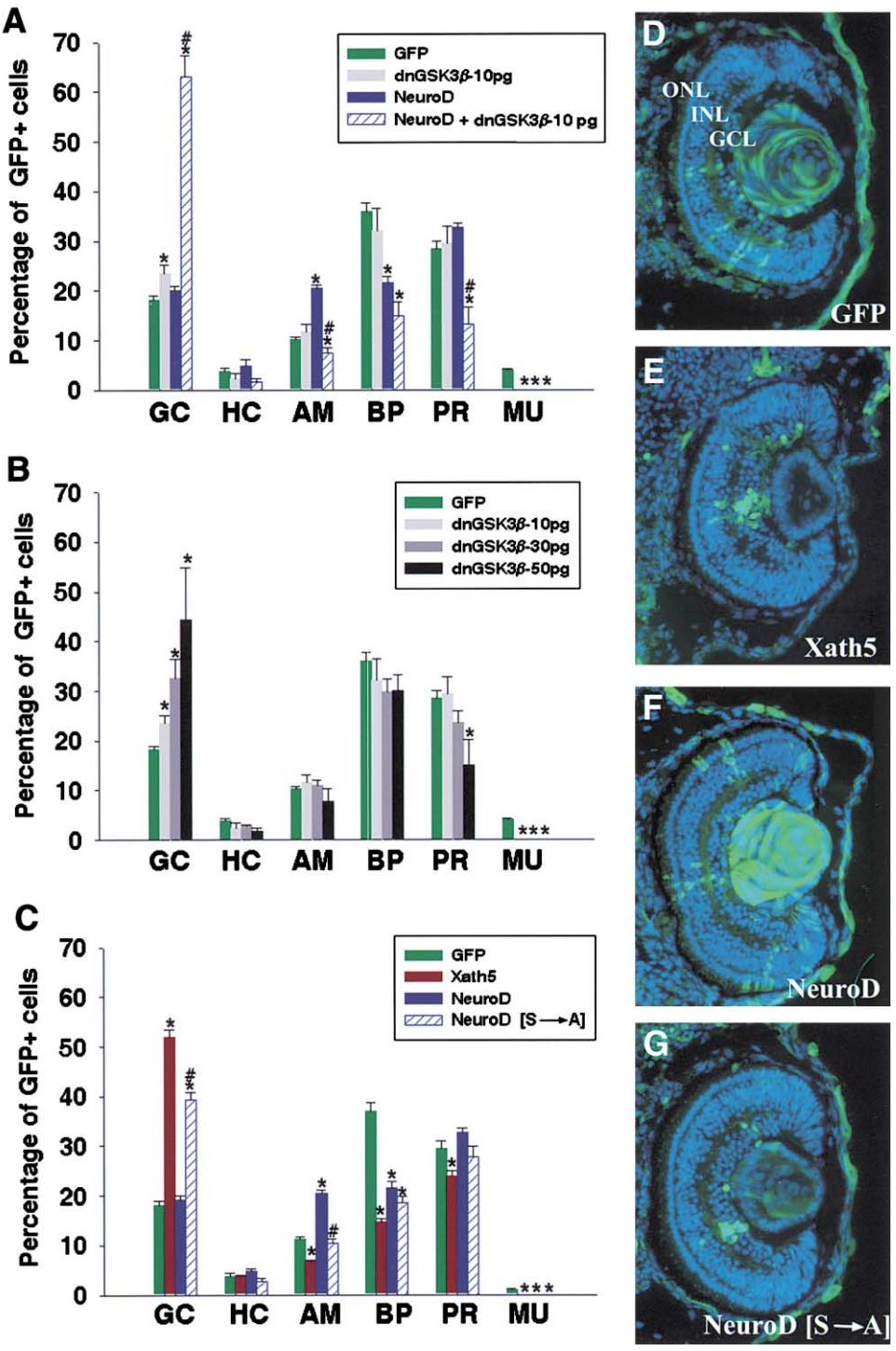

Figure 3. Targeted Overexpression of a GSK3β-Insensitive Form of XNeuroD Increases Differentiated Cells within the Ganglion Cell Layer

(A) Overexpression of XNeuroD by 16-cell RNA injection does not promote an increase in retinal ganglion cell differentiation, compared to control GFP injections, unless GSK3β inhibition is blocked by coexpression of dnGSK3β (10 pg) with XNeuroD. n = 3655 cells from 17 embryos for GFP, n = 2797 cells from 11 embryos for dnGSK3β, n = 2886 cells from 21 embryos for XNeuroD, and n = 338 cells from 5 embryos for XNeuroD + dnGSK3β. Error represents SEM; the asterisks represent significant difference, as compared to GFP controls, while the number signs represent significant difference, as compared to dnGSK3β (10 pg) controls; $p < 0.01$ by Student's t test. GC, ganglion cells; HC, horizontal cells; AM, amacrine cells; BP, bipolar cells; PR, photoreceptor cells; and MU, Müller glial cells.

(B) Overexpression of increasing amounts of dnGSK3β mRNA causes increased retinal ganglion cell differentiation. n = 3655 cells from 17 embryos for GFP, n = 2797 cells from 11 embryos for dnGSK3β (10 pg), n = 270 cells from 3 embryos for dnGSK3β (30 pg), and n = 616 cells from 7 embryos for dnGSK3β (50 pg). Error represents SEM; the asterisks represent significant difference, as compared to GFP controls; $p < 0.01$ by Student's t test.

adopted ganglion cell layer (GCL) fates at the expense of later-born cell types (Figure 3C; $p < 0.001$). This distribution of labeled cell types was similar to that observed with injection of Xath5 mRNA (Figure 3E) but differed from those produced by either XNeuroD or GFP controls (Figures 3D and 3F). Similarly, lipofection of XNeuroD$_{(S\rightarrow A)}$ plasmid into retinal progenitors at optic vesicle stages resulted in 40% $\pm$ 8.0% retinal ganglion cells ($n = 183$ cells from three embryos), which is significantly increased compared to GFP controls (20.3% $\pm$ 1.1%, $n = 1225$ cells from 12 embryos). These data support the hypothesis that phosphorylation of XNeuroD at SPPLSV normally functions to inhibit its ability to promote differentiation of early-born cell types, such as ganglion cells.

In addition, it has previously been shown that both Xath5 and XNeuroD can directly regulate the expression of the POU-homeodomain transcription factor XBrn3d, which is expressed in differentiating and mature retinal ganglion cells (Hutcheson and Vetter, 2001). Our model predicts that the ability of XNeuroD, but not Xath5, to regulate XBrn3d expression should be regulated by GSK3β. To test this, we injected mRNA encoding Xath5, XNeuroD, or XNeuroD$_{(S\rightarrow A)}$ either alone or together with mRNA for GSK3β at the two-cell stage and assayed for XBrn3d expression by in situ hybridization at open neural plate stages. We found that Xath5, XNeuroD, or XNeuroD$_{(S\rightarrow A)}$ could each promote ectopic expression of XBrn3d, but that only XNeuroD could be prevented from doing so by coexpression of GSK3β (data not shown). Thus, the ability of XNeuroD to regulate the expression of a retinal ganglion cell factor depends upon whether it is inhibited by GSK3β.

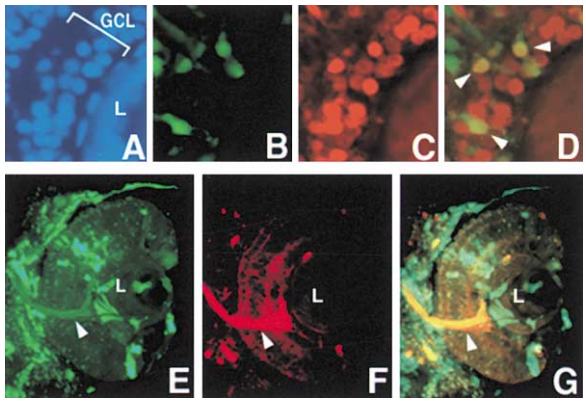

Figure 4. The Cells Promoted by XNeuroD$_{(S\rightarrow A)}$ Are Differentiated Retinal Ganglion Cells

Immunohistochemistry on 14 μm cryosections of retinas from embryos injected with XNeuroD$_{(S\rightarrow A)}$ mRNA at the 16-cell stage.

(A) Hoechst dye labels cell nuclei (blue). The GCL and lens (L) are shown.

(B) The same section showing GFP-labeled retinal ganglion cells in which XNeuroD$_{(S\rightarrow A)}$ is coexpressed.

(C) The same section showing Islet-1 positive cells within the GCL.

(D) A merged image of (A)–(C). Arrowheads indicate GFP-positive/Islet-1-positive cells in the GCL. Islet-1 is expressed in over 95% of GFP-positive cells within the GCL (318/336 cells from five embryos).

(E–G) Labeled cells in the GCL extend axons (arrowhead) that are both positive for GFP (E) and positive for neurofilament (F and G).

GCL, ganglion cell layer; L, lens.

XNeuroD$_{(S\rightarrow A)}$ Promotes the Formation of Differentiated Retinal Ganglion Cells

It was possible that the XNeuroD$_{(S\rightarrow A)}$ mRNA injection was not promoting retinal ganglion cell differentiation but, rather, causing ectopic differentiation of other retinal cell types within the GCL. We confirmed that the labeled cells in the GCL observed with XNeuroD$_{(S\rightarrow A)}$ mRNA injections were indeed differentiated ganglion cells by staining with Islet-1, which marks retinal ganglion cells in the *Xenopus* retina (Dorsky et al., 1997). Over 95% of the GFP-positive cells within the GCL were positive for Islet-1 (Figures 4A–4D; $n = 318/336$ cells in five embryos). In addition, the labeled cells extended GFP-positive axons that also stained positive for neurofilament (Figures 4E–4G) and for Pax6, which marks ganglion cells (data not shown; Belecky-Adams et al., 1997; Mastick et al., 1997). Since manipulation of XNeuroD normally affects amacrine cell populations, we also tested the possibility that the observed increase in cells within the GCL was due, in part, to increased numbers of displaced amacrine cells. However, in the *Xenopus* retina, it has been shown that there are very few displaced amacrine cells in the GCL at the stages we examined (Hiscock and Straznicky, 1990; Huang and Moody, 1997; Rayborn et al., 1981). In addition, only a very small number (<5%) of the GFP positive cells in the GCL were positive for GABA, which stains amacrine cells in the *Xenopus* retina, including displaced amacrine cells (data not shown; Huang and Moody, 1998; Zhu and Straznicky, 1993). Since the number of GFP-positive/GABA-positive cells in the GCL from XNeuroD$_{(S\rightarrow A)}$-injected embryos (average of 4.2 cells per retina from 15 embryos) did not differ from GFP-injected controls (average of five cells per retina from 17 embryos; $p = 0.572$), we conclude that the observed increase in GCL cells is not due to an increase in displaced amacrine cells. Based on three criteria: laminar position, morphology, and the expression of ganglion cell-specific markers, we conclude that the labeled retinal cells within the GCL in the XNeuroD$_{(S\rightarrow A)}$-injected embryos are ganglion cells.

(C) Overexpression of XNeuroD$_{(S\rightarrow A)}$, a GSK3β-insensitive form of XNeuroD, promotes retinal ganglion cell differentiation similar to that seen with overexpression of Xath5. $n = 3655$ cells from 17 embryos for GFP, $n = 2655$ cells from 16 embryos for Xath5, $n = 2886$ cells from 21 embryos for XNeuroD, and $n = 3141$ cells from 15 embryos for XNeuroD$_{(S\rightarrow A)}$. Error represents SEM; the asterisks represent significant difference, as compared to GFP controls, while the number signs represent significant difference between XNeuroD and XNeuroD$_{(S\rightarrow A)}$; $p < 0.01$ by Student's t test.

(D–G) Cryostat sections through the retina of a stage 41 embryo injected at the 16-cell stage with RNA for GFP alone or in combination with RNA for either Xath5, XNeuroD, or XNeuroD$_{(S\rightarrow A)}$, as indicated. Nuclei were stained with Hoechst dye (blue) to visualize the retinal cell layers. Overexpression of Xath5 (E) or XNeuroD$_{(S\rightarrow A)}$ (G) caused an increase in the representation of GFP-labeled cells in the GCL, while overexpression of XNeuroD (F) did not.

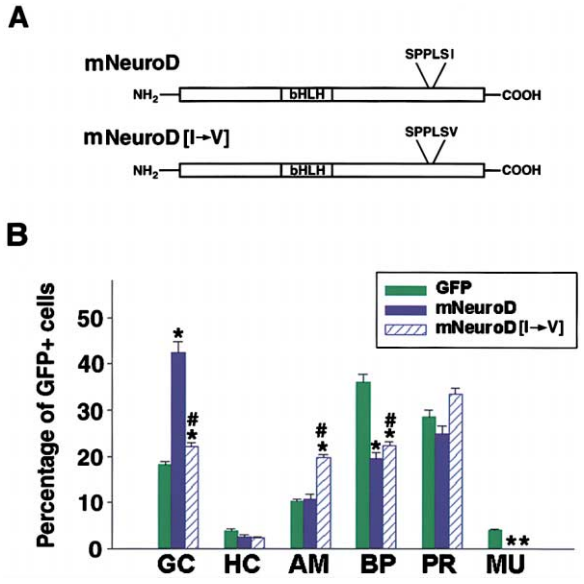

Figure 5. Targeted Overexpression of Mouse NeuroD Promotes Retinal Ganglion Cell Differentiation, While a GSK3β-Sensitive Form of Mouse NeuroD Does Not

(A) mNeuroD lacks a complete consensus phosphorylation site for GSK3β (SPPLSI). This site was mutated to SPPLSV in mNeuroD$_{(I\rightarrow V)}$.

(B) Overexpression of mNeuroD by 16-cell RNA injection promotes an increase in retinal ganglion cell differentiation, compared to control GFP injections, suggesting that it is not inhibited by GSK3β at early stages of retinal development, while mNeuroD$_{(I\rightarrow V)}$, which contains the GSK3β consensus phosphorylation sequence, promotes the differentiation of later-born cell types (such as amacrine cells). n = 3655 cells from 17 embryos for GFP, n = 2583 cells from 10 embryos for mNeuroD, and n = 2264 cells from 7 embryos for mNeuroD$_{(I\rightarrow V)}$.

Error represents SEM; the asterisks represent significant difference, as compared to GFP controls, while the number signs represent significant difference between mNeuroD and mNeuroD$_{(I\rightarrow V)}$; $p < 0.01$ by Student's t test. GC, ganglion cells; HC, horizontal cells; AM, amacrine cells; BP, bipolar cells; PR, photoreceptor cells; and MU, Müller glial cells.

Mouse NeuroD Lacks a Complete GSK3β Consensus Phosphorylation Site and Promotes Retinal Ganglion Cell Differentiation When Overexpressed

Unlike XNeuroD, the NeuroD protein from mouse (mNeuroD) lacks a complete GSK3β consensus phosphorylation site, having the sequence SPPLSI rather than SPPLSV (Figure 5A). If mNeuroD cannot be inhibited by GSK3β phosphorylation, then we would predict that mNeuroD should be active at early stages of retinal development and promote retinal ganglion cell differentiation when overexpressed. Indeed, 42% of neurons derived from blastomeres injected with mNeuroD mRNA adopted GCL fates at the expense of later-born cell types (Figure 5B; $p < 0.001$). This distribution of labeled cell types was similar to that observed with injection of XNeuroD$_{(S\rightarrow A)}$ mRNA (Figure 3B), suggesting that mNeuroD is not being inhibited by GSK3β at early stages of retinal development and can thus promote early-born fates.

Based upon our previous findings, we predicted that introducing a GSK3β consensus phosphorylation sequence into mNeuroD would prevent it from functioning at early stages of retinal development. To test this, we mutated SPPLSI to SPPLSV (mNeuroD$_{(I\rightarrow V)}$; Figure 5A), which is identical to the GSK3β consensus phosphorylation sequence found in XNeuroD. When mNeuroD$_{(I\rightarrow V)}$ was overexpressed by 16-cell RNA injection, only 22% of labeled retinal neurons adopted the ganglion cell fate. Instead, there was a significant increase in amacrine cell differentiation (19.7% ± 0.7%), compared to GFP controls (10.2% ± 0.5%). This is very similar to the effect observed with overexpression of XNeuroD, which results in 21% of labeled cells adopting the amacrine cell fate (Figure 1A). These data are confirmation of our model that the activity of bHLH factors in the retina can be regulated through this GSK3β consensus phosphorylation sequence.

bHLH Factors that Promote Early-Born Cell Fates Promote Early Cell Cycle Exit

Proneural bHLH factors promote neuronal differentiation and play a critical role in regulating the timing of cell cycle exit (Ohnuma et al., 2002). When progenitors are induced to differentiate early in retinogenesis, they go through fewer rounds of cell division, as compared to progenitors that differentiate later. We therefore used clone-size analysis to demonstrate that the timing of bHLH function is regulated during retinal neurogenesis. We examined clone size in embryos injected with GFP either alone or in combination with XNeuroD, XNeuroD$_{(S\rightarrow A)}$, or Xath5 mRNAs into blastomere V1.2.1, which makes a fairly small contribution to the retina (Huang and Moody, 1993). Clones derived from XNeuroD$_{(S\rightarrow A)}$-injected progenitors were 40% smaller (100.25 ± 18.32 cells per clone, n = 5 embryos) than clones in either GFP-injected controls (164.7 ± 14.1 cells per clone, n = 5 embryos, p = 0.01) or XNeuroD-injected embryos (165.0 ± 33.6 cells per clone, n = 5 embryos, p = 0.48), suggesting that XNeuroD$_{(S\rightarrow A)}$ is causing progenitors to exit the cell cycle earlier in retinal development than XNeuroD. A reduced clone size was also observed with overexpression of Xath5 (44.7 ± 8.1 cells per clone, n = 5 embryos, $p < 0.001$). Xath5 clones were significantly smaller than XNeuroD$_{(S\rightarrow A)}$ clones, perhaps due to the fact that Xath5 promoted a larger increase in the representation of labeled retinal ganglion cells (52%), compared to XNeuroD$_{(S\rightarrow A)}$ (40%). Thus, fewer cells in the Xath5 clone remained as proliferating progenitors.

To confirm that early differentiation of progenitors correlates with early cell cycle exit when these genes are introduced directly into the optic vesicle, we have evaluated the number of labeled cells per retina following lipofection of the same constructs. Although these do not represent true "clones," since we cannot ensure that they represent transfection of a single progenitor, they do reflect proliferative effects within the retinal neuroepithelium itself. Consistent with our clone size analysis from the 16-cell injections, lipofection of either Xath5 or XNeuroD$_{(S\rightarrow A)}$ results in 40% fewer labeled cells per retina when compared to NeuroD or GFP (data not shown). This would fit with our model that both Xath5 and XNeuroD$_{(S\rightarrow A)}$ are causing early differentiation of retinal progenitors. Overall, these findings support the idea that proneural genes act to promote cell cycle exit and that the phenotype of neurons that results depends upon the timing of their action.

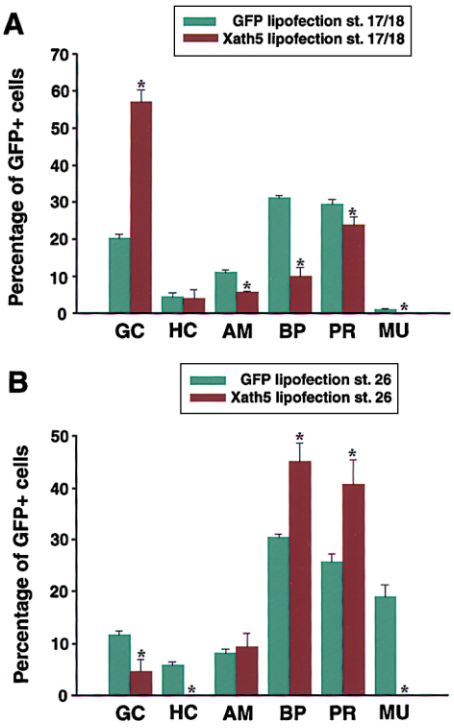

Figure 6. The Timing of Xath5 Expression Influences the Retinal Neuron Type Decision

(A) Lipofection of Xath5 at stage 17/18 promotes an increase in the representation of retinal ganglion cells, the earliest born cell type. n = 1225 cells from 12 embryos for GFP and n = 822 cells from 10 embryos for Xath5.

(B) Lipofection of Xath5 later at stage 26 promotes an increase in the representation of bipolar cells and photoreceptor cells, which are later-born cell types. In both cases, the differentiation of Müller glial cells was suppressed. n = 381 cells from six embryos for GFP and n = 335 cells from nine embryos for Xath5.

Error represents SEM; asterisks represent significant difference, as compared to GFP controls; $p < 0.01$ by Student's t test. GC, ganglion cells; HC, horizontal cells; AM, amacrine cells; BP, bipolar cells; PR, photoreceptor cells; and MU, Müller glial cells.

The Timing of Xath5 Expression Influences Retinal Cell Type Specification

If timing of bHLH function is an important determinant of retinal cell fate, then forced expression of Xath5 later during retinogenesis should not bias toward ganglion cells but, rather, promote the differentiation of later-born cell types. Alternatively, if Xath5 expression dictates the retinal ganglion cell fate, then one might expect that Xath5 would promote ganglion cell differentiation, regardless of the timing of expression. To target Xath5 overexpression to retinal progenitors at later stages of retinogenesis, we used DNA lipofection to allow us to more precisely control the timing of expression (Holt et al., 1990). In general, protein expression becomes detectable approximately 6–8 hr after transfection (Dorsky et al., 1997). Transfection of Xath5 DNA at stage 17/18 (neural fold stage) results in Xath5 overexpression at early stages of retinal neurogenesis (approximately stage 25) and causes an increased proportion of ganglion cells, as compared to GFP controls (Figure 6A; $p < 0.001$, Kanekar et al., 1997). Lipofection of control GFP DNA at stage 26 should result in expression at later stages of retinogenesis (approximately stage 31) and was found to give a different distribution of labeled retinal cell types compared to GFP transfections at stage 17/18 (compare Figures 6A and 6B). The major difference was an increase in Müller glia, the latest born retinal cell type at the expense of ganglion cells, suggesting that progenitors targeted at this later stage are producing later retinal cell types. Next, we lipofected Xath5 into a stage 26 eye and analyzed the effect on retinal differentiation (Figure 6B). Xath5 did not cause an increase in the representation of retinal ganglion cells. Instead, there was an increase in the percentage of transfected bipolar and photoreceptor cells relative to controls (33%, $p < 0.13$; 37%, $p < 0.025$; Figure 6B). There was a compensatory drop in Müller glia (100%, $p < 0.001$). This result is consistent with previous reports that proneural bHLH factors promote neurogenesis at the expense of gliogenesis (Brown et al., 1998; Cai et al., 2000; Kanekar et al., 1997; Morrow et al., 1999). These data demonstrate that the ability of Xath5 to promote the differentiation of specific retinal neuron types is dependent less upon inherent retinal subtype-specific information and more upon the cellular context in which it is active.

The Function of Other Late-Acting bHLH Factors Can Be Modulated by GSK3β

XNeuroD is not the only bHLH factor that appears to affect later-born retinal cell types. Mash1, a mammalian homolog of the *Drosophila* achaete-scute bHLH family, regulates the formation of later-born bipolar neurons (Hatakeyama et al., 2001; Tomita et al., 1996). Expression of Mash1 in *Xenopus* retinal progenitor cells biases retinoblast differentiation toward a bipolar cell fate (Brown et al., 1998; Figure 7). Both Mash1 and its *Xenopus* homolog, Xash1, contain GSK3β consensus phosphorylation sites (SPTISP), making them possible targets for regulation by GSK3β. To test whether Mash1 and Xash1 activities could be modulated by GSK3β, we used 16-cell RNA injection to target overexpression to retinal progenitor cells using 10 pg dnGSK3β mRNA and 10 pg Mash1 or Xash1 mRNA. As before, GFP mRNA was included as a tracer. While Mash1 mRNA alone resulted in a bias toward bipolar cells (34%, $p < 0.001$) at the expense of ganglion cells and horizontal cells, co-injection of Mash1 mRNA with dnGSK3β mRNA resulted in an increase in the representation of ganglion cells relative to controls (Figure 7A). 49% of labeled cells adopted ganglion cell fates at the expense of later-born cell types. This 2.4-fold increase is similar to that observed in Xath5 or XNeuroD $_{(S\rightarrow A)}$-injected embryos. Similarly, Xash1 injection alone resulted in an increase in the percentage of bipolar cells, as compared to controls (39% increase, $p < 0.001$), with decreases in ganglion cells (25% decrease, $p < 0.044$) and photoreceptors (24% decrease, $p < 0.003$; Figure 7B). Co-injection with dnGSK3β only slightly increased the representation of ganglion cells (18% versus 14%, $p = 0.114$). Instead, there appeared to be an increase in the percentage of photoreceptors (35% increase, $p < 0.001$). This observed increase in photoreceptors was due to the increase in cones, another early-born cell type. The num-

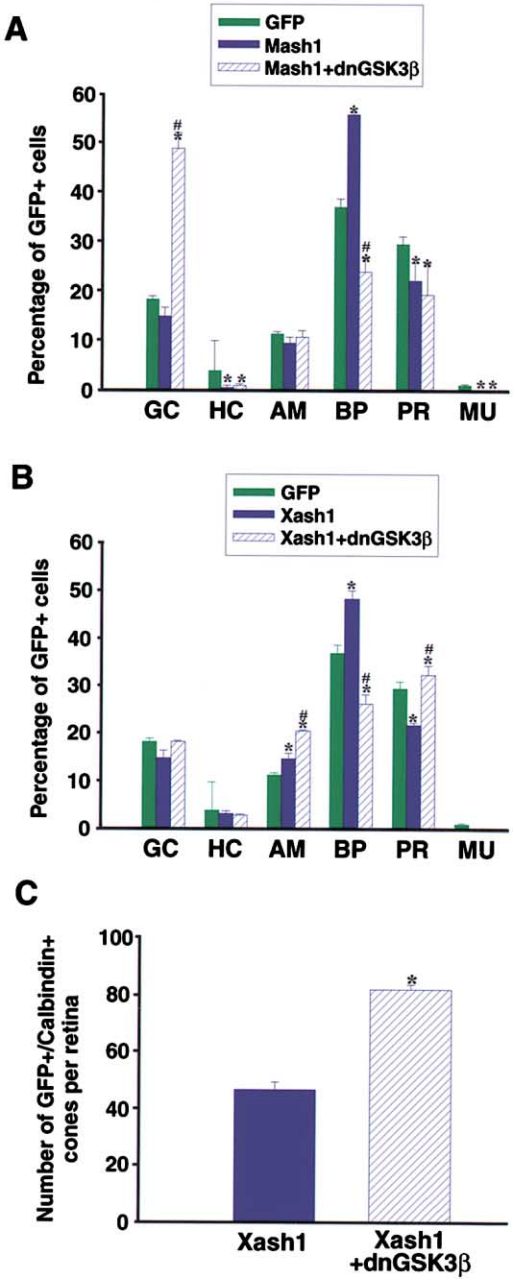

Figure 7. GSK3β Can Regulate the Activity of Mash1 and Xash1 in the *Xenopus* Retina

(A) Overexpression of Mash1 by 16-cell RNA injection promotes an increase in bipolar cell differentiation, compared to GFP control injections. Coexpression of dnGSK3β with Mash1 caused an increase in retinal ganglion cell differentiation. n = 3655 cells from 17 embryos for GFP, n = 793 cells from 5 embryos for Mash1, and n = 410 cells from 5 embryos for Mash1 + dnGSK3β.

(B) Overexpression of Xash1 promotes an increase in bipolar cell differentiation, compared to GFP control injections. Coexpression of dnGSK3β with Xash1 caused an increase in the percentage of labeled cells found in the photoreceptor layer. n = 3655 cells from 17 embryos for GFP, n = 1436 cells from 6 embryos for Xash1, and n = 968 cells from 5 embryos for Xash1 + dnGSK3β.

(C) Coexpression of dnGSK3β with Xash1 caused an increase in the number of GFP-labeled, calbindin-positive cone photoreceptors per retina. n = 5 embryos for Xash1 and n = 5 embryos for Xash1 + dnGSK3β.

ber of photoreceptor cells staining for calbindin, which marks cones in stage 41 retinas (Dorsky et al., 1997), was increased by 38% in retinas from dnGSK3β/Xash1-injected embryos, as compared to the Xash1-injected embryos (Figure 7C). This result indicates that release from inhibition at early stages allows Xash1 to promote early, rather than late, cell fates as well. Similar results were obtained with *Drosophila* scute, which also promotes bipolar differentiation when overexpressed in the *Xenopus* retina, but early-born cell types when coexpressed with dnGSK3β (data not shown). Our results demonstrate that the activities of additional bHLH factors can be altered by inhibition of GSK3β in the retina and are consistent with endogenous GSK3β, serving to regulate the activity of multiple factors that control the differentiation of later-born cell types. Furthermore, these findings demonstrate that bHLH factors from species other than *Xenopus* can be regulated by GSK3β.

Xath5 and XNeuroD Are Independently Regulated during Retinogenesis

We have shown that the late-acting factors XNeuroD, Mash1, and Xash1 are sensitive to inhibition by GSK3β in retinal progenitors, while the early-acting factor Xath5 is not. However, the genesis of early-born neurons is also a coordinated process that is subject to control by negative regulatory pathways. For example, the neurogenic Delta/Notch pathway can regulate retinal ganglion cell differentiation and may play a more general role in regulating the determination of early retinal cell types (Dorsky et al., 1997). In the *Xenopus* retina, coexpression of activated Notch with Xath5 by 16-cell RNA injection can inhibit the ability of Xath5 to promote retinal ganglion cell differentiation (Schneider et al., 2001). In the neural plate, Notch is unable to inhibit XNeuroD function, although this has not been addressed in the retina (Chitnis and Kintner, 1996). This raises the possibility that during retinal neurogenesis there is independent regulation of the function of Xath5 and XNeuroD, which would allow for precise coordination of retinal histogenesis at both early and late stages of development. However, it is possible that Notch can inhibit XNeuroD, but that the inhibitory effect of GSK3β is dominant, masking a Notch effect. To test this, we co-injected mRNAs for either Xath5, XNeuroD, or $XNeuroD_{(S\rightarrow A)}$, along with XΔE, an activated form of the X-Notch-1 receptor lacking the extracellular domain (Coffman et al., 1993), into a dorsal blastomere of 16-cell stage embryos and assayed for effects on the ratios of retinal cell types. Activated Notch alone caused increases in the numbers of undifferentiated cells within the retina (Figure 8A) and was able to inhibit the ability of Xath5 to promote ganglion cell differentiation when coexpressed by 16-cell RNA injection (Schneider et al., 2001; data not shown). In contrast,

Error represents SEM; the asterisks represent significant difference as compared to GFP controls, while the number signs represent significant difference between coexpression of dnGSK3β with either Mash1 or Xash1, as compared to injection of Mash1 or Xash1 alone; $p < 0.01$ by Student's t test. GC, ganglion cells; HC, horizontal cells; AM, amacrine cells; BP, bipolar cells; PR, photoreceptor cells; and MU, Müller glial cells.

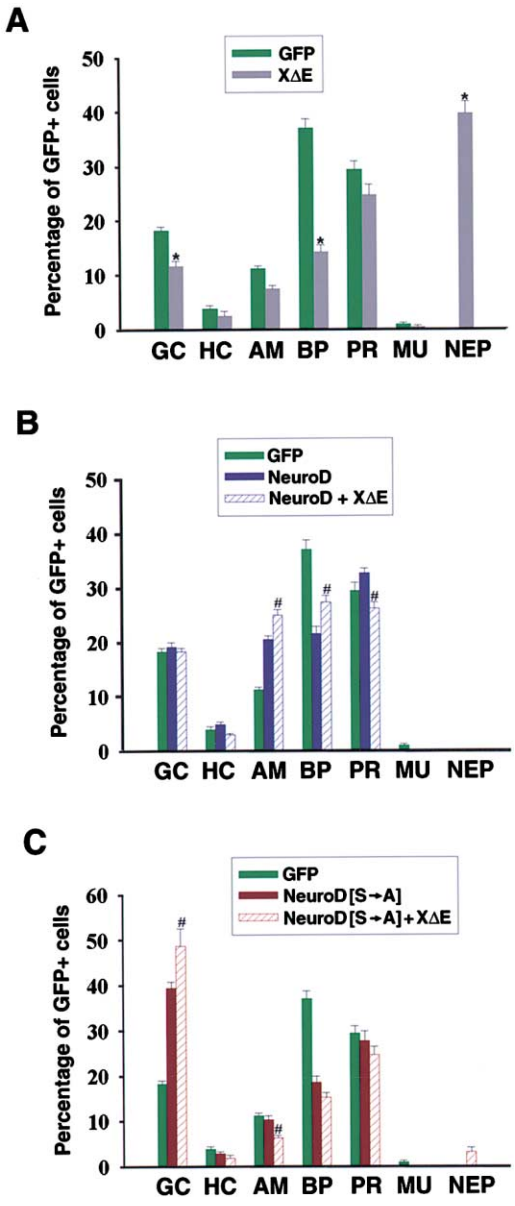

Figure 8. Activated Notch Does Not Inhibit the Function of XNeuroD or $XNeuroD_{(S\rightarrow A)}$ in Retinal Progenitor Cells

(A) Overexpression of XΔE, an activated form of the Notch receptor lacking the extracellular domain, by 16-cell RNA injection suppresses retinal neuron differentiation and causes an increase in undifferentiated neuroepithelial cells (NEP), as compared to GFP controls. n = 3655 cells from 17 embryos for GFP and n = 1090 cells from 6 embryos for XΔE.

(B) Overexpression of XNeuroD alone promotes amacrine cell (AM) differentiation, and this ability is not inhibited by coexpression of XΔE. n = 3655 cells from 17 embryos for GFP, n = 2886 cells from 21 embryos for XNeuroD, and n = 3281 cells from 9 embryos for XNeuroD + XΔE.

(C) Similarly, coexpression of XΔE with $XNeuroD_{(S\rightarrow A)}$ does not inhibit its ability to promote retinal ganglion cell (GC) differentiation. n = 3655 cells from 17 embryos for GFP, n = 3141 cells from 15 embryos for $XNeuroD_{(S\rightarrow A)}$, and n = 605 cells from 5 embryos for $XNeuroD_{(S\rightarrow A)}$ + XΔE.

Error represents SEM; asterisks represent significant difference as compared to GFP controls; $p < 0.01$ by Student's t test. GC, ganglion cells; HC, horizontal cells; AM, amacrine cells; BP, bipolar cells; PR, photoreceptor cells; and MU, Müller glial cells.

activated Notch did not suppress the ability of XNeuroD or $XNeuroD_{(S\rightarrow A)}$ to promote the differentiation of later-born cell types or ganglion cells, respectively (Figures 8B and 8C). These results indicate that Notch differentially regulates Xath5 and XNeuroD in the retina, as well as in the neural plate. The independent regulation of Xath5 and XNeuroD during retinal neurogenesis would allow for precise temporal control over their function and, thus, control over the cell types they produce throughout retinal neurogenesis.

Interestingly, Ohnuma et al. (2002) have shown that when Xath5 or XNeuroD is coexpressed by in vivo lipofection with either activated Notch or other factors that promote cell cycle arrest (such as $p27^{Xic1}$), these factors synergize to promote cell cycle exit and early differentiation of retinal progenitors, giving rise to increases in retinal ganglion cells. Thus, depending upon either the timing of expression or relative levels of expression, Notch may, in some cases, enhance rather than antagonize the differentiation activity of proneural bHLH factors.

Discussion

Neuronal Phenotype and the Timing of Neuronal Differentiation

It has been proposed that the competence of retinal progenitors to generate different retinal cell types changes over time, so that at any given time a progenitor is competent to make only one or a few cell types (Cepko et al., 1996; Livesey and Cepko, 2001). How do bHLH factors contribute to cell fate decisions in the developing retina? Our experiments support a model in which bHLH factors can regulate retinal neuron differentiation, but the neuronal subtype that results depends upon the competence of the progenitor cell in which the bHLH factor functions. Temporal control over bHLH factor activity could be achieved in several ways. Transcriptional regulation can determine when and where a gene is expressed. For example, in the mouse retina, *Math5* is expressed early and is required for the differentiation of retinal ganglion cells, while *NeuroD* is expressed later and is required for the development of amacrine cells (Brown et al., 1998; 2001; Cai et al., 2000; Wang et al., 2001). It is also possible that at a given stage of development, there is heterogeneity of bHLH expression within progenitors, so that not all progenitors differentiate at the same time or adopt the same fate. For example, in the rodent retina, *Mash1* is expressed later in retinogenesis but only in a subset of proliferating progenitors (Jasoni and Reh, 1996). Nieto et al. (2001) have also found that cerebral cortical progenitors are heterogeneous with respect to *ngn2* expression. Here, we show that differential activity of bHLH factors is possible even for factors that are coexpressed, since posttranslational mechanisms can regulate the timing of bHLH factor function.

The effects of timing of bHLH expression and/or function are not limited to the *Xenopus* retina. Overexpression of NeuroD in rat retinas has different effects on retinal neuron differentiation that is, to some extent, dependent upon time of expression (Morrow et al., 1999). Infection of a retrovirus expressing NeuroD in

murine retinas in vivo at postnatal day (P)0 or P4 during the peak of amacrine cell genesis increased the number of amacrine cells, while infection at P7 had no effect on amacrine cells (Morrow et al., 1999). The effect on rod photoreceptor differentiation was different, with no increase in rod photoreceptors following infection at P0 and modest increases observed with infection at P4 and P7 (Morrow et al., 1999). The effects of NeuroD overexpression at earlier stages of retinal development were not tested. It is reasonable to postulate that molecules that either interact with NeuroD or are required in combination with NeuroD also change in expression and/or activity levels over time, resulting in a different profile of retinal cell types. These experiments, together with our findings, suggest that in the developing vertebrate retina, the timing of bHLH factor function is important for determining what neuronal cell type is generated.

bHLH Factors and the Neuronal Subtype Decision

In the developing *Xenopus* retina, we have found that a GSK3β-insensitive form of XNeuroD can specify the same retinal cell type as Xath5, even though Xath5 normally promotes ganglion cells and XNeuroD does not. There are other instances where bHLH factors have similar ability to regulate the development of specific neuronal subtypes. For example, the retinas in *Mash1/Math3* double knockouts completely lack bipolar cells, while the single knockouts have relatively normal bipolar cells, indicating that either gene can complement the loss of the other in the retina (Hatakeyama et al., 2001). Furthermore, overexpression of bHLH factors, such as Mash1, Math3, NeuroD, and ngn2, in mouse retinal explants promotes the differentiation of rods, the predominant cell type being generated at the time of the experiment (Hatakeyama et al., 2001). Similarly, in the murine cerebral cortex, bHLH factors (such as Ngn1, Ngn2, and Mash1) can all induce the differentiation of neurons appropriate to the age of the animal when they are overexpressed, suggesting that layer-specific information is not provided by these bHLH factors (Cai et al., 2000).

However, differences between other bHLH factors have been observed, suggesting that not all bHLH factors have equivalent function. Murine knockout experiments suggest that some aspects of the neuronal phenotype, such as regional identity, may depend on the activity of specific bHLH factors. In both *ngn2* and *ngn1/ngn2* knockout animals, the compensatory upregulation of *Mash1* expression in dorsal cortical progenitors results in conversion of neurons in the dorsal telencephalon to those with ventral characteristics (Fode et al., 2000). In *Drosophila*, there is evidence for specificity in the ability of different proneural bHLH factors to direct the development of different classes of neurons in the peripheral nervous system (reviewed in Bray, 2000). In addition, in *Xenopus*, overexpression of Xath3 promotes the formation of both ganglion cells and cone photoreceptors (Perron et al., 1999), while overexpression of Xath5 only promotes ganglion cell differentiation (Kanekar et al., 1997), even after targeting expression to stages when large numbers of both types of early cells are being generated (data not shown). Furthermore, subtype-specific properties of neurons, such as neurotransmitter phenotypes, may be differentially controlled by distinct bHLH factors or combinations of factors (Matter-Sadzinski et al., 2001).

How do retinal bHLH factors regulate the development of specific subtypes of neurons during normal development? We favor the hypothesis that acquisition of the final retinal phenotype requires unique combinations of transcription factors and their downstream targets. In support of this, it has been shown that acquisition of bipolar cell identity requires the activity of both bHLH factors (such as Mash1 or Math3) and the homeobox factor Chx10 (Hatakeyama et al., 2001). Similarly, during spinal cord development in chick, expression of Ngn1 is insufficient to specify the motor neuron fate unless coexpressed with olig2 (Mizuguchi et al., 2001; Novitch et al., 2001). Thus, in the case of retinal ganglion cell differentiation, bHLH factors (such as Xath5 and XNeuroD) may be able to couple to a retinal ganglion cell-specific program of differentiation, such as activation of XBrn3d expression (Hutcheson and Vetter, 2001), but whether this actually occurs during development may depend upon the complement of other factors expressed in the progenitor cells.

Mechanism of GSK3β Regulation

How is GSK3β regulating the function of bHLH factors, such as XNeuroD? Since the integrity of the GSK3β consensus phosphorylation site is critical for regulating the timing of XNeuroD and $mNeuroD_{(I \rightarrow V)}$ function, it is likely that GSK3β is directly phosphorylating these proteins. However, purified GSK3β protein failed to phosphorylate in vitro translated XNeuroD in an immune-complex kinase assay (data not shown). This is not surprising, since many substrates of GSK3β must first be "primed" or phosphorylated by another kinase at the most carboxy-terminal serine in the consensus phosphorylation sequence before the substrate will be recognized and phosphorylated by GSK3β (reviewed in Frame and Cohen, 2001). It also remains a possibility that XNeuroD is not a direct target for phosphorylation by GSK3β. Nevertheless, our data demonstrate that GSK3β plays a critical role in regulating XNeuroD activity in vivo.

GSK3β is generally active unless specifically inactivated through various signaling pathways (reviewed in Frame and Cohen, 2001). It is not yet known what turns GSK3β regulation off later in retinogenesis, so that XNeuroD and other GSK3β-sensitive bHLH factors can be active to promote differentiation. Candidates include wnt/frizzled signaling (Wodarz and Nusse, 1998), GSK3β binding protein (GBP; Yost et al., 1998), or receptor tyrosine kinase signaling. Further experiments will be necessary to define which signaling pathways regulate GSK3β activity during retinal neurogenesis.

GSK3β Regulation of NeuroD in Other Vertebrates

The consensus sequence for GSK3β phosphorylation is conserved in the NeuroD sequences of *Xenopus* zebrafish and goldfish but is absent in NeuroD sequences from mouse, chick, rat, and human (data not shown). In fish and amphibians, retinogenesis takes place over a short period of time: roughly 24 hr in *Xenopus* and 2–3 days in zebrafish (Holt et al., 1988; Hu and Easter, 1999),

as compared to 11 days in chick and over 2 weeks in mouse (reviewed in Altshuler et al., 1991). Thus, in chick and mouse, transcriptional regulation may ensure that bHLH factors involved in the development of distinct cell types are, for the most part, expressed in sequential and nonoverlapping patterns. For example, *Math5*, which is important for ganglion cell synthesis, is expressed earlier in retinal development than *Mash1*, which is important for bipolar cell differentiation (Brown et al., 1998). In contrast, in the *Xenopus* retina, expression of multiple bHLH factors commences at the same time and often even in the same cells (Kanekar et al., 1997; Perron et al., 1998). Posttranslational regulation would allow progenitor cells to rapidly respond to environmental changes without de novo transcription/translation.

The presence of multiple regulatory pathways is likely critical for regulating the development of a full complement of retinal cell types in a short period of time and may allow the differentiation of different classes of neurons to be regulated in response to extrinsic signals within the developing *Xenopus* retina. However, the fact that bHLH factors from other species (including Mash1 and *Drosophila* scute) contained GSK3β consensus phosphorylation sites and were sensitive to inhibition by GSK3β in our assay suggests that posttranslational regulation of bHLH factor function may be important in multiple species and in tissues other than the retina. In summary, we propose that posttranslational regulation of bHLH function may be one mechanism that is used to generate diversity by modulating when a progenitor will undergo differentiation.

Experimental Procedures

Embryo Collection

Xenopus laevis eggs were obtained by hormone-induced egg laying from adult frogs using standard methods. For 16- or 32-cell injections, only embryos exhibiting stereotypic cleavages (Moody, 1987) were used to consistently identify retina-producing blastomeres across the population of experimental embryos (Huang and Moody, 1993).

Generation of XNeuroD$_{(S\rightarrow A)}$ and mNeuroD$_{(I\rightarrow V)}$ Constructs

To construct a GSK3β-insensitive form of XNeuroD (pCS2+ XNeuroD$_{(S\rightarrow A)}$), amino acid residues 276 and 280 were changed from serine to alanine. XNeuroD$_{(S\rightarrow A)}$ was generated by overlap-extension PCR (Horton et al., 1989), using PFU polymerase (Gibco BRL, Gaithersburg, MD) and specific primers in conjunction with vector primers and pCS2+ XNeuroD as a template. Primers: ND/GSKMUT-5′ CCACCCCTTGCTGTTAACGGGAACTTTAC and ND/GSKMUT-3′ AGCAAGGGGTGGGGCCAGGGGACCATCG. The PCR product was cloned into the pCS2+ vector using EcoRI and XbaI. A GSK3β-sensitive form of mNeuroD (mNeuroD$_{(I\rightarrow V)}$) was generated by changing amino acid residue 276 from an isoleucine to a valine using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The primer used in this mutagenesis was: GCCCGCCGCTCAGCGTCAATGG CAACTTCTC. The final constructs were confirmed by sequencing at the University of Utah Sequencing facility.

Blastomere Injection of mRNAs

Capped mRNAs were synthesized in vitro using a mMessage mMachine SP6 kit according to the manufacturer's protocol (Ambion Inc., Austin, TX). The following constructs in the pCS2+ vector were used: β-galactosidase, GFP, Xath5a (Kanekar et al., 1997), GSK3β (Pierce and Kimelman, 1995), dnGSK3β (Pierce and Kimelman, 1995), XΔE (Coffman et al., 1993), Xash1 (Ferreiro et al., 1993), and Mash1 (Johnson et al., 1990). XNeuroD (Lee et al., 1995) and XNeuroD$_{(S\rightarrow A)}$ were in the pCS2+MT vector. For the two-cell injections, mRNAs were injected as previously described (Kanekar et al., 1997) at the following amounts: 100 pg β-Gal, 400 pg Xath5, 400 pg XNeuroD, 400 pg XNeuroD$_{(S\rightarrow A)}$, and 2 ng GSK3β in a 4 nL volume. β-Gal was used as a lineage tracer in the two-cell experiments. For all experiments, identical results were obtained when full-length, non-myc-tagged XNeuroD in pCS2+ (kind gift of Shin-ichi Ohnuma) was used instead of pCS2+MT XNeuroD. Embryos were allowed to develop in one-tenth full dilution MMR to neural plate stages according to Nieuwkoop and Faber (1994). The embryos were fixed in MEMFA (Harland, 1991) for up to 1 hr, assayed for β-galactosidase activity using previously described methods (Turner and Weintraub, 1994), and processed for whole-mount in situ hybridization, using a digoxigenin-labeled N-tubulin probe, as described in Kanekar et al. (1997).

To determine effects on retinal cell fates, blastomeres at the 16- or 32-cell stage were microinjected with 300 pg of GFP mRNA as a lineage tracer and 10 pg of each individual mRNA in a 1 nL volume. The injected blastomeres were either the major retinal progenitors (D1.1 or D1.2) or for calculating clone size, V1.2.1, a 32-cell stage blastomere (Huang and Moody, 1993).

In Vivo Lipofection of DNA

DNA was transfected into the eye primorida of stage 17–18 or stage 26 embryos as previously described (Holt et al., 1990). pEGFP-C1 plasmid DNA (Clontech) was cotransfected to mark the transfected cells.

Fixation and Retinal Cell Fate Analysis

Embryos were raised in one-tenth full dilution MMR solution until stage 40/41 (Nieuwkoop and Faber, 1994). Embryos were fixed in MEMFA, cryoprotected in sucrose phosphate buffer, embedded in OCT compound (Tissue Tek), and sectioned at 14 μM on a cryostat. Nuclear staining was performed by adding Hoescht dye to the wash solution at a final concentration of 0.0005%. GFP positive cells from central retina sections were identified and counted based on the relative laminar position and morphology, as described in Dorsky et al. (1995; 1997). In some cases, antibody staining was used to confirm cell type. The results were compared using a 95% confidence interval and the Student's t test when the data were normally distributed and the Mann-Whitney U test, when the data did not fit a normal distribution (SigmaStat 2.0, Jandel Scientific).

Immunocytochemistry

Primary antibodies were used at the following dilutions: Islet-1, 1:1, gift of Mahendra Rao, University of Utah; anti-Neurofilament, 1:1, Chemicon; anti-calbindin, 1:100, Sigma; anti-GABA, 1:100, Diasorin; and anti-Pax6, 1:500 (Belecky-Adams et al., 1997; Mastick et al., 1997). An Alexa 568-conjugated donkey anti-mouse secondary antibody was used at 1:300 (Molecular Probes, Inc., Eugene, OR). Nuclear staining was performed by adding Hoescht dye to the wash solution at a final concentration of 0.0005%. For identification of dopaminergic amacrine cells, embryos were raised until stage 44. Anti-tyrosine hydroxylase staining was performed, as previously described (Huang and Moody, 1998).

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