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

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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. These results also suggest that the ratio and patterning of cone types may be determined by TH availability during retinal development.

photoreceptor | retinal development | nuclear hormone receptor

Thyroid hormone (TH) is secreted mostly as thyroxine (T4) from the thyroid gland and converted locally to a transcriptionally active form, 3,5,3′-triiodothyronine (T3), by iodothyronine deiodinating enzymes (1). T3 receptors bind to DNA regulatory elements, often as heterodimers with retinoid X receptors (RXRs), to activate or repress transcription of target genes (for review, see refs. 2 and 3). T3 regulates diverse developmental processes in multiple regions of the central nervous system including the hippocampus, cerebral cortex, inner ear, and cerebellum (4–8). Numerous developmental processes are TH-dependent, including cell differentiation, migration, and dendritic growth (2, 3, 9–11). Perturbations in maternal or fetal TH in humans can lead to clinical syndromes ranging from severe mental retardation and hearing loss to mild or moderate deficits in motor skills, language, memory, and attention (12–14).

In the retina, the TH β2 receptor isoform (TRβ2) is restricted to cone photoreceptors (15, 16), and we previously showed that it is a critical regulator of late-stage cone differentiation. In the first stage of cone differentiation, cones exit the cell cycle and begin to express specific transcription factors, including cone rod homeobox (CRX), TRβ2, and RXRγ (16–20). In the second stage of differentiation, cones express opsin and form specific synaptic connections. Most mammalian cones express short wavelength (S-opsin) or medium wavelength (M-opsin) opsin. However, the spatial arrangement of cone types varies considerably among species. In mice, S-opsin expression begins just before birth and is expressed predominantly in ventral cones. By contrast, M-opsin expression begins at the end of the first postnatal week and is expressed predominantly in dorsal cones (21, 22).

Previous analysis of TRβ2-null mice showed that TRβ2 is required to activate M-opsin and to inhibit S-opsin (23). RXRγ, another nuclear receptor, is also required to inhibit S-opsin, possibly as a heterodimer with TRβ2 (16). Nuclear hormone receptors modulate transcription both in the presence and absence of ligands. Our previous analyses did not reveal whether ligands are involved in the regulation of opsin expression, but we speculated that developmental changes in concentration or regional distribution of ligands could modulate both the ratio of S-opsin- to M-opsin expressing cones and the spatial patterning of cones. The present study confirms the presence of TH in the developing retina. We use *in vitro* and *in vivo* approaches to demonstrate that TH is required to inhibit S-opsin and activate M-opsin expression. Furthermore, TH becomes graded around the time of M-opsin onset, which is consistent with a role for TH in establishing the M-opsin gradient. Together, these results suggest that TH can modulate the M-opsin/S-opsin ratio in the developing retina and that it may play a role in species-specific cone patterning.

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 their normal graded pattern (Fig. 1*A*). 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. 1*B*). 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

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Abbreviations: TH, thyroid hormone; RXR, retinoid X receptor; TRβ2, TH β2 receptor isoform; S-opsin, short wavelength opsin; M-opsin, medium wavelength opsin; P*n*, postnatal day *n*.

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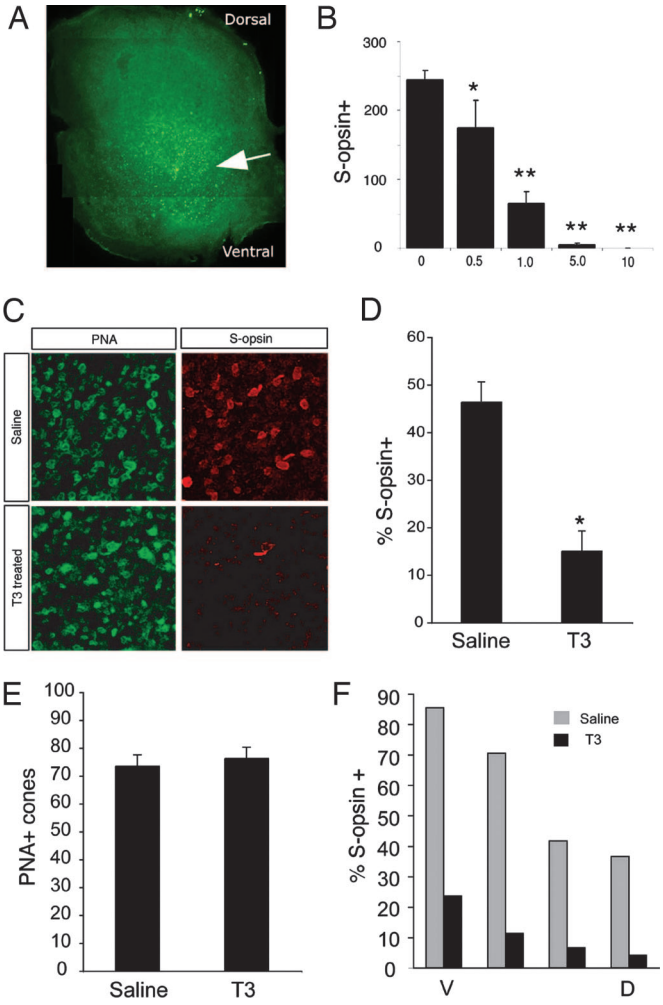

Fig. 1. Exogenous T3 inhibits S-opsin. (*A*) Whole retinas from embryonic day 17 mice cultured for up to 2 weeks. S-opsin immunostaining (green) shows a normal ventral-to-dorsal gradient. (*B*) The number of S-opsin immunolabeled cones were counted in untreated (CTRL) and T3-treated explants after 11 days *in vitro* (*, $P < 0.01$; **, $P < 0.001$). (*C–F*) Newborn mice (P0) were injected s.c. with either saline or T3 (1.5 μg) 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-μm^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 (*, $P < 0.015$ 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.

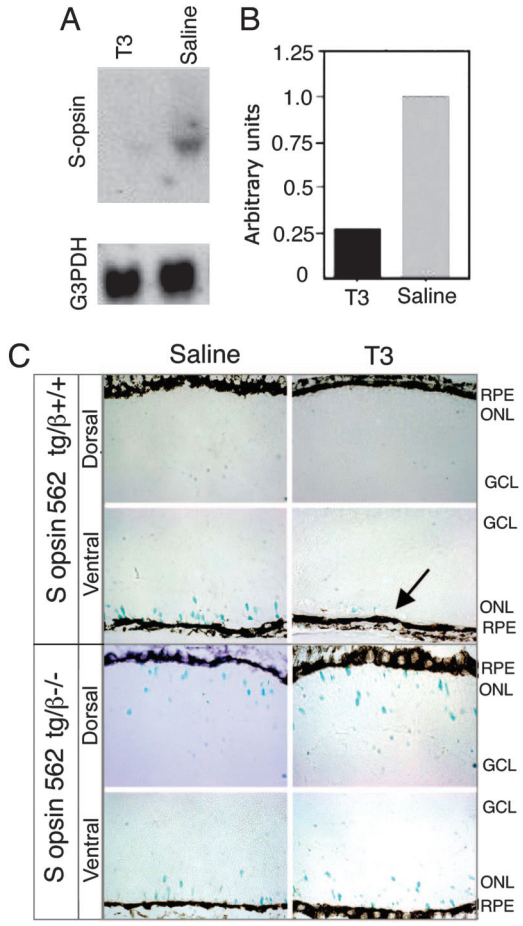

Fig. 2. T3 inhibits S-opsin *in vivo*. A 1.5-μg dose of T3 was injected into mice once daily, from P0 to P3. (*A*) Northern blots show that S-opsin transcript is reduced in wild-type mice that were treated with T3. (*B*) Digital quantification of band intensity shows a 75% decrease in S-opsin transcript in mice injected with T3. (*C*) T3 reduces the number of S-opsin-expressing cones *in vivo*. Transgenic mice expressing LacZ under the control of the S-opsin promoter show a typical S-opsin gradient, with β-galactosidase expression highest in the ventral retina (*Upper Left*). Injecting T3 into reporter mice from P0 to P3 dramatically reduces the number of cones expressing S-opsin (*Upper*, arrow). Injecting T3 into S-opsin reporter mice lacking the TRβ2 receptor did not effect the number of S-opsin-expressing cones (*Lower*). ONL, outer nuclear layer; GCL, ganglion cell layer; RPE, pigment epithelium.

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. 1*C* 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. 1*D*) 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. 1*E*), 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. 1*F*).

Northern blots reveal that T3 treatment of neonatal mice similarly reduces S-opsin transcript by $\approx$75% (Fig. 2 *A* and *B*). To confirm that T3 acts through the TRβ2 receptor *in vivo*, we injected T3 into wild-type or TRβ2-null mice, each crossed onto an S-opsin promoter/β-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 TRβ2-null mice (Fig. 2*C*), confirming that TRβ2 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

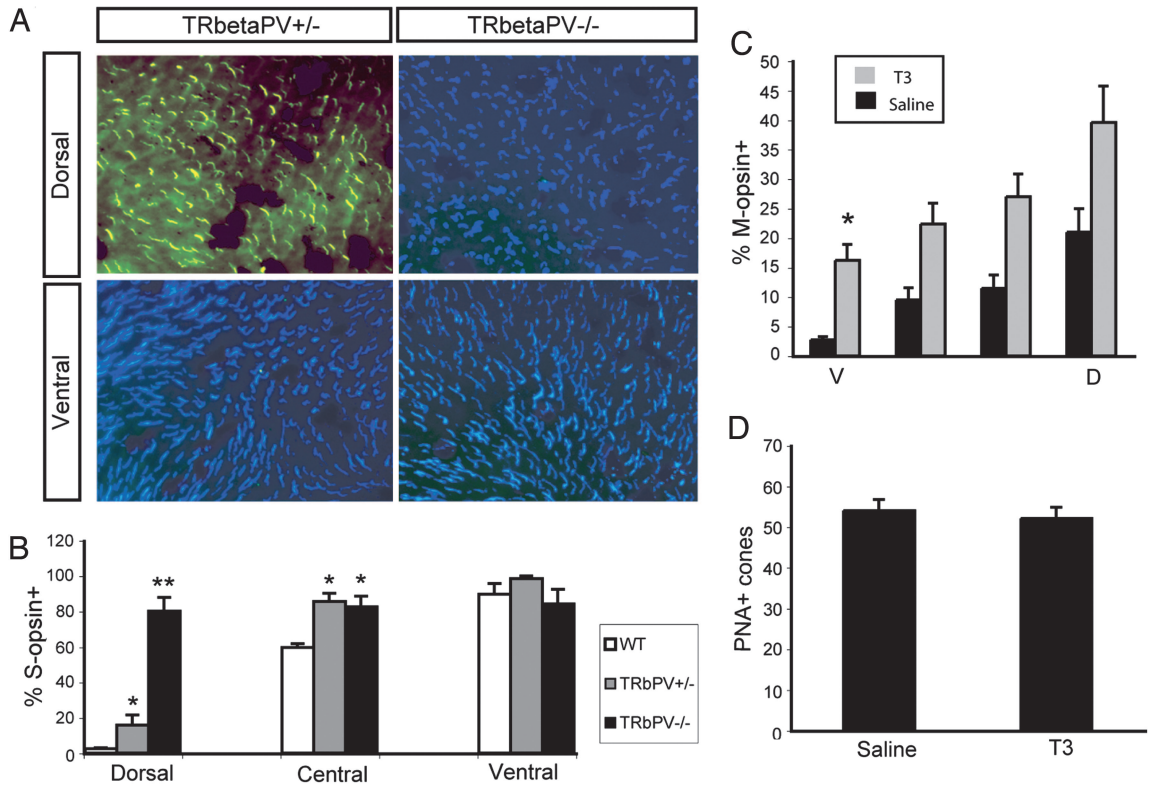

Fig. 3. T3 binding is required for opsin patterning. (*A*) Whole-mount retinas from adult WT, TR$\beta^{PV/+}$ (not shown), and TR$\beta^{PV/PV}$ were double-labeled with antibodies to M-opsin (green) and S-opsin (blue). Wild-type retinas show almost exclusive expression of M-opsin in the dorsal retina and S-opsin in the ventral retina. TRβ^{PV} retinas, which have a targeted mutation that blocks T3 binding to TRβ, have no M-opsin expression, and S-opsin is ectopically expressed in dorsal cones. (*B*) Quantification of the percentage of S-opsin immunoreactive cones shows statistically significant changes in S-opsin for both the homozygous mutant as well as the TRβPV$^{+/-}$ retinas. *, $P < 0.005$; **, $P = 9.3E$. (*C*) T3 up-regulates M-opsin expression. P7 pups were injected s.c. with T3 for 4 days and then killed, and their retinas were flat-mounted for immunolabeling with M-opsin and PNA ($n = 4$ for saline and $n = 5$ for T3-treated). The percentage of M-opsin$^+$ cones was increased in each region of the retina, but most significantly in the ventral retina (*, regions compared with ANOVA $P < 0.01$; pairwise comparison, $P < 0.05$ for *D*). (*D*) Total cone number is not affected by T3 treatment in P7–P10 mice; total number of PNA$^+$ cones per field shown.

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 expression in whole-mounted adult wild-type, TR$\beta^{PV/+}$, and TR$\beta^{PV/PV}$ 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 TR$\beta^{PV/PV}$ cones, where T3 cannot bind to TRβ, all cones expressed S-opsin (Fig. 3*A*). This finding suggests that endogenous T3 is required to inhibit S-opsin in dorsal cones. Furthermore, we could not detect M-opsin in TR$\beta^{PV/PV}$ 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. 3*B*). S-opsin is increased in all regions of the TR$\beta^{PV/PV}$ 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 TR$\beta^{PV/PV}$ retinas. We also found a small increase in dorsal S-opsin-expressing cones in the TR$\beta^{PV/+}$ 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. 3*C*); 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. 3*D*).

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 bisected 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 between P0 and P10, and a downward trend for T3 and T4 in the ventral retina after P4. At P10, the time of M-opsin onset, both T3 and T4 were more than twice as concentrated in the dorsal

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

	P0	P4	P10
T4, ng/g			
Dorsal	7.4 ± 1.3	8.3 ± 1.6	11.7 ± 1.7*
Ventral	7.8 ± 2.1	7.5 ± 1.7	6.6 ± 2.4
T3, ng/g			
Dorsal	4.3 ± 1.0	5.2 ± 1.4	5.3 ± 1.0
Ventral	4.0 ± 1.2	5.9 ± 1.5	2.7 ± 1.6

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. $P < 0.05$.

retina than in the ventral retina (Fig. 5*A* and Fig. 6 and Table 2, which are published as supporting information on the PNAS web site).

Discussion

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

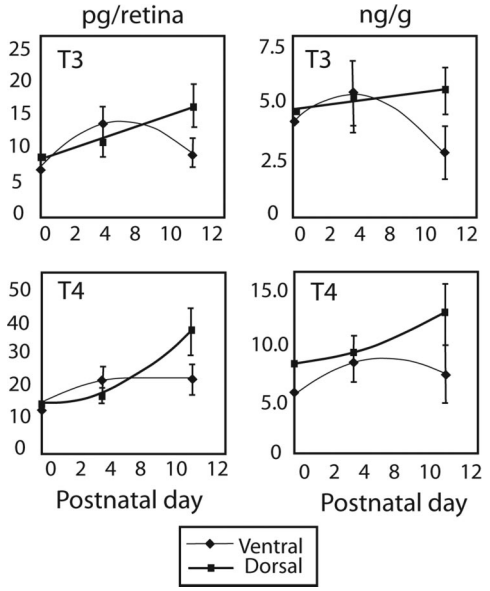

Fig. 4. Changes in thyroid hormone during mouse retinal development between P0 and P10. Retinas were bisected into dorsal and ventral halves and pooled for measurement of T3 and T4 by RIA. The concentrations are expressed per retina (*Left*) and per gram of retinal tissue (*Right*). T3 and T4 increase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

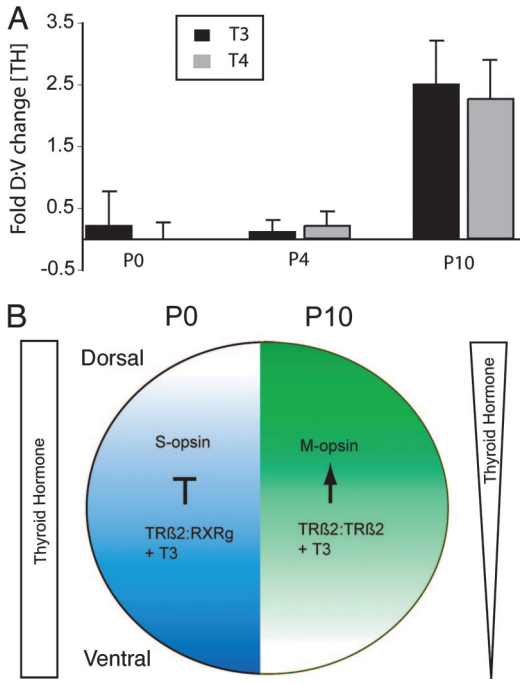

Fig. 5. Ligand gradients in the developing retina. (*A*) There is no difference in the ratio of dorsal–ventral T3 or T4 at P0 or P4. At P10, both T3 and T4 were elevated in the dorsal retina. (*B*) Model of opsin regulation by thyroid hormone. At S-opsin onset (schematized on the left), TRβ2, RXRγ, and 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.

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. 5*B*. 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 web site). Here, we show 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 TR$\alpha 1^{-/-}$ 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

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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 opsin, which is the fish cone opsin most closely related to S-opsin in mice (40, 41). It is also possible that the gradient of S-opsin is controlled by a transcription factor, and not by a diffusible factor. A candidate is COUP-TFII, which is known to repress signaling mediated by TR, RXR, and RAR (42, 43). COUP-TFII is more abundant in the dorsal retina at the time of S-opsin onset (44), and could play a role in repressing S-opsin.

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 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 TRβ^{PV} 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 nM Hepes, 0.11% $NaHCO_3$, 25 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 30 nM selenium, 20 nM progesterone, 800 nM 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% CO_2, 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 562-bp 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/TRβ-null mice were generated by crossing with TR$\beta^{-/-}$ 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 (TR$\beta^{+/+}$, TR$\beta^{PV/+}$, and TR$\beta^{PV/PV}$) 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).

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