The Developmental Progression of Eight Opsin Spectral Signals Recorded from the Zebrafish Retinal Cone Layer Is Altered by the Timing and Cell Type Expression of Thyroxin Receptor β2 (trβ2) Gain-Of-Function Transgenes

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Abstract
Zebrafish retinal cone signals shift in spectral shape through larval, juvenile, and adult development as expression patterns of eight cone-opsin genes change. An algorithm extracting signal amplitudes for the component cone spectral types is developed and tested on two thyroxin receptor β2 (trβ2) gain-of-function lines crx:mYFP-2A-trβ2 and gnat2:mYFP-2A-trβ2, allowing correlation between opsin signaling and opsin immunoreactivity in lines with different developmental timing and cell-type expression of this red-opsin-promoting transgene. Both adult transgenics became complete, or nearly complete, “red-cone dichromats,” with disproportionately large long-wavelength-sensitive (LWS)1 opsin amplitudes as compared with controls, where LWS1 and LWS2 amplitudes were about equal, and significant signals from SWS1, SWS2, and Rh2 opsins were detected. But in transgenic larvae and juveniles of both lines it was LWS2 amplitudes that increased, with LWS1 cone signals rarely encountered. In gnat2:mYFP-2A-trβ2 embryos at 5 d postfertilization (dpf), red-opsin immunoreactive cone density doubled, but red-opsin amplitudes (LWS2) increased <10%, and green-opsin, blue-opsin, and UV-opsin signals were unchanged, despite co-expressed red opsins, and the finding that an sws1 UV-opsin reporter gene was shut down by the gnat2:mYFP-2A-trβ2 transgene. By contrast both LWS2 red-cone amplitudes and the density of red-cone immunoreactivity more than doubled in 5-dpf crx:mYFP-2A-trβ2 embryos, while UV-cone amplitudes were reduced 90%. Embryonic cones with trβ2 gain-of-function transgenes were morphologically distinct from control red, blue or UV cones, with wider inner segments and shorter axons than red cones,

Significance Statement
As different combinations of eight cone-opsin mRNAs are successively expressed during zebrafish development and maturation, the composite cone-ERG spectral signal shifts. Amplitudes of each of the eight resulting cone signals are inferred computationally from the composite signal, both in controls and in two thyroxin receptor β2 (trβ2) gain-of-function transgenics, crx:mYFP-2A-trβ2 and gnat2:mYFP-2A-trβ2, trβ2 being a transcription factor required for expression of the red-cone opsins long-wavelength-sensitive (LWS1) and LWS2. Adult transgenics become red cone dichromats with excess LWS1 amplitudes, but larvae and juveniles evoke excess LWS2 amplitudes. Controls retain five to six cone signals of changing composition throughout development. The progression of transgene-induced amplitude alterations is slower in gnat2:mYFP-2A-trβ2, with supernormal red-opsin antigenicity not immediately correlating with red-cone signaling.
suggesting cone spectral specification, opsin immunoreactivity and shape are influenced by the abundance and developmental timing of trβ2 expression.

Key words: developmental biology; ERG; opsin absorbance; opsin antibodies; red cones; spectral sensitivity

Introduction

Spectral patterns in the zebrafish cone ERG shift with development (Saszik et al., 1999; Nelson et al., 2019). These shifts are determined in part by factors regulating opsin expression. We develop an algorithm to extract the electrical contributions of each of the eight zebrafish cone opsins from massed ERG cone signals and use this tool to examine alterations in cone-signal development brought about by perturbations in expression of the regulatory factor thyroid hormone receptor β2 (trβ2). The process examines the correlation of opsin expression with opsin signals during perturbations in the levels of a key transcription factor for cone development. Thyroid hormone receptor β2, a splice variant of the thrb gene, is selectively expressed in vertebrate retinal cones (Ng et al., 2001). When deleted, cones expressing opsins in the long-wave-length-sensitive (LWS) subfamily of the opsin molecular phylogenetic tree (Terakita, 2005) fail to be produced. This includes the MWS cones of mouse (Ng et al., 2001; Roberts et al., 2006), human MWS and LWS cone function (Weiss et al., 2012), and both LWS1 and LWS2 red-cone signals of zebrafish (Deaveu et al., 2020). The LWS cone subfamily senses the longest wavelengths a species detects, with opsin spectral peaks ranging from 511 nm in mouse (Jacobs et al., 1991) to 625 nm in goldfish (Marks, 1965).

Two gain-of-function transgenics, cxr:mYFP-2A-trβ2 (cxr:trβ2) and gnat2:mYFP-2A-trβ2;mpv17→+/− (gnat2:trβ2; Suzuki et al., 2013), are used to perturb both the developmental timing and the cellular locus of trβ2 expression. The first (cxr:trβ2) is active by day 2 in larval development, in embryonic retinal progenitor cells (Shen and Raymond, 2004; Suzuki et al., 2013). The second (gnat2:trβ2), trβ2 is active only in differentiated cone cells. The gnat2 gene is the promoter of the cone transducin α subunit (Brockerhoff et al., 2003; Kennedy et al., 2007). In the embryonic larvae of each transgenic Suzuki et al. (2013) found an excess of red-opsin immunoreactive cones. In cxr:trβ2 the larval densities of green-opsin, blue-opsin, and ultraviolet (UV) opsin immunoreactive cones were reduced, whereas in gnat2:trβ2 excess red-opsin immunoreactivity found a home in cones expressing other opsins, forming mixed-opsin cones. These transgenic mixed-opsin cones were thought to model rodent cones that natively express both UV and MWS opsins (Applebury et al., 2000). We here confirm the immunoreactive patterns of these transgenics raised under the same conditions used for electrophysiological recordings of spectral signals to determine whether altered opsin patterns have a parallel in cone spectral signals. The physiological consequences of altered trβ2 expression are unknown, and might result in either retinal disease, or a spectrally unique visual system.

Although trβ2 might be thought of as a binary ON/OFF switch for LWS-cone development, it clearly has other actions, and the activity level and cell-type expression of trβ2 may influence the action. Dilution of trβ2 in adult zebrafish trβ2 +/− animals made red cones less dense and depressed long-wavelength sensitivity (Deaveu et al., 2020). In embryonic and juvenile zebrafish, while unbound trβ2 sufficed for red-cone development, binding of exogenous thyroid hormone (TH) shifted larval cones expressing LWS2 opsin to expression of LWS1 opsin, and athyroidism switched them back (Mackin et al., 2019). TH depressed the expression of both SWS1- (UV-opsin) and SWS2- (blue-opsin) message (Mackin et al., 2019). TH and trβ2 appear further to be involved in the preferential expression of several of the tandem quadruplicate Rh2 cone opsins in zebrafish (Mackin et al., 2019). It appears likely that TH and trβ2 are upstream regulators in the specification of all cone types, though only for red cone neuronal specification is trβ2 essential (Deaveu et al., 2020). Therefore, the developmental impact on electrical signaling in all eight zebrafish cone types is investigated.

Materials and Methods

Zebrafish (Danio rerio) husbandry and experimental design

The transgenic gain-of-function and reporter-line zebrafish cxr:mYFP-2A-trβ2, gnat2:mYFP-2A-trβ2;mpv17→+/−, trβ2:tdTomato;sws1:GFP, sws1:nlsBmCherry and sws2: GFP (Takechi et al., 2003, 2008; Suzuki et al., 2013) were generously provided by the Rachel Wong Lab (University of Washington). In gain-of-function studies, larvae were
obtained by outcrosses such that transgenics were heterozygotes and controls were siblings (Fig. 1A), studied at random on the same developmental days postfertilization (dpf). The transgenic gnat:mYFP-2A-trβ2 was maintained on a mppv17−/−/−/− (roy orbison) background (D’Agati et al., 2017; Fig. 1C). There are several studied mutations of mppv17 all with loss of irridophores (Bian et al., 2021). Here, we use the naturally occurring 19-base-pair intronic deletion of the roy orbison mutant (D’Agati et al., 2017). The lack of reflective iridophores in the iris of this mutant facilitated phenotyping of pupil fluorescence. Pineal fluorescence was also a gnat2:mYFP-2A-trβ2;mpv17+−−− gain-of-function phenotype is studied in a roy orbison (mpv17−−−−) background strain. The darkly pigmented, nonreflective iris of this control strain aids in visualizing the dim transgenic fluorescence of the pupil (p). The yolk sac (y) is autofluorescent. D–F, Live confocal imaging of retinas in 6-dpf larvae. D, WT red (red) and UV (green) cone morphology visualized with trβ2:tdTomato and wss1:GFP fluorescent reporter transgenes. E, The mYFP construct in the gnat2:mYFP-2A-trβ2 transgene causes cones (C) and bipolar cells (BC) to fluorescence. F, The mYFP construct in the transgene gnat2:mYFP-2A-trβ2 marks only cone cells.

Figure 1. Reporter fluorescence of zebrafish larvae. A, Larvae from outcrosses of crx-mYFP-2A-trβ2 heterozygotes are either heterozygous or wild-type controls (WT). The heterozygotes are recognized by both pupil (p) and heart (h) fluorescence. The yolk sac (y) is autofluorescent. B, The cornea of a 5-d transgenic eye isolated from a crx:mYFP-2A-trβ2 larva is penetrated with a patch electrode for ERG recordings. The eye is <0.5-mm diameter. C, The gnat2:mYFP-2A-trβ2;mpv17−−−−−−−− gain-of-function phenotype is studied on a roy orbison (mpv17−−−−−−−−) background strain. The darkly pigmented, nonreflective iris of this control strain aids in visualizing the dim transgenic fluorescence of the pupil (p). The yolk sac (y) is autofluorescent. D–F, Live confocal imaging of retinas in 6-dpf larvae. D, WT red (red) and UV (green) cone morphology visualized with trβ2:tdTomato and wss1:GFP fluorescent reporter transgenes. E, The mYFP construct in the gnat2:mYFP-2A-trβ2 transgene causes cones (C) and bipolar cells (BC) to fluorescence. F, The mYFP construct in the transgene gnat2:mYFP-2A-trβ2 marks only cone cells.
nitrocellulose filter paper (Millipore, 0.45 mm pore, catalog #HABP02500, MilliporeSigma), and decapitated (without anesthetic) using a long (37 mm) insect pin (Carolina Biological Supply). Using a binocular microscope (M212-5; Leica Microsystems), a longitudinal, dorsal-ventral cut through the head propitosed and isolated larval eyes, which were positioned facing up, taking care not to touch the eye directly. In the recording chamber, larval eyes mounted on the nitrocellulose chip were perfused at 0.1 ml/min with Minimal Essential Medium (MEM; ThermoFisher Scientific catalog #11090-099, equilibrated with 95% O2 and 5% CO2) using a syringe pump (New Era 500L; Braintree Scientific) and a 28-gauge microfil syringe needle as applicator (MF28G67; World Precision Instruments). The chamber was an inverted lid for a 35-mm culture dish (ThermoFisher Scientific), with a disk of 41-μm-mesh nylon filter (Millipore) covering the bottom to wick away perfusate. The perfusion applicator was positioned on the nylon mesh; 20 mM L-aspartate (Sigma-Aldrich), added to the MEM perfusate, blocked postsynaptic, glutamatergic, photoreceptor mechanisms leaving only photoreceptor signals (Silliman et al., 1969). Aspartate medium blocks cone synaptic transmission through saturation of glutamatergic receptors of three types: ionotropic cation channels, metabotropic-mediated cation channels, and glutamate-transporter-mediated anionic channels (Grant and Dowling, 1995, 1996; Connaughton and Nelson, 2000).

Patch electrodes (3-μm tip) were inserted trans-coneally (Fig. 1B) to record the isolated cone ERG signals (cone PIII; Wong et al., 2004). Adult eyecups were prepared from eyes removed from fish decapitated with a fresh, single-edged razor. Corneas and lenses were removed from the isolated eyes mounted upright on a 5- to 10-mm square of black nitrocellulose paper, and the preparation was placed in a recording chamber (as above). The perfusion applicator was placed directly above the retina, oxygenating the vitreal surface with MEM containing 10 mM L-aspartate at 0.3 ml/min. Microelectrodes broken to 300 μm tip diameter placed in the eyecup recorded cone-PIII ERG signals (cone PIII; Wong et al., 2004).

Live imaging of larval retinas

Transgenic larvae were raised at 28°C in 300 μM phenylthiourea (PTU; Sigma-Aldrich) to prevent melanin formation in the pigment epithelium and allow imaging of the retina in vivo. At 6 dpf, each larva was anesthetized with Tricaine Methanesulfonate (MS222, Sigma-Aldrich) and mounted individually in 1.5% agarose (Sigma-Aldrich type VII-A) on an eight-channel slide with the right eye against the cover glass floor of the chamber. Eyes were imaged in z-stacks on Zeiss 880 confocal microscope at either 25× or 40× magnification at 1024 × 1024-pixel resolution. Cone morphometrics were measured for 5 or 6 fish in each transgenic line in Fiji (ImageJ) on the optical slice that offered the longest stretch of resolved cones. These measurements were analyzed for differences using a one-way ANOVA and Tukey’s post hoc test. Fluorescent reporters identified the morphology of wild-type (WT) red and UV cones in eyes from trβ2:tdTomato;sws1:GFP larvae (Fig. 1D) and of transgenic gnat:mYFP-2A-trβ2 cones (Fig. 1F). In crx: mYFP-2A-trβ2, reporter fluorescence appeared in both cones and bipolar cells (Fig. 1E) as previously seen in antibody staining for Crx in zebrafish (Shen and Raymond, 2004).

Immunohistochemistry

Larvae were euthanized by icing, and then fixed in 4% paraformaldehyde (PFA) in 0.1 M PBS, pH 7.4, for 25 min at room temperature. Retinas were dissected in PBS using a pair of 30-gauge syringe needles, blocked in a solution containing 5% normal donkey serum and phosphate-buffered Triton X-100 0.5% (PBT) for 1–2 h and then incubated with primary antibodies. Triton X-100 was added to enhance antibody penetration. Primary antibodies included anti-ultraviolet opsin (rabbit, 1:500, kindly provided by David Hyde), anti-ultraviolet opsin (rabbit, 1:5000, kindly provided by Jeremy Nathans), anti-blue opsin (rabbit, 1:5000, kindly provided by Jeremy Nathans) and anti-rod opsin (1D4, mouse, 1:100, Santa Cruz: sc-57 432, RRID:AB_785511), the later raised against bovine rhodopsin but recognizing red-cone outer segments in zebrafish (Yin et al., 2012). These were diluted into the blocking solution. After incubating for 4 d at 4°C, samples were washed three times, 15 min each, in PBT and incubated for 1 d with secondary antibodies (DyLight 649 donkey anti-mouse, and Alexa 594 donkey anti-rabbit, RRID: AB_2340621, 1:500 each, Jackson ImmunoResearch) diluted in blocking solution. After three, 15-min washes (PBT), immunostained retinas were mounted in 0.5% agarose and coverslipped with Vectashield mounting medium (Vector, RRID: AB_2336789). Confocal image stacks were acquired on a Leica SP8 microscope using a 1.0 NA 63× oil-immersion objective lens. Images were typically acquired with an XY resolution of 0.077 μm per pixel and 0.25-μm-thick Z slices (Yoshimatsu et al., 2014).

Spectral stimulus protocol

Larval eyes and adult eyecups were stimulated with nine wavelengths ranging from 330 to 650 nm (20-nm half-width interference filters, 40-nm increments, Chroma Technology). Seven intensities were presented at each wavelength (UV compliant neutral density filters, 0.5 log unit increments covering 3 log units, Andover Corporation). A calibrated photodiode (Newport Corporation) was used to determine stimulus irradiance in quanta units. This was placed in the plane of the cornea for larval eyes or the plane of the retina for adult eyecups. All spectral model calculations are based on absolute, wavelength-specific photodiode calibrations of quanta delivered to the eye. The light source was a 150-W OFR Xenon arc with two optical channels gated by Uniblitz shutters (Vincent Associates). The stimulus channel passed through three Sutter filter wheels, through a UV-visible compliant liquid light guide (Sutter Instruments), through the epifluorescence port of the BX51WI upright microscope (Olympus–Life Science Solutions), and through either a 10× UPlanFLN/0.3 microscope objective (larvae) or a 4× UPlanSApo/0.16 objective (adults). The second optical channel passed through hand-
inserted filters and an infrared (IR) compliant liquid light guide (Newport Corporation) providing infrared (IR) side illumination for visualization and “dark” backgrounds, or through a 627-nm interference filter for red adapting backgrounds.

The spectral protocol was a fixed sequence of 280- and 300-ms monochromatic light flashes of different irradiances and wavelengths delivered by computer using in-house software. Among these stimuli were 64 unique irradiance-wavelength combinations and six replicates delivered 10 min apart as an amplitude-stability check during the 20 min protocol. The 280 stimuli created a set of 70, 2.5 and 6 s, with the longer intervals separating the brighter stimuli. The maximal irradiances in log(quantum·μm⁻²·s⁻¹) in the stimulus protocol were 7.2 (650 nm), 6.3 (610 nm), 6.4 (570 nm), 6.3 (530 nm), 6.4 (490 nm), 6.1 (450 nm), 5.7 (410 nm), 5.7 (370 nm), and 5.2 (330 nm).

To record a spectral dataset, the stimulating objective was positioned over the eye with a translation stage (MT-800; Sutter Instrument). Microelectrodes were inserted into eyes or eyecups with a micro-positioner (Sutter Instrument, MPC-385). ERG signals from the microelectrode were amplified by 10,000 (World Precision Instruments, DAM80, 0.1–Hz to 1.0-kHz bandpass), and digitized (2000 Hz) with an Axon instruments 1440A (Molecular Devices) using Clampex 10 software. Setting the Clampex averaging feature to retain all amplitude data points for each spectral dataset. Datasets associated in Origin with the wavelengths and irradiances of the stimuli, providing 70 wavelength, irradiance, and amplitude data points for each spectral dataset. Datasets with unstable response amplitudes over the collection period were rejected. For each genetic variant, multiple datasets from ~10 eyes were normalized to the maximal response of each dataset to form a cumulative dataset, which was fit to a spectral algorithm. The normalization weighted the individual datasets making up the cumulative dataset equally. Combining datasets from many eyes separated the trends in genetic alterations of spectral properties from the variations among individual eyes. Nonlinear fits of models to spectral datasets used the Levenberg–Marquardt iteration algorithm provided by Origin. The algorithm finds the best spectral model and extracts amplitudes of various cone signals together with standard errors of estimate (SEs) from the cumulative cone-PIII response.

The above spectral protocol does not record zebrafish rod signals as the retinas are not dark adapted, saturating stimuli are used, and the interstimulus intervals are short (Bilotta et al., 2001). There are four rods surrounding each UV cone in adult zebrafish (Fadool, 2003) but less than one rod for every four UV cones in 5-dpf larvae (Alvarez-Delfin et al., 2009). Rod b-waves amount to <10% of maximal b-wave amplitude in larval zebrafish and can only be discerned as a slower peaking threshold response after 2 h of dark adaptation (Venkatraman et al., 2020). Slow-peaking (~400 ms) rod b-waves are recorded in dark adapted adults by avoiding suprathreshold stimuli and with interstimulus intervals of 15 s or greater (Bilotta et al., 2001).

**Analysis of ERG signals**

Datasets were imported into Origin analysis software and processed using Origin LabTalk scripts (Origin, various versions; Originlab Corporation). The four replicate-waveforms at each of the 70 wavelength and irradiance combinations were averaged and boxcar filtered (17 ms, one 60-Hz line-frequency cycle). Peak-to-rough amplitudes were extracted during the 850-ms following stimulus onset, an interval including both the hyperpolarizing trough and repolarizing peak of the aspartate-isolated cone-PIII response. There was no b-wave component in this signal (Nelson and Singla, 2009). Each amplitude was associated in Origin with the wavelengths and irradiances of the stimuli, providing 70 wavelength, irradiance, and amplitude data points for each spectral dataset. Datasets with unstable response amplitudes over the collection period were rejected. For each genetic variant, multiple datasets from ~10 eyes were normalized to the maximal response of each dataset to form a cumulative dataset, which was fit to a spectral algorithm. The normalization weighted the individual datasets making up the cumulative dataset equally. Combining datasets from many eyes separated the trends in genetic alterations of spectral properties from the variations among individual eyes. Nonlinear fits of models to spectral datasets used the Levenberg–Marquardt iteration algorithm provided by Origin. The algorithm finds the best spectral model and extracts amplitudes of various cone signals together with standard errors of estimate (SEs) from the cumulative cone-PIII response.

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**Statistical analysis**

We use t tests, F tests, and ANOVAs to compare results from different treatments or measurements. To determine the most appropriate spectral models, F tests on residual variances for different spectral models were employed. For statistical tests, GraphPad Prism software (RRID:SCR_002798), web-based calculators, and statistical functions addressable within Originlab Labtalk software were used.

**Finding the cone combinations best representing spectral datasets**

Eight cone opsins are expressed in zebrafish retina (Chinen et al., 2003). The algorithm to identify the opsin signals generating the ERG spectral shape is based on the axiom that the tiny radial photocurrents of individual cones sum linearly in extracellular space to produce a net extracellular current which, by traveling through extracellular resistivity, generates an ERG photovoltage. We assume individual cone photocurrents relate to irradiance through Hill functions of exponent 1.0 and semi-saturation irradiance σ (Baylor and Fuortes, 1970) and that σ varies with wavelength in inverse proportion to each opsin absorbance. This scheme is represented in Figure 2A (Eq. 1). V is the net summed photovoltage in the cone ERG (cone PIII) which depends on V the stimulus irradiance in quanta and w the wavelength. The Vmi values are nonlinear fit values, the maximal or saturation voltages for each of the i cone types. The semi-saturation irradiance for the i = k, cone is k, as evaluated at the i = k, cone absorbance maximum. The ki values differ among cone types relative to each other. The relative ki values, expressed as log(ki) relative to UV-cone sensitivity, are literature values (Nelson et al., 2019) listed in Figure 2C. The UV-cone value for log(ki) is fit by the algorithm. A(wmax, w) is absorbance as a function of wavelength (w) for the i = k cone, whose wavelength maximum is wmax. The maximum wavelengths are literature values (Nelson et al., 2019). The Dartnall nomogram (Dartnall, 1953) is used as the absorbance function A(wmax, w). This approximation has the convenience of making opsin absorbance across wavelengths a function of a single variable, wmax. The Dartnall nomogram posits that opsin absorbance shape is constant
when plotted on a reciprocal wavelength axis. The template nomogram functions derive from suction electrode recordings of cones in *Danio aequipinnatus* (Palacios et al., 1996), a relative of zebrafish (*Danio rerio*). These templates are represented as 8th order polynomials. We use a single template polynomial (Hughes et al., 1998) for all red, green, and blue cones listed in Figure 2C, but a separate polynomial for UV cones (Palacios et al., 1996). The resulting absorbance shapes appear in Figure 2B. Altogether there are 255 unique combinations of eight cones, each combination is a candidate to best model cone spectral responses in the ERG. B, Spectral shapes of opsin absorbances \([A(wl_{max}, \omega l)]\) are generated from 8th-order template polynomials (Palacios et al., 1996; Hughes et al., 1998) using Dartnall nomogram translations along the wavelength axis to represent opsins of different peak wavelengths (Dartnall, 1953). C, Parameters for each of the \(i\) cone types are numbered in order from short to long wavelengths. SWS, short-wavelength-sensitive opsins; RH2, rhodopsin-like green-cone opsins; LWS, long-wavelength-sensitive opsins.

**Figure 2.** Algorithm for determining the signal strengths of the different cone types contributing to ERG spectral data. A, Equation 1. Aspartate-isolated cone signals from zebrafish eyes are a summation of signals from eight cone types, each distinguished by different maximal amplitudes (\(V_{mi}\)), semi-saturation irradiances (\(k_i\)), and opsin peak absorbances (\(wl_{max}\)). There are 255 unique combinations of eight cones, each combination is a candidate to best model cone spectral responses in the ERG. B, Spectral shapes of opsin absorbances \([A(wl_{max}, \omega l)]\) are generated from 8th-order template polynomials (Palacios et al., 1996; Hughes et al., 1998) using Dartnall nomogram translations along the wavelength axis to represent opsins of different peak wavelengths (Dartnall, 1953). C, Parameters for each of the \(i\) cone types are numbered in order from short to long wavelengths. SWS, short-wavelength-sensitive opsins; RH2, rhodopsin-like green-cone opsins; LWS, long-wavelength-sensitive opsins.
Results

Cone distributions in the larval retinas of wild-type and crx:trβ2 transgenics

Suzuki et al. (2013) developed the crx:mYFP-2A-trβ2 gain-of-function transgenic as a rescue line to restore red-cones during a morpholino blockade of native trβ2 that abolished their formation. In zebrafish the crx gene promoter becomes active at the retinal progenitor stage (~2 dpf; Shen and Raymond, 2004). The gain-of-function crx:trβ2 transgene replaces the missing retinal trβ2, but not in the same cell types or at the same developmental stage. In the experiments of Suzuki et al. (2013), it was nonetheless effective. Red-opsin immunoreactive cones were restored with supernormal density, but there was suppression of green-opsin, blue-opsin, and UV-opsin immunoreactive cones (Suzuki et al., 2013).

In Figure 3A,B, the experiment of Suzuki et al. (2013) is repeated on crx:mYFP-2A-trβ2 larvae without morpholino suppression of native trβ2. In wild-type retinas, immunoreactive mosaics of both UV cones (Fig. 3Aii) and red cones (Fig. 3Aiii) stain with opsin antibodies. Superposition of both mosaics (Fig. 3Aiii) show no opsin overlaps, with each cone type expressing a single opsin. The situation is similar for blue cones, which exist in a mosaic pattern separate from red cones (Fig. 3Aiv,v,v).

Red cones are denser in the retinas of the crx:mYFP-2A-trβ2 larvae (Fig. 3Bii,v) than in WT (Fig. 3Aii,v). Based on Figure 3A,B, the density of red-opsin immunoreactive cones in these 5-dpf crx:trβ2 transgenics (188,000 mm⁻¹) is significantly greater than WT (72,500 mm⁻¹; t(10) = 18.0, p = 6.0 × 10⁻⁹). The density of crx:trβ2 UV opsine immunoreactive cones (7900 mm⁻¹; Fig. 3B) is significantly less than WT (42,000 mm⁻¹; Fig. 3A); t(6) = 7.0, p = 0.00043). One of the three crx:trβ2 UV cones (Fig. 3B) stains for both UV and red opsins (Fig. 3Biii–arrowhead), indicating co-expression of red and UV opsins. The density of crx:trβ2 blue-opsin immunoreactive cones (13,800 mm⁻¹; Fig. 3Biv) is significantly less than WT (30,500 mm⁻¹; Fig. 3Aiv; t(6) = 4.7, p = 0.0033). Of four transgenic blue cones illustrated (Fig. 3Biv), one is immunoreactive for red opsin (Fig. 3Biv–arrowhead), evidence for co-expression of red and blue opsins in a single cone. The alterations in densities of cone types resemble findings obtained in the presence of morpholino blockade of native trβ2 (Suzuki et al., 2013) suggesting the crx:trβ2 transgene alters cone developmental patterns regardless of the activity of the native trβ2 gene. The difference is the mixed opsin cones, which were not earlier described.

Cone spectral signals from wild-type and crx:trβ2 retinas in larvae and adults

The spectral pattern of aspartate-isolated cone signals from larval crx:mYFP-2A-trβ2 eyes parallels the altered cone densities. With short and UV wavelength stimulation (410 and 330 nm; Fig. 3C), an isolated WT eye responds with substantial signals for three of the brightest stimulus irradiances (green, dark yellow or red traces) but at these same wavelengths, signals from a crx:trβ2 eye (Fig. 3D) are evident at only the two brightest irradiances (dark yellow and red traces) and are of lesser amplitude than WT. Maximal amplitudes were evoked at 490 nm in the WT eye (Fig. 3C), while amplitudes in the crx:trβ2 eye plateaued at longer wavelengths (490, 570, and 650 nm; Fig. 3D). The loss of amplitudes at 490 nm (Fig. 3D) is not a rod signal loss, as no change in the time to peak of threshold signals is apparent.

Adult spectral differences in crx:mYFP-2A-trβ2 and WT eyecups were even more pronounced than those in larval eyes. In the WT traces, the maximal amplitude occurred at 490 nm (Fig. 4A), whereas the maximal amplitude from a crx:trβ2 eyecup was seen at 650 nm (Fig. 4B). Amplitudes at 410 and 330 nm (Fig. 4B) were greatly reduced as compared with the WT control (Fig. 4A). At these short wavelengths WT signals were more sensitive than crx:trβ2 signals, with the dimmer stimuli (Fig. 4A,B, gray and blue traces) evoking deflections in WT, but only overlapping baselines in crx:trβ2 eyecups. The slower-peak, mid-spectral threshold signals of rods are not evoked in either WT or crx:trβ2.

Wild-type and crx:trβ2 waveforms in larvae and adults

The crx:mYFP-2A-trβ2 transgene subtly changes the amplitudes and kinetics of aspartate-isolated cone signals (cone PIII). In larvae a greater mean amplitude and greater spread of amplitudes were found (Fig. 4C). The mean of maximal peak-to-trough responses in 6-dpf spectral datasets for 10 WT larval eyes was 89.6 ± 8.7 μV (28 datasets, mean and SE), while the mean of maximal amplitudes for 12 crx:trβ2 eyes was 145.0 ± 18.7 μV (29 datasets). The crx:trβ2 amplitudes were somewhat larger (t(65) = 2.65, p = 0.0103; Fig. 4C). The dispersion of larval amplitudes was also significantly greater for crx:trβ2 (F(4.77,28,27) = 5.6 × 10⁻⁵, p = 0.00011). But on reaching adulthood, crx:trβ2 maximal PIII amplitudes became indistinguishable from WT counterparts (Fig. 4C). In 14 WT eyecups the mean of maximal peak-to-trough amplitudes was 34.4 ± 5.1 μV (20 datasets). In 16 crx:trβ2 eyecups the mean of maximal peak-to-trough amplitudes was 32.4 ± 6.1 μV (21 datasets). The difference was insignificant (t(38) = 0.248, p = 0.806). The variance in maximal amplitudes was similar (F(1.51,20,19) = 0.813, p = 0.375). Lesser amplitudes occur in adult eyecups than intact larval eyes as photocurrents escape around eyecup edges, whereas the larval eye is an electrically sealed system. Neither larval nor adult crx:trβ2 amplitude distributions (Fig. 4C) show evidence of amplitude loss, a characteristic associated with retinal degenerations.

“PIII ON trough” is the time interval from stimulus onset to the minimum in the vitreal negative phase of the cone PIII signal. In larvae, PIII-ON trough latencies for the crx:mYFP-2A-trβ2 waveforms were similar to WT, but in adults the latency to the PIII ON trough was shorter (Fig. 4D). Response peak and trough timings were measured on the mean waveform for all 280 responses in a spectral dataset. In this average response, noise is minimized and interferes least with determination of extrema timing. For larval eyes the latency to the cone PIII trough for 29 crx:trβ2 datasets (12 eyes) was 135 ± 6 ms from stimulus...
onset (mean and SE). The trough time for 28 WT datasets (10 eyes) was $134 \pm 5$ ms. The WT and transgenic trough times were not significantly different ($t_{(55)} = 0.218, p = 0.828$) and the trough-time variances were also similar ($F_{(1.80,28,27)} = 0.935, p = 0.133$; Fig. 4D). In 16 adult cxcr:trβ2 eye cups the mean latency to PIIl ON troughs was $111 \pm 3$ ms (21 datasets). In 14 WT eye cups the PIIl trough latency was $124 \pm 5$ ms (20 datasets). The adult cxcr:trβ2 transgenics were somewhat quicker

Figure 3. Opsin distributions and spectral responses in embryonic wild-type and cxcr:mYFP-2A-trβ2 larval eyes. Ai, UV opsin (SWS1) immunoreactive cones in a WT retina. Aii, Avi, Red opsin (LWS1, LWS2) immunoreactive cones in WT retinas. Aiv, Blue-opsin (SWS2) immunoreactive cones in a WT retina. Al, UV and red opsins are expressed in separate cones in a WT retina. Au, Red and blue opsins are expressed in separate cones in a WT retina. Bi, UV-opsin immunoreactive cones in a cxcr:trβ2 retina. Bii, Bvi, Red-opsin immunoreactive cones in cxcr:trβ2 retinas. Biv, Blue-opsin immunoreactive cones in a cxcr:trβ2 retina. There are fewer UV and blue cones in cxcr:trβ2 retinas than in WT retinas. Bii, One cxcr:trβ2 cone is immunoreactive for both UV and red opsins (arrowhead). Bv, Arrowhead points to a cxcr:trβ2 cone immunoreactive for both red and blue opsins. C, Cone signals from a WT larval eye respond to all stimulus wavelengths with largest amplitudes at 490 nm. D, A larval cxcr:trβ2 retina responds with maximal amplitudes at wavelengths 490, 570, and 650 nm but is less responsive than WT for 330, 410, and 490 nm (arrows), wavelengths that stimulate blue and UV cones. A, B, 5-dpf larvae. C, D, 6-dpf larvae. Perfusion medium contains 20 mM aspartate to isolate photoreceptor signals in the ERG. Five of nine stimulus-protocol wavelengths are illustrated. The stimulus irradiances [units of log(quanta·μm$^{-2}$·s$^{-1}$)] appear in legends to the right of stacked irradiance-response traces.
to peak ($t_{(46)} = 2.133, p = 0.039$). The variances in onset latencies were similar in $crx:tr\beta2$ and WT adults ($F_{(2.11,19,20)} = 0.947, p = 0.106$; Fig. 4D). For WT controls, larval and adult trough latencies did not differ significantly ($t_{(46)} = 1.45, p = 0.155$). For $crx:tr\beta2$ transgenics, trough times for adults were significantly quicker than for larvae ($t_{(46)} = 3.23, p = 0.0022$).

**Figure 4.** Adult cone spectral signals in WT and $crx:mYFP-2A-tr\beta2$ retinas. **A.** In WT eyecups, all wavelengths and most irradiances evoke signals, with a maximal response at 490 nm. **B.** Cone signals from $crx:tr\beta2$ eyecups are maximal at 650 nm, where they are relatively greater than WT (up arrow). Response amplitudes to wavelengths that stimulate blue and UV cones (330, 410, and 330 nm) are relatively less than WT (down arrows). In **A** and **B**, adults are 8–18 months. The perfusion medium contains 10 mM aspartate to isolate photoreceptor signals. The nested responses are irradiance-response series at each wavelength, with irradiances given in legends to the right of the traces in units of log(quantum·μm⁻²·s⁻¹). **C–E.** Properties of ERG cone-PIII waveforms in larval and adult, WT and $crx:tr\beta2$ retinas. **C.** Distributions of maximal trough-to-peak amplitudes in larval and adult spectral datasets. **D.** Cone-signal latency from stimulus onset to the minimum of the ON trough of the cone signal are measured on the mean responses of each 280-stimulus spectral dataset. **E.** Cone-signal latency to the OFF peak in the mean responses of spectral datasets. OFF latencies are measured from stimulus offset. Asterisks (n.s., not significant) are probabilities that WT and $crx:tr\beta2$ distributions differ in larvae or adults (GraphPad Prism convention, statistics given in text).
OFF signal. The larval PIII-OFF-peak latencies for crx:mYFP-2A-trb2 (360 ± 36 ms) did not differ significantly from WT controls (313 ± 17 ms, t(65) = 1.717, p = 0.247), although the variance in crx:trb2 timing was significantly greater (F(4,48,28.27) = 0.999, p = 1.3 × 10⁻⁴). In adults the crx:mYFP-2A-trb2 OFF peak (262 ± 14 ms), like the ON trough, occurred significantly sooner than in WT (329 ± 20 ms, t(33) = 2.728, p = 0.0095). The variances of adult OFF-peak latencies were similar in crx:trb2 and WT adults (F(1.99,19.20) = 0.932, p = 0.135; Fig. 4E). For WT controls, larval and adult PIII OFF peak timing was not significantly different (t(46) = 0.600, p = 0.551). For crx:trb2 transgenics PIII-OFF-peak times for adults were somewhat quicker than for larvae (t(48) = 2.234, p = 0.030). Taken together, the latency distributions (Fig. 4D,E) show little evidence of waveform abnormalities associated with errors in cone phototransduction or retinal disease. The quicker waveforms of crx:trb2 adults may result from different weighting of contributing cone types.

In crx:trb2 larvae, red-cone signals increase but UV-cone and blue-cone signals diminish

To determine the larval cone contributions affected by the crx:mYFP-2A-trb2 transgene, 255 models comprising all combinations of eight cone spectral types, were fit to crx:trb2 and WT cumulative spectral datasets. For the 6-dpf WT and crx:trb2 larvae, the cumulative datasets included 1858 response amplitudes (28 datasets) and 1860 amplitudes (29 datasets), respectively. Optimal models differed (WT, #111; crx:trb2, #202). The residual variance of the WT model (#111) as fit to the crx:trb2 cumulative dataset differed from the residual variance of the crx:trb2 dataset fit to its own optimal model 202 (F(9,16,1821,1825) = 1, p = 0), indicating that the WT model is not an equivalently good representation of crx:trb2 data.

In Figure 5A,B, amplitudes are plotted against irradiance for four of the nine test wavelengths. Continuous curves are calculated from the optimal models for WT and crx:trb2 datasets. These curves are generated from a global model fit to all wavelength data, not just the illustrated data. The adherence of irradiance-response curves to datapoints for individual wavelengths is an index of the ability of the global models to represent the cumulative spectral data. The distribution of points and curves is more compressed along the irradiance axis for crx:trb2 larvae (Fig. 5B) than for WT controls (Fig. 5A). The points and curve at 370 nm (magenta) lie to the left of the 490-nm points and curve (green) for WT spectral signals (Fig. 5A) but to the right of the 490-nm curve and points for crx:trb2 larvae (Fig. 5B), suggesting a loss of sensitivity for 370 nm-sensing cones (UV cones). The log of semi-saturation irradiance calculated for the R1 and R2 cones was 4.56 ± 0.018 for WT larvae and 4.53 ± 0.027 for crx:trb2 (Fig. 5A,B). These semi-saturation irradiances did not significantly differ (t(3681) = 1.858, p = 0.063).

The inferred Vm values (Fig. 2, Eq. 1) relate closely to functional strength for the individual cone types comprising the 6-dpf larval cone-PIII signal. In Figure 5C, the modeled Vm values are compared for WT and crx:mYFP-2A-trb2 larvae. Six Vm, cone signals significantly contributed to WT cone PIII ERGs: Vu358-UV, Vb415-B1, Vb440-B2, Vg460-G1, Vg500-G4, Vr556-R2. Only four cone contributions were identified in crx:trb2 larvae: Vb415-B1, Vg460-G1, Vr556-R2, Vr575-R1. Missing in the transgenic are UV cone signals (Vu358), B2 cone signals (Vb440), and G4 signals (Vg500). But an extra red-cone signal, R1 (Vr575), not detected in WT, became significant in crx:trb2 larvae. Unaffected were the R2 (Vr556) cone signal, the B1 signal (Vb415) and the G1 signal (Vg460). Overall, the modeling algorithm shows that UV-cone amplitudes were significantly reduced, red-cone amplitudes significantly increased, and among four blue and green cones signals, two were significantly reduced and two were not significantly affected.

Modeled spectral sensitivities represent the impact not only of Vm but also k values and include Hill-function response compressions, which flatten cone spectral functions for bright stimulation (Eq. 1; Fig. 2A). This is the fullest model representation, but less interpretable in terms of individual cone contributions. The spectral curves of Figure 5D are modeled response amplitudes for three levels of constant quantal stimulation across the spectrum. The strong UV signal in WT larval eyes (Fig. 5A) appears as an ultraviolet spectral peak (~370 nm) in Figure 5D, regardless of stimulus brightness, despite the larger saturation amplitudes of the R2 (Vr556) cones, which, in WT controls, manifest as a long-wavelength bulge more prominent with greater constant-quantal irradiances, which better stimulate the higher semi-saturation characteristics of R2 physiology. The failure to find UV cone signals in the crx:trb2 larval transgenics precludes an ultraviolet spectral peak at any quantal-irradiance and allows red-cone signals to create spectral peaks (540–550 nm). The residual blue and green signals of crx:trb2 cooperate to create a 444-nm peak at the dimmest level of stimulation (Fig. 5D).

With 255 models tested, there is not always a single best-fitting model for each dataset as judged by residual variance. For the larval WT datasets, model 95, also a six-cone model, was indistinguishable from model 111 (F(0.99995,1851,1851) = 0.491, p = 0.982). This model replaces the low amplitude G4 (Vg500) cone amplitude with a similar amplitude G3 (Vg480) signal. The WT five-cone model 103 was also indistinguishable (F(0.99719,1851,1851) = 0.476, p = 0.952). This model omits the low-amplitude G1 (Vg460) cone signal. For the crx:trb2 datasets, model 201 was indistinguishable from model 202 (F(0.99995,1856,1856) = 0.491, p = 0.9822). This model substitutes a low-amplitude UV-cone signal (Vu358) for the low-amplitude B1-cone signal (Vb415).

Adult crx:trb2 zebrafish are red cone dichromats

Only the red R1 (Vr575) and R2 (Vr556) cone signals made significant contributions to the adult cone-PIII responses of crx:mYFP-2A-trb2 (Fig. 6C). A five-cone WT model including blue, green, and red cones best represented adult controls. These cone signals are Vb415-B1, Vg460-G1, Vg500-G4, Vr556-R2, Vr575-R1. The cumulative datasets included 14 eyecups from WT adults and 16 from crx:trb2 adults (WT, 20 datasets, 1400 responses; crx:trb2, 21 datasets, 1470 responses). When the adult
WT model (#234) is fit to the adult crx:trβ2 dataset, the residual variance is larger than the variance for the optimal crx:trβ2 model (#192; F(1,923,1443,1445) = 1, p = 0) indicating different cone combinations are operating. The crx:trβ2 transgene alters the positioning of adult irradiance-response data points and model curves (Fig. 6A,B). The 370- and 490-nm curves and points (magenta and green) lie to the left side of the 570-nm curve and points (yellow) in WT adults but shift to the right side of the 570-nm curve and points in crx:trβ2 adults, a diminution of UV-wavelength sensitivity. The calculated log of semi-saturation irradiance for long-wavelength-sensitive cones (R1, R2) was 4.49 ± 0.033 for WT adults and 4.69 ± 0.022 for crx:trβ2 adults (Fig. 6A,B), crx:trβ2 being significantly less sensitive (t(2818) = 5.17, p = 2.5 × 10⁻⁵). The modeled amplitudes of cones with significant signals appear in Figure 6C. In the adult WT model, significant blue-cone and green-cone signals (B1, Vb415 and G1, Vg460) are detected, but these signals are not significant in adult crx:trβ2 transgenics, where there are only red-cone signals (R2, Vr556 and R1, Vr575). In crx:trβ2, R1 cones contribute a significantly larger amplitude signal than R2 cones (180%, t(2818) = 3.92, p = 0.000092), whereas in the WT controls, R1 and R2 signal amplitudes were not significantly different.

The modeled spectral curves for the crx:mYFP-2A-trβ2 adult red-cone dichromat (Fig. 6D) show long-wavelength-peak functions at 565 nm at all quantal stimulation levels, while WT spectral peaks occur between 430 and 522 nm, depending on stimulus brightness. Except at wavelengths >570 nm, WT control amplitudes are greater than transgenic, due both to the greater sensitivity of WT cones overall, and to the presence of mid-wavelength and

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**Figure 5.** Spectral models of cumulative data from 6-dpf embryonic crx:mYFP-2A-trβ2 and wild-type eyes. A, WT irradiance-response datapoints, SEs, and optimal model (#111) curves as fit to 1858 spectral response amplitudes combined from 28 normalized datasets taken from 10 eyes (Fig. 1A, Eq. 1 algorithm); 370 nm, n = 23–28; 490 nm, n = 27–28; 570 nm, n = 23–28; 650 nm, n = 23–55. B, crx:trβ2 irradiance-response datapoints and optimal model (#202) curves fit to 1860 spectral points combined from 29 datasets taken from 11 eyes. Individual points: 370 nm, n = 22–28; 490 nm, n = 23–28; 570 nm, n = 27–28; 650 nm, n = 24–55. The crx:trβ2 transgene moves curves and points along the irradiance axis as compared with WT. C, In WT, six cone opsins types (gray bars) were detected by the optimal model. In crx:trβ2, four cone opsin signals were detected (magenta bars). Cone saturation amplitudes [Vm values (Eq. 1; Fig. 2A) ± SE] are fractions of dataset maximal amplitudes. Asterisks denote significance of differences between WT and crx:trβ2 (one-sample or two-sample t tests; n.s., not significant; GraphPad Prism convention). Vu358 (UV, one-sample test): t(1822) = 17.8, p = 1.2 × 10⁻⁶; Vb415 (B1): t(3647) = 0.958, p = 0.338; Vb440 (B2): t(1822) = 4.27, p = 2.0 × 10⁻⁵; Vg460 (G1): t(3647) = 1.87, p = 0.062; Vg500 (G4, one-sample test): t(1822) = 3.33, p = 8.8 × 10⁻⁴; Vr556 (R2): t(3647) = 0.318, p = 0.751; Vr575 (R1): t(1822) = 2.72, p = 0.0067. D, Spectral peaks shift to longer wavelengths for crx:trβ2 (magenta) as compared with WT (black). Spectral curves are the modeled amplitudes that would be evoked by three different irradiances of constant quantal stimulation across the spectrum [3.4, 4.0, and 4.6 log(quanta·μm⁻²·s⁻¹)]. Spectral shapes differ with stimulus brightness. A, B. The log(kR1R2) values are modeled R1-cone and R2-cone semi-saturation irradiances in log(quanta·μm⁻²·s⁻¹). A–D, 20 mm asparagine medium.
short-wavelength (blue and green) cones, absent in the transgenic. As judged by residual variance no other models were indistinguishable from the illustrated ones for either crx:trβ2 or WT adults (F tests for all other models, p, 0.95).

Larval gnat2:trβ2 cone types and spectral signals

The gnat2:mYFP-2A-trβ2;mpv17 transgenic was developed to test whether red cones could be restored to a population of mid and short wavelength cones already differentiated under morpholino blockade of the native trβ2 gene, which prevented red-cone development (Suzuki et al., 2013). The gnat2 locus codes for cone transducin α, a gene product only expressed in differentiated and functional cone cells. It is expressed by four dpf. Suzuki et al. (2013) found the gnat2:trβ2 transgene induced a supra-normal 5-dpf density of red-opsin immunoreactive cones in the absence of native trβ2, but unlike the crx:mYFP-2A-trβ2 rescue, green-opsin (Rh2), blue-opsin (SWS2), and UV-opsin (SWS1) immunoreactive cone densities were normal (Suzuki et al., 2013).

The pattern of embryonic larval and juvenile opsin immunoreactivity in gnat2:trβ2 transgenics is similar to that seen in larvae with morpholino blockade of native trβ2. In the 12-dpf control retina (roy orbison mpv17+/−/−/−, both UV and red cones (Fig. 7 Ai, iii) stain with opsin antibodies. Superposition of red-cone and UV-cone mosaics (Fig. 7 Aii) shows no red-opsin and UV-opsin overlap, with immunoreactivity for each opsin expressed in a separate cone cell. The control larval blue cones also exist in a

Figure 6. Adult crx:mYFP-2A-trβ2 transgenics are red-cone dichromats. A, Wild-type cone-PIII irradiance-response curves and datapoints at four stimulus wavelengths. The 370-, 490-, 570-, and 650-nm amplitudes are means (+SE) from the cumulative dataset (n = 20 except at 650 nm where n = 20 or 40). Curves are generated from the optimal model (#234) fit to a cumulative 1400 responses at seven irradiances for each of nine wavelengths as compiled from 20 spectral datasets acquired in 14 WT adult eyecups.

B, The crx:trβ2 gain-of-function transgene causes irradiance-response points and model curves to bunch together as compared with WT. The model generating the curves (#192) is fit to 1470 cumulative responses compiled from 21 datasets acquired from 18 crx:trβ2 eyecups. The amplitudes (+SE) are means (370, 490, 570, and 650 nm, n = 21 except for 650 nm, n = 21 or 42). Signals from five blue, green, or red cone types were detected in WT adults (gray bars), but signals from only the two red-cone types were detected in crx:trβ2 eyecups (magenta bars). Fit values of cone saturation amplitudes (Vm, Eq. 1; Fig. 2A) are plotted on a dataset-normalized scale (+SE). Except for Vr556 (LWS2) cone types differed significantly in amplitudes between WT and crx:trβ2 (asterisks, GraphPad Prism convention, n.s., not significant). Vb415 (B1, one-sample test): t(1373) = 3.84, p = 1.4 × 10−5; Vg500 (G3, one-sample test): t(1373) = 4.36, p = 1.4 × 10−5; Vg460 (G1, one-sample test): t(1373) = 2.30, p = 0.021; Vr556(R2): t(1373) = 0.782, p = 0.434; Vr575 (R1): t(1373) = 3.91, p = 9.2 × 10−5. C, Signals from five blue, green, or red cone types were detected in WT adults (gray bars), but signals from only the two red-cone types were detected in crx:trβ2 eyecups (magenta bars). Fit values of cone saturation amplitudes (Vm, Eq. 1; Fig. 2A) are plotted on a dataset-normalized scale (+SE). Except for Vr556 (LWS2) cone types differed significantly in amplitudes between WT and crx:trβ2 (asterisks, GraphPad Prism convention, n.s., not significant). Vb415 (B1, one-sample test): t(1373) = 3.84, p = 1.4 × 10−5; Vg500 (G3, one-sample test): t(1373) = 4.36, p = 1.4 × 10−5; Vg460 (G1, one-sample test): t(1373) = 2.30, p = 0.021; Vr556(R2): t(1373) = 0.782, p = 0.434; Vr575 (R1): t(1373) = 3.91, p = 9.2 × 10−5. D, Model spectral curves for adult WT (black) and crx:trβ2 (magenta) eyecups. The crx:trβ2 transgene shifts sensitivity peaks to long wavelengths at all stimulus irradiances. Curves are modeled for constant quantal stimuli at 3.4, 4.0, and 4.6 (logquanta·μm−2·s−1). A, B, The log(kR1R2) values are the irradiance semi-saturation values in log(quanta·μm−2·s−1) for both R1 and R2 cones. A–D, Eight- to 18-month adults, 10 mM aspartate medium.
mosaic pattern separate from red cones (Fig. 7Aiv,v,vii). The gnat2:trβ2 retina shows dense representations of UV-opsin (SWS1) and blue-opsin immunoreactive cones in control (mpv17−/−) retina. Aiv, Blue-opsin (SWS2) immunoreactive cones in control retina. Aiii, Av, Red (LWS1, LWS2) opsin immunoreactive cones in control retina. Aii, UV and red opsins are expressed in separate cones in control retina. A, B, 12 dpf. C, D, Cone signals in mpv17−/− control and gnat2:trβ2 retinas are robust at all wavelengths, with greatest amplitudes at 490 nm and similar spectral patterns. The perfusion medium contains 20 mM aspartate to isolate cone signals. Stimulus irradiances \(\log(\text{quanta} \cdot \mu\text{m}^2 \cdot \text{s}^{-1})\) appear in the legends to the right of irradiance-response trace stacks; 6-dpf larvae.

The gnat2:trβ2 retina shows dense representations of UV-opsin and blue-opsin immunoreactive cones (Fig. 7Bii, iv). Red-opsin immunoreactive cones appear denser in gnat2:trβ2 than in mpv17−/− control retinas (Fig. 7Biii,v). The gnat2:trβ2 red cone density (95,500 mm\(^{-2}\)) was significantly greater than the control strain (51,400 mm\(^{-2}\); \(t_{1,4} = 6.7, p = 0.000011\); Fig. 7Aiii,Avi,Biii,Bvi). The densities of UV-opsin and blue-opsin immunoreactive cones were similar in gnat2:trβ2 and controls. The gnat2:trβ2 UV-cone density (34,252 mm\(^{-2}\)) was not significantly different from mpv17−/− (23,435 mm\(^{-2}\); \(t_{6} = 1.7, p = 0.143\), nor was the gnat2:trβ2 blue-cone density (21,633 mm\(^{-2}\)) different from mpv17−/− (27,041 mm\(^{-2}\); \(t_{6} = 1.6, p = 0.168\). In

**Figure 7.** Juvenile cone distributions and spectral responses in gnat2:myYFP-2A-trβ2;mpv17−/− and mpv17−/− control retinas. A, UV opsin (SWS1) opsin immunoreactive cones in control (mpv17−/−) retina. Aiv, Blue-opsin (SWS2) immunoreactive cones in control retina. Aiii, Av, Red (LWS1, LWS2) opsin immunoreactive cones in control retina. Aii, UV and red opsins are expressed in separate cones in control retina. A, B, 12 dpf. C, D, Cone signals in mpv17−/− control and gnat2:trβ2 retinas are robust at all wavelengths, with greatest amplitudes at 490 nm and similar spectral patterns. The perfusion medium contains 20 mM aspartate to isolate cone signals. Stimulus irradiances \(\log(\text{quanta} \cdot \mu\text{m}^2 \cdot \text{s}^{-1})\) appear in the legends to the right of irradiance-response trace stacks; 6-dpf larvae.
these 12-dpf *gnat2:trβ2* juveniles (Fig. 7B), UV-opsin and blue-opsin immunoreactive cones were frequently immunoreactive for red opsin (arrowheads), with 2/3 of cones being mixed opsin types.

Densities of cone types and mixed opsin patterns were similar earlier in development. In 7-dpf larvae, the density of red-opsin immunoreactive cones in *gnat2:trβ2* was 70,800 mm⁻¹, significantly greater than the *mpv17−−/−* control (52,300 mm⁻¹; *t*(10) = 13.0, *p* = 1.3 × 10⁻⁹). The densities of blue-opsin immunoreactive cones were similar (*gnat2:trβ2*, 22,000 mm⁻¹; *mpv17−−/−*, 24,900 mm⁻¹, *t*(1) = 1.9, *p* = 0.096), but the density of 7-dpf *gnat2:trβ2* UV immunoreactive cones was slightly less (*gnat2:trβ2*, 36,900 mm⁻¹; *mpv17−−/−*, 46,900 mm⁻¹, *t*(3) = 3.4, *p* = 0.010). Mixed blue-red and UV-red opsin expression was noted in ~10% of 7-dpf blue-opsin or UV-opsin immunoreactive cones.

**Larval and adult cone spectral signals from control and *gnat2:trβ2* retinas**

Despite greater red-cone density and numerous mixed opsin cones (Fig. 7B), the spectral patterns of larval, aspartate-isolated cone signals (cone-Pill) from *in vitro* *gnat2:mYFP-2A-trβ2;mpv17−−/−* eyes are similar to the *mpv17−−/−* controls. In the trace recordings of Figure 7C, an *mpv17−−/−* control larval eye produces substantial signals at all wavelengths, with maximal amplitudes evoked by the 490-nm stimulus. A *gnat2:trβ2* larval eye gives a similar amplitude pattern for the test wavelengths and irradiances (Fig. 7D), with maximal amplitudes at 490 nm.

In an adult *mpv17−−/−* control eyecup (Fig. 8A), the spectral pattern of cone-Pill ERG waveforms shows a with a broad range of responsiveness across wavelengths, and the 490-nm stimuli yielding an amplitude peak. An adult *gnat2:mYFP-2A-trβ2;mpv17−−/−* eyecup also shows a broadly responsive spectral pattern (Fig. 8B), but greater amplitudes are evoked at long wavelengths (570 and 650 nm; Fig. 8B, arrows), suggesting that the underlying input signals from red, green, blue, and UV cone types have undergone a late developmental transformation under the influence of *gnat2:trβ2*.

**Similar waveforms from control and *gnat2:trβ2* larvae and adults**

There was little difference in the distribution of amplitudes or waveform kinetics between the *gnat2:mYFP-2A-trβ2;mpv17−−/−* transgenic and the *mpv17−−/−* control strain (Fig. 8C–E). In both larvae and adults, the maximal ERG Pill amplitudes were similar for the *gnat2:trβ2* transgenics and controls. The 6-dpf larval mean of dataset-maximum peak-to-trough responses (23 datasets, 15 eyes) for *mpv17−−/−* controls was 110.5 ± 12.1 μV (mean and SE). For *gnat2:trβ2* larvae, the mean of dataset-maximum peak-to-trough amplitudes was 130.3 ± 16.5 μV (22 datasets, 17 eyes). Maximal amplitudes of the larval *gnat2:trβ2* signals were not significantly different from those of the background strain (*t*(43) = 0.969, *p* = 0.338). The variances in maximal dataset amplitudes were similar in transgenic and control larvae (*F*(1,79,21,22) = 0.908, *p* = 0.185).

Adult *gnat2:mYFP-2A-trβ2;mpv17−−/−* maximal cone Pill amplitudes were not distinguishable from controls (Fig. 8C). In 16 *mpv17−−/−* control adult eyecups the mean of dataset-maximum peak-to-trough amplitudes was 22.5 ± 3.1 μV (30 datasets). In 15 *gnat2:trβ2* eyecups the mean of dataset-maximum peak-to-trough amplitudes was 28.2 ± 7.2 μV (24 datasets). The difference was not significant (*t*(50) = 0.780, *p* = 0.439). The variance in adult transgenic peak amplitudes was greater (*F*(4,38,23,29) = 0.9999, *p* = 0.00025).

Examining kinetics of the larval and adult dataset mean waveforms, the latency to the cone-Pill trough minimum and the variance in its timing differed little between 6-dpf larval *gnat2:trβ2* eyes and the *mpv17−−/−* controls (Fig. 8D). The onset time for the larval cone-Pill trough for *gnat2:trβ2* eyes (29 datasets, 17 eyes) was 152 ± 5 ms from stimulus onset (mean and SE). The cone-Pill trough latency for larval eyes from the *mpv17−−/−* controls (26 datasets, 16 eyes) was 139 ± 4 ms. The trend, though not significant, for larval *gnat2:trβ2* onset kinetics was toward greater delay in the Pill ON trough (*t*(50) = 1.89, *p* = 0.065). Larval *gnat2:trβ2* eyes trended, though not significantly, toward greater variability in trough latencies (*F*(2,9,28,29) = 0.9669, *p* = 0.067).

For adults the mean time to the Pill ON trough was 122 ± 4 ms in *gnat2:trβ2* eyecups (24 datasets, 15 eyes) and 128 ± 4 ms in the *mpv17−−/−* controls (26 datasets, 16 eyes). The differences were not significant (*t*(48) = 1.11, *p* = 0.274). The variances in adult trough latencies for *gnat2:trβ2* and background strain were similar (*F*(1,23,25,29) = 0.689, *p* = 0.623). Adult trough latencies were significantly quicker than larvae for both *mpv17−−/−* controls and *gnat2:trβ2* transgenics (*mpv17−−/−*: *t*(50) = 2.03, *p* = 0.047; *gnat2:trβ2* *t*(51) = 4.39, *p* = 0.000057).

The mean cone-Pill OFF peak timing and variance in larval and adult *gnat2:mYFP-2A-trβ2 mpv17−−/−* did not differ from controls (Fig. 8E). The mean waveforms for the 6-dpf *gnat2:trβ2* larval datasets peaked at 321 ± 22 ms after stimulus offset (29 datasets, 15 eyes). For the larval *mpv17−−/−* controls, the OFF peak occurred at 292 ± 18 ms (26 datasets, 16 eyes). These latencies were not significantly different (*t*(50) = 0.980, *p* = 0.332). The variances in larval OFF peak timing for transgenic and control were similar (*F*(1,83,28,29) = 0.935, *p* = 0.132). For adult datasets, the OFF peak of the *gnat2:trβ2* mean waveforms gave delays of 308 ± 17 ms from stimulus offset (24 datasets, 15 eyes). In the adult *mpv17−−/−* control datasets, the delay was 309 ± 14 ms. The *gnat2:trβ2* adult OFF-peak timing did not differ from controls (*t*(48) = 0.031, *p* = 0.976). The variances in adult OFF peak timings were not different (*F*(1,34,23,25) = 0.763, *p* = 0.473). Adult OFF-peak latencies did not differ significantly from larvae for either *mpv17−−/−* controls or *gnat2:trβ2* transgenics (*mpv17−−/−*: *t*(50) = 0.75, *p* = 0.459; *gnat2:trβ2*: *t*(51) = 0.43, *p* = 0.670). Overall, the *gnat2:trβ2* transgene has little effect on cone-Pill amplitudes or waveforms. There is no electrical evidence of degenerative disease.

In *gnat2:trβ2* larvae the transgene increases R2-cone signals

The larval cone Pill spectral responses of *gnat2:mYFP-2A-trβ2;mpv17−−/−* are similar to the *mpv17−−/−* control
Signal loss in the UV and signal gain at long wavelengths, as seen in crx:mYFP-2A-trβ2 larvae (Fig. 3C,D), are not as evident. Modeling suggests subtle changes. The optimal model for gnat2:trβ2 is #79, and the optimal control model is #77. Applying the control model 77 to the gnat2:trβ2 dataset gives a greater residual variance than model 79 ($F_{1.21,1506,1511} = 0.999, p = 0.00021$), indicating that cone inputs generating the gnat2:trβ2 strain (Fig. 7C,D).
Figure 9. Embryonic spectral properties of cone signals from gnat2:myFP-2A-trβ2;mpv17+/– and mpv17+/– control retinas. A, Control irradiance-response points and optimal model curves. The WT model (#77) was fit to 1945 spectral response amplitudes, combined from 28 datasets taken from 15 eyes. The points are subsets taken from the cumulative data. The 370-, 490-, and 570-nm amplitudes are means (±SE), n = 27 or 28; the 650 nm means, n = 28 or 56. B, The gnat2:trβ2 irradiance-response points and model curves at 370, 490, and 570 nm are more bunched together than in the control. The gnat2:trβ2 model (#79) was fit to 1540 spectral points, combined from 22 datasets taken from 17 eyes. The 370-, 490-, and 570-nm amplitudes are means (±SE), n = 22; 650 nm, n = 22 or 44. C, Four cone opsin types contributed significantly to cone-signals isolated from mpv17+/– controls (gray bars) but five types proved significant in gnat2:trβ2 eyes (magenta bars). Cone saturation amplitudes (Vm, Eq. 1; Fig. 2A) are fractions of maximal dataset amplitudes (±SE). Asterisks represent the significance of amplitude differences between mpv17+/–– control and gnat2:trβ2 (GraphPad Prism convention, n.s., not significant). Vu358 (UV): t(3422) = 5.37, p = 8.7 x 10^{-8}; Vb415 (B1, one-sample test): t(1311) = 2.90, p = 0.0038; Vb440 (B2): t(3422) = 2.62, p = 0.0081; Vg460 (G1): t(3422) = 0.851, p = 0.395; Vs556 (R2): t(3422) = 9.94, p = 5.6 x 10^{-3} D, Model spectral curves for mpv17+/– controls and gnat2:trβ2 retinas for constant quantal irradiances of 3.4, 4.0, and 4.6 log(quantum-μm^{-2}·s^{-1}). There is greater long-wavelength sensitivity, and lesser short-wavelength sensitivity in the embryonic gnat2:trβ2 gain-of-function transgenic, although the gains and deficits are much less severe than in the crx:trβ2 larvae. A, B, The log(k_{r1D}) values are the modeled R1-cone or R2-cone semi-saturation irradiances in log(quantum-μm^{-2}·s^{-1}). A–D, 6-dpf larvae, 20 mM aspartate medium.

Cumulative dataset differ from control. In Figure 9A,B, amplitudes are plotted against irradiance for four of the nine test wavelengths and compared with continuous curves calculated from best-fitting models. These are fit to 1945 points (15 eyes, 28 spectral datasets) for mpv17+/–– controls and 1540 points (17 eyes, 22 spectral datasets) for gnat2:trβ2. The distribution of points and curves at 570, 490 and 370 nm are more overlapping along the irradiance axis for gnat2:trβ2 larvae than are the same wavelength points and curves for the control strain. Points and curves at 370 and 490 nm (magenta, green) lay to the left of the 570-nm points and curve (yellow) for mpv17+/–– controls but coincide with the 570-nm curve for gnat2:trβ2, suggesting less sensitivity from short-wavelength-sensitive and mid-wavelength-sensitive cones. The logs of semi-saturation irradiances for the red (R1, R2) cones were 4.50 ± 0.02 for mpv17+/–– larvae and 4.51 ± 0.02 for gnat2:myFP-2A-trβ2;mpv17+/–– (Fig. 9A,B), nearly identical (t(3422) = 0.251, p = 0.802).

Four cone signals are identified in 6-dpf larval mpv17+/–– controls, but five were found in gnat2:myFP-2A-trβ2 siblings (Fig. 9C). Significantly reduced in gnat2:trβ2 are UV (Vu358) and B2 (Vb440) cone amplitudes. Significantly increased are B1 (Vb415) and R2 (Vs556) cone signals. The G1 cone signal (Vg460) was not significantly affected. The larval mpv17+/–– control and gnat2:trβ2 model-generated spectral sensitivities are similar (Fig. 9D), but with increased long-wavelength and decreased short-wavelength sensitivities in gnat2:trβ2. This leads to long-wavelength spectral peaks between 535 and 549 nm for gnat2:trβ2, not seen in the mpv17+/–– controls. While there is a definite spectral shift toward long-wavelength sensitivity in gnat2:trβ2 larvae...
cone semi-saturation irradiances in log(quanta·
(GraphPad Prism convention). Vu358 (UV, one-sample test):
to long wavelengths for all constant-quantal irradiances [3.4, 4.0, and 4.6 log(quanta·t
amplitudes (=2 3 o r 4 6 .
Four were found in gnat2;trβ2 adults (magenta bars). Cone saturation amplitudes (∝, Eq. 1; Fig. 2A) are fractions of dataset maximum amplitudes (∝SE). Asterisks (or n.s., not significant) represent the significance of differences between control and gnat2;trβ2 cone signals (GraphPad Prism convention). Vu538 (UV, one-sample test): t1(1579) = 4.81, p = 1.7 × 10-6; Vb415 (B1): t2(2983) = 2.97, p = 0.0030; Vg460 (G1); t2(2983) = 2.26, p = 0.024; Vg480 (G3, one-sample test): t1(1579) = 6.59, p = 6.1 × 10-11; Vr556 (R2); t2(2983) = 1.52, p = 0.127; Vr757 (R1); t2(2983) = 4.67, p = 1.2 × 10-6. D. Adult spectral curves for mpv17–/– control and gnat2;trβ2 transgenics. The transgene shifts sensitivity peaks to long wavelengths for all constant-quantal irradiances [3.4, 4.0, and 4.6 log(quanta·s-1)]. A–D, Eight- to 18-month adults, 10 mM aspartate medium.

Figure 10. Adult spectral signals from gnat2:trβ2;mpv17–/– transgenics and mpv17–/– controls. A, Control (mpv17–/–) irradiance–response amplitudes and model curves at four wavelengths. The optimal model (#219) was fit to 1610 responses (all wavelengths) combined from 23 datasets taken from 13 eyecups. The 370-, 490-, and 570-nm amplitudes are means (∝SE, n = 23; 650 nm, n = 23 or 46. B, Irradiance-response amplitudes, and model curves, for gnat2;trβ2. The transgene shifts 370- and 490-nm irradiance-response functions from the left of the 570-nm curve (control) to the right of the 570-nm curve (transgenic). The spectral algorithm was fit to 1400 points combined from 20 datasets accumulated from 11 eyecups. The 370-, 490-, and 570-nm amplitudes are means (∝SE), n = 20; 650 nm, n = 20 or 40. C, In adult mpv17–/– control eyecups (gray bars), significant signals from six cone types were detected. Four were found in gnat2;trβ2 adults (magenta bars). Cone saturation amplitudes (∝, Eq. 1; Fig. 2A) are fractions of dataset maximum amplitudes (∝SE). Asterisks (or n.s., not significant) represent the significance of differences between control and gnat2;trβ2 cone signals (GraphPad Prism convention). Vu538 (UV, one-sample test): t1(1579) = 4.81, p = 1.7 × 10-6; Vb415 (B1): t2(2983) = 2.97, p = 0.0030; Vg460 (G1); t2(2983) = 2.26, p = 0.024; Vg480 (G3, one-sample test): t1(1579) = 6.59, p = 6.1 × 10-11; Vr556 (R2); t2(2983) = 1.52, p = 0.127; Vr757 (R1); t2(2983) = 4.67, p = 1.2 × 10-6. D, Adult spectral curves for mpv17–/– control and gnat2;trβ2 transgenics. The transgene shifts sensitivity peaks to long wavelengths for all constant-quantal irradiances [3.4, 4.0, and 4.6 log(quanta·s-1)]. A–D, Eight- to 18-month adults, 10 mM aspartate medium.

(Fig. 9D), the extent is subtle compared with cxr;trβ2 larvae (Fig. 5D).

Of the 255 models fit to the 6-dpf larval gnat2:trβ2 combined dataset none were deemed to fit equally as well as model 79 based on residual variance, that is by an F test with p > 0.95. Two were indistinguishable from the best-fit model 77 for the mpv17–/– controls. Model 103 (F0.9992,1919,1940) = 0.493, p = 0.986), employed an additional 5th cone substituting G4 for G1 and adding B1. Model 101 (F0.9972,1940,1940) = 0.475, p = 0.95) substituted G4 for G1. All three indistinguishable control models agreed on the presence of UV, B2, and R2 cone signals in mpv17–/– controls.

Adult gnat2:trβ2 retinas generate large R1 red-cone signals
To determine the cone-type composition of adult gnat2:trβ2 retinal cone signals we searched for the best models to represent the gnat2:trβ2 cumulative spectral dataset. Trough-to-peak amplitudes for all eyes and datasets (mpv17–/–, 13 eyes, 23 datasets, 1610 responses; gnat2:trβ2, 11 eyes, 20 datasets, 1400 responses) were fit to the 255 combinations of eight cones. Model 219 fit best for mpv17–/– control eyecups, and model 202, for gnat2:trβ2 (Fig. 10A,B). When the adult gnat2:trβ2 cumulative dataset was fit to the control model 202, the residual variance was significantly greater than with the optimal gnat2:trβ2 model, indicating the cones signals contributing to the gnat2:trβ2 gain-of-function dataset differed significantly from the control (F1(1.942,1370,1374) = 1, p = 0).

For both points and optimal model curves the gnat2:trβ2 transgene alters adult irradiance–response characteristics (Fig. 10A,B). In the control dataset (mpv17–/–), the 570-nm points and curve (yellow) show less sensitivity than either the 370- or 490-nm points and curves (magenta,
green), but show greater sensitivity in gnat2:trβ2. In gnat2:trβ2, brighter stimuli are required to elicit responses in the UV (370 nm) and mid spectrum (490 nm). The fit of log of semi-saturation irradiances for the red cones (R1, R2) were 4.30 ± 0.026 for adult controls and 4.43 ± 0.026 for gnat2:trβ2 adults (Fig. 10A,B), a significantly greater sensitivity (~35%) than for gnat2:trβ2 adults (t(2953) = 3.44, p = 0.0006).

In the best-fitting model for gnat2:mYFP-2A-trβ2; mpv17−/− adults, the long-wavelength R1 cone (Vr575) contributes the largest amplitude signal with the R2 amplitude (Vr556) being only 22% as large, significantly less than R1 (t(2748) = 5.23, p = 2.6 × 10−11), and the gnat2:trβ2 R1 amplitudes are only marginally significant (t(1379) = 2.02, p = 0.044; Fig. 10C). In the mpv17−/− control, both R1 and R2 red cone amplitudes were highly significant (Vr575-R1, t(1580) = 6.01, p = 4.1 × 10−18; Vr556-R2, t(1580) = 5.52, p = 8.0 × 10−17) and did not significantly differ in amplitude (t(3156) = 0.042, p = 0.966; Fig. 10C). A six-cone model best fit adult control datasets (Vu358-UV, Vb415-B1, Vg460-G1, Vg480-G3, Vr556-R2, Vr575-R1). A four-cone model sufficed for gnat2:trβ2 adults (Vb415-B1, Vg460-G1, Vr556-R2, Vr575-R1). Unlike gnat2:trβ2 larvae (Fig. 9C), UV-cone signals were not significant in gnat2:trβ2 adults (Fig. 10C).

At all levels of constant quantal stimulation, long-wavelength spectral peaks between 560 and 570 nm are modeled for gnat2:mYFP-2A-trβ2; mpv17−/− (Fig. 10D). Models of the mpv17−/− control peak spectrally between 441 and 493 nm depending on stimulus brightness. Except at wavelengths >540 nm, control amplitudes were greater than transgenic, due both to the greater cone sensitivities (lesser half-saturation irradiances), and to the significantly greater amplitudes of numerous mid-wavelength and short-wavelength cones (Vu358-UV, Vb415-B1, Vg460-G1, Vg480-G3).

As judged by residual variance no models were indistinguishable from the illustrated ones for gnat2:mYFP-2A-trβ2; mpv17−/− adults (F tests for other models, p < 0.95). Model 235 was indistinguishable from model 219 (the best model for the mpv17−/− control adults (F(0.978,1603,1603)= 0.482, p = 0.96). Models #235 and #219 both employed six cones, but model 235 substituted the G4 (Vg500) for the G3 (Vg480) cone.

The larval dual opsin UV cone functions as a UV cone in trβ2 gain-of-function transgenics

Many cones in gnat2:mYFP-2A-trβ2; mpv17−/− larvae express mixed opsins, overlaying a red opsin on the na-

tive expression of a shorter-wavelength opsin (Fig. 7B). A few dual-opsin cones also occur in crx:mYFP-2A-trβ2 lar-

vae (Fig. 3B). The spectral physiology and molecular development of such cones is of interest. The wide spectral separation of red and UV opsins makes the two opsins in dual-opsin UV cones amenable to separate stimulation. One proposal is that zebrafish transgenic dual-opsin cones are analogous to the stable mws-swss “dual phys-

iology” cone configuration seen in rodents (Jacobs et al., 1991; Applebury et al., 2000; Nikonov et al., 2005; Suzuki et al., 2013). Another proposal is that the mixed-opsin cones in gnat2:trβ2 zebrafish are transitional states, later to develop into red cones. The tendency to lose UV-opsin signals in trβ2 gain-of-function transgenics suggests an adverse action on UV opsins, and a comparable model in mouse development is that trβ2 changes differentiated S cones to M cones (Swaroop et al., 2010).

To observe the effects of excess trβ2 on sws1 (UV opsin) gene activity, UV opsin immunoreactivity was examined in double transgenic gnat2:trβ2 larvae to which a fluorescent sws1 opsin reporter gene was added (gnat2-

mYFP-2A-trβ2;sws1:nfsBmCherry;mpv17−/−). Confocal micrographs of tangential planes through the cone photo-

receptor layers in control and the double transgenic larvae show that at 5 dpf (Fig. 11D,H) red-opsin immunoreactive cones are about twice as dense in gnat2:trβ2 larvae (148,000 ± 12,000 mm−2) as in controls (60,000 ± 14,000 mm−2; t(6) = 5.52, p = 0.0015), but at this stage, the density of UV-opsin immunoreactive cones (Fig. 11B,F) is not affected (gnat2:trβ2: 48,000 ± 3400 mm−2; control: 52,000 ± 4100 mm−2; t(6) = 0.77, p = 0.47).

For the mpv17−/− control, all UV-opsin immunoreactive cones (Fig. 11A–C, green) express the sws1:nfsBmCherry UV-opsin gene reporter as a magenta inner segment halo surrounding the narrower green immunofluorescence of the UV-cone outer segment (Fig. 11A). UV-opsin and red-opsin immunoreactivities are segregated, being expressed in separate cone cells (Fig. 11C), the naive larval opsin ex-

pression pattern (Allison et al., 2010). In the larval gnat2:

trβ2 double transgenic not all UV-opsin immunoreactive cones show the magenta halo of the sws1:nfsBmCherry re-

porter gene. In some, the sws1 reporter gene generates no fluorescence (Fig. 11E, red and green arrowheads), sug-

ggesting the native sws1 gene locus is inactive, and UV opsin, while still present, is no longer being synthesized. Only legacy SWS1 immunoreactivity remains. In Figure 11G, patterns of gnat2:trβ2 immunoreactivity for UV opsin and red opsin are compared. The arrowheads point to cone cells where both UV and red opsin are co-expressed. A comparison of Figure 11F with E reveals that UV-opsin immunoreactive cones with inactive sws1 reporter genes are the same cones that are double immunoreactive for both UV and red opsins. The expression of red opsin in a UV cone by the gain-of-function gnat2:trβ2 transgene appears incompatible with continued expression of UV-

opsin, suggesting that the overabundance trβ2 transgene

appears incompatible with continued expression of UV-

opsin, as in rodents, but would be limited by the catabolism of previously expressed, but not renewed, UV-opsin.

At the same embryonic developmental stage (5 dpf), the functional properties of UV cones with co-expression of red opsin were explored. In this earliest embryonic dataset, the same 5-cone model (#79) was the best fit for both control and gnat2:trβ2 gain-of-function larvae (Fig. 11I), and the residual variance of the gnat2:trβ2 dataset was not signif-

icantly greater when modeled with the control amplitude distribution (F(1,031,1230,1235)= 0.705, p = 0.591), although the R2-cone saturation amplitude (Vr556) tended greater (9%) in gnat2:trβ2 (t(3091) = 3.39, p = 0.0007). Saturation am-

plitudes of shorter-wavelength cone signals (Vu358-UV,
Vb415-V1, Vb440-B2, Vg460-G1) in gnat2:trβ2 and control were not significantly different. UV-cone saturation amplitude at 5 dpf appeared unaffected by numerous of its members both lacking sws1 gene activity and co-expressing red opsin. Red chromatic adaptation should desensitize UV/red mixed opsin cones to UV stimulation. 650 nM red stimuli are not absorbed by UV opsins, but 370-nm UV stimuli are strongly absorbed. We generated irradiance-response functions at 650 and 370 nm in the presence of red or IR backgrounds (Fig. 11J,K). For 370-nm stimuli, the control, irradiance-response curves overlap in both datapoints and Hill-function curve fits regardless of red or IR background illumination (Fig. 11J). Hill function fits give semi-saturation values of 4.53 log(quantum·μm⁻²·s⁻¹) for the IR background and 4.59 for the 627-nm background, not distinguishable (t_{290} = 1.26, p = 0.210), an expected result as UV-opsin does not absorb either the 627 nm or the IR adapting light. As the 650-nm stimuli (Fig. 11J) are not as efficiently transduced, even by red cones, the 650-nm

Figure 11. The UV-opsin reporter is inactive in embryonic cones co-expressing UV and red opsins, but these red opsins produce no UV-desensitizing signal. A, UV-opsin (SWS1) immunoreactivity (green) and florescence of a reporter transgene for sws1 (sws1:nfsBmCherry, magenta) colocalize in mpv17+/− control UV cones. B, UV-opsin immunoreactivity in control retina. C, In the control retina UV-opsin (green) and red-opsin (red) immunoreactivity localize in separate cones. D, Red-opsin immunoreactivity in the control retina. E, In gnat2:trβ2 cones, UV-opsin immunoreactivity (green) is always found when there is sws1 reporter gene fluorescence (sws1:nfsBmCherry, magenta), but not all UV-opsin immunoreactive cones show reporter-gene fluorescence (red and green checkered arrowheads). F, UV-opsin immunoreactive cones in the gnat2:trβ2 retina. G, Co-expression of UV-opsin (green) and red-opsin (red) immunoreactivity in gnat2:trβ2 cones. White arrowheads (F, G, H) point to cones expressing both UV opsin (green) and red opsin (red). The dual opsin cones are the UV-opsin immunoreactive cones in E, that lack sws1 reporter fluorescence (red and green checkered arrowheads). H, Red-opsin immunoreactive cones in gnat2:trβ2 retina. I, Saturation amplitudes (Vmi ± SE) of signals from five cone types in control (gray) and gnat2:trβ2 (magenta) eyes. Control: Spectral algorithm fit to 1890 datapoints from 27 datasets recorded in 15 eyes; gnat2:trβ2, 1260 datapoints from 18 datasets recorded in 11 eyes. In each case, the optimal fit was model 79. Asterisks give significance of differences (two sample t tests, n.s., not significant) in cone-signal amplitudes (Vu358: t_{3091} = 0.56, p = 0.57; Vb415: t_{3091} = 0.25, p = 0.81; Vb440: t_{3091} = 1.05, p = 0.29; Vg460: t_{3091} = 0.084, p = 0.93; Vr556, t_{3091} = 3.39, p = 0.0007). J, Control strain (mpv17+/−) irradiance-response curves at 370 and 650 nm in the presence of infrared (IR; black) or 627-nm (red) backgrounds (bkgd). Points are means ± SE (370-nm stimulus: IR background, n = 27; red background, n = 15; 650-nm stimulus: IR background, n = 54 or 27; red background, n = 30 or 15). K, gnat2:trβ2 irradiance-response curves at 370 and 650 nm in the presence of IR (black) or red backgrounds. Points are means ± SE (370-nm stimulus: IR background, n = 18; red background, n = 12; 650-nm stimulus: IR background, n = 18 or 36; red background, n = 12 or 24). J, K, Hill function curves fit at each background and wavelength are constrained to have equal maximal amplitudes and exponents. Cone-PIII ERG signals isolated with 20 mM aspartate. A–K, Eyes and retinas are from 5-dpf embryos.
irradiance-response functions shift to greater irradiances. On the IR background the 650-nm semi-saturation irradiance is 6.38 log(quanta·m\(^{-2}\)·s\(^{-1}\)/C\(_0\)) but increases to 6.53 log(quanta·m\(^{-2}\)·s\(^{-1}\)/C\(_0\)) with the 627-nm background, a significant desensitization (\(t_{(583)} = 3.66, p = 0.0003\)). The absorption of the red background by the red-cone red opsin reduces red-cone sensitivity (Fig. 11J).

What was unexpected was that the 370-nm irradiance functions for the gnat2::tr\(\beta\)2 gain-of-function larvae, with co-expression of red opsin in some UV cones, were unaffected by the red background (Fig. 11K). The red opsin in the mixed-opsin UV cones, which would be activated by the 627-nm background, does not desensitize 370-nm UV signals. For 370-nm stimuli, Hill fits give semi-saturation irradiances of 4.42 log(quanta·m\(^{-2}\)·s\(^{-1}\)) on the IR background and 4.44 log(quanta·m\(^{-2}\)·s\(^{-1}\)) on the 627-nm background, values not significantly different (\(t_{(205)} = 0.300, p = 0.765\)). But the same red background significantly desensitizes red-opsin signals from gnat2::tr\(\beta\)2 red cones, as seen with 650-nm stimuli (Fig. 11K). The Hill semi-saturation changed from 6.26 log(quanta·m\(^{-2}\)·s\(^{-1}\)) on the IR background to 6.55 log (quanta·m\(^{-2}\)·s\(^{-1}\)) on the 627-nm background, a significant sensitivity loss (\(t_{(410)} = 5.01, p = 1.5 \times 10^{-12}\)), which demonstrates the effectiveness of this background for red opsins (Fig. 11K). The failure of long-wavelength backgrounds to affect UV signals from mixed-opsin cones has also been observed in ERG spectra of rodents (Jacobs et al., 1991). In zebrafish the spectral physiology of UV cones appears, at least early in development at 5 dpf, not to be affected by the introduction of transgenic red opsins into many UV-cone members, or by inactivation of the UV-opsin gene.

Thyroxin receptor \(\beta\)2 gain-of-function transgenes alter cone morphology

Thyroid hormone receptor \(\beta\)2 is required in zebrafish for both the development of red cones and the expression of red opsins (Deveau et al., 2020). Morphologically, adult red cones are the principal members of zebrafish double cones (Engstrom, 1960; Raymond et al., 1993). Double cones failed to develop in tr\(\beta\)2 mutants (Deveau et al., 2020). The impact of tr\(\beta\)2 in determining the course of cone morphologic development is seen in transverse optical sections of individual transgenic cones from in vivo 6-dpf larvae (Fig. 12A,C). These are higher magnifications taken from larval retina confocal image stacks such as seen in Figure 1D–F. In the Figure 12 examples, the fluorescent-reporter shapes of tr\(\beta\)2 gain-of-function cones are distinct from the control morphologies of red, blue, and UV cones, the latter marked by the reporter fluorescent

![Figure 12.](https://doi.org/10.1523/JNEUROSCI.0326-22.2022)
proteins expressed in *sws2:GFP* (blue cones) and *sws1:GFP;trβ2:tdTomato* (UV and red cones).

The maximal widths of the five sorts of cone inner segments illustrated differed (ANOVA, $F_{(4,356)} = 36.98$, $p < 0.00001$). The maximal widths for cone inner segments of *cnx:mYFP-2A-trβ2* labeled cones were significantly wider than those of red cones (post hoc Tukey, $p < 0.000001$), as are the maximal widths of cone inner segments of *gnat2:mYFP-2A-trβ2*; mpv17→−/− labeled cones (post hoc Tukey, $p = 0.00024$). Neither trβ2 gain-of-function cone is as wide as the inner segments of UV cones (post hoc Tukey: *cnx:mYFP-2A-trβ2*, $p = 0.000005$; *gnat2:mYFP-2A-trβ2*; mpv17→−/−, $p < 0.000001$). The inner segment widths of the *cnx:mYFP-2A-trβ2* cones were not distinguishable from blue cones (post hoc Tukey: $p = 0.126$), while *gnat2:mYFP-2A-trβ2* widths were marginally narrower than blue cones (post hoc Tukey: $p = 0.00764$).

The lengths of axons connecting the base of cone inner segments and the apex of cone synaptic pedicles differed among the cone types (ANOVA, $F_{(4,290)} = 9.692$, $p < 0.00001$; Fig. 12D). In the trβ2 gain-of-function cones, the length of the axon is significantly shorter than in red cones (post hoc Tukey: *cnx:mYFP-2A-trβ2*, $p = 0.00644$; *gnat2:mYFP-2A-trβ2*; mpv17→−/−, $p = 0.00883$; Fig. 12D). The lengths are not distinguishable from blue cone axons (post hoc Tukey: *cnx:mYFP-2A-trβ2*, $p = 0.212$; *gnat2:mYFP-2A-trβ2*; mpv17→−/−, $p = 0.175$) or UV cones axons (post hoc Tukey: *cnx:mYFP-2A-trβ2*, $p = 0.108$; *gnat2:mYFP-2A-trβ2*; mpv17→−/−, $p = 0.134$).

On morphometrics, the mYFP-marked trβ2 gain-of-function cones have not attained red-cone shape at 6 dpf, but nonetheless enhance red cone signal amplitudes. Gain-of-function trβ2 morphologically alters larval cones and appears to shift the metrics toward a larval blue-cone pattern.

Impact of excess thyroid hormone receptor β2 on development of zebrafish spectral signals

During zebrafish maturation, patterns of cone opsin mRNA expression change through interactions with a “developmental factor” (Takechi and Kawamura, 2005). Green-cone and red-cone spectral peaks shift from shorter to longer wavelengths by adulthood (Nelson et al., 2019) as different members of gene-duplicated green (Rh2) and red (lws) opsin groups are sequentially expressed. In the present control data, the R2 opsin signal (Vr556) is largest in embryonic (5, 6 dpf) and juvenile (12 dpf) ages but the R1 opsin signal (Vr575) attains equal (Vr556) is largest in embryonic (5, 6 dpf) and juvenile (12 dpf) and adult developmental stages. R2 cone amplitudes (Vr556) increase at both 5 and 12 dpf (12 dpf: $t_{(1556)} = 6.29, p = 3.9 \times 10^{-10}$) and R1 cone signals (Vr575) increase for 6-dpf embryos and for adults (6 dpf: $t_{(1826)} = 2.72, p = 0.0067$; Adult: $t_{(2818)} = 3.92, p = 9.2 \times 10^{-5}$; Fig. 13A).

For the gnat2 promoter, the consequences of gain-of-function trβ2 are more gradual in developmental course (Fig. 13B). In the 5dpf *gnat2:mYFP-2A-trβ2* embryonic larvae, the overall distribution of signal amplitudes from cone types is not significantly different from those found in the control (mpv17→−/−) siblings, but R2 cone signals (Vr556) trend larger (Fig. 13B, 5dpf). At 6dpf, cone signal distribution differs significantly and enhancement of R2 amplitudes increases (6 dpf: 22%, $t_{(3422)} = 9.42, p = 5.6 \times 10^{-23}$). In 12 dpf juveniles, R2 amplitude increases by 50% ($t_{(2950)} = 2.65, p = 0.0082$), but in adults red-cone enhancement switches from R2 (Vr556) to R1 (Vr575), where a 241% enhancement is seen ($t_{(2953)} = 4.86, p = 1.2 \times 10^{-6}$), with no significant change seen in R2-cone amplitudes ($t_{(2953)} = 1.52, p = 0.127$). In gnat2:trβ2, UV cone signals (Vr358) persist through larval and juvenile stages, with amplitudes either not significantly different (5 dpf: $t_{(3091)} = 0.563, p = 0.574$; 12 dpf: $t_{(2549)} = 0.594, p = 0.553$) or less than control (6 dpf: 45%, $t_{(3422)} = 5.36, p = 8.7 \times 10^{-5}$), but unlike the significant UV signals of control adult ($t_{(1579)} = 4.81, p = 1.7 \times 10^{-5}$), no UV-signal was detected in gnat2:trβ2 adults (Fig. 13B). B1-cone and G1-cone signals persist in gnat2:trβ2 adults but are significantly reduced in amplitude as compared with controls (B1: 37%; $t_{(2953)} = 2.97, p = 0.0030$; G1, 50%; $t_{(2953)} = 2.23, p = 0.024$).

Discussion

Modeling of the massed cone signals from zebrafish retinas yielded estimates of amplitude contributions from eight spectrally distinct cone types during embryonic, juvenile, and adult developmental stages. The technique provided a window on the impact of transgene-induced overabundance in the red-cone transcription factor trβ2 for the balance among electrical signals from red-cone opsins and all other opsin types. Two trβ2 gain-of-function transgenics were studied. In *cnx:mYFP-2A-trβ2*, the *cnx* promoter introduced trβ2 into retinal progenitor cells, whether ultimately fated to become cone cells or other types, such as bipolar cells (Shen and Raymond, 2004). In *gnat2:mYFP-2A-trβ2*; mpv17→−/−, the gnat2 promoter increased trβ2 levels only in differentiated cone types, including green, blue, and UV cones where it is not native,
as well as adding an extra dose to the red-opsin cones where it is normally expressed (Ng et al., 2001; Suzuki et al., 2013). Neither transgene caused major alterations in the amplitudes or kinetics of massed cone signals as isolated from the ERG by blockade of cone synapses. In cxr: mYFP-2A-trβ2 larvae, response amplitudes were larger than WT controls, as was the variance in amplitudes. In the adults, peak times of both onset and offset waveform elements were significantly faster. Neither alteration suggested a major net influence on phototransduction. In gnat2:mYFP-2A-trβ2;mpv17−/− eyes, there were no significant changes either in amplitudes or in onset and offset kinetics. But in both transgenics, significant changes were found in the relative contributions from different cone types to retinal spectral responses.

**Opsin signals in cxr:trβ2 transgenics**

For cxr:trβ2 transgenics, the net amplitude of red opsin signals increased at all developmental stages, as did the densities of red-opsin immunoreactive cones, as seen
previously by Suzuki et al. (2013), suggesting increased cone numbers led to increased signal strength. But LWS2 (R2) opsin signals were favored in larvae and juveniles, while LWS1 (R1) signal amplitudes became disproportionately large in adults. The latter suggests that, in addition to augmentation of red cone abundance and signal amplitude, excess trβ2 favored R1 opsin expression but to do so required a cofactor expressed only late in development. Individual zebrafish red cones express either one or the other of the lws1 or lws2 opsin genes but not both (Tsujimura et al., 2010) and thyroid hormone, the trβ2 ligand, can induce a cone to switch opsins from LWS2 to LWS1 (Mackin et al., 2019). In situ hybridization indicates that red cones in embryonic and larval zebrafish choose mainly the lws2 opsin gene, while adult red cones are more likely to transcribe lws1, particularly in peripheral retina (Takechi and Kawamura, 2005). The WT and mpv17+/-- control cone-ERG spectral analyses support a direct proportionality to the red-opsin transcript development. LWS2 (R2) opsin signals were the largest, and most often the only, red-cone signals detected in embryos and juveniles of control animals, but about equal amplitudes of both R1 and R2 signals appeared in adult controls. In gain-of-function crx:trβ2 larvae and juveniles, with one exception, it was the early development R2 signals that trβ2 overabundance increased. In crx:trβ2 adults, it was the late development R1 signals that increased, becoming the largest-amplitude cone spectral signal, and shifting cone ERG spectral peaks from mid-spectrum in controls to long wavelengths in crx:trβ2. Augmentation of LWS1 transcript occurs in adult six7 transcription factor knock-outs (Ogawa et al., 2015), suggesting a model where this transcription factor inhibits lws1, but might itself be inhibited by trβ2. Alternatively thyroid hormone has been shown to favor expression of LWS1 over LWS2 transcript (Mackin et al., 2019) and a different model would propose greater thyroid hormone levels in adults would lead to greater levels of bound trβ2, proportionally favoring a switch to LWS1 cone physiology.

Zebrafish UV (SWS1) cones are the molecular phylogenetic relatives of mammalian S-cones (Terakita, 2005). In crx:trβ2 transgenics the amplitude of UV cone signals was greatly decreased or eliminated at all developmental stages, suggesting the diminished densities of UV opsin immunoreactive cones noted herein and by Suzuki et al. (2013), led directly to decreased UV-cone signal strength. With the progression of developmental stages in teleosts, the role of UV cones typically diminishes (Cheng et al., 2006; Carleton, 2009; Nelson et al., 2019). Juvenile trout lose UV sensitivity as they mature. The process is thyroid hormone sensitive (Brownman and Hawryshyn, 1994) and correlates with regional loss of SWS1 UV-opsin immunoreactivity and UV cone morphologies. Based on regional maturation in thyroid hormone and thyroid hormone receptor levels within retinal quadrants, together with experimental treatments with thyroid hormone (T4), Raine and Hawryshyn (2009) proposed that regional loss of UV-cone signaling was caused by regional increases in both in T4 and trβ. In zebrafish, similarly, T4 reduced UV-opsin transcript levels (Mackin et al., 2019). Present results add that increased levels of trβ2 receptor itself reduce the signal amplitude and numbers of UV cones and that trβ2 is a potential candidate regulating their density. How this might occur in the absence of a transgene is less clear, as trβ2 is not normally expressed by UV cones (Suzuki et al., 2013).

**Opsin signals in gnat2:trβ2 transgenics**

For gnat2:trβ2 5-d embryos, introduction of trβ2 into functional cones of all spectral types doubled the numbers of red-opsin immunoreactive cones without changing the densities of cones with other opsin immunoreactivity, a result first noted by Suzuki et al. (2013) and repeated here. But in the present cone ERG analysis, red-cone signal amplitude increased <10%. In this counterexample, the densities of red-opsin immunoreactive cones and signal strength were not proportional. Densities of green-opsin, blue-opsin, and UV-opsin immunoreactive cones, and the distribution of opsin signals among them, were unchanged. As discovered by Suzuki et al. (2013) and confirmed here, much of the increase in density for red-opsin immunoreactive cones is accounted for by co-expression. Suzuki et al. (2013) found red-green and red-UV immunoreactive cones. To this we add cones immunoreactive for both red and blue opsins. Red opsins induced in differentiated gnat2:trβ2 green, blue, and UV cones evidently do not immediately result in a greater red-cone electrical signal at the 5-d larval stage. By adulthood the spectral signals gnat2:trβ2 do come to resemble those of adult crx:trβ2 red opsin dichromats, with largest amplitudes originating from LWS1 opsins and green blue and UV amplitudes reduced or suppressed. This suggests the introduction of trβ2 into differentiated cones quickly expresses red opsins but is much slower to generate electrical signals than is the case with crx:trβ2, which introduces trβ2 into cone progenitors.

In embryonic gnat2:trβ2 transgenics the amplitude of UV cone signals was not affected by co-expression of red opsins. To examine red-UV immunoreactive cones further we examined sws1 reporter gene expression and the sensitivity of UV cones to adaptation by red backgrounds. In controls, sws1 reporter activity and UV-opsin immunoreactivity co-localized, but in gnat2:trβ2 transgenics UV-opsin immunoreactive cones that co-expressed red opsin lost sws1 reporter activity, suggesting that, even without transcriptional resupply of UV opsin, long-lived previously synthesized UV-opsin survived in the cone disks and functioned for some time after synthesis was suppressed. We suggest that the co-expressed red opsins remain electrically dormant during this initial period, as red backgrounds which desensitize red-cone signals failed to desensitize UV-cone signals in 5-dpf gnat2:trβ2. In the longer term UV-opsin would be lost to disk shedding (O’day and Young, 1978). This may be a path by which UV/red mixed opsin cones are gradually lost, and/or converted to red cones, similar to an important model in mouse M-cone embryogenesis from primordial UV cones (Swaroop et al., 2010).
blue-cone (SWS2) signals. Vb415 (B1, SWS2) and Vg460 (G1, Rh2-1) signals were significant in cxr:trβ2 embryos and juveniles but lost in adults. G1-cone signals might be either increased or decreased compared with controls in gnat2:trβ2 embryos or juveniles but both were reduced in adults. In adult zebrafish trβ2+/− mutants (Deveau et al., 2020) green-cone signals increased significantly in amplitude. These observations suggest a late-stage inhibitory effect of trβ2 on green and blue cones. Knock-out of the zebrafish homeobox transcription factor six7 eliminates green-cone Rh2 transcripts in adults and six6 knock-outs adversely affected blue-cone SWS2 transcript (Ogawa et al., 2015, 2019). An association of these transcription factors with blue and green cones was made by single-cell sequencing and machine learning methods (Ogawa and Corbo, 2021). Speculatively there is an inhibitory action of trβ2 on these homeobox genes.

Changes in cone morphology

Larval cone morphologies in both trβ2 gain-of-function transgenics were altered. In gnat2:mYFP-2A-trβ2, where the transgene was expressed in all cone types, larval cones expressing the mYFP fluorescent transgene reporter did not look like red cones, resembled none of the control cone-type morphologies, but had a transgenic shape closest in morphometrics to, but visually distinguishable from, the control larval blue cones. There appears to be an early alteration of native morphologies induced by activity of the gain-of-function transgene. In cxr:mYFP-2A-trβ2 the mYFP transgene reporter was more sparsely expressed in the cone layer, nonetheless revealing altered cone morphology with inner segment widths and axon lengths similar to gnat2:mYFP-2A-trβ2 cones. The basis of these trβ2-induced shape changes is not known, but despite altered shape, the cones are robustly functional. Clearly trβ2 is importantly involved in large swaths of cone development, including the formation of adult double cones, with characteristic Arrestin 3a antigenicity (Deveau et al., 2020). Thyroid hormone receptor β2 pathways are not yet fully elaborated, but studies of zebrafish mutants and gain-of function transgenics expand the inventory of physiological, morphological, and genetic targets and provide insight into further roles in development.

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