Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2009 | 108 | 91–101 doi: 10.1111/j.1471-4159.2008.05739.x

Retinoic acid receptor-related orphan receptor α regulates a subset of cone genes during mouse retinal development

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Abstract

Color vision is supported by retinal cone photoreceptors that, in most mammals, express two photopigments sensitive to short (S-opsin) or middle (M-opsin) wavelengths. Expression of the *Opn1sw* and *Opn1mw* genes, encoding S-opsin and M-opsin, respectively, is under the control of nuclear receptors, including thyroid hormone receptor β2 (TRβ2), retinoid X receptor γ (RXRγ), and RORβ, a member of the retinoic acid receptor-related orphan receptor (ROR) family. We now demonstrate that RORα, another member of the ROR family, regulates *Opn1sw*, *Opn1mw*, as well as *Arr3* (cone arrestin) in the mouse retina. RORα expression is detected in cones by postnatal day 3 and maintained through adulthood. The retinas of *staggerer* mice, carrying a null mutation of RORα, show significant down-regulation of *Opn1sw*, *Opn1mw*, and *Arr3*. RORα acts in synergy with cone-rod homeobox transcription factor (Crx), to activate the *Opn1sw* promoter *in vitro*. Chromatin immunoprecipitation assays reveal that RORα directly binds to the *Opn1sw* promoter, *Opn1mw* locus control region, and the *Arr3* promoter *in vivo*. Our data suggest that RORα plays a crucial role in cone development by directly regulating multiple cone genes.

Keywords: arrestin, cone photoreceptor, opsin, retina, RORα, *staggerer*.

J. Neurochem. (2009) **108**, 91–101.

The vertebrate retina contains two types of photoreceptors: rods that function in dim light and cones that mediate color vision in bright light. Most mammals have dichromatic color vision, supported by cones expressing two opsin photopigments sensitive to short (S-opsin) or middle (M-opsin) wavelengths (Ebrey and Koutalos 2001). Humans and other primates have an additional cone photopigment sensitive to long wavelengths (L-opsin), and thus have trichromatic color vision. Mutations affecting the cone opsin genes, as well as other genes involved in cone phototransduction, are responsible for color vision defects of varying degrees of severity (Deeb 2004).

The expression of the cone opsin genes is under distinct spatial and developmental controls. In mice, S-opsin expression starts at the late embryonic stage (Ng *et al.* 2001; Applebury *et al.* 2007), while M-opsin expression is induced during the second postnatal week (Fei 2003). The majority of cones in the mature mouse retina co-express both S- and M-opsins (Applebury *et al.* 2000). However, S-opsin expression is more concentrated in ventral cones, whereas M-opsin is expressed predominantly in dorsal cones (Szél *et al.* 1992; Applebury *et al.* 2000).

Several transcription factors have been identified that regulate the spatial and temporal patterning of cone opsin expression. Thyroid hormone receptor β2 (TRβ2) and retinoid X receptor γ (RXRγ) act in concert to repress S-opsin expression during the embryonic period and in the dorsal region of the mature retina (Ng *et al.* 2001; Roberts *et al.* 2005). It has been suggested that the postnatal down-regulation of both TRβ2 and RXRγ promotes the onset of S-opsin

Received July 22, 2008; revised manuscript received October 8, 2008; accepted October 10, 2008.

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Abbreviations used: ChIP, chromatin immunoprecipitation; LCR, locus control region; PNA, peanut agglutinin; ROR, retinoic acid receptor-related orphan receptor; RORE, ROR response element; RXR, retinoid X receptor; TR, thyroid hormone receptor.

expression (Ng *et al.* 2001; Roberts *et al.* 2005). TRβ2 is also required to induce M-opsin expression (Ng *et al.* 2001). As TRβ2 is uniformly expressed in developing cones, the dorsal–ventral gradient of M-opsin expression has been attributed to the graded concentration of thyroid hormone within the retina (Roberts *et al.* 2006). Recently, RORβ, a member of the retinoic acid receptor-related orphan receptor (ROR) family, has emerged as a transcriptional activator of S-opsin (Srinivas *et al.* 2006). Thus, the orchestrated action of nuclear receptors seems to control the timing and spatial arrangement of opsin expression in developing cones.

RORα, another member of the ROR family, has been implicated in the differentiation of cerebellar Purkinje neurons (Hamilton *et al.* 1996; Steinmayr *et al.* 1998; Gold *et al.* 2003) and the maintenance of circadian rhythms in the suprachiasmatic nuclei of the hypothalamus (Sato *et al.* 2004; Akashi and Takumi 2005). In the adult retina, RORα has been localized to the ganglion cell layer and inner nuclear layer (Steinmayr *et al.* 1998; Ino 2004). However, the precise adult cell types expressing RORα is unclear, nothing is known about its developmental pattern of expression in the retina, and its function in this tissue is also unexplored. We now demonstrate that RORα is expressed in postnatal cones and that it plays a critical role in cone development by regulating a subset of cone genes, including S-opsin and M-opsin.

Materials and methods

Mice

Heterozygous *Staggerer* mice (B6.Cg-Rorasg/J) obtained from the Jackson Laboratory (Bar Harbor, ME, USA) were bred, and the progeny were genotyped using a 3-primer PCR assay, as previously described (Gold *et al.* 2003). Animals were maintained under a 12 : 12 h light/dark photoperiod and killed by decapitation or cervical dislocation in the middle of the light phase at various developmental stages. *Nrl*−/− mice are described by Mears *et al.* (2001). C57BL/6J mice were obtained from Charles River Laboratories Japan (Yokohama, Japan). All procedures were approved by the local animal care committees.

Immunohistochemistry

Goat polyclonal antibody to RORα (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit (Chemicon International, Temecula, CA, USA) and goat (Santa Cruz) polyclonal antibodies to S-opsin, and rabbit polyclonal antibody to M-opsin (Chemicon) were used as primary antibodies. Alexa Fluor 568 donkey anti-goat IgG and Alexa Fluor 488 donkey anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies. Fluorescein peanut agglutinin (PNA) (Vector Laboratories, Burlingame, CA, USA) was used to label all cones. Immunohistochemistry was performed as described elsewhere (Fujieda and Sasaki 2008). Immunohistochemical controls were performed by using primary antibody preabsorbed with an excess amount of immunogen peptide (for RORα, sc-6062P; Santa Cruz) or by omission of primary antibody. Dilutions of primary antibodies were as follows: RORα 1 : 600, S-opsin (rabbit) 1 : 2000, S-opsin (goat) 1 : 500, and M-opsin 1 : 2000. All secondary antibodies were diluted 1 : 1000. Fluorescence signals were examined by confocal laser scanning microscope (LSM510 META; Carl Zeiss, Jena, Germany).

Quantitative (real-time) RT-PCR

Total RNA was extracted from the retina using the RNAqueous-4PCR kit (Ambion, Austin, TX, USA). First strand cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). cDNA templates were subjected to quantitative PCR by using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR contained 1 μL each of cDNA sample or serially diluted standard cDNA, 1× SYBR Green PCR master mix (Applied Biosystems), and 300 nM of each primer (see supporting information Table S1 for the list of primers) in a final volume of 25 μl. Amplification involved a two-step PCR with denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min for 40 cycles. Control reactions were performed by using the PCR mixture without cDNA template or using RNA samples without RT. *Gapdh* expression, which was not affected by the *staggerer* mutation, served as an internal control for data normalization.

Luciferase assays

Human embryonic kidney cells were co-transfected with plasmid DNA [2 μg of the reporter vector pGL4.10 (containing the mouse *Opn1sw* promoter (−507 to +54)), 400 ng of expression plasmid(s) (*Crx*, *Rora* or empty control) and 20 ng of the *Renilla* luciferase control vector (pGL4.74)] using Express-In transfection reagent (Open Biosystems, Huntsville, AL, USA). Deletion and site-directed mutants of the reporter constructs were kind gifts from Dr. D. Forrest, National Institutes of Health/NIDDK, Bethesda, MD, USA (Srinivas *et al.* 2006). 48-h post-transfection luciferase activities were measured using the dual luciferase reporter assay system (Promega, Madison, WI, USA) with a 20/20 luminometer (Turner Biosystems, Sunnyvale, CA, USA). Normalized luciferase activities were then calculated and averaged across 3-wells per condition tested. Experiments were repeated in triplicate and *t*-tests performed on the resulting data.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described (Dorval *et al.* 2006), with partial modification. Retinas cross-linked with 4% formaldehyde in phosphate-buffered saline were sonicated to an average DNA size of 1 kb. 20 μL of each chromatin sample was removed and DNA purified to prepare the input DNA as a reference. Chromatin samples equivalent to 3 whole retinas were incubated for 1 h with 1 μg of anti-RORα antibody or normal goat IgG, followed by an incubation with protein G-Sepharose (Sigma, St Louis, MO, USA) for an additional 1 h. After extensive washes, samples were eluted, cross-links reversed, and DNA fragments purified by High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) with a final elution product of 100 μL. Real-time PCR (Applied Biosystems) was used to amplify 2 μL of the final ChIP product, as well as the input, and the copy number was quantified by comparison to a standard curve for each primer set generated with serially diluted genomic DNA. All primers used are shown in supporting information Table S1.

Results

RORα is expressed in cones

As an initial step in analyzing RORα function in the retina, we performed immunohistochemical localization of RORα in the 3-week-old mouse retina. Intense nuclear staining for RORα was observed in the ganglion cell layer and inner nuclear layer (Fig. 1a and b), in agreement with the previous report (Ino 2004). In addition, we observed an immunoreactive cell population scattered along the outer margin of the outer nuclear layer (arrowheads in Fig. 1b). The low cell density and position within the outer nuclear layer suggested that these cells may be cone photoreceptors. The entire thickness of the outer nuclear layer was also marginally stained. Control sections reacted with primary antibody preabsorbed with antigen peptide exhibited no immunoreactivity (data not shown). To further confirm the specificity of primary antibody, we performed RORα immunohistochemistry in the retina of 3-week-old *staggerer* mice, containing a loss-of-function mutation in *Rora*, the gene encoding RORα. As previously reported for RORα-null mutant mice (Steinmayr *et al.* 1998), no apparent morphological alterations were observed in the retina of *staggerer* mutants (Fig. 1c). No specific immunoreactivity for RORα was observed in the mutant retina, supporting the specificity of immunoreaction obtained in the wild-type mice (Fig. 1d).

To determine whether cone photoreceptors express RORα, double immunolabeling was performed using antibodies to cone-specific opsins in combination with anti-RORα antibody. RORα was co-localized with both S-opsin and M-opsin, indicating that RORα is indeed expressed in cones (Fig. 1e–j). Virtually all cones seemed to express RORα, and no gradient of RORα expression was observed along the dorsal–ventral axis of the retina.

Developmental expression of RORα in the mouse retina

RORα expression in the mouse retina was further examined during embryonic and postnatal development. No RORα

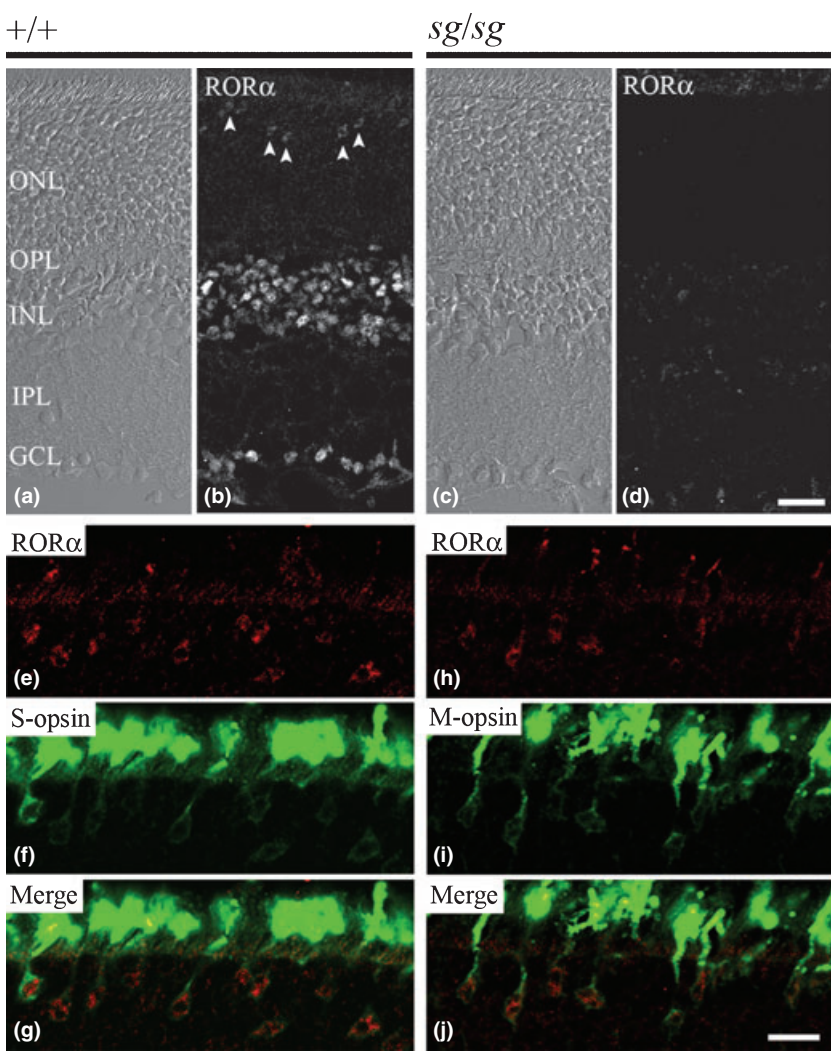

Fig. 1 Immunohistochemical localization of RORα in the retina of wild-type (a, b, e–j) and *staggerer* (c and d) mice. (a) Differential interference contrast (DIC) image of the wild-type (+/+) retina. GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer. (b) RORα immunoreactivity in the wild type retina. Arrowheads depict presumptive cones exhibiting RORα immunoreactivity. (c) DIC image of the *staggerer* mutant retina (*sg*/*sg*). (d) RORα immunoreactivity is absent in the *staggerer* retina. Scale bar (a–d) = 20 μm. (e–g) Co-localization of RORα and S-opsin indicating RORα expression in cones. (h–j) RORα is also co-localized with M-opsin. Scale bar (e–j) = 10 μm.

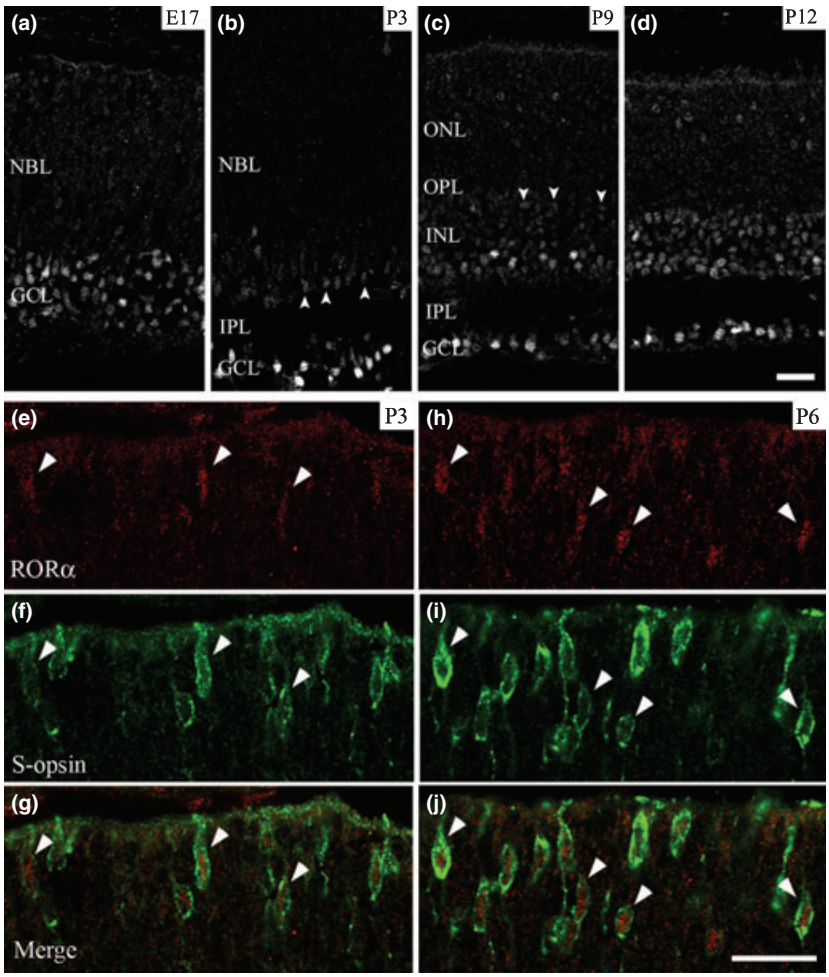

Fig. 2 Immunohistochemical localization of RORα in the developing retina. The ganglion cell layer becomes positive for RORα by E17 (a). RORα immunoreactivity is detected in presumptive amacrine cells by P3 (arrowheads in b) and presumptive bipolar cells by P9 (arrowheads in c). RORα expression exhibits the adult pattern by P12 (d). Scale bar (a–d) = 20 μm. Double immunolabeling shows developing cones immunoreactive for both S-opsin and RORα at P3 (arrowheads in e–g) and P6 (arrowheads in h–j). Scale bar (e–j) = 20 μm. GCL: ganglion cell layer; NBL: neuroblastic layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer.

immunoreactivity was observed in the E14 mouse retina (data not shown). At E17, cells in the developing ganglion cell layer were immunolabeled for RORα while the neuroblastic layer, composed mostly of mitotic progenitor cells and early-born neurons such as cones and horizontal cells, was free of immunoreactivity (Fig. 2a). The cellular pattern of RORα expression did not change significantly until P3, when faint immunoreactivity was first observed at the inner border of the neuroblastic layer (composed of developing amacrine cells) (Fig. 2b), along with the outer border of the same layer (presumptive cones) (Fig. 2e). Immunoreactivity in presumptive cones was increased in intensity by P6 (Fig. 2h) and maintained during subsequent development. The neuroblastic layer splits into the inner and outer nuclear layers by P6. The outer half of the inner nuclear layer, composed mostly of developing bipolar cells, became faintly immunoreactive by P9 (Fig. 2c). The adult pattern of RORα expression was established by P12 (Fig. 2d).

To unambiguously determine whether RORα-positive cells observed in the outer retina during postnatal development are developing cones, double immunolabeling was conducted using RORα and S-opsin antibodies at P3 and P6. At both stages, virtually all RORα-positive cells located near the outer margin of the retina co-localized with S-opsin (arrowheads in Fig. 2e–j), indicating that developing cones start to express RORα by P3.

The expression of certain cone genes, including S-opsin, M-opsin, and cone arrestin, has been shown to be graded across the dorsal–ventral axis of the mouse retina (Szél *et al.* 1992; Applebury *et al.* 2000; Corbo *et al.* 2007). To confirm that RORα is symmetrically expressed across the retina, we performed quantitative RT-PCR to analyze the transcript levels of *Rora*, as well as other cone-related genes, in the dorsal and ventral halves of the mouse retina during postnatal development (Supporting information Fig. S1). No dorsal-ventral differences in *Rora* and *Rorb* (encoding RORβ) expression were observed throughout postnatal development. By contrast, the transcript levels of *Thrb2* (encoding TRβ2) were significantly higher in the dorsal retina compared to the ventral retina at all ages examined except for P0. The graded expression of *Opn1sw* (S-opsin), with higher levels in the ventral retina was

observed throughout postnatal development, in agreement with previous reports (Roberts *et al.* 2005; Applebury *et al.* 2007). No *Opn1mw* (M-opsin) gradient of expression was observed at P0 or P6, but higher expression in the dorsal retina was observed at P12 and P21. A similar pattern was observed for *Arr3* (cone arrestin). No dorsal–ventral differences in *Gnat2*, *Pde6h*, and *Gngt2* were observed throughout postnatal development.

RORα mutation affects cone gene expression

Expression of cone opsin genes is regulated by nuclear receptors, such as TRβ2 (Ng *et al.* 2001) and RORβ (Srinivas *et al.* 2006). Prompted by our observations that RORα is expressed in cones, we hypothesized that RORα may also be involved in cone development by regulating the transcription of cone genes. To test this hypothesis, we first quantitated the cone cell density in the wild-type and *staggerer* retinas at P21 using PNA labeling (Fig. 3a–c). There were no significant differences in the density of PNA-labeled cones between genotypes. Similar results were observed at P35 (data not shown), indicating that the RORα mutation does not affect the generation or survival of cones during this period of development.

Next, P21 wild-type and *staggerer* retinas were subjected to double immunolabeling for S- and M-opsins to examine the effects of *staggerer* mutation on cone opsin expression. Most cones exhibited both S- and M-opsin immunoreactivity in the wild-type mouse retina, as reported previously (Applebury *et al.* 2000). S-opsin immunoreactivity was more concentrated in ventral cones compared to dorsal cones, whereas M-opsin immunoreactivity was more intense in dorsal cones compared with ventral cones (Fig. 3d–i). In the *staggerer* retina, immunoreactivity for both opsins was notably reduced throughout the retina. (Fig. 3j–o). S-opsin immunoreactivity was detected at reduced levels in ventral cones and was undetectable in most dorsal cones. M-opsin immunoreactivity, on the other hand, was detected at reduced levels in dorsal cones while only few cones in the ventral retina displayed M-opsin immunoreactivity.

We next performed quantitative RT-PCR to analyze the expression of a series of cone genes in the retinas of wild-type, heterozygous *staggerer* and homozygous *staggerer* mutants at P21 (Fig. 3, lower panel). The transcript levels of *Opn1sw, Opn1mw* and *Arr3* were significantly reduced in the *staggerer* retina when compared with wild-type littermates. By contrast, other cone genes examined (*Gnat2*, *Pde6c*, *Pde6h*, *Gngt2*, *Cngb3*) did not show significant genotype effects. Expression of the rod-specific gene *Rho* (encoding rhodopsin) was also unaffected (data not shown). To examine the effects of RORα mutation on the expression of nuclear receptors known to regulate cone genes, the transcript levels of *Thrb2*, *Rxrg* (encoding RXRγ), *Rorb*, as well as *Rora*, were also analyzed by quantitative RT-PCR. *Thrb2* was marginally up-regulated in the *staggerer* retina, whereas no significant differences were found in *Rxrg* and *Rorb* expression between genotypes. The mutant *Rora* transcripts were expressed at reduced levels in the *staggerer* retina. *Shh* (encoding sonic hedgehog) and *Pcp2* (encoding Purkinje cell protein 2) are significantly down-regulated in the *staggerer* cerebellum (Gold *et al.* 2003), but these genes were unaffected by the mutation in the retina (data not shown). Taken together, these findings suggest that RORα is required for the full expression of a subset of cone genes, including *Opn1sw*, *Opn1mw*, and *Arr3*.

Developmental profile of the effects of RORα mutation

Next, we examined cone gene expression in the wild-type and *staggerer* retinas during postnatal development by quantitative RT-PCR (Fig. 4). *Opn1sw*, *Opn1mw*, *Arr3*, and *Thrb2* were selected for analysis, because these genes were shown to be RORα-dependent when examined at P21 (Fig. 3). *Opn1sw* transcript levels in the *staggerer* retinas were significantly lower than those in the wild-type retinas at all ages examined except P0 (Student's *t*-test, $p < 0.01$). The effects of RORα mutation on *Opn1mw* expression were significant at P6 (wild-type 0.0064 vs. *staggerer* 0.0015; $p < 0.01$) and the later postnatal ages ($p < 0.01$). Interestingly, fold reduction of *Opn1mw* transcripts in the *staggerer* retina was greatest during the period between P6 and P12 (4.3, 5.0 and 4.0-fold reduction at P6, P9, and P12, respectively), when *Opn1mw* expression is dramatically induced in the wild-type retina (approximately 2000-fold increase from P3 to P12). *Arr3* expression in the *staggerer* retinas was significantly reduced compared with the wild-type retinas at all ages examined except P0 (P3: wild-type 0.021 vs. *staggerer* 0.0069 ($p < 0.05$), P9: wild-type 0.17 vs. *staggerer* 0.14 ($p < 0.05$), $p < 0.01$ at P6, P12, P21 and P35). *Thrb2* was the only cone gene found to be up-regulated in the *staggerer* retina. However, temporal analysis demonstrated that the up-regulation of *Thrb2* was significant only in the mature retina (P21 and P35). Taken together, RORα is required for proper *Opn1sw*, *Opn1mw*, and *Arr3* expression at P3 and later postnatal ages, consistent with the timing of RORα expression in wild-type cones (Fig. 2).

RORα activates the *Opn1sw* promoter in synergy with Crx

Our data described above raise the possibility that *Opn1sw*, *Opn1mw*, and *Arr3* are RORα target genes in the mouse retina. The *Opn1sw* promoter contains two consensus motifs for the ROR family, RORE1 and RORE2 (−305 and −210 relative to the start codon) (Srinivas *et al.* 2006), and thus we employed transient transfection assays to test whether RORα can regulate the transcription of this gene. Co-transfection of RORα together with the *Opn1sw* promoter (−507 to +54)/luciferase reporter constructs led to a dose-dependent induction of luciferase activity in human embryonic kidney cells

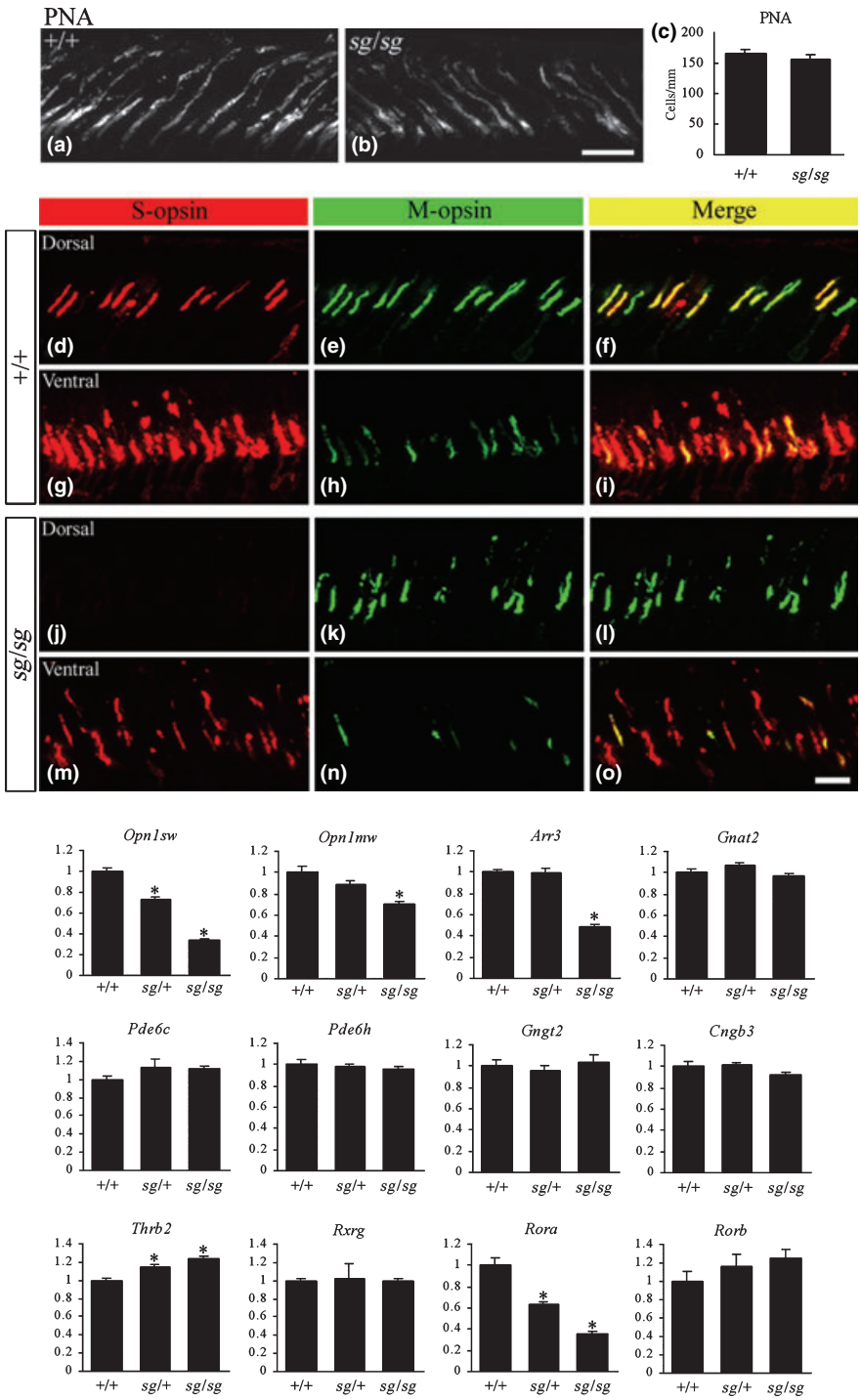

Fig. 3 Upper panel (a–o): Effects of RORα mutation on cone cell density and cone opsin immunoreactivity. Vertical sections (confocal z-stack images at 1 μm interval) of the wild-type (a) and *staggerer* (b) retinas stained with peanut lectin (PNA). Scale bar (a and b) = 20 μm. The density of PNA-labeled cones was calculated in the central part of the retina (c). Bars represent the mean ± SEM (n = 3). Vertical sections (confocal z-stack images at 1 μm interval) of the wild-type (d–i) and *staggerer* (j–o) retinas double-immunolabeled for S- and M-opsins. Immunoreactivity in both dorsal and ventral cones is shown for comparison. Scale bar (d–o) = 10 μm. Lower panel: Quantitative RT-PCR analysis of cone-related genes in the retinas of wild-type (+/+), heterozygous *staggerer* (*sg*/+) and homozygous *staggerer* mutants (*sg*/*sg*) at postnatal day 21. The transcript levels of each gene are expressed relative to wild-type controls (=1) after normalization to *Gapdh* levels. Each bar represents the mean ± SEM (n = 5). Asterisks denote statistically significant expression differences between wild-type and *staggerer* genotypes (*sg*/+ or *sg*/*sg*) (Student's *t*-test; $^*p < 0.01$).

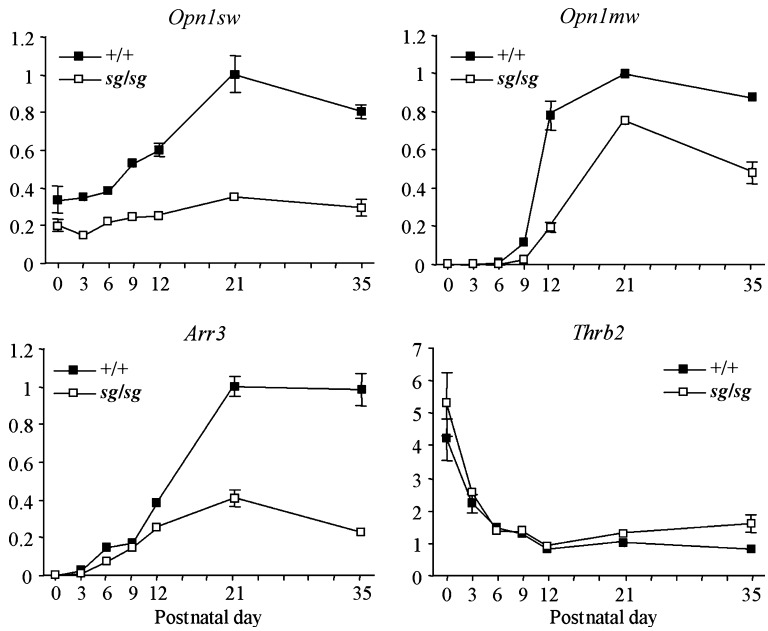

Fig. 4 Quantitative RT-PCR analysis of cone genes in the wild-type (+/+) and *staggerer* (*sg*/*sg*) retinas during postnatal development. The transcript levels of each gene are shown relative to wild-type levels at P21 (=1) after normalization to *Gapdh* levels. Each plot represents the mean ± SEM (n = 3).

(Fig. 5, left). The *Opn1sw* promoter was activated by both RORα (two-fold) and Crx (10-fold), and a synergistic response (44-fold) was observed in the presence of both expression vectors (Fig. 5, right). This synergy was significantly impaired by site-directed mutagenesis of both RORE1 and RORE2, and by deletion to position −200 removing both ROREs. These results suggest that RORα activates *Opn1sw* transcription in cooperation with Crx and that the ROREs in the *Opn1sw* promoter are required for this activation.

RORα binds to the regulatory regions of cone genes *in vivo*

To determine whether *Opn1sw*, *Opn1mw*, and *Arr3* are direct targets of RORα *in vivo*, we next performed ChIP assays using anti-RORα antibody. Primers were designed for the promoter regions of *Opn1sw*, *Opn1mw* and *Arr3*, as well as the locus control region (LCR) of *Opn1mw*. The promoters of *Hbb* (encoding hemoglobin beta) and *Shh* were also examined as negative controls. The first analysis using C57BL/6 mouse retinas failed to detect RORα at the given genomic regions (Fig. 6c), but this may reflect the paucity of cones in the wild-type mouse retina, which represent only 3% of the photoreceptor layer and 2% of the total retina. To overcome this difficulty, we performed ChIP assays on *Nrl*−/− retina, in which all rods are transformed to functional cones (Mears *et al.* 2001; Daniele *et al.* 2005). In the *Nrl*−/− retina, the whole thickness of the outer nuclear layer exhibited intense RORα immunoreactivity, providing

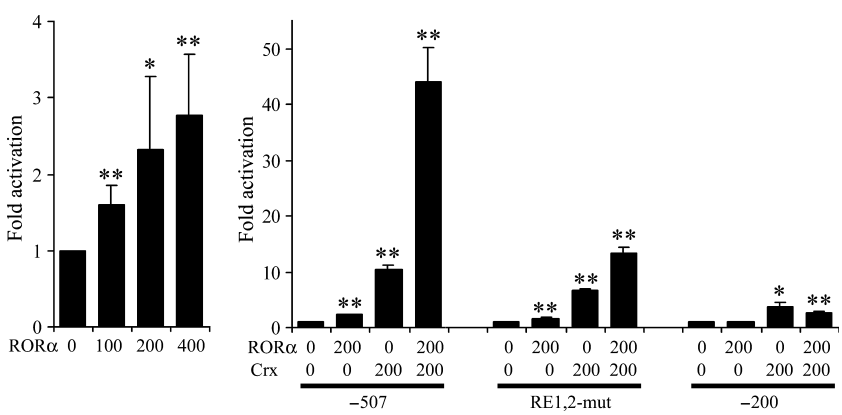

Fig. 5 Co-transfection assays demonstrate the activation of the *Opn1sw* promoter by RORα. The *Opn1sw* promoter (−507 to +54)/luciferase reporter is activated by RORα in a dose-dependent manner (left, 0–400 ng). The presence of both RORα and Crx leads to a synergistic activation (right, −507), which is impaired by site-directed mutations (RE1,2-mu) or deletion to −200 (−200). Data are shown as fold activation over basal activity and represent the mean ± SEM (n = 3). Asterisks denote statistically significant differences compared to basal activity (Student's t-test; *p < 0.05, **p < 0.01).

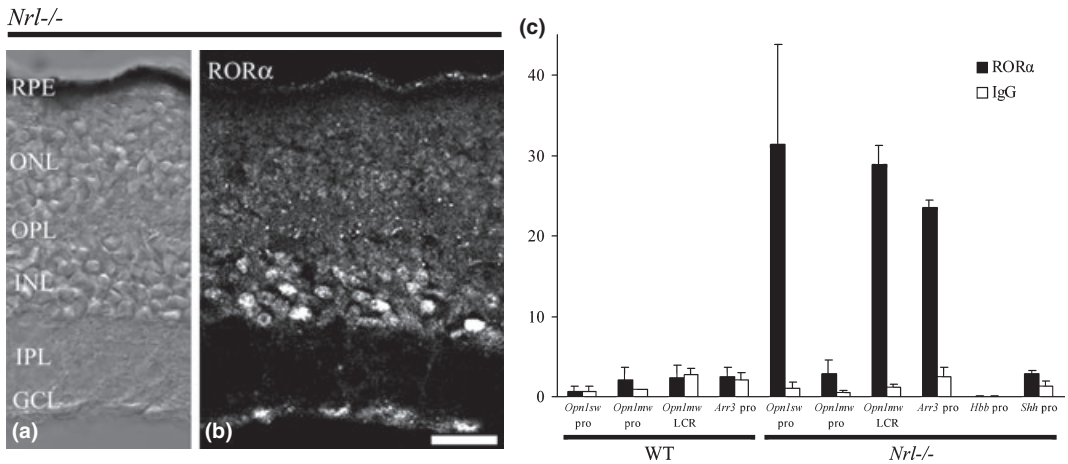

Fig. 6 *In vivo* ChIP assays using cone-rich *Nrl*–/– retina reveal RORα binding to the regulatory regions of cone genes. (a) Differential interference contrast (DIC) image of the *Nrl*–/– retina. GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; RPE: retinal pigment epithelium. (b) RORα immunoreactivity in the *Nrl*–/– retina. The whole thickness of the ONL containing cones is immunoreactive. Scale bar (a and b) = 20 μm. (c) ChIP assays using the *Nrl*–/– retinas demonstrate RORα binding to the *Opn1sw* promoter (*Opn1sw* pro), *Opn1mw* locus control region (*Opn1mw* LCR), and *Arr3* promoter (*Arr3* pro), but not *Opn1mw* promoter (*Opn1mw* pro). DNA fragments bound to immunoprecipitated RORα were quantitated by real-time PCR. Data are expressed relative to the IgG control (*Opn1sw* pro, *Nrl*–/– retinas), which is assigned a value 1. Each bar represents the mean ± SEM (n = 3).

additional evidence that RORα is present in cones (Fig. 6a and b). Strikingly, ChIP assays using the cone-enriched *Nrl*–/– retina detected significant levels of RORα at the *Opn1sw* promoter, LCR of *Opn1mw*, and *Arr3* promoter, but not *Opn1mw* promoter (Fig. 6c). RORα was absent from the *Hbb* and *Shh* promoters which, together with the negligible signal obtained with IgG at all sites, proved specificity. These results strongly suggest that RORα binds to the regulatory regions of *Opn1sw*, *Opn1mw*, and *Arr3* and thus that the down-regulation of these genes in the *staggerer* mouse is a direct result of its absence at these cone gene regulatory elements.

Discussion

RORα as a novel regulator of cone genes

This study is the first, to our knowledge, to address the role of RORα in the retina, and we herein demonstrate that RORα is involved in cone photoreceptor development by regulating a subset of cone genes. This is supported by our findings that (i) RORα is expressed in cones, (ii) the expression of S-opsin, M-opsin and cone arrestin is significantly impaired in the absence of RORα, (iii) RORα mutation affects cone gene expression from P3 onward, temporally corresponding with the timing of RORα expression in wild-type cones, (iv) RORα activates the S-opsin gene promoter in cooperation with Crx *in vitro*, and (v) RORα binds to the regulatory regions of the S-opsin, M-opsin and cone arrestin genes *in vivo*. While RORβ and TRβ2 selectively activate S- and M-opsins, respectively (Ng *et al.* 2001; Srinivas *et al.* 2006), RORα is required to activate both cone opsins, as well as cone arrestin. Thus, in contrast to RORβ and TRβ2, which play key roles in the determination of opsin identity in cones, RORα may be involved in the terminal differentiation of cones by promoting expression of specific genes in all cones. Although RORα targets several core components of the cone phototransduction pathway, it is probably not required for the survival of cones, because no apparent degeneration or loss of cones was observed in the RORα mutant retina during the first 5 weeks of postnatal life. The longer term effects of mutation could not be evaluated due to increased lethality of mutant animals.

Regulation of S-opsin expression

Srinivas *et al.* (2006) have recently reported that RORβ transactivates mouse *Opn1sw* by binding to two ROR response elements (RORE) located in the promoter region. Although RORβ occupies the RORE during the late embryonic period, these authors suggested the presence of other factors that bind these RORE at later developmental stages. RORE are composed of a half-site core motif PuGGTCA preceded by a short AT-rich sequence, and RORα as well as RORβ activates transcription by binding to these elements (Giguère *et al.* 1994). Indeed, our reporter assays demonstrated that these elements are required for the promoter activation by RORα, and ChIP assays using the pure-cone retinas of the *Nrl*–/– mice detected RORα at this promoter region *in vivo*, suggesting that RORα may also transactivate *Opn1sw* by binding to the RORE in this region. The promoter activity was only modestly activated by

RORα alone but was strikingly enhanced by cooperation with Crx. Interestingly, a similar synergistic response has been reported between Crx and RORβ, and such cooperation between homeodomain factors and orphan nuclear receptors has been proposed to enhance their target gene specificity (Srinivas *et al.* 2006). How RORα and RORβ, which seem to share the same binding sites in the *Opn1sw* promoter, cooperate to regulate transcription is a matter of great interest and warrants further investigation.

Regulation of M-opsin expression

Our quantitative RT-PCR analysis has demonstrated a dramatic activation of M-opsin expression in the wild-type mouse retina from P3 to P12 (Fig. 4). Although M-opsin expression requires TRβ2 and its ligand thyroid hormone (Ng *et al.* 2001; Roberts *et al.* 2006), the mechanism of M-opsin activation by TRβ2 remains poorly understood. Mouse retinal explants are capable of expressing M-opsin only when harvested from P3 or older pups, indicating that the commitment of cones to an M-opsin identity occurs around P3 (Söderpalm *et al.* 1994; Wikler *et al.* 1996). TRβ2 transcript levels peak during the embryonic period and are down-regulated after birth (Ng *et al.* 2001; Applebury *et al.* 2007), showing no apparent temporal correlation with M-opsin induction. However, a detailed immunohistochemical analysis showed that TRβ2 protein is transiently down-regulated between E19 and P2, followed by re-expression in cones by P5 (Roberts *et al.* 2005). This re-expression of TRβ2 temporally coincides with the commitment of cones to M-opsin expression. Interestingly, our data show that RORα is expressed in cones from P3, again coinciding with the timing of the commitment to M-opsin expression. These findings lead to a hypothesis that the up-regulation of TRβ2 and RORα around P3 commits postnatal cones to express M-opsin, resulting in the subsequent dramatic activation of M-opsin (Fig. 7).

Although the lack of RORα results in four to five-fold reductions in M-opsin transcript levels at P6-P12, a much smaller reduction (1.3-fold) was observed at P21. This suggests that the reduction in M-opsin expression may be partially compensated by P21. TRβ2 is most likely involved in this process, because TRβ2 transcripts become significantly up-regulated in the RORα mutant retina by P21, possibly by a feedback mechanism triggered by the impaired activation of M-opsin.

Our ChIP assays using the *Nrl*−/− retina have demonstrated that RORα binds to the LCR of *Opn1mw*, but not to the promoter region. The LCR, located between 3.1 and 3.7 kb upstream of the human L/M-opsin gene array, is an enhancer region that plays an essential role in both L and M-opsin transcription (Wang *et al.* 1992; Smallwood *et al.* 2002). In mouse, transcription factors known to regulate photoreceptor genes, such as Crx and Otx2, have been shown to bind this region *in vivo* to regulate M-opsin expression (Peng and Chen 2005; Peng *et al.* 2005). The present evidence that RORα regulates M-opsin transcription through a direct interaction with the LCR further supports the notion that the LCR is a critical regulatory region for M-opsin expression.

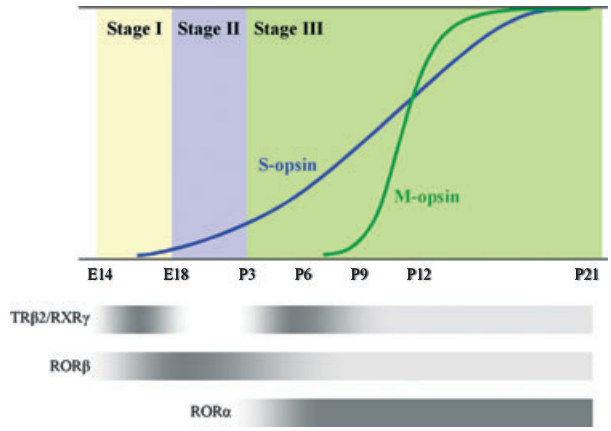

Fig. 7 Three stages of cone development defined by the expression of nuclear receptors regulating the cone opsin genes. In stage I, TRβ2 and its heterodimeric partner RXRγ repress S-opsin expression. In stage II, transient down-regulation of TRβ2 and RXRγ and up-regulation of RORβ lead to S-opsin induction. In stage III, the re-expression of TRβ2 and up-regulation of RORα cooperatively induce M-opsin expression. RORα also acts to amplify S-opsin expression.

The present study is the first to show the dorsal-ventral difference in TRβ2 expression in the retina. Because previous immunohistochemical and *in situ* hybridization studies have failed to identify a graded pattern of TRβ2 expression (Ng *et al.* 2001; Roberts *et al.* 2005, 2006), establishment of the M-opsin gradient has been attributed to a gradient in TRβ2 ligand, thyroid hormone. However, our observation that TRβ2 expression is higher in the dorsal retina raises the possibility that not only the thyroid hormone gradient, but also the gradient of TRβ2 expression may contribute to shaping the M-opsin gradient. Although we observed higher TRβ2 expression in the dorsal retina as early as P6, M-opsin expression was not graded at this stage. This implies that the TRβ2 gradient alone may not be sufficient to form an M-opsin gradient at least at this stage.

Regulation of cone arrestin expression

Despite the recent progress in our understanding of the transcriptional regulation of cone opsin genes, regulatory factors for the expression of other cone genes are largely unknown. Cone arrestin plays an important role in cone phototransduction by binding to light-activated, phosphorylated cone opsins (Zhu *et al.* 2003). Our data provide the first evidence that RORα regulates cone arrestin expression through a direct interaction with the cone arrestin promoter. The levels of cone arrestin transcripts are extremely low at birth, but increase during the postnatal

period to reach adult levels by P21. This postnatal induction of cone arrestin is significantly impaired by the RORα mutation, indicating that RORα is required for the maximal expression of cone arrestin. However, a gradual increase in cone arrestin transcripts was still observed in the absence of RORα, suggesting that other factor or factors may be involved to induce cone arrestin expression in cooperation with RORα. Indeed, previous studies using human retinoblastoma cell lines showed that cone arrestin expression is up-regulated by retinoic acid or thyroid hormone treatment, suggesting the possible involvement of nuclear receptors, such as RXRγ and TRβ2, in the transcriptional control of cone arrestin gene (Li *et al.* 2002; Liu *et al.* 2007).

Developmental sequence of cone opsin regulation

On the basis of our findings and those previously reported, we propose a model in which cone development is divided into at least three stages defined by the expression of nuclear receptors regulating the cone opsin genes (Fig. 7). Cones at stage I are characterized by the expression of TRβ2 and RXRγ and repression of cone opsin genes. Both TRβ2 and RXRγ are expressed in newly generated cones from E14.5 and act cooperatively to repress S-opsin expression (Ng *et al.* 2001; Roberts *et al.* 2005). The factor that represses M-opsin until the appropriate developmental stage has not been discovered. At stage II, both TRβ2 and RXRγ are transiently down-regulated, leading to the de-repression of S-opsin beginning around E18 (Roberts *et al.* 2005). RORβ probably acts to induce S-opsin at this stage although the temporal expression pattern of RORβ in cones remains to be determined. M-opsin expression is still repressed, and thus cones in stage II express only S-opsin. Stage III is initiated by the re-expression of TRβ2 and up-regulation of RORα. TRβ2 and RORα now cooperate to induce M-opsin. RORα also acts to amplify S-opsin expression. The role of RORβ in S-opsin induction at this stage is unclear as RORβ in photoreceptors is down-regulated during the postnatal period (Chow *et al.* 1998; Srinivas *et al.* 2006).

Unlike TRβ2 and RORβ, which are significantly down-regulated during postnatal development, RORα expression persists in mature cones, implying that RORα may be involved not only in the developmental induction but also the maintenance of cone opsin genes. RORα has been implicated in the circadian clock function within the suprachiasmatic nucleus (Sato *et al.* 2004; Akashi and Takumi 2005). It has been reported that RORα expression in the retina exhibits a circadian oscillation (Kamphuis *et al.* 2005; Tosini *et al.* 2007) and that the transcription of S-opsin in the mouse retina is also under circadian control (von Schantz *et al.* 1999). It will be interesting to test the hypothesis that RORα may control the circadian expression of its target genes, including cone opsins, in the mature retina.

Acknowledgments

The authors thank Douglas Forrest for plasmids and Keqin Yan for technical assistance. This work was partly supported by Grant-in-Aid for Scientific Research, No. 17500239 (to H.S.) and No. 18591936 (to H.F.), from Japan Society for the Promotion of Science.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Quantitative RT-PCR analysis of RORα and other cone-related gene expression in the dorsal and ventral halves of the wild-type retinas during development.

Table S1 PCR primers used in RT-PCR or ChIP assays.

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