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The Basic Helix-Loop-Helix Transcription Factor *neuroD* Is Expressed in the Rod Lineage of the Teleost Retina

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ABSTRACT

Persistent rod genesis in the retinas of teleost fish was first described over 2 decades ago, but little is known regarding the underlying genetic and molecular mechanisms that govern this phenomenon. Because of its function in the developing mammalian retina and persistently mitotic adult tissues, we sought to characterize the cellular expression of the basic helix-loop-helix (bHLH) transcription factor *neuroD* in the persistently neurogenic retina of adult teleosts. We show here that, in the adult retina of the goldfish, *neuroD* is expressed by putative amacrine cells, nascent cones, and the mitotically active cells of the rod lineage. *neuroD* is the first gene shown to be expressed by rod precursors, the immediate antecedents of rod photoreceptors. In contrast to the vertebrate classes described previously, *neuroD* is not expressed in multipotent progenitors in the teleost retina. Combining *neuroD* in situ hybridizations with cell-cycle-specific markers suggests that, in rod precursors, *neuroD* expression is cell cycle specific. J. Comp. Neurol. 477:108–117, 2004.

Indexing terms: neurogenesis; cell cycle; proliferation; gene expression; cell fate; development

Generating neuronal diversity in the developing nervous system is regulated by a complex interplay of extrinsic and intrinsic molecular events occurring coordinately in time and space. Transcriptional regulation is a key intrinsic mechanism, and from flies to mammals members of the basic helix-loop-helix (bHLH) family of transcription factors play essential roles. Prominent among the vertebrate bHLH factors is *neuroD* (also known as *BETA2*; Lee et al., 1995), one of several vertebrate homologues of the *Drosophila* gene *atonal* (Brennan and Moses, 2000). The function of *neuroD* has been examined in numerous tissues and cell lines. Forced expression of *neuroD* induces dividing cells to withdraw from the cell cycle (Farah et al., 2000) and can impart a neuronal fate to nonneuronal cells (Lee et al., 1995; Farah et al., 2000). From this, *neuroD* was hypothesized to be a key regulator of both proliferation and cellular differentiation in the developing brain. This function appears to be conserved in neurogenic regions of the adult brain, e.g., olfactory epithelium and hippocampus, as well as persistently mitotic nonneural tissues, e.g., small intestine (Naya et al., 1997; Miyata et al., 1999; Lee et al., 2000; Liu et al., 2000; Nibu et al., 2001).

Not surprisingly, *neuroD* is expressed in the developing retina, where it appears to regulate multiple functions. Loss- and gain-of-function studies in rodents suggest that *neuroD* regulates neuron vs. glial cell fate (Morrow et al., 1999; but see Inuoe et al., 2002; Pennesi et al., 2003), is determinative for amacrine cells (Morrow et al., 1999; Moore et al., 2002; but see Inuoe et al., 2002), and acts as a differentiation and survival factor for photoreceptors (Ahmad et al., 1998; Morrow et al., 1999; Pennesi et al., 2003). In the chick, *neuroD* appears to be determinative for photoreceptors (Yan and Wang, 1998, 2004; Fischer et al., 2004).

Similarly to the case in all vertebrates, the teleost retina differentiates from a sheet of neuroepithelial cells. However, in contrast to virtually all homeothermic ani-

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mals, retinal differentiation occurs when the animal and the retina are small (e.g., posthatch day 2.5 in zebrafish; Schmitt and Dowling, 1999; Li et al., 2000) and the number of retinal neurons is a tiny fraction of the adult total. In teleosts and frogs (Perron and Harris, 2000) and to a lesser extent birds (Fischer and Reh, 2000; see also Moshiri and Reh, 2004), a vestige of the retinal neuroepithelium is retained at the retinal margin, and this thin annulus of progenitors supplies the vast majority of the neurons found in the adult (Müller, 1952; Lyall, 1957; Johns, 1977; Meyer, 1978; Marcus et al., 1999). This annulus of cells, the circumferential germinal (or marginal) zone (CGZ), is a perpetually self-renewing neuroepithelium sustained by stem cells (for review see Easter and Hitchcock, 2000; Easter and Malicki, 2002). In addition, rod photoreceptors are generated in situ throughout the mature retina by a delayed but equally prolonged wave of neurogenesis. After the retina differentiates, progenitors within the inner and outer retinal layers continue to divide, giving rise exclusively to rods, which are insinuated into the existing lawn of photoreceptors (Johns and Fernald, 1981; Raymond and Rivlin, 1987; Hagedorn and Fernald, 1992). The rod lineage consists of stationary stem cells in the inner nuclear layer (INL) that express *pax6* (Otteson et al., 2001), migratory progenitors (INL progenitors) that move from inner to outer nuclear layers, and rod precursors, the immediate antecedents of rod photoreceptors, which reside in the outer nuclear layer (Otteson et al., 2001; see also Johns and Fernald, 1981; Johns, 1982; Julian et al., 1998; for review see Otteson et al., 2003). Cells of this lineage are distinguished by their laminar position, nuclear morphology, and differing rates of division.

Because of its function in the developing mammalian retina and persistently mitotic adult tissues, we sought to determine whether *neuroD* plays a role in the persistent neurogenesis in the retinas of teleosts by examining its cellular pattern of expression. This study revealed that *neuroD* is expressed in a subset of differentiated amacrine cells, nascent cone photoreceptors, and the INL progenitors and rod precursors of the rod lineage. *neuroD* is the second transcription factor mapped to this lineage of cells, and the first shown to be expressed by rod precursors. In contrast to that described for the mammals and amphibians, in teleosts *neuroD* is not expressed by multipotent retinal progenitors. A series of double-labeling experiments suggests that, in cells of the rod lineage, the expression of *neuroD* is cell cycle specific.

MATERIALS AND METHODS

Protocols for animal husbandry, anesthesia, and sacrifice were approved by the Unit for the Use and Care of Animals at the University of Michigan and conform to NIH guidelines.

Systemic bromodeoxyuridine treatment

Mitotically active cells in the retina were labeled by systemic exposure to bromodeoxyuridine (BrdU; Otteson et al., 2001). Briefly, animals were housed for 4 or 24 hours in a solution containing 5 mM BrdU, 0.67 g/liter NaCl, 0.1 g/liter Neutral Regulator (Seachem Laboratories, Stone Mountain, GA), and 0.03 g/liter tetracycline. It is assumed that BrdU is taken up through the gills and is distributed to various tissues through the vasculature. Housing animals in BrdU for as briefly as 2 hours is adequate to label dividing cells in the retina (Hitchcock, unpublished observations). Animals were immediately sacrificed after removal from the BrdU solution.

Growth hormone injections

Selected animals received an intraperitoneal injection of growth hormone (0.5 μg/g body wt.) to stimulate proliferation of retinal progenitors (Otteson et al., 2002). Forty-eight hours later, these animals were exposed to systemic BrdU for 24 hours. Eye cups were then prepared and processed as described below.

Anesthesia and tissue processing

Anesthesia was induced by immersing animals in 0.1% tricaine methanesulfonate (MS222) until gill movements stopped. Animals were sacrificed by exanguination.

Eye cups (whole eyes lacking the cornea and lens) were fixed by one of two methods, both of which are compatible with in situ hybridization. First, for sections processed for in situ hybridization followed by immunostaining with antibodies against proliferating cell nuclear antigen (PCNA), eye cups were fixed overnight in alcoholic formaldehyde (see Vihtelic and Hyde, 1998). Second, for sections processed for in situ hybridization followed by immunostaining with antibodies against either phosphohistone H3 or BrdU, eye cups were fixed for 3–5 hours by immersion in ice-cold 4% paraformaldehyde freshly prepared in 100 mM phosphate buffer, pH 7.2. Regardless of fixation solution, after fixation, eye cups were infiltrated overnight in 20% sucrose in phosphate buffer and embedded in Optimal Cutting Temperature media (OCT; Sakura Finetek, Torrance, CA). Cryosections were cut at 5 μm, and every other section was mounted on glass slides (Colorfrost/plus; Fisher Scientific, Pittsburgh, PA).

In situ hybridization

In situ hybridization was performed as described previously (Hitchcock et al., 2001; Otteson et al., 2002). Briefly, the full-length *neuroD* cDNA (Korzh et al., 1998) was linearized by using the restriction enzyme BamH1, and a digoxygenin-labeled riboprobe was synthesized by in vitro translation with T7 RNA polymerase and an RNA labeling kit (Roche Diagnostic Corp., Indianapolis, IN). The precipitated riboprobe (approximately 10 μg) was resuspended in 100 μl of RNase-free water and stored at –20°C.

Immediately after cryosectioning, sections were desiccated for 1 hour, rehydrated in ethanols, treated with 0.1 M proteinase K followed by acetic anhydride, and dehydration in ethanols. The sections were then dried for at least 1 hour. The riboprobe was diluted 1:500 in probe buffer, and 80 μl of this solution (approximated 16 ng of probe) were puddled onto each slide, sealed with a coverslip, and hybridized overnight at 55°C. On the following day, sections were washed for 60 minutes at 55°C in a solution containing equal volumes of 1× SSC and dionized formamide plus 0.1% Tween-20. The hybridized probes were visualized according to manufacturer's instructions (Roche Diagnostic Corp.) by using an alkaline phosphatase-conjugated antibody against digoxygenin and a colorimetric reaction with NBT-BCIP as the enzymatic substrate. The color reaction was stopped, generally after 2–5 hours, by rinsing sections in 100 mM Tris-HCl buffer, pH 7.2. In situ hybridizations were followed imme-

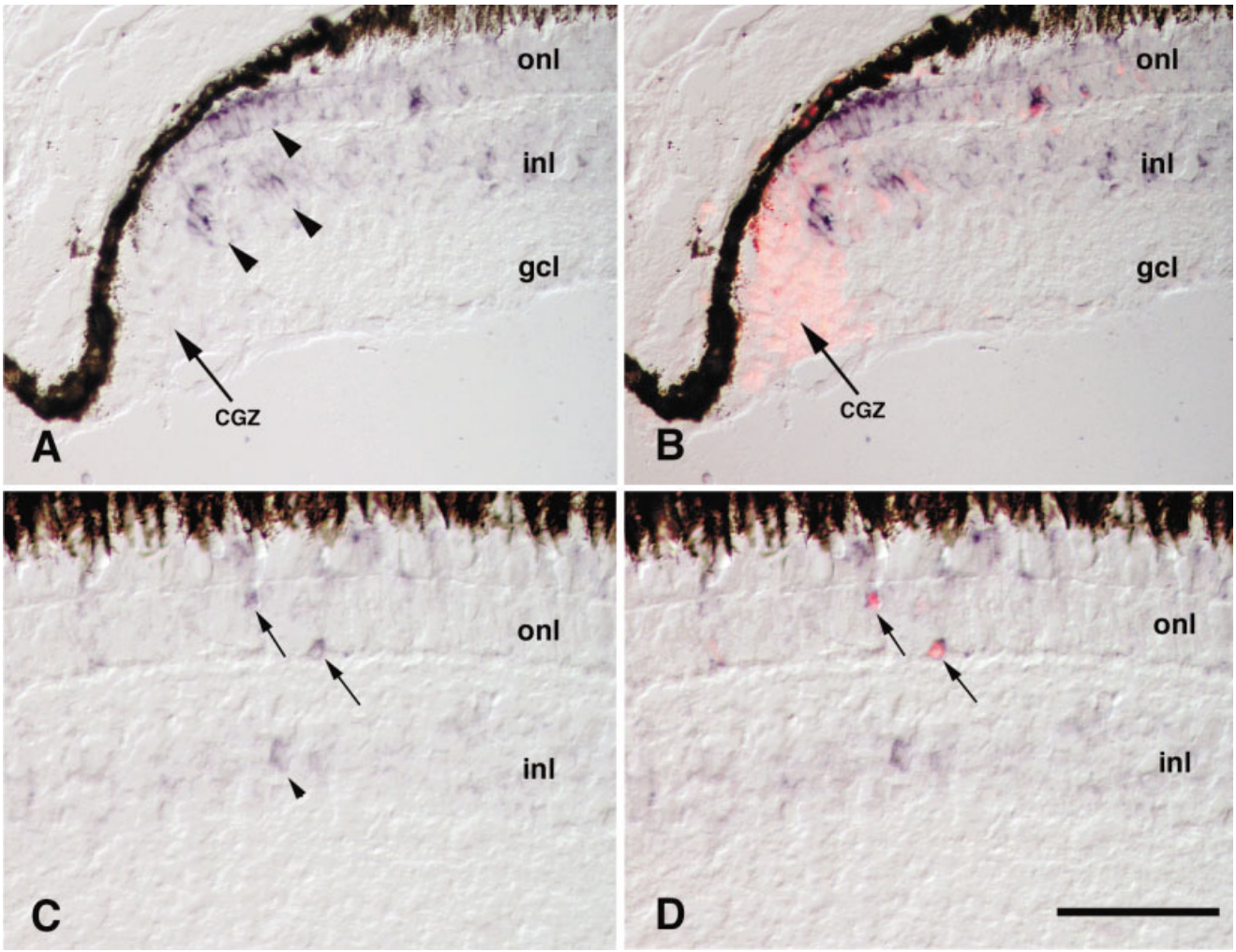

Fig. 1. *neuroD* expression at the margin of the teleost retina. **A,B:** At the retinal margin, *neuroD* is expressed in newly postmitotic cone photoreceptors within the ONL (arrowheads in ONL) and cells in the overlying INL of the circumferential larval zone (arrowheads in INL). *neuroD* is not expressed by the dividing, multipotent cells of the CGZ, which are labeled with antibodies against PCNA. **C,D:** Within the mature retina, *neuroD* is expressed by putative amacrine cells in the INL (arrowhead) and PCNA-positive rod precursors within the ONL (arrows). CGZ, circumferential germinal zone; onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer. Scale bar = 25 μm in D (applies to C,D); 50 μm for A,B.

diately by immunostaining with one of three antibodies (see below).

Double in situ hybridizations were performed with probes for *neuroD* and *cyclin D1* (Yarden et al., 1995). The basic protocol was identical to that described above, but with the following modifications. Flurescein-labeled riboprobes for *cyclin D1* were synthesized and used at a dilution of at 1:2,000 (approximately 4 ng probe per slide). Both *neuroD* and *cyclin D1* probes were diluted in buffer and hybridized simultaneously to the sections. After posthybridization washes, the sections were immunostained with a cocktail of antibodies against digoxigenin conjugated to alkaline phosphotase and antibodies against fluorescein conjugated to peroxidase. The sections were then rinsed, and the *neuroD* probes were visualized with fast red (Roche Diagnostic Corp.) as the substrate, which produces a fluorescent precipitate visible with the rhodamine filter set on a fluorescence microscope. The sections were then rinsed thoroughly, and the *cyclin D1* probes were visualized by using the Tyramide Signal Amplification Kit (Perkin Elmer, Norwalk, CT) according to the manufacturer's instructions with streptavidin-Alexa fluor 488 (Molecular Probes, Eugene, OR), which was visible with the fluorescein filter set on a fluorescence microscope.

Immunostaining

Standard techniques were used for all immunostaining (Hitchcock et al., 1992). PCNA was labeled with a monoclonal antibody (Sigma-Aldridge, St. Louis, MO) diluted 1:3,000. Cells in the M-phase of the cell cycle were labeled with a polyclonal antibody against phosphohistone H3 (Upstate Biotechnology, Lake Placid, NY) diluted 1:200. BrdU-labeled cells were immunostained with a monoclonal antibody (Becton Dickinson Immunocytochemistry Systems, San Jose, CA) diluted 1:100. The primary antibodies were visualized with secondary antibodies raised in

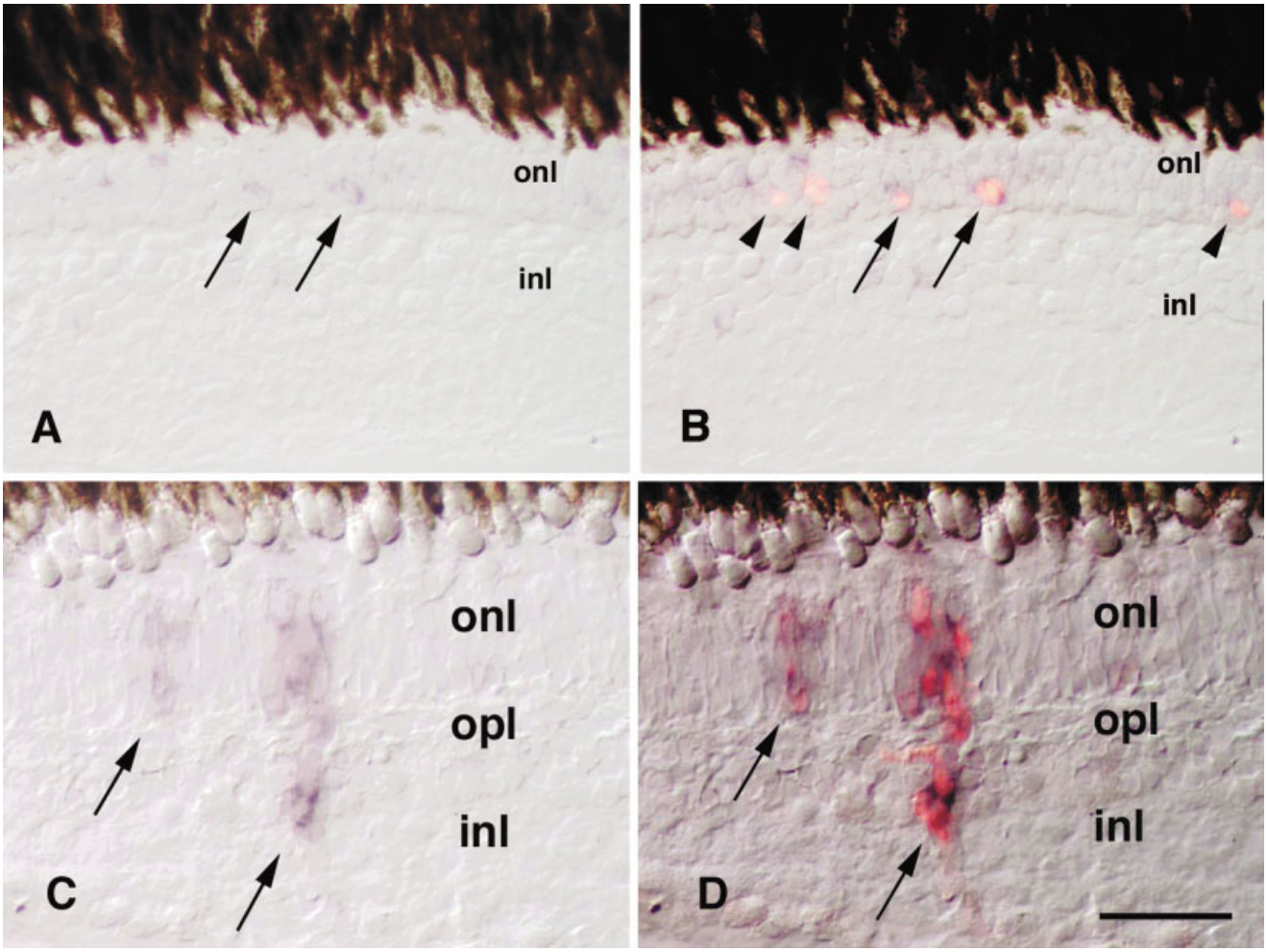

Fig. 2. *neuroD* expression and BrdU immunostaining within the ONL. **A,B:** In the ONL, *neuroD*-expressing rod precursors are most frequently observed at the vitread boundary of the ONL (arrows). The *neuroD*-expressing cells (A) are also immunostained with antibodies against BrdU (B). Note, however, that not all BrdU-containing rod precursors express *neuroD* (arrowheads). **C,D:** *neuroD* is also expressed by mitotically active rod progenitors within the INL. onl, outer nuclear layer; inl, inner nuclear layer; opl, outer plexiform layer. Scale bar = 25 μm.

goat that recognize either mouse or rabbit immunoglobulins and conjugated to fluorescent labels.

Photography

Images were captured with a Nikon DMX 1200 digital camera and either transmitted or indirect fluorescence illumination. Digital overlays and final figures were assembled in Adobe Photoshop.

Cell counts

Labeled rod precursors within the ONL were counted in 20 nonadjacent sections from a minimum of three eyes from three different animals (60 sections total). Rod precursor labeled with the antibodies or fluorescein isothiocyanate (FITC)-*cyclin D1* probes were identified by using fluorescence illumination; then, by switching between fluorescence and transmitted light or the second fluorescence filter set, cells were scored for whether they also expressed *neuroD*.

RESULTS

In situ hybridizations reveal a complex and temporally dynamic pattern of *neuroD* expression in the teleost retina. First, *neuroD* is expressed by cells lying in the inner half of the INL that have large somata and are regularly spaced across the section (Figs. 1, 2, 5), characteristics indicative of amacrine cells, which commonly express developmental regulatory genes (Hitchcock et al., 1996; de Melo et al., 2003). Second, *neuroD* is expressed by nascent cone photoreceptors (Fig. 1). In the teleost retina, immature cones lie in a rod photoreceptor-free annulus between the CGZ and mature (rod-containing) retina (Raymond and Rivlin, 1987; Stenkamp et al., 1997). Third, *neuroD* is expressed by mitotically active cells of the rod-photoreceptor lineage, INL progenitors and rod precursors (Figs. 1, 2). These cells are identified by their laminar address, nuclear morphologies, and, most importantly, markers of proliferation (PCNA, phosphohistone H3, or

incorporation of BrdU during S phase of the cell cycle; Figs. 1, 2, 5). Within the INL, the *neuroD*-expressing rod progenitors are generally observed as isolated, single cells, although they occasionally appear as a radial column spanning the inner and outer nuclear layers (Fig. 2).

Among the cells of the rod lineage, rod precursors within the ONL are the most abundant and, compared with other cells in the lineage, divide relatively rapidly (Otteson et al., 2001). Therefore, subsequent experiments evaluated the expression of *neuroD* in rod precursors only. It is assumed that the observations made for this population of cells are common to other members of the rod lineage.

As a second approach to confirm that *neuroD* is expressed in rod precursors, we took advantage of the wide variation in the number of rod precursors that are labeled in individual animals following exposure to BrdU. This variation in the number of labeled cells presumably reflects the momentary growth rate of the animal (Otteson et al., 2001). We also took advantage of the ability to stimulate the proliferation of rod precursors by intraperitoneal injections of growth hormone (Otteson et al., 2002). If *neuroD* is expressed in rod precursors, it was anticipated that, across a group of animals in which the number of BrdU-labeled rod precursors is variable, the number of rod precursors labeled with BrdU and the number expressing *neuroD* should covary. In this experiment, several animals were exposed to BrdU for 24 hours and killed or injected with recombinant trout growth hormone and 48 hours later exposed to BrdU for 24 hours. In sample sections taken from an eye of each animal, the number of BrdU-labeled rod precursors in each retina was qualitatively evaluated. A subset of these retinas were then selected to span a broad range of BrdU-labeled rod precursors (few cells per section to many). Additional sections from each eye were then processed for *neuroD* in situ hybridization, followed by BrdU immunostaining. Separately counting rod precursors in the ONL that were labeled with BrdU and rod precursors expressing *neuroD* showed that there is a linear relationship between the number of cells expressing *neuroD* and the number labeled with BrdU (Fig. 3). This result is consistent with the conclusion that *neuroD* is expressed by rod precursors and indicates that the expression of *neuroD* by these cells is tied to their rate of proliferation and responsive to the extrinsic molecules that govern this.

While we performed the experiments described above (illustrated in Figs. 1–3), it was apparent that not all rod precursors in the ONL immunostained with antibodies against PCNA or labeled by 24 hr exposure to BrdU also express *neuroD*. For example, the slope of the regression line passing through the data in Figure 3 is about 0.5. These observations indicate that *neuroD* is not a ubiquitous marker of rod precursors. The observation that not all dividing rod precursors express *neuroD* suggested that *neuroD* expression in these cells may be cell cycle specific. To evaluate this possibility further, the proportion of rod precursors that were labeled either with PCNA or with BrdU and also expressing *neuroD* was quantified. PCNA is a protein found within dividing cells that increases during late G1 phase of the cell cycle, reaches a maximum during S, and declines during G2/M (Kurki et al., 1986, 1988; Coltrera and Gown, 1991). When in situ hybridization was combined with immunostaining for PCNA, the cell counts showed that 44.8% ± 3.4% of PCNA-positive rod precursors (n = 1,303) express *neuroD* (Fig. 4). In

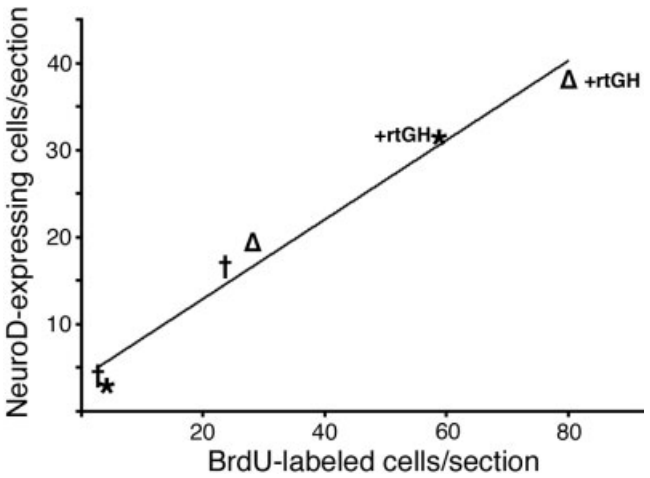

Fig. 3. Graph illustrating the relationship between the average number of BrdU-labeled rod precursors observed in a section and the average number of *neuroD*-expressing cells. The linear relationship between the number of dividing rod precursors and the number of *neuroD*-positive cells in the ONL is evidenced by the regression line drawn through the data. Note that the slope of the line is ~0.50, indicating that there are many more BrdU-labeled cells/section than *neuroD*-expressing cells/section. The common symbols represent sections from pairs of eyes that were mounted and processed on the same slides.

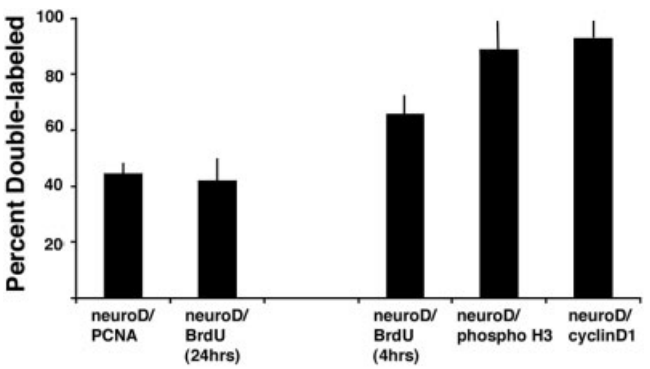

Fig. 4. Bar graphs illustrating the proportion of rod precursors labeled with various markers of cell proliferation that also express *neuroD*. Each bar represents the average values, plus one standard deviation. The labels beneath each bar indicate the label combined with in situ hybridization for *neuroD*: PCNA, proliferating cell nuclear antigen; BrdU, systemic exposure to bromodeoxyuridine for 24 and 4 hours; Phos H3, phosphohistone H3.

animals exposed to BrdU for 24 hours, an interval expected to mark cells at all phases of the cell cycle, 42.6% ± 8.2% of BrdU-labeled rod precursors (n = 1679) expressed *neuroD* (Fig. 4). These data suggest that fewer than half the cycling rod precursors express *neuroD*. This led to the tentative conclusion that *neuroD* expression may be cell cycle specific.

It was predicted that, if *neuroD* expression fluctuates with the cell cycle, by combining markers for specific phases of the cell cycle with *neuroD* in situ hybridizations, the proportion of double-labeled cells should increase during those phases of the cell cycle when *neuroD* is expressed. Therefore, three additional double-labeling experiments were undertaken.

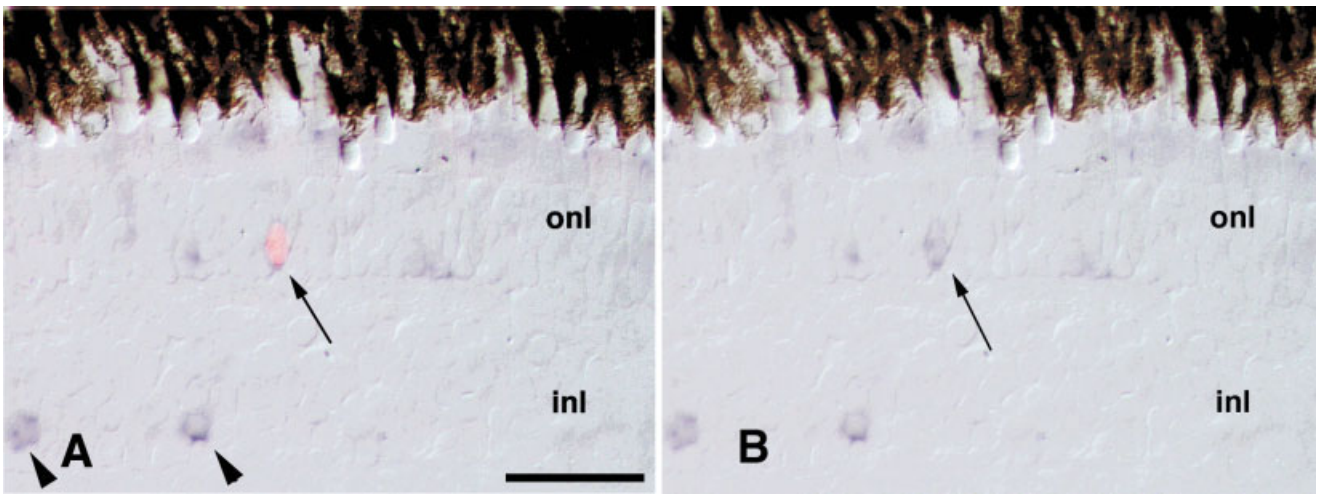

Fig. 5. Rod precursors express *neuroD* during the mitotic phase of the cell cycle. Rod precursors that are labeled with antibodies against phosphohistone H3 (arrow; **A**) express *neuroD* (arrow; **B**). Note the *neuroD*-expressing amacrine cells in the INL (arrowheads; A). onl, Outer nuclear layer; inl, inner nuclear layer. Scale bar = 25 μm.

First, animals were sacrificed after exposure to BrdU for 4 hours, a duration judged to label cells within S phase or slightly beyond, and the proportion of BrdU-labeled cells expressing *neuroD* was quantified. In these animals, relatively few cells were BrdU positive (n = 236 in 60 sections), as would be expected, but 65.7% ± 6.4% of these cells expressed *neuroD* (Fig. 4). Next, a similar experiment was performed with antibodies against the phosphorylated form of the core histone H3. Histone H3 is specifically phosphorylated during mitosis and is a marker of cells in the M phase of the cell cycle (Hans and Dimitrov, 2001). These cells are rare (~1–2/section), and phosphohistone-positive cells were counted in 120 sections from three retinas (rather than 60) and scored for whether they also express *neuroD*. This experiment showed that 87.6% ± 9.8% of M-phase cells (n = 222) expressed *neuroD* (Fig. 4). Finally, double in situ hybridizations were performed with probes to *neuroD* and *cyclin D1*. *cyclin D1* is a member of a family of cyclin genes whose products govern the progression of mammalian cells through G1 phase and the G1/S transition (Han et al., 1999; Ohnuma and Harris, 2003). Previous studies showed that, in mammalian cells, *cyclin D1* mRNA oscillates during the cell cycle and is typically induced in mid-G1 (Motokura et al., 1991, 1992; Matsushime et al., 1991; Bianchi et al., 1994). From these data, we infer that, in the teleost retina, *cyclin D1* expression also oscillates during the cell cycle and is induced in G1; therefore, *cyclin D1* in situ hybridization was used here to label cells in G1. In the teleost retina, *cyclin D1* is expressed by a small cluster of cells in the CGZ (see Fig. 6). These *cyclin D1*-expressing cells are generally found in the middle of the CGZ and represent a subset of the retinal progenitors at the margin. *cyclin D1* is also expressed by rod precursors within the ONL (Fig. 6). These cells are rare (~1–2 cells/section, similar in number to rod precursors labeled with the mitosis-marking antibodies; see above). The restricted expression of *cyclin D1* to a subset of retinal progenitors and the scarcity of rod precursors expressing this gene are consistent with our inference that *cyclin D1* is expressed within a restricted phase of the cell cycle. If the expression of *cyclin D1* was not cell cycle specific, *cyclin D1*-expressing cells should be at least as numerous as the PCNA-immunostained cells (Fig. 1), but this was not observed.

Because of the relatively few labeled rod precursors per section, *cyclin D1*-expressing rod precursors were scored in 120 sections from three retinas (rather than 60). The double in situ hybridizations showed that 93.3% ± 7.0% of cells expressing *cyclin D1* (n = 149) also express *neuroD* (Fig. 6).

DISCUSSION

The cellular expression of *neuroD* in the teleost retina is spatially complex but readily interpretable based on the structure and growth characteristics of this tissue. First, *neuroD* is expressed by a subset of putative amacrine cells. Four lines of evidence lead to this conclusion. 1) These *neuroD*-expressing cells are generally found within the inner tier of the INL. 2) They are relatively evenly spaced along the length of the retinal section, reflecting the regularity characteristic of amacrine-cell mosaics. 3) These cells are never labeled with markers of cell cycle proteins (PCNA, phosphohistone H3, cyclin D1), and they do not incorporate BrdU into their nuclei, consistent with their being postmitotic and not cells of the rod lineage. 4) In sections from any given animal, they are relatively invariable in number, irrespective of the large variations in the number rod precursors and INL progenitors observed between animals. It is common for developmental regulatory genes to have constitutive patterns of expression in the adult brain (Stoykova and Gruss, 1994), and *neuroD* is expressed abundantly in fully differentiated neurons in the vertebrate nervous system (Lee et al., 1995, 2000; Mueller and Wullimann, 2002), including amacrine cells in the retina (Acharya et al., 1997; Pennesi et al., 2003).

Second, *neuroD* is transiently expressed in immature cone photoreceptors. In the teleost retina, newly born cones lie in a rod-free annulus of the ONL located between

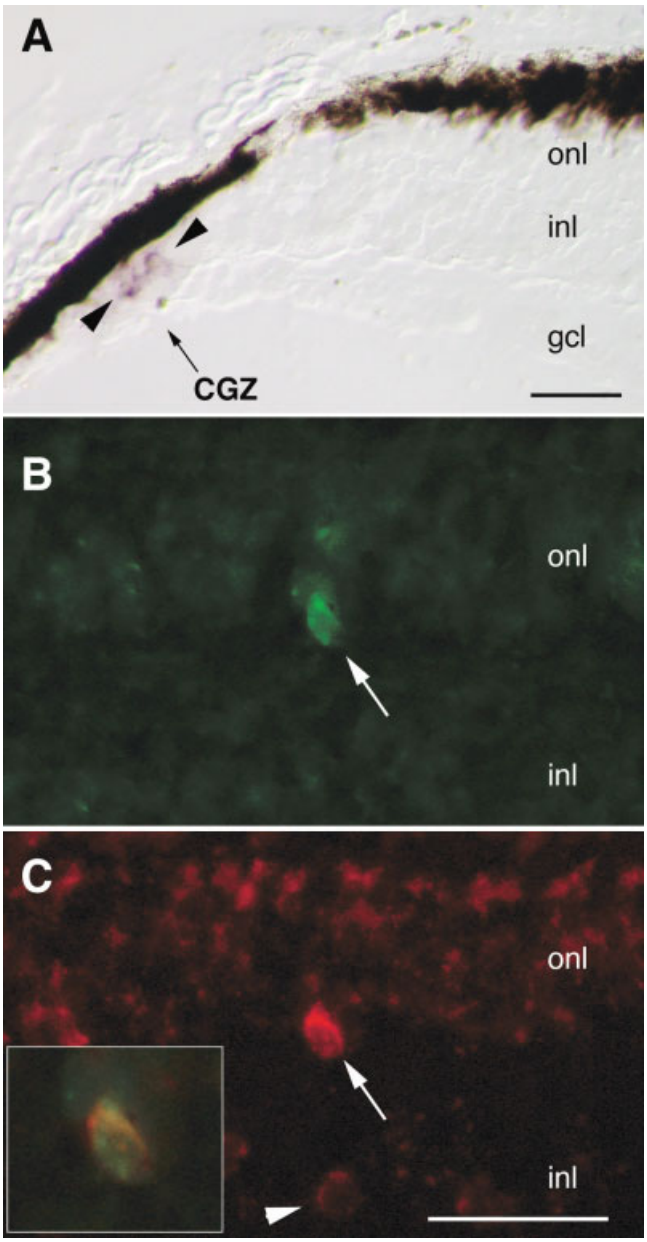

Fig. 6. *cyclin D1*-expressing cells in the teleost retina. **A:** *cyclin D1* is expressed by a small cluster of cells within the circumferential germinal zone. **B:** Example of a rod precursor within the ONL that expresses *cyclin D1*. **C:** This same cell also expresses *neuroD*. The **inset** is a digital overlay of the cell illustrated in B and C. CGZ, circumferential germinal zone; onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer. Scale bars = 25 μm in A; 25 μm in C applies to B,C.

the CGZ and mature (rod-containing) retina (Raymond and Rivlin, 1987; Stenkamp et al., 1997; Otteson et al., 2001). The expression of *neuroD* in cones is transient, because, with continual genesis at the margin, each generation of cones is displaced centrally by the subsequent generation, and *neuroD* appears not to be expressed by the central cones. The transient expression in teleost cones suggests that *neuroD* plays a role in the differentiation and/or maturation of cone photoreceptors. The details of *neuroD* function in these nascent cones remain to be determined.

Third, *neuroD* is expressed by cells of the rod photoreceptor lineage. These *neuroD*-expressing cells are mitotically active and display all the features characteristic of cells of the rod photoreceptor lineage (Johns and Fernald, 1981; Johns, 1982; Raymond and Rivlin, 1987; Hagedorn and Fernald, 1992; Julian et al., 1998; Otteson et al., 2001, 2002). Rod genesis in teleosts is unique among vertebrates. In these vertebrates, the vast majority of rods are generated after the differentiation of the early retinal neuroepithelium and from a lineage of progenitors that gives rise exclusively to this cell type. Furthermore, rod genesis in fish persists throughout the life of the animal (Otteson et al., 2003). The expression of *neuroD* in cells of the rod lineage suggests that this gene plays a critical role in generating this single cell type and that the teleost retina can serve as a valuable model for finding the fundamental cellular function of this gene. Furthermore, the present study is the first demonstration of a gene expressed in rod precursors, a cell identified over 2 decades ago (Johns and Fernald, 1981) but to date identified only by markers of mitotic activity.

Finally, it is noteworthy that, in contrast to that reported for *Xenopus* and mammals, *neuroD* in teleosts is not expressed by multipotent retinal progenitors. The absence of *neuroD* in cells of the CGZ indicates that this gene does not regulate cell fate determination in the teleost retina. The family of bHLH genes is large (Cepko 1999), however, and it can be reasonably assumed that other members provide proneural activity to specify cell fates among retinal progenitors in teleosts. Also, in the teleost retina, *neuroD* is not expressed in differentiated rods. In mammals, *neuroD* is constitutively expressed in rods (Morrow et al., 1999; Pennesi et al., 2003) and is required for their survival (Pennesi et al., 2003). The absence of *neuroD* expression in rods indicates that, whereas this gene likely functions to generate this cell type (see below), it is not required for their function or survival.

Rod precursors are a singular population of neural progenitors in the teleost retina that can be identified by their laminar position and nuclear morphology. These cells were first identified many years ago (Johns and Fernald, 1981) and were shown to be a class of neural progenitors giving rise exclusively to rod photoreceptors. Combining *neuroD* in situ hybridizations with PCNA and BrdU immunocytochemistry showed that *neuroD* is not ubiquitously expressed by rod precursors. Greater than half of those cells expressing PCNA or labeled with BrdU following a 24-hour exposure do not express this gene. If *neuroD* plays a role in persistent rod genesis in teleosts, and the cellular pattern of expression suggests that this is so, what might account for the fact that a significant fraction of the cycling rod precursors does not express this gene? There are at least two potential explanations. One is that there are two pools of rod precursors in the ONL, one pool that is replicating without expressing *neuroD* and a second pool that is also dividing and does express this gene. A known function of *neuroD* in mitotically active cells is to govern withdrawal from the cell cycle (Mutoh et al., 1998; Farah et al., 2000), and *neuroD* could play this role in a subset of rod precursors as they prepare to exit the cell cycle to differentiate into rods. The possibility that there are two pools of dividing cells in the ONL is inconsistent,

however, with the observation that almost every rod precursor in M phase of the cell cycle expresses *neuroD*. If every rod precursors that is PCNA-positive will undergo mitosis, then the possibility of separate pools of rod precursors is excluded. A second explanation for the heterogeneous expression of *neuroD* by rod precursors is that *neuroD* expression is cell cycle specific; *neuroD* is expressed at some phases of the cell cycle and not at others. This possibility was examined further by combining *neuroD* in situ hybridization with a second labeling method selected to identify cells at different phases of the cell cycle, phosphohistone H3 to label cells in M phase, *cyclin D1* to label cells in G1, and 4-hour BrdU exposure to label cells in S. The expectation was that, if *neuroD* is expressed in M, G1, or S phases, the proportions of double-labeled cells would increase over the proprtion observed for the more ubiquitous markers of proliferation, PCNA and 24-hour exposure to BrdU. These experiments showed that *neuroD* is expressed by 84% of rod precursors in M phase, over 90% of those cells in G1, and nearly 70% of rod precursors in S. These data are taken as evidence that, in rod precursors, *neuroD* is expressed during each of these phases of the cell cycle. Our interpretation of these data is that rod precursors express *neuroD* as they pass through M, G1, and S phases of the cell cycle, and those mitotic rod precursors that do not express *neuroD* are in G2, and perhaps also in late S and early M.

Although we view the interpretation of our data as the most parsimonious, our conclusions must be considered tentative. First, too little is known about the cell cycle kinetics of rod precursors to do more than indirectly identify different phases of the cell cycle. Unlike the case for cells in vitro, techniques to synchronize the cell cycle (see, e.g., Motokura et al., 1992) and thereby study a homogeneous population of dividing cells are not available. Second, the markers used here may not faithfully identify specific phases of the cell cycle. The inference that PCNA immunostaining marks cells in G1, S, and G2 is based on work from mammalian cell lines and has not been examined for teleosts. For example, if PCNA is present is cells that have withdrawn from the cell cycle (G0), this could account for some of the PCNA-positive cells that do not express *neuroD*. Also, although cyclin D1 protein synthesis is tightly coupled to the cell cycle, it is not completely documented that *cyclin D1* gene expression is transcriptionally regulated in a similar manner. That *cyclin D1* in situ hybridization marks those rod precursors in the G1 phase is inferred but has yet to be proved. Third, markers for cells in G2 were not used, precluding a direct demonstration that *neuroD* is not expressed by rod precursors in G2. Finally, the transcriptional regulation of *neuroD* expression, as revealed by in situ hybridization, might not accurately reflect the translational regulation of neuroD. Whereas *neuroD* transcription may be transient, the neuroD protein may be present throughout the cell cycle and function in an invariant manner as cells progress through the cell cycle. If, however, the regulation of neuroD protein reflects the proposed cell-cycle-specific transcription, transcriptional events regulated by this protein may be linked to specific stages of the cell cycle.

The relationship between cell cycle activation and cell fate determination is well recognized (Ohnuma and Harris, 2003), and *neuroD* plays a determinative role in specifying fates of retinal progenitors in the mammalian, frog, and avian retinas. However, in a lineage of cells dedicated to generating a single cell type *neuroD* may have only a limited function. Analogously to the expression of *neuroD* in the rod lineage, in postnatal mammals, *neuroD* is expressed by a variety of tissue-specific progenitors that generate single cell types, e.g., dentate gyrus of the hippocampus (Miyata et al., 1999; Liu et al., 2000; Seki, 2002), external granule layer of the cerebellum (Lee et al., 2000), olfactory epithelium (Cau et al., 1997; Suzuki et al., 2003), and small intestine (Naya et al., 1997; Mutoh et al., 1998). Gain- and loss-of-function studies suggest that, in these tissues, *neuroD* acts primarily to regulate mitotic activity. The similarity between rod genesis in teleosts and cell-type-specific genesis in postnatal mammals suggests that in tissue-specific lineages, where the fates available to cycling progenitors are limited, *neuroD* may be utilized in a common, but limited function.

Finally, we observed that *cyclin D1* is expressed in both retinal progenitors within the CGZ and rod precursors. This is not surprising; the protein encoded by this gene is a ubiquitous regulator of the cell cycle in mitotically active cells (Dyer and Cepko, 2001; Coqueret, 2002; Ohnuma and Harris, 2003; Murray, 2004). The observation that the *cyclin D1*-positive cells reside in a small cluster in the midst of the CGZ is consistent with that reported for *Xenopus*, in which cell-cycle activators are coordinately expressed within subsets of progenitors within the circumferential marginal zone (CMZ; Ohnuma et al., 2002). *cyclin D1* expression in cells of the rod lineage suggests that a common repertoire of activators also governs the cell cycle in these cells. We used *cyclin D1* expression as a marker of cells in the G1 phase of the cell cycle, and its restricted spatial expression among retinal progenitors in fish (present study) and frogs (Ohnuma et al., 2002) suggests that it can be used for this purpose. The coincidence in the expression of *cyclin D1* and *neuroD* in rod precursors, however, is in marked contrast to the case in the teleost CGZ, where *neuroD* is not expressed, and the CMZ in *Xenopus*, where these two genes are hierarchically ordered and expressed in spatially separate cellular domains (Ohnuma et al., 2002). This observation, again, speaks to the potentially restricted function that *neuroD* may play in cells of the rod lineage.

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LITERATURE CITED

Acharya HR, Dooley CM, Thoreson WB, Ahmad I. 1997. cDNA cloning and expression analysis of NeuroD mRNA in human retina. Biochem Biophys Res Commun 233:459–463.

Ahmad I, Acharya HR, Rogers JA, Shibata A, Smithgall TE, Dooley CM. 1998. The role of NeuroD as a differentiation factor in the mammalian retina. J Mol Neurosci 11:165–178.

Bianchi S, Fabiani S, Muratori M, Arnold A, Sakaguchi K, Miki T, Brandi ML. 1994. Calcium modulates the cyclin D1 expression in a rat parathyroid cell line. Biochem Biophys Res Commun 204:691–700.

Brennan CA, Moses K. 2000. Determination of *Drosophila* photoreceptors: timing is everything. Cell Mol Life Sci 57:195–214.

Cau E, Gradwohl G, Fode C, Guillemot F. 1997. Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. Development 124: 1611–1621.

Cepko CL. 1999. The roles of intrinsic and extrinsic cues and bHLH genes in the determination of retinal cell fates. Curr Opin Neurobiol 9:37–46.

Coltrera MD, Gown AM. 1991. PCNA/cyclin expression and BrdU uptake define different subpopulations in different cell lines. J Histochem Cytochem 39:23–30.

Coqueret O. 2002. Linking cyclins to transcriptional control. Gene 299:35–55.

de Melo J, Qiu X, Du G, Cristante L, Eisenstat DD. 2003. Dlx1, Dlx2, Pax6, Brn3b, and Chx10 homeobox gene expression defines the retinal ganglion and inner nuclear layers of the developing and adult mouse retina. J Comp Neurol 461:187–204.

Dyer MA, Cepko CL. 2001. Regulating proliferation during retinal development. Nat Rev Neurosci 2:333–342.

Easter SS Jr, Hitchcock PF. 2000. Stem cells and regeneration in the retina: what fish have taught us about neurogenesis. Neuroscientist 6:454–464.

Easter SS Jr, Malicki JJ. 2002. The zebrafish eye: developmental and genetic analysis. Results Probl Cell Differ 40:346–70.

Farah MH, Olson JM, Sucic HB, Hume RI, Tapscott SJ, Turner DL. 2000. Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. Development 127:693–702.

Fischer AJ, Reh TA. 2000. Identification of a proliferating marginal zone of retinal progenitors in postnatal chickens. Dev Biol 220:197–210.

Fischer AJ, Wang SZ, Reh TA. 2004. NeuroD induces the expression of visinin and calretinin by proliferating cells derived from toxin-damaged chicken retina. Dev Dyn 229:555–563.

Hagedorn M, Fernald RD. 1992. Retinal growth and cell addition during embryogenesis in the teleost *Haplochromis burtoni*. J Comp Neurol 321:193–208.

Hitchcock PF, Lindsey Myhr KJ, Easter SE Jr, Mangione-Smith R, Dwyer Jones D. 1992. Local regeneration in the retina of the goldfish. J Neurobiol 23:187–203.

Hitchcock PF, Macdonald RE, VanDeRyt JT, Wilson SW. 1996. Antibodies against Pax6 immunostain amacrine and ganglion cells and neuronal progenitors, but not rod precursors, in the normal and regenerating retina of the goldfish. J Neurobiol 29:399–413.

Hitchcock PF, Otteson DC, Cirenza PF. 2001. Expression of the insulin receptor in the retina of the goldfish. Invest Ophthalmol Vis Sci 42: 2125–2129.

Inoue T, Hojo M, Bessho Y, Tano Y, Lee, JE, Kagayama R. 2002. *Math3* and *NeuroD* regulate amacrine cell fate specification in the retina. Development 129:831–841.

Johns PR. 1977. Growth of the adult goldfish eye. III. Source of the new retinal cells. J Comp Neurol 176:343–357.

Johns PR. 1982. Formation of photoreceptors in larval and adult goldfish. J Neurosci 2:178–198.

Johns PR, Fernald RD. 1981. Genesis of rods in teleost fish retina. Nature 293:141–142.

Julian D, Ennis K, Korenbrot JI. 1998. Birth and fate of proliferative cells in the inner nuclear layer of the mature fish retina. J Comp Neurol 394:271–282.

Korzh V, Sleptsova I, Liao J, He J, Gong Z. 1998. Expression of zebrafish bHLH genes ngn1 and nrd defines distinct stages of neural differentiation. Dev Dyn 213:92–104.

Kurki P, Vanderlaan M, Dolbeare F, Gray J, Tan EM. 1986. Expression of proliferating cell nuclear antigen (PCNA)/cyclin during the cell cycle. Exp Cell Res 166:209–219.

Kurki P, Ogata K, Tan EM. 1988. Monoclonal antibodies to proliferating cell nuclear antigen (PCNA)/cyclin as probes for proliferating cells by immunofluorescence microscopy and flow cytometry. J Immunol Methods 109:49–59.

Lee JE, Hollenberg SM, Snider L, Turner DL, Lipnick N, Weintraub H. 1995. Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science 268:836–844.

Lee JK, Cho JH, Hwang WS, Lee YD, Reu DS, Suh-Kim H. 2000. Expression of neuroD/BETA2 in mitotic and postmitotic neuronal cells during the development of nervous system. Dev Dyn 217:361–367.

Li Z, Hu M, Ochocinska MJ, Joseph NM, Easter SS Jr. 2000. Modulation of cell proliferation in the embryonic retina of zebrafish (*Danio rerio*). Dev Dyn 219:391–401.

Liu M, Pleasure SJ, Collins AE, Noebels JL, Naya FJ, Tsai MJ, Lowenstein DH. 2000. Loss of BETA2/NeuroD leads to malformation of the dentate gyrus and epilepsy. Proc Natl Acad Sci U S A 97:865–870.

Lyall AH. 1957. The growth of the trout retina. Q J Microsc Sci 98:101–110.

Marcus RC, Delaney CL, Easter SS Jr. 1999. Neurogenesis in the visual system of embryonic and adult zebrafish (*Danio rerio*). Vis Neurosci 16:417–424.

Matsushime H, Roussel MF, Sherr CJ. 1991. Novel mammalian cyclins (CYL genes) expressed during G1. Cold Spring Harbor Symp Quant Biol 56:69–74.

Meyer RL. 1978. Evidence from thymidine labeling for continuing growth of retina and tectum in juvenile goldfish. Exp Neurol 59:99–111.

Miyata T, Maeda T, Lee JE. 1999. NeuroD is required for differentiation of the granule cells in the cerebellum and hippocampus. Genes Dev 13: 1647–1652.

Moore KB, Schneider ML, Vetter ML. 2002. Posttranslational mechanisms control the timing of bHLH function and regulate retinal cell fate. Neuron 34:183–195.

Morrow EM, Furukawa T, Lee JE, Cepko CL. 1999. NeuroD regulates multiple functions in the developing neural retina in rodent. Development 126:23–36.

Moshiri A, Reh TA. 2004. Persistent progenitors at the retinal margin of ptc+/− mice. J Neurosci 24:229–237.

Motokura T, Bloom T, Kim HG, Juppner H, Ruderman JV, Kronenberg HM, Arnold A. 1991. A novel cyclin encoded by a bcl1-linked candidate oncogene. Nature 350:512–515.

Motokura T, Keyomarsi K, Kronenberg HM, Arnold A. 1992. Cloning and characterization of human cyclin D3, a cDNA closely related in sequence to the PRAD1/cyclin D1 proto-oncogene. J Biol Chem 267: 20412–20415.

Mueller T, Wullimann MF. 2002. Expression domains of neuroD (nrd) in the early postembryonic zebrafish brain. Brain Res Bull 57:377–379.

Müller H. 1952. Bau und Wachstum der Netzhaut des Guppy (*Lebistes reticulatus*). Zool Jahrb 63:275–324.

Murray AW. 2004. Recycling the cell cycle: cyclins revisited. Cell 116:221–234.

Mutoh H, Fung BP, Naya FJ, Tsai MJ, Nishitani J, Leiter AB. 1997. The basic helix-loop-helix transcription factor BETA2/NeuroD is expressed in mammalian enteroendocrine cells and activates secretin gene expression. Proc Natl Acad Sci U S A 94:3560–3564.

Naya FJ, Huang HP, Qiu Y, Mutoh H, DeMayo FJ, Leiter AB, Tsai MJ. 1997. Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. Genes Dev 11:2323–2334.

Nibu K, Kondo K, Ohta Y, Ishibashi T, Rothstein JL, Kaga K. 2001. Expression of NeuroD and TrkB in developing and aged mouse olfactory epithelium. Neuroreport 12:1615–1619.

Ohnuma S, Harris WA. 2003. Neurogenesis and the cell cycle. Neuron 40:199–208.

Ohnuma S, Hopper S, Wang KC, Philpott A, Harris WA. 2002. Coordinating retinal histogenesis: early cell cycle exit enhances early cell fate determination in the *Xenopus* retina. Development 129:2435–2446.

Otteson DC, Hitchcock PF. 2003. Stem cells in the teleost retina: persistent neurogenesis and injury-induced regeneration. Vis Res 43:927–936.

Otteson DC, D'Costa AR, Hitchcock PF. 2001. Putative stem cells and the lineage of rod photoreceptors in the mature retina of the goldfish. Dev Biol 232:62–76.

Otteson DC, Cirenza PF, Hitchcock PF. 2002. Persistent neurogenesis in the teleost retina: evidence for regulation by the growth-hormone/insulin-like growth factor-I axis. Mech Dev 117:137–149.

Pennesi ME, Cho JH, Yang Z, Wu SH, Zhang J, Wu SM, Tsai MJ. 2003. BETA2/NeuroD1 null mice: a new model for transcription factor-dependent photoreceptor degeneration. J Neurosci 23:453–461.

Perron M, Harris WA. 2000. Determination of vertebrate retinal progenitor cell fate by the Notch pathway and basic helix-loop-helix transcription factors. Cell Mol Life Sci 57:215–223.

Perron M, Kanekar S, Vetter ML, Harris WA. 1998. The genetic sequence of retinal development in the ciliary margin of the *Xenopus* eye. Dev Biol 199:185–200.

Raymond PA, Rivlin PK. 1987. Germinal cells in the goldfish retina that produce rod photoreceptors. Dev Biol 122:120–138.

Raymond PA, Barthel LK, Curran GA. 1995. Developmental patterning of rod and cone photoreceptors in embryonic zebrafish. J Comp Neurol 359:537–550.

Schmitt EA, Dowling JE. 1999. Early retinal development in the zebrafish, *Danio rerio*: light and electron microscopic analyses. J Comp Neurol 404:515–536.

Seki T. 2002. Expression patterns of immature neuronal markers PSA-NCAM, CRMP-4 and NeuroD in the hippocampus of young adult and aged rodents. J Neurosci Res 70:327–334.

Stenkamp DL, Barthel LK, Raymond PA. 1997. Spatiotemporal coordination of rod and cone photoreceptor differentiation in goldfish retina. J Comp Neurol 382:272–284.

Stoykova A, Gruss P. 1994. Roles of Pax-genes in developing and adult brain as suggested by expression patterns. J Neurosci 14:1395–412.

Suzuki Y, Mizoguchi I, Nishiyama H, Takeda M, Obara N. 2003. Expression of Hes6 and NeuroD in the olfactory epithelium, vomeronasal organ and non-sensory patches. Chem Senses 28:197–205.

Vihtelic TS, Hyde DR. 2000. Light-induced rod and cone cell death and regeneration in the adult albino zebrafish (*Danio rerio*) retina. J Neurobiol 44:289–307.

Yan RT, Wang SZ. 1998. neuroD induces photoreceptor cell overproduction in vivo and de novo generation in vitro. J Neurobiol 36:485–496.

Yan RT, Wang SZ. 2004. Requirement of neuroD for photoreceptor formation in the chick retina. Invest Ophthalmol Vis Sci 45:48–58.

Yarden A, Salomon D, Geiger B. 1995. Zebrafish cyclin D1 is differentially expressed during early embryogenesis. Biochim Biophys Acta 1264: 257–260.