Making the gradient: Thyroid hormone regulates cone opsin expression in the developing mouse retina

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Edited by Jeremy Nathans, Johns Hopkins University School of Medicine, Baltimore, MD, and approved March 1, 2006 (received for review November 17, 2005)

Most mammals have two types of cone photoreceptors, which contain either medium wavelength (M) or short wavelength (S) opsin. The number and spatial organization of cone types varies dramatically among species, presumably to fine-tune the retina for different visual environments. In the mouse, S- and M-opsin are expressed in an opposing dorsal–ventral gradient. We previously reported that cone opsin patterning requires thyroid hormone β2, a nuclear hormone receptor that regulates transcription in conjunction with its ligand, thyroid hormone (TH). Here we show that exogenous TH inhibits S-opsin expression, but activates M-opsin expression. Binding of endogenous TH to TRβ2 is required to inhibit S-opsin and to activate M-opsin. TH is symmetrically distributed in the retina at birth as S-opsin expression begins, but becomes elevated in the dorsal retina at the time of M-opsin onset (postnatal day 10). Our results show that TH is a critical regulator of both S-opsin and M-opsin, and suggest that a TH gradient may play a role in establishing the gradient of M-opsin and S-opsin expression. These results also suggest that the ratio and patterning of cone types may be determined by TH availability during retinal development.

Results

Exogenous T3 Inhibits S-Opsin Expression in Developing Cone Photoreceptors. To assess the effects of TH on cone opsin development, we first used an in vitro approach (24). Retinas from E17 embryos become can be cultured as explants and develop normal lamination and cell-type specific marker expression (25, 26). Although photoreceptor outer segments do not form in culture, S-opsin could be detected in cell bodies and processes in the outer nuclear layer after 5 days in vitro, and the S-opsin-expressing cone photoreceptors develop in a normal graded pattern (Fig. L4). As previously documented by others, we could not detect M-opsin by immunohistochemistry in mouse explant cultures (27, 28). We treated explants with increasing concentrations of T3 for 10 days in vitro. We found a dose-dependent reduction in the number of S-opsin immunolabeled cones in T3-treated retinas (Fig. 1B). Concentrations as low as 500 pM were sufficient to cause a small, but statistically significant, reduction in S-opsin-expressing cones, and 1 nM caused a 75% reduction in the number of S-opsin-expressing cones. At T3 concentrations above 5 nM, few cones expressed S-opsin. T3 did not repress S-opsin in TRβ2-deficient retinal explants, indicating that repression of S-opsin by T3 is mediated specifically by the TRβ2 receptor (data not shown).

To confirm that T3 can inhibit S-opsin in the intact developing retina, we experimentally increased T3 by injecting mouse pups...
in vitro cones were counted in untreated (CTRL) and T3-treated explants after 11 days. The Student’s t test; mean and SEM shown in graph. \( \text{**P}<0.01; \text{***P}<0.001 \). (C–F) Newborn mice (P0) were injected s.c. with either saline or T3 (1.5 \( \mu \)g) for 3 days. The animals were killed, and the retinas were flat-mounted and labeled with S-opsin (red) and PNA (green) to label the total population of cones. Images were collected with a Zeiss Pascal confocal microscope at \( \times 40 \), and both S-opsin and total cones (PNA \(^+\)) were quantified from four 900-\( \mu \)m\(^2\) regions in each of three saline and three T3-treated retinas. A total of 881 cones were counted in the saline group and 914 for the T3-treated group. (C and D) There was a significant decline in the number of S-opsin-expressing cones in the T3-treated retinas (\( \text{**P}<0.015 \); Student’s t test; mean and SEM shown in graph). (E) There was no significant difference in the total number of PNA \(^+\) cones between the groups (saline, 73.4 \( \pm \) 6.45 SD; T3-treated, 76.16 \( \pm \) 4.06 SD). (F) The T3-treated retinas still demonstrate a very shallow gradient in the number of S-opsin cones per field, but in all regions there were many fewer S-opsin-expressing cones than in the saline-injected animals.

s.c. with T3 beginning at P0. After 3 days of saline or T3 injections, mice were killed and the retinas prepared for flat-mount immunolabeling or for mRNA analysis. Fig. 1C shows whole mounts labeled with an S-opsin antibody (red) and peanut lectin (PNA, green), which labels all cones. There was a significant reduction in the number of S-opsin-labeled cones (Fig. 1D) in the retinas of the T3-treated animals, as compared with the saline-injected animals, but no reduction in the total number of PNA \(^+\) cones (Fig. 1E), indicating that the T3 treatment did not selectively kill cones or alter the number of progenitors that differentiated into cones. As noted above, the normal retina has a ventral-to-dorsal gradient of S-opsin \(^+\) cones. Although the number of S-opsin \(^+\) cones was reduced >4-fold in retinas of T3-treated animals as compared with saline-treated animals, there was still a slight gradient in their distribution (Fig. 1F).

Northern blots reveal that T3 treatment of neonatal mice similarly reduces S-opsin transcript by \( \approx 75\% \) (Fig. 2A and B). To confirm that T3 acts through the TR\( \beta \)2 receptor in vivo, we injected T3 into wild-type or TR\( \beta \)2-null mice, each crossed onto an S-opsin promoter/\( \beta \)-galactosidase reporter. After 3 days of T3 injections, we found that the number of cones expressing the S-opsin reporter was dramatically reduced in T3-injected retinas of wild-type mice, but not in those of TR\( \beta \)2-null mice (Fig. 2C), confirming that TR\( \beta \)2 mediates the T3-mediated repression of S-opsin in vivo. These transgenic results further suggest that the T3-mediated repression occurs at the transcriptional level.

**A Mouse Model of Human Thyroid Hormone Resistance Has Altered Cone Opsin Expression.** To determine the role of endogenous TH signaling in cone development, we analyzed a mouse model of...
resistance to thyroid hormone (RTH) syndrome, characterized by a reduced sensitivity to thyroid hormone and elevated levels of serum T4. A targeted mutation (PV) was introduced into the TRβ gene that causes a frame-shift in the ligand-binding domain of both TRβ1 and TRβ2. The mutated gene produces a receptor that cannot bind ligand, but can still bind to DNA response elements and affect transcription in its unliganded form (29).

We looked for changes in the expression of S-opsin and M-opsin in whole-mounted adult wild-type, TRβPV+/+ , and TRβPV/PV retinas by colabeling with antibodies to S-opsin and M-opsin. Heterozygous retinas had similar S-opsin and M-opsin gradients to those of wild-type animals. However, in the TRβPV/PV cones, where T3 cannot bind to TRβ, all cones expressed S-opsin (Fig. 3). This finding suggests that endogenous T3 is required to inhibit S-opsin in dorsal cones. Furthermore, we could not detect M-opsin in TRβPV/PV cones, which is consistent with a requirement for endogenous T3 to activate M-opsin.

We quantified the percentage of cones (labeled with peanut lectin), in dorsal, central, and ventral regions of whole-mounted retinas that were colabeled with S-opsin (Fig. 3B). S-opsin is increased in all regions of the TRβPV/PV retina. This increase is most pronounced in the dorsal retina, where <3% of dorsal cones express S-opsin in wild-type retinas and 84% of cones express S-opsin in TRβPV/PV retinas. We also found a small increase in dorsal S-opsin-expressing cones in the TRβPV/+ retina. Thus, transcriptional regulation of both M- and S-cone opsin requires binding of the ligand, T3, to the TRβ2 receptor with a functional ligand binding domain.

To confirm that TH regulates M-opsin expression, we also made injections of T3 into postnatal day 7 (P7) mice for 4 days during the developmental period of onset of M-opsin expression. The retinas of the T3-treated animals had an increase in the percentage of M-opsin+ cones (of total PNA+ cones) in all regions, when compared with their saline-treated littermates (Fig. 3C); however, this effect was most pronounced in the ventral retina, where the number of M-opsin+ cones is the lowest in normal mice. Thus, experimentally increasing TH levels during the time of onset of M-opsin significantly activates its expression, but has no effect on the total number of cones (Fig. 3D).

Endogenous T3 Becomes Graded in the Postnatal Retina. Because TRβ2 regulates both S- and M-opsin expression in developing cones, we hypothesized that a gradient in thyroid hormone could establish the opposing dorsal-ventral gradient of S- and M-opsin in the mouse retina. To determine whether thyroid hormone is graded, we bisectioned mouse retinas into dorsal and ventral hemispheres at P0, P4, and P10, and quantified T3 and T4 by RIA. As shown in Table 1, T3 ranged from 4.0 to 5.9 ng per gram of tissue, and T4 ranged from 6.6 to 11.7 ng per gram of tissue. Fig. 4 shows an upward trend for T3 and T4 in the dorsal retina (Fig. 3C); however, this effect was most pronounced in the ventral retina, where the number of M-opsin+ cones is the lowest in normal mice. Thus, experimentally increasing TH levels during the time of onset of M-opsin significantly activates its expression, but has no effect on the total number of cones (Fig. 3D).
that TH is graded at the time of M-opsin onset, but not at the time of S-opsin onset. These results suggest that a TH may establish the M-opsin gradient, but that additional factors are required to establish the S-opsin gradient.

Establishing Gradients. Our model of cone opsin expression is shown in Fig. 5B. Although TRβ2 and RXRγ are required to regulate cone opsin expression, neither receptor shows a graded dorsal-ventral pattern of expression that correlates with the gradients of opsin (refs. 16 and 23; see Fig. 7, which is published as supporting information on the PNAS website). Here, we show that TH becomes graded in the retina by P10, and suggest that the M-opsin gradient may be established by a TH-dependent up-regulation of M-opsin in the dorsal retina. This model is consistent with the developmental expression of an M-opsin reporter, which is first expressed symmetrically across the retina, and is only up-regulated in the dorsal retina after P6 (32), at the time when we detect increased TH in the dorsal retina.

In this study, we showed that mice with a targeted mutation in TRβ (PV) that prevents the binding of T3 do not express M-opsin (29). Although some phenotypes observed in mice with the PV mutation are due to dominant-negative competition with wild-type TRα1 (33), opsin expression is normal in TRα1−/− mice (D.F. and M.S., unpublished observations). Therefore, the phenotypes that we observe are most likely due to direct effects of unliganded TRβ. Moreover, the fact that both S- and M-opsin are regulated in a complementary manner by exogenous T3 is consistent with the hypothesis that a lack of T3-binding by the mutant receptor is responsible for the observed phenotype.

Previous studies support a role for T3 in establishing gradients: T3 is required to establish transcriptional gradients in the developing retina and that T3 is required for normal development of both S and M cones. We show that exogenous T3 inhibits S-opsin when experimentally elevated at the time of S-opsin onset, and activates M-opsin when animals are treated at the time of M-opsin onset. Analysis of a mouse with a mutation in the ligand binding domain of TRβ indicates that binding of endogenous TH to the TRβ receptor is required to inhibit S-opsin and to activate M-opsin in vivo. These results, along with our previous analysis of TRβ2-deficient mice (23) and in vitro studies of rat retinal cultures (30, 31), show a requirement for TH in the developmental regulation of cone opsins. Finally, we show that TH is graded at the time of M-opsin onset, but not at the time of S-opsin onset. These results suggest that a TH may establish the M-opsin gradient, but that additional factors are required to establish the S-opsin gradient.

Table 1. T3 and T4 measured by radioimmunoassay as described in Methods and reported as nanograms per gram of retinal tissue

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<td>T3, ng/g</td>
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<tr>
<td>Dorsal</td>
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One-way ANOVA and the protected least significant difference post hoc test were used for multiple comparisons of T3 and T4 data, after validation of the homogeneity of variances by the Bartlett–Box F test. Results are expressed as means ± SEM. *Significantly greater than both P0 and P4 values. P < 0.05.

Discussions
Thyroid Hormone Is Required for Cone Opsin Patterning. We previously reported that two nuclear hormone receptors, TRβ2 and RXRγ, regulate the developmental expression of cone opsins in the mouse retina (16, 23). In this study, we analyzed whether the TR ligand, thyroid hormone, regulates cone opsin expression. This report demonstrates that both T3 and T4 are present in the developing retina and that T3 is required for normal development of both S and M cones. We show that exogenous T3 inhibits S-opsin when experimentally elevated at the time of S-opsin onset, and activates M-opsin when animals are treated at the time of M-opsin onset. Analysis of a mouse with a mutation in the ligand binding domain of TRβ indicates that binding of endogenous TH to the TRβ receptor is required to inhibit S-opsin and to activate M-opsin in vivo. These results, along with our previous analysis of TRβ2-deficient mice (23) and in vitro studies of rat retinal cultures (30, 31), show a requirement for TH in the developmental regulation of cone opsins. Finally, we show that TH is graded at the time of M-opsin onset, but not at the time of S-opsin onset. These results suggest that a TH may establish the M-opsin gradient, but that additional factors are required to establish the S-opsin gradient.

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Fig. 5. Ligand gradients in the developing retina. (A) There is no difference in the ratio of dorsal-ventral T3 or T4 at P0 or P4. At P10, both T3 and T4 were elevated in the dorsal retina. (B) Model of opsin regulation by thyroid hormone. At S-opsin onset (schematized on the left), TRβ2, RXRγ, and T3 are required to inhibit S-opsin expression in dorsal cones. However, TH is not graded in the retina at this time, indicating that another factor (X) is required to establish the S-opsin gradient. TRβ2 is required for M-opsin onset around P10 (schematized on the right). T3 is highest in the dorsal retina at this time, which likely promotes M-opsin expression in the dorsal retina.

Fig. 4. Changes in thyroid hormone during mouse retinal development between P0 and P10. Retinas were bisected into dorsal and ventral halves and pooled for measurement of T3 and T4 by RIA. The concentrations are expressed per retina (Left) and per gram of retinal tissue (Right). T3 and T4 increase in the dorsal retina between P0 and P10.

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developing rat caudate (34), and a T3 gradient in the Xenopus eye mediates dorsal-ventral growth during metamorphosis (35). This gradient in Xenopus is established during metamorphosis by increased expression of the T3-inactivating enzyme, type 3 deiodinase (D3), in the dorsal retina (35). D3 is abundant in the developing mouse retina, whereas type 1 and type 2 enzymes are at or below the detection limit (M.S. and D.F., unpublished data). Unlike in Xenopus, we did not see an obvious gradient of D3 in mouse. Thus, in contrast to the Xenopus retina, the T3 gradient in mouse retina may be due to asymmetric distribution of TH binding proteins or transporters rather than asymmetric distribution of deiodinases.

**Multiple Factors Regulate S-opsin Expression.** Opsin gradients have been observed in many species, including the house mouse, insectivores, and the hyena (36–38). In all of these species, S-opsin is enriched in the ventral retina, suggesting that a conserved factor either promotes S-opsin expression in the ventral retina or inhibits it in the dorsal retina. Although our data demonstrates that T3 is necessary to inhibit S-opsin in dorsal cones, T3 is not graded at the time of S-opsin onset, indicating that another factor is required to establish the S-opsin gradient. Likely candidates are ligands for RXRs or retinoic acid receptors (RARs). The RAR ligand, all-trans retinoic acid (ATRA), is an attractive candidate because it has a well-characterized gradient in the developing retina (39). A recent study of zebrafish cone development showed that ATRA represses UV opsins, which is consistent with the idea that another factor is required to establish the S-opsin gradient.

**Functional Significance.** Transgenic reporter mice for human S-opsin and L/M opsin show mouse-specific reporter gradients (45, 46), suggesting that the same factors that control opsin expression in mice likely affect cone opsin expression in humans. In contrast to the mouse cone pattern, humans have a central to peripheral cone gradient, with an area of S-cone exclusion in the central fovea (47). It will be interesting to determine whether TH signaling is involved in establishing this gradient in humans. Disruptions in TH signaling caused by diet, genetics, or exposure to environmental chemicals, such as polychlorinated biphenyls (PCBs), in pregnant mothers and newborns could potentially affect diverse developmental processes in the CNS, including color vision (48, 49). A recent report shows that hypothyroid infants or infants born to hypothyroid mothers develop reduced contrast sensitivity, suggesting that TH may also be required for proper cone opsin expression in humans (50). Additionally, the children of women who were exposed to high levels of organic solvents during pregnancy showed decreased visual acuity, deficits in red/green color discrimination, and an increased risk of red/green color blindness (51). Elucidating the molecular mechanisms of thyroid hormone signaling during neural development may help to prevent or treat these mental and visual deficiencies caused by environmental or genetic alterations in the thyroid signaling pathway.

**Methods**

**Animals and in Vivo Treatments.** All animal experiments followed approved protocols of the host institutions. Transgenic TRβnull mice were a gift from Sheue-yann Cheng (National Cancer Institute, Frederick, MD) (29). For in vivo studies, newborn (P0) or P7 pups were injected s.c. with 1.5 μg of T3 or saline vehicle every 24 h for 4 days. Pups were killed 2 h after the last injection and were either frozen for RNA extraction or fixed for β-galactosidase assay and whole-mount opsin immunohistochemistry.

**Explant Cultures.** Whole retinas were cultured on a nitrocellulose membrane for up to 2 weeks in vitro, in a method modified from ref. 24. Briefly, retinas from embryonic day 17 (E17) mice were dissected free from the lens, pigmented epithelium, and extraocular tissue, four small incisions were made in the peripheral retina to allow flattening, and retinas were placed photoreceptor side down on a Millicell-CM 0.4 μm filter insert (catalog no. PICM03050). Filters were placed into a six-well plate containing 1 ml of explant media [DMEM:F12 (GIBCO catalog no. 895108EA), 0.6% glucose, 5 mM Heps, 0.11% NaHCO3, 25 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 30 mM selenium, 20 mM progesterone, 800 mM L-glutamine, penicillin and streptomycin (GIBCO), N2 supplement (GIBCO), and 10% dialyzed FBS (GIBCO)]. Explants were cultured at the gas–liquid interface at 37°C, 5% CO2, and media were replaced every other day. T3 (10 nM; Sigma) was added to culture medium of some wells for the duration of the experiments.

**S-opsin Transgene.** The S-opsin 562 transgenic construct includes the promoter and 5′ flanking region of the mouse S-opsin gene (Opn1sw) fused in frame with a lacZ cassette at the ATG translational start codon of the first exon of the Opn1sw gene (52) as described in detail elsewhere (M.S. and D.F., unpublished data). Transgenic founders were derived on the B6D2/F1J strain at the Mount Sinai transgenic facility and were identified by Southern blot analysis using a lacZ probe. Carriers were backcrossed with wild-type B6D2/F1J mice. Of the seven founders, three showed expression. S-opsin 562 transgene/TRβnull mice were generated by crossing with TRβnull mice (53).

**RNA Analysis.** Total RNA was prepared from pools of at least eight eyes. For Northern blot analysis, 15 μg of total RNA was loaded per lane and filters were probed for S-opsin, as described (23). As a loading control, the same filters were subsequently hybridized with a GAPDH probe.

**Quantitative PCR.** cDNA was prepared from retinas cultured for 11 days with or without T3, and the relative levels of cone arrestin transcripts were measured by quantitative PCR as described (54) and normalized to β-actin. Primer sequences are available upon request.

**β-Galactosidase Staining.** Eyes were fixed in 2% paraformaldehyde in PBS for 2 h at 4°C, followed by cryoprotection in 30% sucrose in PBS at 4°C for 1 h and embedded in optimal cutting temperature medium (O.C.T.; VWR, West Chester, PA). Cryosections (10 μm thick) were stained for β-galactosidase activity by incubating in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) substrate.

**Immunohistochemistry and Quantification of Cone Gradients.** Whole retinas from at least three animals from each genotype (TRβnull, TRβnull+/−, and TRβnull+/+) were immunolabeled with opsin antibodies and quantified as described (16).

**Radioimmunoassays.** Retinas from P0, P4, or P10 mice were dissected free of the pigment epithelium, lens, and choroid in cold PBS and quick-frozen in an Eppendorf tube in dry ice. At least 50 mg of tissue was pooled for each sample (>15 retinas at P0 and eight retinas at P10). Measurements of T4 and T3 for at least three samples at each age were determined by highly sensitive and specific radioimmunoassays after extensive extraction and purification of the iodothyronines from tissues, modified from procedures described elsewhere (55, 56). To increase recovery of the very small amounts of the iodothyronines that...
were expected in the very small tissue samples, we excluded the initial methanol/chloroform extraction and back-extraction into an aqueous phase, and purified the initial methanol extract of the retinas directly on the resin columns. To confirm that the small amounts of TH that we detected were not artifacts, we tested separate pools of sample extracts at three to five successive 2-fold dilutions and showed that the extracts behaved in the RIA in the same manner as the T3 and T4 standard solutions (see Fig. 8, which is published as supporting information on the PNAS web site).

We thank Sheue-yann Cheng for the TRPV/PV mice; Chris McGuire, Paige Etter, Melissa Philips, Maria Jesus Presas, and Socorro Duran for technical assistance; and Paige Etter for helpful comments on the manuscript. This work was supported by National Institutes of Health Grants T32 EY07031 and NS28308 (to M.R. and T.A.R.), by the National Institute on Deafness and Other Communication Disorders, a Hirshl award, and the intramural program at National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases (to M.S. and D.F.), and by Instituto de Salud Carlos III, Red de Centros de Metabolismo y Nutricion (03/08) and Instituto de Saludo Carlos III P1031417 (03/1417) (to G.M.D.E.).