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SCIENCE DIRECT

Developmental Brain Research 151 (2004) 193–197

DEVELOPMENTAL BRAIN RESEARCH

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Short communication

Timing and location of rhodopsin expression in newly born rod photoreceptors in the adult teleost retina

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Accepted 6 April 2004
Available online 8 June 2004

Abstract

Labeling of newly divided retinal cells with bromodeoxyuridine (BrdU) and a rhodopsin mRNA probe revealed that rhodopsin is first expressed by new rod photoreceptors 2 days after cell birth in an adult cichlid fish. Most new cells that expressed rhodopsin had nuclei located in the vitreal half of the outer nuclear layer (ONL), lending further support to the hypothesis that movement from scleral to vitreal ONL is associated with rod differentiation.
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Theme: Development and regeneration
Topic: Cell differentiation and migration

Keywords: Haplochromis burtoni; *Astatotilapia burtoni*; Rod progenitor; Rod precursor; Opsin; Retinal development

New retinal cells are continuously produced in the adult teleost retina through the division of progenitor cells located in the ciliary marginal zone [5,7,15,16], the inner nuclear layer [9,20], and the outer nuclear layer (ONL) [5,8,15], allowing the fish eye to grow in proportion to its body without sacrificing visual sensitivity or acuity [3].

Progenitor cells in the ONL normally only produce rod photoreceptors [8,10,21] and therefore have been termed rod progenitors. In an African cichlid, *Haplochromis burtoni*, the cell bodies of dividing rod progenitors are interspersed among mature rods in the scleral ONL, adjacent to the cone photoreceptor layer. After terminal mitosis, their cell bodies move through multiple rows of mature rods to the vitreal edge of the ONL, adjacent to the outer plexiform layer [12–14]. Based on these data, it has been hypothesized that rod progenitors that remain in the scleral ONL continue to proliferate, while those that move to the vitreal ONL differentiate into functional rod photoreceptors [13]. This is analogous to the development of other neural structures, such as the cerebral cortex, where proliferative cells stay within a germinal zone while cells that migrate away from the germinal zone differentiate into neurons [22].

Although it has been shown that newly divided cells in the *H. burtoni* ONL ultimately become rod photoreceptors based on morphology [8] and the expression of rhodopsin protein [11], it was not known when they first express rhodopsin mRNA and where in the ONL their cell bodies are located when rhodopsin expression begins. Understanding whether rhodopsin expression occurs before, during, or after movement to the vitreal ONL is an important step in understanding how rod differentiation is regulated in these newly added cells. Cell fate in the developing retina is controlled by a combination of intrinsic and extrinsic factors [1]. By moving within the ONL, these cells may experience different local environments that could potentially influence their differentiation. Alternatively, movement could occur after differentiation has already begun.

To discover when and where newly born cells begin to express rhodopsin in adult *H. burtoni*, we labeled dividing retinal cells with bromodeoxyuridine (BrdU), sacrificed fish at different times after BrdU administration, and then

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0165-3806/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.devbrainres.2004.04.001

located and counted cells in the ONL that expressed both BrdU and rhodopsin mRNA.

H. burtoni bred from wild-caught stock [4] were maintained in aquaria (12 h:12 h light/dark cycle, 27 °C, pH 8.0). Animals were treated in accordance with the NIH and Stanford University A-PLAC Committee protocols for animal experimentation. To label newly divided cells in the retina, adult fish (3.0–4.1 cm length) were injected intraperitoneally with 0.3 mg/g body weight of BrdU (Sigma) in phosphate buffered saline (PBS) at either 1–2 h after lights off or 1–2 h after lights on. Fish were killed by rapid cervical transsection at selected times after BrdU injection (0.5 to 8 days, 2 animals at each time point, 16 animals total) at 1–2 h after lights on. After removal of the cornea and lens, eyes were fixed in 4% paraformaldehyde in PBS overnight at 4 °C, then incubated in 30% sucrose overnight at 4 °C. Eyes were then cryosectioned in the nasal-temporal plane at 10–12 μm.

The sections were then processed for rhodopsin in situ hybridization and BrdU immunohistochemistry. A full-length *H. burtoni* rhodopsin cDNA was cloned from a *H. burtoni* retinal cDNA library (D. Anderson and R. Fernald, GenBank accession number AF315354). Antisense digoxigenin (DIG)-labeled riboprobes (~120 bp) were synthesized (Maxiscript; Ambion). Slides were rehydrated in sodium citrate buffer (SSC), rinsed in 50% ethanol, and incubated with antisense DIG-labeled rhodopsin probe (1 μg/ml in hybridization buffer) at 55 °C overnight. Slides were then incubated with 20 μg/ml RNAse A for 15 min at 37 °C, washed in decreasing concentrations of SSC including a

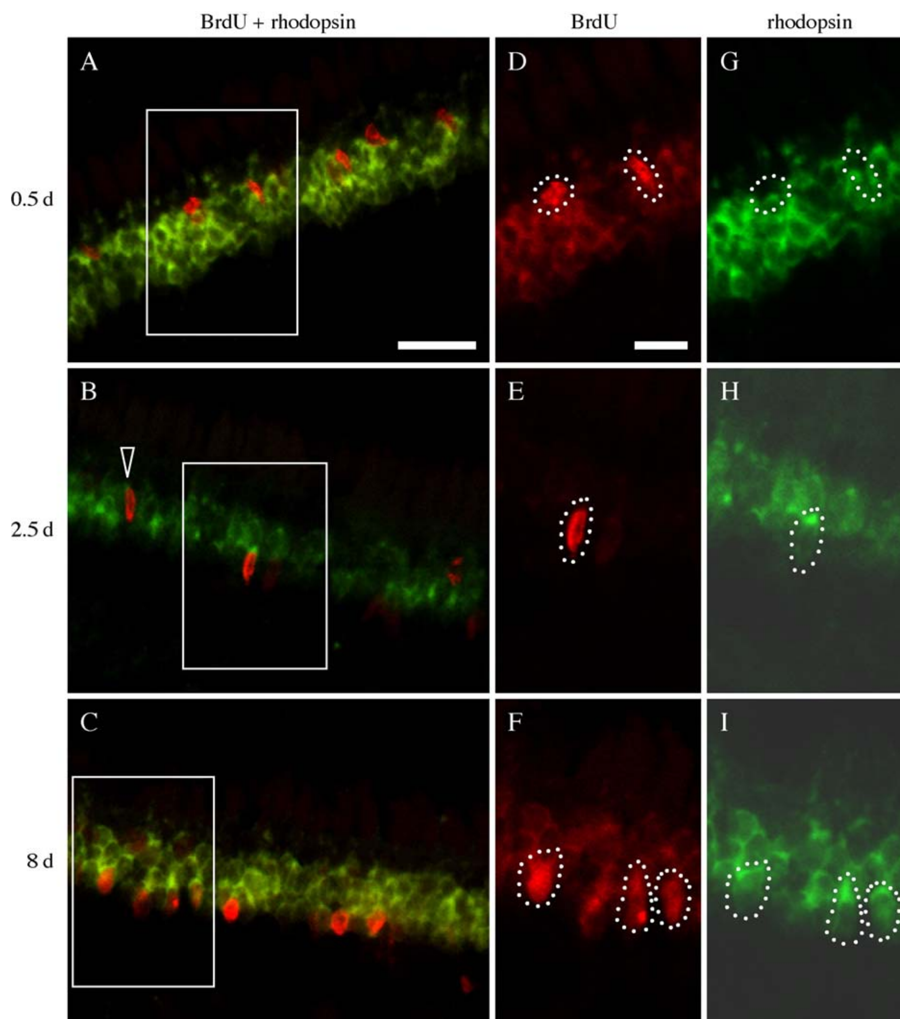

Fig. 1. Rhodopsin expression occurs after newly divided cells move to the vitreal ONL. BrdU (red) and rhodopsin (green) labeling is shown at 0.5 days (top row), 2.5 days (middle row), and 8 days (bottom row) after BrdU injection. D–I are higher magnification photomicrographs of the tissue shown in the boxes in A–C. Dotted outlines indicate the corresponding location of BrdU-labeled nuclei. All panels are oriented so that sclerad is towards the top, vitread is towards the bottom. Top row: 0.5 days after BrdU injection. There is no association between the BrdU-labeled nuclei (D) and the pattern of rhodopsin staining (G), indicating that these cells do not express rhodopsin. All of the BrdU-labeled cells are located in the scleral half of the ONL. Middle row: 2.5 days after BrdU injection. Box in (B) shows a BrdU-labeled cell that expresses rhodopsin in the vitreal ONL; open arrowhead in (B) indicates a BrdU-labeled cell that does not express rhodopsin in the scleral ONL. Bottom row: 8 days after BrdU injection. The cells in (F) and (I) all express rhodopsin. All of the BrdU-labeled cells are located in the vitreal half of the ONL. Scale bar in A–C = 20 μm. Scale bar in D–I = 10 μm.

high stringency wash of 0.2× SSC at 55 °C for 30 min, washed in 0.05% Tween-20 in PBS (PBS/Tween) and blocked for 1 h in 0.5% blocking reagent (TSA kit; NEN Life Sciences) in PBS (PBSB). Slides were incubated in 1:50 or 1:100 anti-DIG-HRP (Roche) in PBSB at 4 °C overnight, washed in PBS/Tween, and incubated in 1:50 biotinyl tyramide (NEN Life Sciences) for 7–14 min. After washing in PBS/Tween, slides were incubated in 1:200 or 1:500 avidin–fluorescein (Vector Labs) in PBSB for 1–2 h and washed in PBS/Tween. Slides incubated with sense DIG-labeled riboprobe showed no fluorescent signal above background (data not shown). To detect incorporated BrdU, slides were incubated in 4 N HCl for 30 min, washed in PBS with 0.3% Triton X-100 (PBS/Tx), blocked for 1–2 h in 10% normal goat serum in PBS/Tx, and then incubated in 10% rat anti-BrdU (Accurate Chemical & Scientific) in PBS/Tx with 0.2% BSA at 4 °C overnight. Slides were then washed in PBS/Tx, incubated in 1:200 anti-rat Texas Red (Jackson Immunoresearch) in PBS for 2–3 h, and washed in PBS. Slides were coverslipped with Fluoromount (Southern Biotechnology Associates).

Retinal sections were analyzed on a laser scanning confocal microscope using 1 μm optical sections (Molecular Dynamics MultiProbe 2010). Since BrdU is located in the nucleus and rhodopsin mRNA is located in the cytoplasm, cells were considered double labeled if they had a BrdU-labeled nucleus surrounded by a ring of rhodopsin signal of the same shape. Cells considered not double labeled had no corresponding rhodopsin ring around the BrdU-labeled nucleus in any optical section (see Fig. 1 for examples). A total of 464 BrdU-labeled cells, sampled evenly from across the nasal-temporal plane of the retina, were analyzed (mean = 29 cells per animal). For each BrdU-labeled cell analyzed, its position within the ONL was designated as either "scleral" or "vitreal" based on the location of its nucleus within the ONL. The extent of the ONL was defined by the region of rhodopsin staining, since the ONL primarily contains mature rod photoreceptors (see Fig. 1).

BrdU-labeled cells did not express rhodopsin between 0.5 and 1.5 days after BrdU injection ($n = 181$ cells; Figs. 1 and 2A). The earliest BrdU/rhodopsin double labeled cells were observed at 2 days after BrdU injection (mean = 4.2% of BrdU-labeled cells; $n = 59$ cells, Fig. 2A). At 2.5 days after BrdU injection, 18.5% of BrdU-labeled cells expressed rhodopsin (mean; $n = 97$ cells; Figs. 1 and 2A), and the proportion of double labeled cells increased thereafter (Fig. 2A). At the latest time observed, 8 days, 85.7% (mean; $n = 37$ cells) of BrdU-labeled cells expressed rhodopsin (Figs. 1 and 2A). The percentage of BrdU-labeled cells that expressed rhodopsin increased linearly over time, as shown by a regression analysis of the means ($y = 12.54x - 12.68$; $R^2 = 0.97$).

In agreement with previous reports [12–14], we found that as survival time increased, BrdU-labeled nuclei were located at increasingly more vitread positions within the ONL (Figs. 1 and 2B). After 0.5 days following BrdU injection, 9.0% (mean; $n = 68$ cells) of BrdU-labeled cells were located in the vitreal ONL but after 8 days, 94.6% (mean; $n = 37$ cells) were located in the vitreal ONL (Fig. 2B). This relationship also exhibited a linear trend, as shown by a regression analysis of the means ($y = 9.94x + 29.00$; $R^2 = 0.73$). While vitread movement likely accounts for most of this shift in cellular location, some of the decline in the number of BrdU-labeled cells in the scleral ONL may be due to rod progenitors re-entering the cell cycle [13], which would dilute their BrdU label and make them difficult to detect.

BrdU-labeled cells in the vitreal ONL were significantly more likely to express rhodopsin than those in the scleral ONL (data from 2 to 8 days after BrdU injection; $\chi^2(1) = 57.1$; $p < 0.001$; Fig. 3). Only 2.1% (2/95) of scleral BrdU-labeled cells expressed rhodopsin 2–8 days after BrdU injection, in contrast to 46.3% (87/188) of vitreal BrdU-labeled cells from the same time period (Fig. 3). This

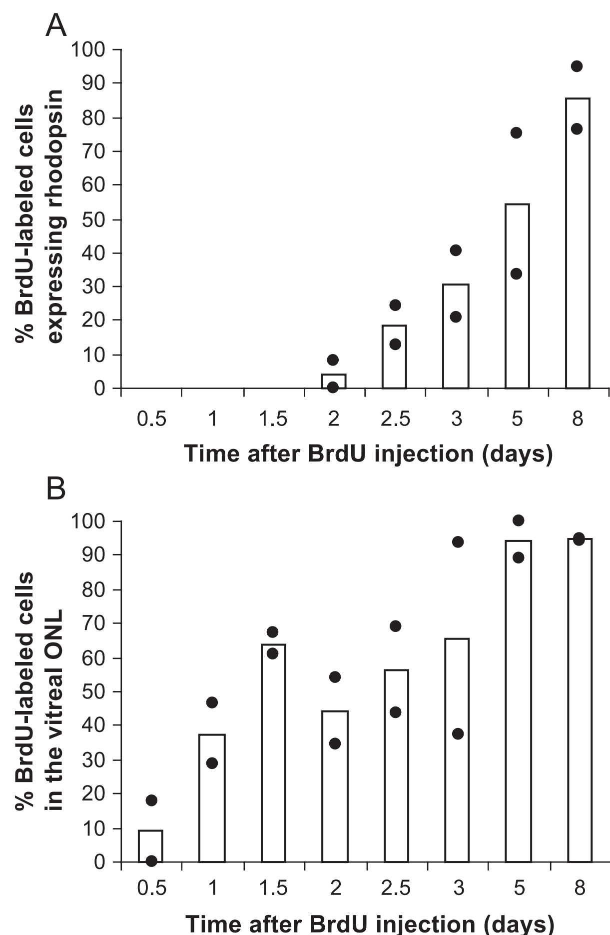

Fig. 2. Newly born cells begin to express rhodopsin 2 days after cell birth. Over time, the percentage of new cells expressing rhodopsin increases (A) and the cells move further vitread (B). (A) The percentage of BrdU-labeled cells expressing rhodopsin is graphed as a function of time after BrdU injection. (B) The percentage of BrdU-labeled cells located in the vitreal half of the ONL is graphed as a function of time after BrdU injection. Black points indicate individual values for each animal, white bars represent the mean of the two animals.

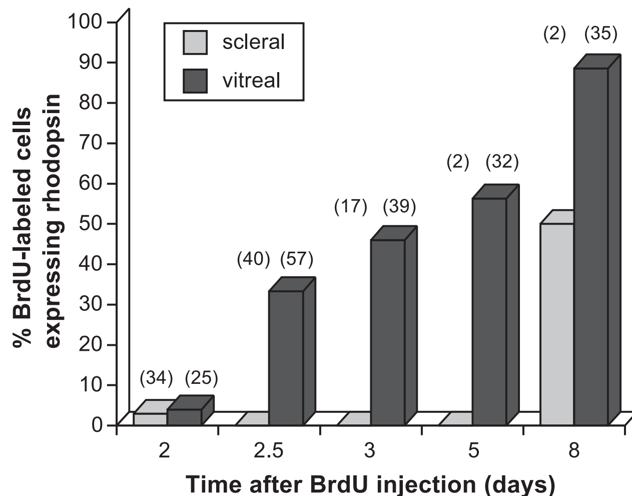

Fig. 3. Newly born cells located in the vitreal ONL are more likely to express rhodopsin than those located in the scleral ONL, regardless of survival time. The percentage of BrdU-labeled cells expressing rhodopsin in each location (gray bars = scleral ONL, black bars = vitreal ONL) is graphed as a function of time after BrdU injection. Each bar represents data from two animals. Numbers in parentheses above the bars indicate the number of cells analyzed at each location and time point. The bar representing scleral location at 8 days appears high because there were only two cells, one of which expressed rhodopsin.

difference was not due to an absence of scleral cells at ages when rhodopsin is expressed. For example, at 2.5 days after BrdU injection, there were 40 BrdU-labeled scleral cells, none of which expressed rhodopsin. This is despite the fact that 33.3% (19/57) of vitreal cells expressed rhodopsin at that age (Fig. 3). A typical example is shown in Fig. 1B where a cell that is labeled with both BrdU and rhodopsin (in box) is located in the vitreal ONL, while a BrdU-labeled cell that is not labeled with rhodopsin (arrowhead) is located in the scleral ONL.

We have shown that rhodopsin mRNA is not expressed until 2 days after cell birth in the adult *H. burtoni* retina. This timing is similar to that found in the adult goldfish, in which new rods first expressed rhodopsin mRNA between 1 and 3 days after cell birth [23], and in developing animals when the first expression of rhodopsin protein was shown to be 2.5 days after cell birth in embryonic *H. burtoni* [6] and 48–54 h in neonatal rat [18,24].

We have also shown that the percentage of newly born cells expressing rhodopsin increases over time, reaching 85.7% at 8 days after BrdU injection (mean; Fig. 2A). This could be due to an increasing number of cells exiting the cell cycle over time, or differences in the timing of rhodopsin expression in cells that become post-mitotic at the same time. Since some of these newly born cells may re-enter the cell cycle instead of differentiating directly into rod photoreceptors [13], it is not surprising that some of these BrdU-labeled cells never expressed rhodopsin, even after 8 days.

Interestingly, we have shown that the vast majority (97.8% = 87/89; Fig. 3) of all BrdU-labeled cells that expressed rhodopsin were located in the vitreal, rather than the scleral ONL, regardless of time after cell birth. Therefore, younger cells that reached the vitreal ONL were more likely to express rhodopsin than older cells that remained in the scleral ONL. This further supports the hypothesis that cells that re-enter the cell cycle and stay undifferentiated remain in the scleral ONL, and those that differentiate into rod photoreceptors move to the vitreal ONL [13].

The association between vitreal location and rhodopsin expression could be the result of at least two distinct mechanisms. Cells that move to the vitreal ONL may have already started differentiating into rods, but do not express rhodopsin until they reach the vitreal ONL because other cell-intrinsic upstream events need to occur before rhodopsin can be expressed. For example, the genes Nrl and Crx are expressed prior to rhodopsin in the developing retina [19] and can regulate rhodopsin expression [17]. Alternatively, external factors in the vitreal region of the ONL could be actively involved in instructing new cells to express rhodopsin. One possible factor is basic fibroblast growth factor (FGF-2), which has been shown to promote rhodopsin expression in *H. burtoni* [11] and is expressed in the outer plexiform layer, adjacent to the vitreal ONL [2]. Although our experiments cannot directly answer the question of whether the decision to differentiate occurs before or after movement, it is intriguing that vitreal movement precedes rhodopsin expression by at least 1.5 days (compare Fig. 2A and B). This could be sufficient for factors in the region of the vitreal ONL to play a role in rod differentiation.

Future studies using markers that identify rod differentiation earlier or that block movement within the ONL could help elucidate whether this movement of new cells is a prerequisite for, or a consequence of, rod photoreceptor differentiation.

Acknowledgements

The authors thank D. Anderson for cloning the *H. burtoni* rhodopsin gene, Dr. W. Chang for the rhodopsin mRNA probe, and Drs. A. Greenwood, K. Hoke, C. Zygar, A. Ettinger, and H. Hofmann for valuable discussions and comments on the manuscript.

This work was supported by a Stanford Graduate Fellowship/Lucille P. Markey Biomedical Research Fellowship to RGH and NIH EY 05051 to RDF.

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