

Retinal network adaptation to bright light requires tyrosinase

Patrick S Page-McCaw¹⁻³, S Clare Chung^{1,3}, Akira Muto¹, Tobias Roeser^{1,2}, Wendy Staub¹, Karin C Finger-Baier¹, Juan I Korenbrot¹ & Herwig Baier¹

The visual system adjusts its sensitivity to a wide range of light intensities. We report here that mutation of the zebrafish *sdY* gene, which encodes tyrosinase, slows down the onset of adaptation to bright light. When fish larvae were challenged with periods of darkness during the day, the *sdY* mutants required nearly an hour to recover optokinetic behavior after return to bright light, whereas wild types recovered within minutes. This behavioral deficit was phenocopied in fully pigmented fish by inhibiting tyrosinase and thus does not depend on the absence of melanin pigment in *sdY*. Electroretinograms showed that the dark-adapted retinal network recovers sensitivity to a pulse of light more slowly in *sdY* mutants than in wild types. This failure is localized in the retinal neural network, postsynaptic to photoreceptors. We propose that retinal pigment epithelium (which normally expresses tyrosinase) secretes a modulatory factor, possibly L-DOPA, which regulates light adaptation in the retinal circuitry.

Although light intensity in an animal's environment may vary by ten orders of magnitude, visually driven behaviors remain stable. Light adaptation allows the retinal neural network to remain sensitive to small differences in light intensity with respect to the background, regardless of its absolute intensity. Within the retina, adjustments of the dynamic range of light sensitivity begin with the phototransduction cascade¹, which adapts on the timescale of milliseconds to seconds. Additional, slower processes adjust the relative weight of synaptic signals in the neural circuitry. In the fish retina, for instance, cone photoreceptors actively contract in response to light, bringing their outer segments into the focal plane of the optical apparatus, while the more sensitive rods slide past them in the opposite direction². These 'retinomotor movements' are reversed in darkness. Similarly, upon light exposure, horizontal cells extend finger-like protrusions toward cone pedicles, called 'spinules', to enhance synaptic communication between cones and interneurons^{3,4}. These and a multitude of other network adaptations take minutes to hours.

Retinal network adaptations are thought to be controlled by light-dependent changes in neuromodulator levels. Dopamine acts as a paracrine factor in dynamic balance with melatonin⁵. In fish, exogenous dopamine mimics the effects of ambient light, including retinomotor movements and spinule formation, on the dark-adapted outer retina, whereas melatonin opposes the dopaminergic effect and mimics darkness⁶. Dopamine is synthesized in two sequential steps. First, the amino acid L-tyrosine is hydroxylated to L-dihydroxyphenyl alanine (L-DOPA). This precursor is then decarboxylated to dopamine. The two enzymes commonly implicated in dopamine synthesis, tyrosine hydroxylase (TH) and aromatic amino acid decarboxylase (AADC),

are localized in a sparse class of widely branched retinal interneurons called dopaminergic interplexiform cells (IPCs)⁷. The IPCs seem to be in a unique position to supply dopamine to the retinal circuits, although it remains unclear whether this role in adaptation is exclusive to these cells⁸⁻¹⁵.

Several zebrafish mutants have been described that lack melanin in their eyes and in the rest of their body^{16,17}. In a systematic behavioral examination of ~400 zebrafish mutants previously isolated in large-scale morphological screens, we discovered that mutations in one of the albino genes, *sdY* (encoding Sandy), severely affected visual performance¹⁸. The *sdY* visual defect could not be attributed to the ocular anomalies commonly found in albino mammals, such as a lack of a fovea, a rod deficit, or a misrouting of the optic pathway¹⁹. We report here that the visual impairment is conditional and reversible. Retinal light adaptation, as measured in a behavioral assay and electrophysiologically, is disrupted in *sdY* mutants. Because the *sdY* gene encodes tyrosinase (which, like tyrosine hydroxylase, converts L-tyrosine to L-DOPA), we propose that tyrosinase may be important in a novel pathway for dopamine production in the retina.

RESULTS

Visual behavior of *sdY* depends on previous light exposure

Visual abilities of *sdY* mutants were assessed with a behavioral assay at six or seven days post-fertilization (dpf). At this age, zebrafish can see well, but their visual circuitry is still immature^{4,20}. Visual performance is also driven exclusively by cone photoreceptors; substantial rod contributions are observed only after 15 dpf²¹. We could therefore test only photopic vision in these larvae. Furthermore, light adaptation is slower

¹University of California, San Francisco, Department of Physiology, Program in Neuroscience, 513 Parnassus Ave., San Francisco, California 94143-0444, USA.

²Present addresses: Rensselaer Polytechnic Institute, Troy, New York 12180, USA (P.P.-M.) and Isenbruck and Partners, Technologiepark Heidelberg, Im Neuenheimer Feld 582, 69120 Heidelberg, Germany (T.R.). ³These authors contributed equally to this work. Correspondence should be addressed to H.B. (hbaier@itsa.ucsf.edu).

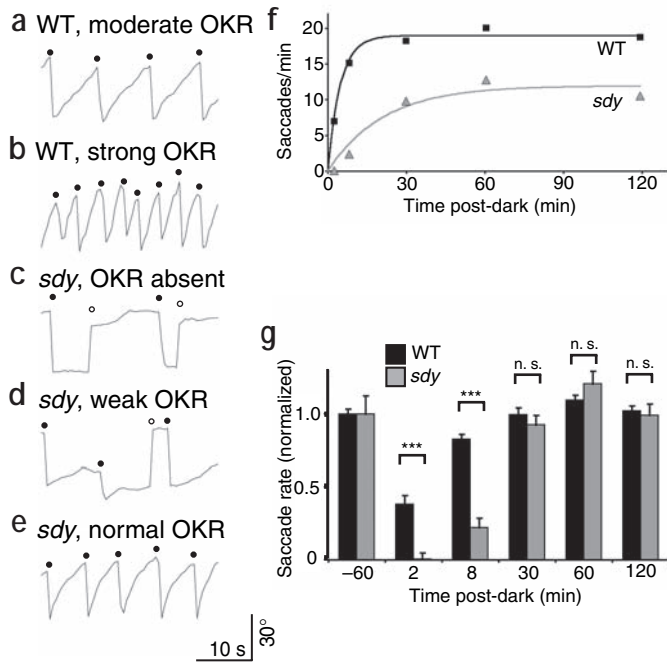


Figure 1 Variability and dark-pulse sensitivity of the *sdly* OKR. (a–e) OKR traces from wild types (WT, a,b) and *sdly* mutants (c–e) in response to a constant motion stimulus (high-contrast sinewave grating; 36° per cycle; moving at a velocity of 18°/s). In c–e, saccades are indicated by filled circles (against the motion of the stimulus) or by open circles (with the motion). Note the apparently random saccades in c. (f,g) Mutants are slow to recover from a 45-min dark pulse, ending at time 0. Their OKR reaches the pre-dark level an hour after light onset, whereas wild-type siblings recover more quickly. In f, mean saccade rates of wild types (black squares) and *sdly* mutants (grey triangles) were fitted with an exponential function (solid lines) to calculate the time constants of recovery. Responses of *sdly* were suppressed to 60% of wild type in this recording. In g, saccade rates were normalized to pre-dark levels for each genotype and plotted as bar graphs, to emphasize the adaptation kinetics in the period immediately after light onset. We tested 18 *sdly* and 18 wild type at most time points. Error bars, s.e.m. Statistical significance was calculated with Student's *t*-test ($\alpha = 0.05$; two-tailed, assuming unequal variance). * $P < 0.05$; *** $P < 0.001$; n.s., not significant.

in larvae than in adult animals; it occurs on the order of minutes (see below). During the optokinetic response (OKR), zebrafish move their eyes in pursuit of a moving grating composed of alternating dark and bright stripes^{20,22}. We previously determined that the strength or gain of the OKR in response to a constant stimulus correlates with the frequency of reset saccades, in which the eyes snap back to their original positions between pursuits²³ (Methods). The saccade rate was therefore measured and used to quantify the ability of the fish to detect the stimulus. We noted that spontaneous eye movements (saccades during exposure to a stationary grating) were very similar in wild types and mutants (2.8 ± 0.4 /min for *sdly*, $n = 129$, and 2.5 ± 0.3 /min for wild type, $n = 79$; mean \pm standard error), suggesting that oculomotor control is not affected by the mutation.

When the fish were raised in a standard incubator with sporadic exposure to light, their OKR performance was variable (Fig. 1a,b). For *sdly* mutants, this variability was particularly pronounced, ranging from apparent blindness to a wild-type-like response (Fig. 1c–e). This raised the possibility that differences between individuals were due to variations in previous exposure to ambient light. We therefore tested the visual functions of fish raised under a controlled lighting regime. Larvae

were maintained under cyclic lighting conditions (14 h of light, 10 h of dark) and were tested in the afternoon of the seventh day after fertilization. Under these conditions, all wild types and all mutants displayed a vigorous OKR. Depending on the stimulus, however, *sdly* responses were always reduced to about 60–80% of the wild-type level.

The finding that the OKR became more consistent in *sdly* mutants raised in cyclic lighting conditions compared to those exposed to more sporadic lighting suggested that the defect may be in the time course of adaptation to the onset (or offset) of steady illumination. We tested this possibility by examining the behavioral consequence of periods of darkness in the otherwise typical afternoon light environment. Fish larvae were placed into OKR test chambers in the afternoon and kept for 45 min in darkness (Fig. 1f). Upon return to bright light, wild-type larvae showed a reduced OKR 2 min after the end of the dark pulse (the first time point measured) and returned to their normal (pre-dark pulse) response with a time constant of ~3 min (Fig. 1f). In contrast, *sdly* mutants were unresponsive after 2 min in the light, barely responsive at 8 min, and required 15 min to recover to 68% of the pre-dark pulse response (Fig. 1f). The difference in the time course of adaptation is clearest when OKR gain is normalized to pre-dark levels (Fig. 1g). Qualitatively similar results were obtained with shorter pulses of darkness (5 min), although *sdly* mutants recovered more quickly and the wild-type OKR was not detectably affected (data not shown). These experiments suggest that in *sdly* mutants the adaptation mechanism necessary to cope with a sudden transition from darkness to bright light is impaired.

The *sdly* gene encodes tyrosinase

To begin to understand the cellular underpinnings of the light-adaptation defect, we cloned the *sdly* gene. The *sdly* mutation was mapped to the same region of chromosome 15 as tyrosinase (Methods). Wild-type tyrosinase cDNAs and two mutant alleles (*sdly*^{s3555} and *sdly*^{tk20}, which are phenotypically indistinguishable) were sequenced. The *sdly*^{s3555} mutation eliminates the initiation codon (Fig. 2a), and because no other initiation codon in the sequence could produce a functional protein, we expected the mutation to result in a complete loss of function. In *sdly*^{tk20}, a glutamate codon adjacent to the catalytically essential copper-binding domain B is mutated to encode lysine (Fig. 2a). This position is conserved in all tyrosinase and tyrosinase-related proteins from fungi to vertebrates (Fig. 2b). As the mutation changes both the charge and the size of the encoded amino acid, we expected it to likewise encode a non-functional protein. Human tyrosinase mutations within the same protein domain²⁴, and even in the same amino acid²⁵, are associated with oculocutaneous albinism (Fig. 2a).

To confirm that *sdly* mutations disrupt the tyrosinase gene, the wild-type locus was used to rescue the *sdly* phenotype. We injected a DNA construct containing the full-length tyrosinase-coding region under the control of 5.5 kb upstream sequence²⁶ into *sdly* mutants at the one-cell stage. The construct rescued melanin synthesis in 56% of injected mutant embryos in a mosaic fashion ($n = 27$; Fig. 2c–f). In rescued animals, individual melanocytes appeared black in otherwise albino fish (Fig. 2e,f). A control construct, carrying a frame-shift mutation in the tyrosinase gene, was unable to rescue pigmentation ($n = 20$; Fig. 2d). The mapping, sequence and rescue data demonstrate that *sdly* encodes tyrosinase.

Light adaptation requires tyrosinase, but not melanin

Tyrosinase may have an acute physiological role in light adaptation. Alternatively, it may exert its effect on visual behavior indirectly via its role in melanin synthesis. To distinguish between these possibilities, we blocked the enzymatic activity of tyrosinase, starting at 4 dpf (after the larvae have become fully pigmented), using the specific inhibitor phenylthiourea (PTU; 0.2 mM). This late PTU treatment did not visibly

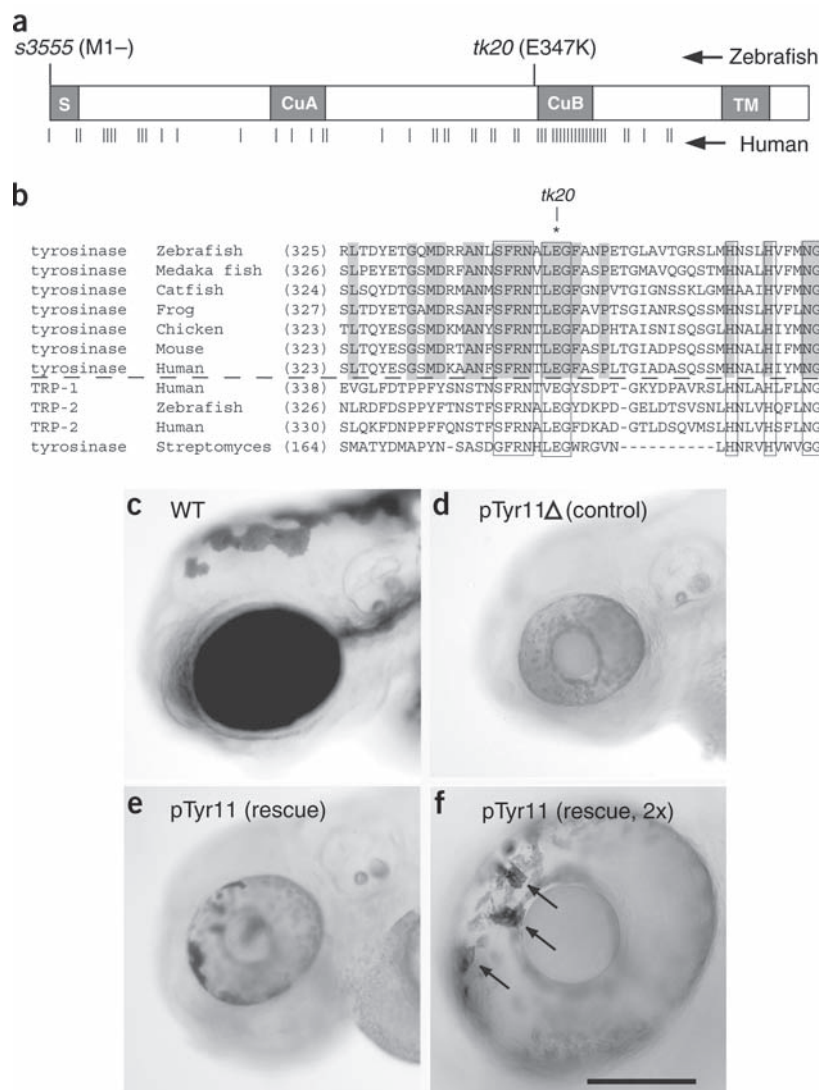


Figure 2 Molecular identification of *sdyl* tyrosinase. (a) Domain structure of tyrosinase. Vertical lines indicate positions of point mutations in zebrafish *sdyl* alleles, *s3555* and *tk20*, and in human oculocutaneous (OCA1) albinos²⁴. Only human missense mutations are shown. S, signal peptide; CuA, CuB, copper-binding domains; TM, transmembrane domain. (b) A conserved glutamate is changed to a lysine in the *tk20* allele. The shaded amino acids are conserved in vertebrates (above hatched line). The boxed amino acids are conserved in tyrosinases and tyrosinase-related proteins (TRPs) of eukaryotes. (c–f) Heads of 3-day old zebrafish embryos (lateral view). (c) Wild type. (d) *sdyl* mutant, injected with control plasmid. (e) *sdyl* mutant, injected with tyrosinase expression construct. (f) Same fish as in e, magnified. Note the mosaic rescue of eye pigmentation. Scale bar (shown in f), 200 μ m (a–e); 100 μ m (f).

ever, appeared to have normal ERG waveforms (8 of 38; **Fig. 4d**). Bath application of L-DOPA (30 μ M, added 30 min before recording) removed the oscillations in 4 of 5 *sdyl* mutants tested (**Fig. 4e**). Both *sdyl* and *alb* dark-adapted retinas were ~ 2.5 log units more sensitive to light than those from wild types, as measured by the *b*-wave peak amplitude dependence on light intensity (**Fig. 4f**). Hypersensitivity was in part normalized by L-DOPA (**Fig. 4f**). These results show that, although both forms of albinism increase retinal light sensitivity, only the *sdyl* form disrupts retinal signaling.

Based on the OKR phenotype, we hypothesized that the *sdyl* mutation compromises the ability of retinal circuits to reset their sensitivity under varying light conditions. We used an ERG protocol to directly test this possibility. Zebrafish larvae were thoroughly dark-adapted and then exposed to two light flashes of equal intensity. The first (conditioning) flash exposed the dark adapted animals to light, whereas the second (test) flash assessed their ability to adapt to the first flash. We only used larvae that showed a recognizable *b*-wave for this experiment. When the two flashes were bright (452 Cd/m²), there was a clear difference in the response to the second flash between *sdyl* mutants on the one hand and wild type and *alb* on the other (**Fig. 5a**). In *sdyl* mutants, the *b*-wave amplitude elicited by the second flash was smaller than that to the first one, when the two flashes were delivered in 15 s or 30 s intervals. When longer inter-flash intervals were used (45 and 60 s), however, no difference between *sdyl* and wild type was seen. When the first flash was tenfold dimmer (44 Cd/m²), the response to the second flash of the same intensity was unchanged for all genotypes tested (*sdyl*, *alb*, and wild type) and for all intervals used (15, 30, 45, and 60 s; **Fig. 5b**). These findings suggest that the dark-adapted *sdyl* retina does not properly reset its gain when challenged by a bright-light stimulus.

In the paired-flash ERG protocol used here, wild-type eyes recovered from the first flash almost instantaneously, as measured by the *b*-wave, whereas mutant eyes required over 30 s. In the behavioral experiments, recovery of a full-scale OKR occurred on a substantially slower time scale of several minutes, even in wild type. It was therefore important to ask if ERG recordings reflect the changes measured with the OKR. An

affect pigmentation, but closely phenocopied the OKR deficit of *sdyl* mutants following a dark pulse (**Fig. 3**). PTU did not affect the OKR, when the fish remained in continuous light. These experiments support the hypothesis that a product of tyrosinase activity other than melanin is required for bright-light adaptation.

sdyl mutants fail to reset retinal sensitivity

To identify a neural correlate of the observed behavioral deficit in the *sdyl* retina, we turned to electrophysiology. Electroretinograms (ERGs) were recorded from 6–7 dpf wild-type and *sdyl* larvae (Methods). We also tested another albino mutant, *alb*, to determine to what extent the visual phenotype is specific to *sdyl*. The *alb* mutation causes a similar phenotype to *sdyl*, while preserving tyrosinase activity¹⁶. The ERG waveform in response to short light flashes is typically composed of a cornea-negative *a*-wave and a larger, cornea-positive *b*-wave (**Fig. 4a**). The *a*-wave is generated by light-induced suppression of photoreceptor currents, whereas the *b*-wave reflects summed activity in the inner retina, mostly of bipolar cells. We find that dark-adapted *sdyl* retinas are clearly responsive to light. However, while the ERG waveforms of all *alb* mutants tested (**Fig. 4b**) were similar to wild type, those of most *sdyl* mutants (30 of 38) were variable and appeared desynchronized (**Fig. 4c**). In these mutants, distinct, single *b*-waves were replaced by large, irregular oscillations. A minority of *sdyl* mutants, how-

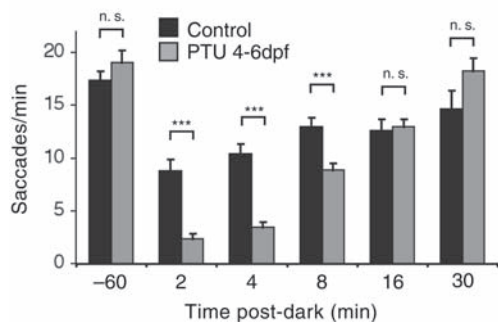


Figure 3 PTU phenocopies the light-adaptation deficit of the *sdv* mutation. Fully pigmented wild-type zebrafish larvae (4 dpf) were incubated in PTU (0.2 mM) for 2 days before OKR measurements. PTU-treated fish were morphologically and behaviorally indistinguishable from controls, except for a suppressed OKR after a dark pulse. Data were plotted as in **Figure 1g**, but were not normalized.

obvious difference between these two experiments is the duration of the light stimulus: In the paired-flash ERG measurement, the conditioning flash was only 10 ms, whereas in the OKR measurement the first recording was made 2 min after onset of continuous light. We more closely simulated in the electrophysiological experiments the circumstances of the OKR measurements by testing conditioning light pulses of varying duration (between 500 and 7000 ms), thus increasing the amount of the adapting light. These experiments were carried out only in wild type. We found that the *b*-wave response elicited by the test flash was suppressed, when longer conditioning steps were applied (**Fig. 6**). The extent of suppression increased with duration of the conditioning light following a first-order exponential function with a time constant of 2.9 s. The fact that the kinetics of recovery of *b*-wave responses depends on the intensity of the conditioning light therefore likely accounts for the slow return of the OKR following darkness.

Phototransduction is normal in *sdv* mutants

Light adaptation occurs in both photoreceptors and in the retinal network downstream of the phototransduction cascade. We asked if we could localize the change in the *sdv* retina more precisely by isolating the photoreceptor response from the rest of the retina. We measured ERG in fish bathed in 2-amino-4-phosphono butyric acid (APB), a glutamate agonist that blocks signaling through the *on* pathway by hyperpolarizing certain bipolar cells. Bath application of this drug isolates the *a*-wave generated by photoreceptors^{27,28}. We first asked if the amplitude and shape of the *a*-wave were changed by the *sdv* mutation. The latency and initial downswing of the *a*-wave were similar between wild

type and mutants, indicating that phototransduction is unimpaired in *sdv* mutants (**Fig. 7a,b**). However, later (> 50 ms), APB-insensitive components of the summed potential are less synchronized resulting in irregular waveforms between trials (**Fig. 7b**). We next asked if *sdv* photoreceptors were hypersensitive by plotting their *a*-wave amplitudes as a function of flash intensity (**Fig. 7c**). Perhaps surprisingly, given the absence of melanin pigment around the eye, photoreceptor light sensitivity was unaltered in *sdv* mutants. The hypersensitivity observed for the ERG *b*-wave (**Fig. 4f**) must therefore be a physiological property of the retinal network, not an optical effect resulting from lack of melanization.

Next we asked if adaptation of phototransduction was impaired by challenging the dark-adapted, APB-treated retina with a pair of short light flashes, as done before for the *b*-wave. We used an interflash interval of 15 s throughout. Even at bright intensities, the *a*-wave response to the second flash was not different between *sdv* and wild type (**Fig. 7d**). This is in contrast to the *b*-wave response of untreated *sdv* to the second flash, which, under the same conditions, had not fully recovered from the first flash (see **Fig. 5a**). Our observations on the isolated *a*-wave place the light-processing deficit of *sdv* mutants firmly in the retinal network, at a cellular stage postsynaptic to photoreceptors.

Network adaptation is out of balance in the *sdv* retina

Dark adaptation and light adaptation in the retinal network are in a dynamic push-and-pull equilibrium, which is controlled by the levels of ambient light. Based on our behavioral and electrophysiological find-

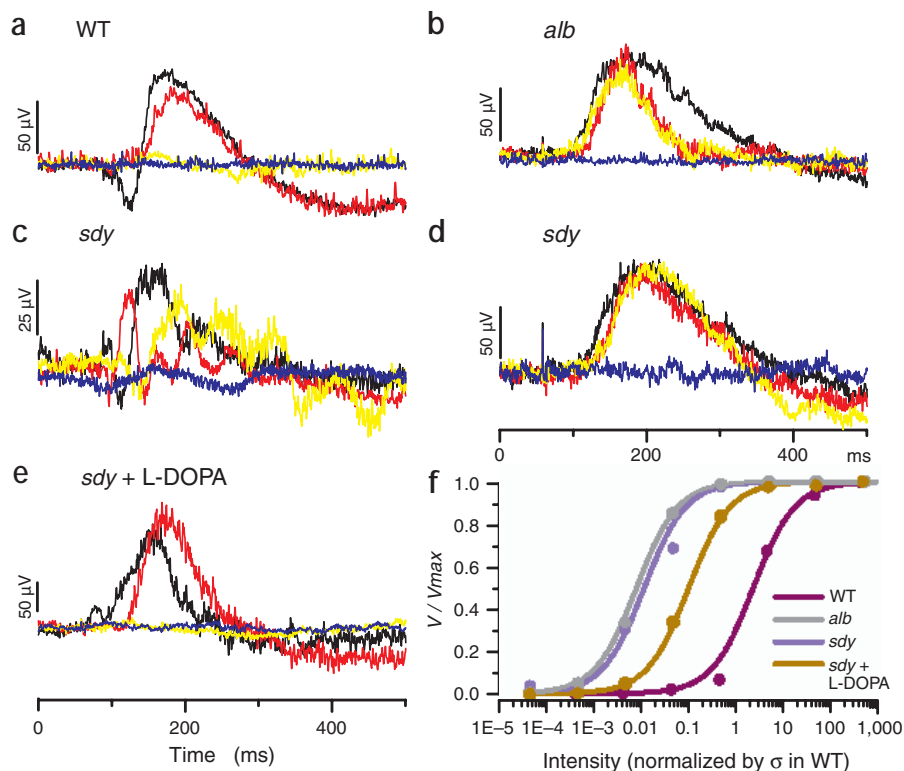


Figure 4 Abnormal electrophysiological light responses of the *sdv* retina. (**a–e**) ERG traces in response to light flashes of varying intensity. Intensities were increased in 2 log₁₀ steps. Traces are color-coded from dimmest to brightest light in the sequence blue, green, red and black. In wild type (WT; **a**), *alb* mutants (**b**) and a minority of *sdv* mutants (**d**), normal *b*-waves and occasionally *a*-waves were detected. In (**c**) most *sdv* mutants, the ERG waveforms were irregular. This deficit could be rescued by applying exogenous L-DOPA (100 μM, bath-applied 30 min before ERG recordings; **e**). (**f**) *b*-wave amplitudes, normalized to maximum, as a function of light intensity, fitted by the Naka-Rushton relationship (Methods). *sdv* and *alb* retinæ are about 2 log units more sensitive than wild type. Hypersensitivity is partially rescued by exogenous L-DOPA (100 μM).

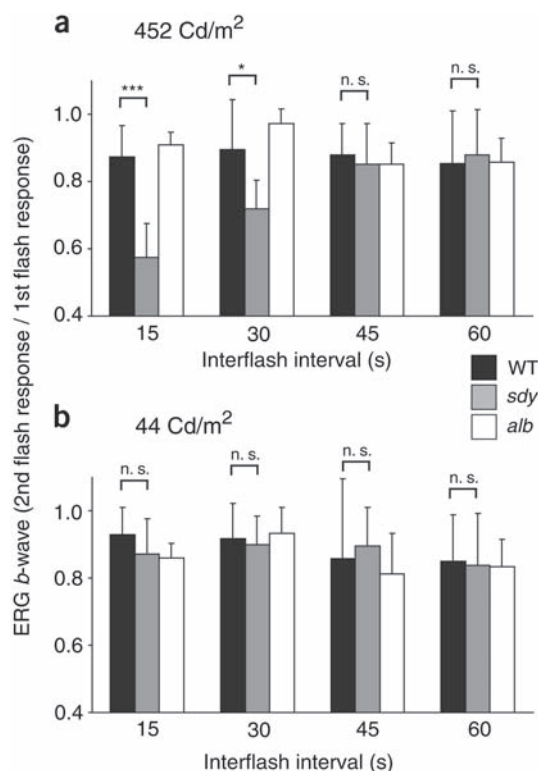


Figure 5 Recovery from a light flash is disrupted in the *sd*y retina. Two flashes (10 ms) of equal intensity were applied, spaced 15, 30, 45 or 60 s. To measure recovery between flashes, the *b*-wave peak value to the second stimulus was divided by the one to the first. **(a)** Response to a pair of flashes of bright light (452 Cd/m²). *sd*y (*n* = 8) responses do not fully recover when the second flash is presented 15 or 30 s after the first. Wild type (*n* = 8) and *alb* (*n* = 6) are unaffected. **(b)** Response to a pair of flashes of tenfold dimmer light (44 Cd/m²). All genotypes, wild type (*n* = 9), *sd*y (*n* = 9) and *alb* (*n* = 5), respond with undiminished strength to the second flash. Significance was calculated for the entire data set using repeated-measures ANOVA ($P < 0.001$). For pairs of data, we used a two-tailed Student's *t*-test. * $P < 0.05$; *** $P < 0.001$; n. s., not significant. Error bars, s.d.

a striking inability to properly set its light sensitivity as background luminance changes. This defect manifests itself when the dark-adapted larval retina is challenged by bright light. Following light onset, *sd*y mutants are initially unresponsive and recover with a sluggish time course. The same defect is seen in fully pigmented animals, when they are treated before the experiment with the tyrosinase inhibitor PTU, and it is not observed in *alb* mutants, which lack melanin but have functional tyrosinase¹⁶. These experiments argue that a tyrosinase product other than melanin is responsible for the enzyme's function in light adaptation.

The neural correlate of the adaptation deficit was traced, by ERG recordings, to the retinal network postsynaptic to the photoreceptors. The dark-adapted *sd*y retina exhibits irregular ERG *b*-wave responses and fails to recover from short light flashes. We could not detect a difference in sensitivity or adaptation of the isolated *a*-wave, suggesting that phototransduction is unaffected. The *b*-wave defect is worsened by increasing either the intensity or the duration of the light stimulus, closely simulating the behavioral effect of continuous light in the OKR protocol. Taken together, these data argue that our electrophysiological measurements have identified the neural substrate (and thus the cause) of the observed behavioral changes.

Both our behavioral and electrophysiological findings suggest that a signal necessary to adapt to bright light is missing in *sd*y mutants. The light sensitivity of the retinal network is adjusted by ambient light, acting via secreted factors^{30–32}. The dark-adaptive neuromodulator melatonin is in a dynamic push-and-pull equilibrium with light-adaptive factors. Failure to light adapt, as shown here following mutation of tyrosinase, is therefore expected to render the retina overly susceptible to melatonin. This tendency was confirmed by supplying exogenous melatonin to *sd*y mutants, which resulted in rapid loss of visual responses. Conversely, blocking melatonin signaling partially rescued visual responses following a dark pulse in *sd*y

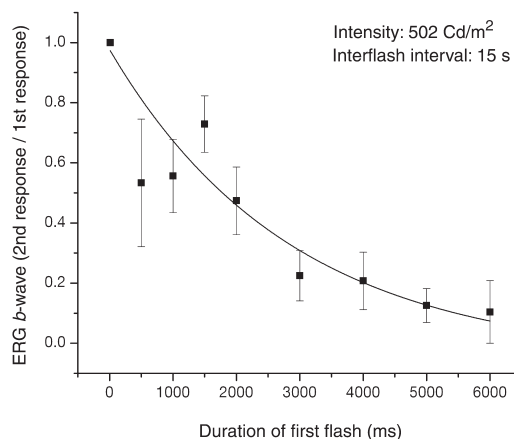
ings, we predicted that the *sd*y mutation might shift the balance in favor of dark adaptation. If this prediction holds, visual behavior following a dark pulse should recover more quickly, when dark adaptation is blocked. Moreover, the effect of the dark pulse should be simulated, in continuous light, by addition of an agent that promotes dark adaptation.

In support of the prediction, the effect of a dark pulse on the OKR was mimicked pharmacologically by supplying melatonin (1 mM) in the light (**Fig. 8**). Upon repeated stimulation with a moving grating, we observed that *sd*y mutants became unresponsive within 5 min after addition of melatonin (data not shown), whereas wild type remained responsive for over 30 min. Furthermore, the *sd*y OKR was partially rescued by supplying DH-97 (2 mM), a specific antagonist of retinal melatonin receptors²⁹, for one hour before and during the dark pulse (**Fig. 8**). Under all treatment conditions, *alb* mutants behaved very similarly to wild type (**Fig. 8**). These experiments suggested that the *sd*y retina has a propensity to dark-adapt, either because it overproduces a dark-adaptive signal or, more likely, because it lacks a light-adaptive signal that opposes the action of melatonin.

DISCUSSION

The *sd*y gene encodes the zebrafish homolog of tyrosinase, an enzyme that functions in melanin synthesis. We demonstrate here, using behavioral and electrophysiological measurements, that the *sd*y mutant has

Figure 6 Recovery of sensitivity in the light is slowed down as the duration of the adapting light increases. A paired-flash protocol was used, similar to **Figure 5a**, but here the duration of the first (conditioning) flash was varied from 500 to 7000 ms (compared to 10 ms in **Fig. 5**). Bright light flashes (502 Cd/m²) and an interflash interval of 15 s were used in all experiments. The amplitude of the second *b*-wave response divided by that of the first *b*-wave response was plotted as a function of first-flash duration, and the data were fitted by a single exponential with a time constant of 2.9 s. Only wild-type fish (*n* = 15) were tested. Error bars, s.e.m.



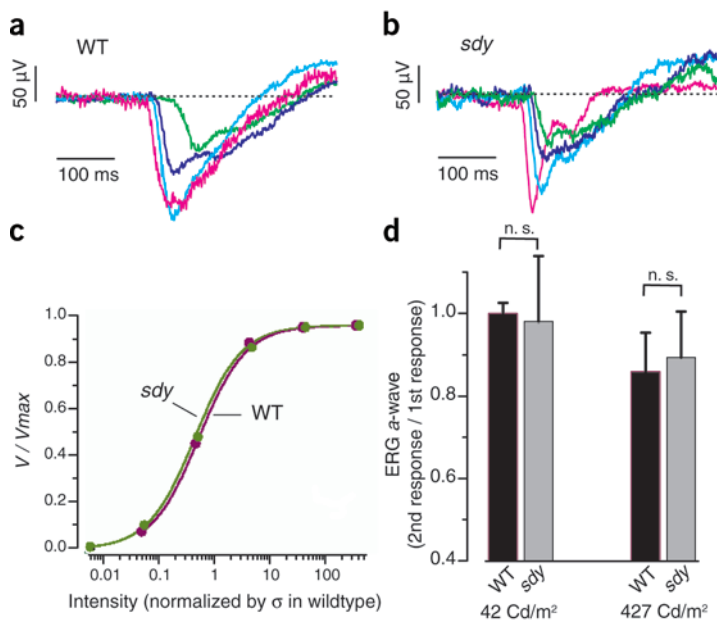


Figure 7 Photoreceptors function normally in *sd*y mutants. (a,b) Typical ERG signals recorded in individual, dark-adapted larval zebrafish (a, WT; b, *sd*y) treated with 1 mM APB (Methods). Selected, non-averaged signals were generated by flashes (10 ms) of the following intensities (in Cd/m²): 0.38 (green line), 3.8 (blue), 38.3 (aqua) and 383 (red). (c) Naka-Rushton functions (Methods) optimally fit to data from wild type ($n = 6$) and *sd*y ($n = 5$). Sensitivity of phototransduction (expressed as the intensity required to elicit half-maximal response, σ) is similar between wild types (0.31) and mutants (0.28). (d) Paired-flash stimulation, as in Figure 3, for two light intensities and with a constant interflash interval of 15 s. Recovery of the *a*-wave from the conditioning flash is normal in *sd*y mutants (two-tailed *t*-test, $P = 0.73$). Data are from 11 *sd*y and 11 wild type at the high light intensity and 4 *sd*y and 3 wild type at the lesser intensity. Error bars, s.d.

mutants. Hence, the main problem of the *sd*y retinal network seems to be an imbalance between light adaptation and dark adaptation.

The molecular nature of *sd*y suggests that the light-adaptive signal missing in the mutant could be dopamine. Dopamine secretion is thought to be critical for light adaptation⁵. Tyrosinase is expressed in the retinal pigment epithelium (RPE), which surrounds and supports the photoreceptor cells. It catalyzes the hydroxylation of tyrosine to L-DOPA, as well as the subsequent oxidation of L-DOPA to dopaquinone. In zebrafish, we have observed that AADC, the enzyme that converts L-DOPA to dopamine, is expressed in photoreceptors, dopaminergic IPCs and possibly horizontal cells (data not shown). In a simple model, the RPE may produce L-DOPA and secrete it into the extracellular space surrounding the photoreceptors, which take it up and convert it to dopamine. Dopamine may then be released into the extracellular space, where it acts on dopamine receptors at several sites in the retinal circuitry. This signal might reset the retinal gain, enabling the visual circuitry to cope with brighter light. Any of the steps required for synthesis, release or uptake of L-DOPA could be regulated by light. Indeed, several cell types in the retina, in addition to photoreceptors, are light-sensitive and express opsins, including the RPE itself^{33,34}. Loss-of-function mutations of tyrosinase are expected to remove the supply of L-DOPA derived from the RPE, resulting in a chronic deficiency of dopamine.

Consistent with this hypothesis, bath application of L-DOPA normalized ERG *b*-waves in *sd*y mutants, although the rescue was only

partial. We also tested whether OKR behavior could be restored by L-DOPA. These experiments remained inconclusive, because bath-applied L-DOPA, as well as various dopamine agonists, were found to block eye movements altogether, probably by a direct effect on brain-stem premotor circuits^{35,36} (H.B., unpublished observations). It could be useful to know the dopamine concentrations in the retinas of mutant and wild-type zebrafish larvae, although these measurements are technically difficult and may not have sufficient spatial resolution to reveal

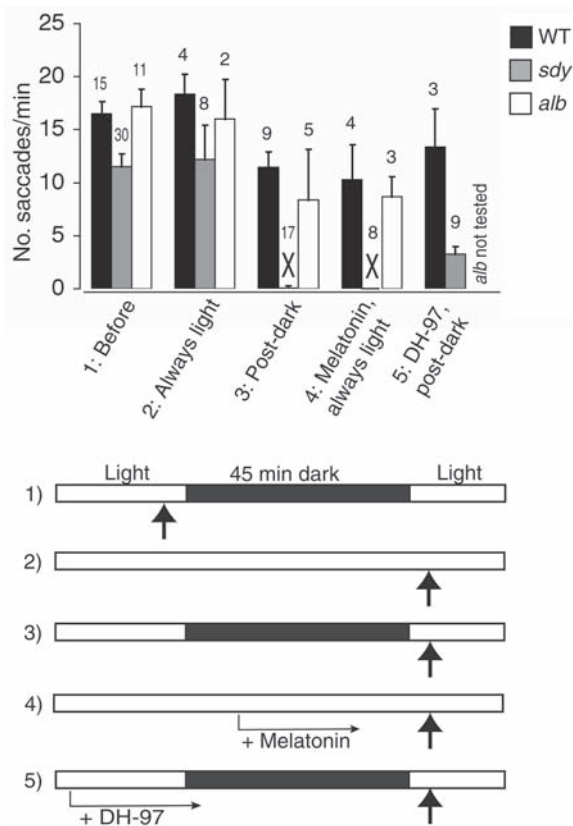


Figure 8 Dark and light adaptation are out of balance in *sd*y mutants. X indicates absence of a measurable OKR. The various treatment regimes are depicted below the graph, with horizontal arrows indicating presence of drugs. The horizontal arrow indicates the time of OKR measurement. Conditions 1 and 3: A dark pulse (45 min) abolishes the OKR of previously responsive mutants for the first 5 min after light onset. Condition 2 (control): Fish that are embedded in methyl cellulose, but remain in continuous light, show undiminished responses. Condition 4: Melatonin (1 mM, 30 min) mimics the effect of a dark pulse, even in continuous light. Condition 5: The melatonin antagonist DH-97 (2 mM, applied 1 hour before the dark pulse) partially rescues the after-dark OKR of *sd*y mutants. The *alb* mutants were not tested in condition 5. Number of fish tested is given above the bars. Error bars, s.e.m.

a difference. As dopamine is known to have multiple, localized effects on retinal circuitry⁵, including effects on the inner retina that promote dark adaptation¹², a direct test of the dopamine hypothesis will have to await the development of better methods to manipulate dopaminergic signaling in the zebrafish retina.

Others have reported that tyrosinase contributes to dopamine synthesis in the striatum of mice³⁷, complementing the canonical pathway via TH. A similar additive effect of the tyrosinase and TH pathways may be at work in the retina. Although the retinas of *sdv* mutants switch poorly from dark to light, they do not become trapped in the dark state indefinitely, possibly because the TH-expressing IPCs continue to supply dopamine. (Additionally, melatonin is downregulated during the day, thus releasing its 'grip' on the retinal network.) This model explains why, after transition from dark to light, *sdv* mutants first lose, but then slowly regain, their vision.

When applied to the dark-adapted fish retina, dopamine triggers the biochemical and structural changes in the retinal network that are characteristic of light adaptation, such as spinule formation and retinomotor movements^{6,38}. In mice lacking the D4 dopamine receptor in photoreceptors, adjustment of gain in the transmission of light responses from photoreceptors to inner retinal neurons is disrupted³⁹ (similar to the phenotype observed by us in *sdv* mutants). In contrast to the *sdv* mutation in zebrafish and the *D4* knockout in mice, ablation of the dopaminergic IPCs by 6-hydroxydopamine (6-OHDA) has a relatively moderate effect, although brightness perception is clearly increased¹¹. In these dopamine-depleted retinas, ERG waveforms are normal^{10,12}, spinules may still form (although at suppressed levels)^{14,15}, and retinomotor movements are intact^{8,9}. These observations have been taken as evidence that dopamine is sufficient, but not necessary for light adaptation. Our model provides an alternative interpretation by proposing that there are two independent dopamine-synthetic pathways in the retina, only one of which is removed by IPC ablation. Interestingly, a lack of L-DOPA during development seems to cause some aspects of the ocular syndrome in albino mammals^{40,41}. Moreover, dopaminergic signaling starts in the embryonic chick retina before TH is detectable⁴². Thus, evidence is converging on a tight link between tyrosinase activity and dopamine in the vertebrate retina.

METHODS

Animals. All experiments were done on zebrafish larvae at 6–7 dpf. The *sdv* and *alb* mutations used in this study were induced by ethyl nitrosourea and result in complete melanin deficiency. The *sdv*^{tk20} allele was described earlier¹⁷. The *sdv*^{s355}, *sdv*^{s901}, *alb*^{s3567} and *alb*^{s1154} alleles were discovered in a *de novo* mutagenesis screen (H.B., unpublished data).

OKR assay. Details of the larval OKR assay have been described²³. Briefly, larvae were mounted in 2.5% methylcellulose and placed in a circular drum. Motion stimuli were generated on a computer using NIH Object Image (<http://rsb.info.nih.gov/nih-image/>) and projected into the drum with an LCD projector (InFocus LP-755). The stimulus was a high-contrast sinewave grating, with a period of 36° per cycle, moving at a velocity of 18°/s. Mean ambient light level in the drum was 200–400 Cd/m², as measured with a calibrated luminometer. Eye movements were recorded for 1 min, at 2 frames per second, and analyzed using Object Image²³. The OKR was measured by counting the number of reset saccades against the direction of motion, corrected for random eye movements by subtracting the number of saccades with the motion.

Cloning of the *sdv* gene. The *sdv* mutation was genetically mapped to a 2-centimorgan interval on chromosome 15, near the microsatellite markers *z9773* and *z7216*. The zebrafish tyrosinase gene was cloned by degenerate PCR and found to localize between these markers on the physical map, using the T51 radiation-hybrid panel⁴³. For the rescue experiments, we used the previously described medaka tyrosinase construct pTyr11, kindly provided by H. Inagaki and H. Hori²⁶. The frame-shift mutant, pTyr11Δ, was constructed by digesting

pTyr11 with BstBI, filling in the ends with T4 DNA polymerase, and ligating. The resulting plasmid has a truncated open reading frame.

ERG recordings. Anesthetized larvae (0.003% tricaine) were placed on a water-soaked filter paper connected to ground with a platinum wire electrode. Control experiments demonstrated that the anesthetic had no effect on the ERG. Under dim red light, a saline-filled glass capillary, pulled to a 5–10 μm tip and connected to the recording amplifier via a Ag/AgCl electrode, was placed on the larval eye, just above the iris. The fish were then allowed to thoroughly dark-adapt for 20 min and ERG-recorded in response to flashes of varying intensity. Fish larvae were uniformly illuminated from above with a collimated light beam (about 4 mm in diameter). Stimulus light was generated with a DC-operated 150 W Xenon lamp. The light spectrum was nearly flat in the visible range, as is typical for Xenon emission. Stimulus intensity was measured with a calibrated luminometer placed at the position of the larva in the recording apparatus. Differential signals between the active electrode (selected to be positive) and ground were recorded with a high-gain, high-impedance amplifier (Model 1800, A-M Systems) and bandpass-filtered between 1 and 1,000 Hz. Signals were digitized online at 2 kHz, stored, and later analyzed using pClamp (Axon Instruments). When using dim light stimuli, 8–16 signals were averaged to reduce noise. Designated functions were fit to experimental data with non-linear, least-square minimization algorithms (Origin software, MicroCal). The light sensitivity of the *b*-wave is well described by the Naka-Rushton relation $V(I) = V_{max} * I / (I + \sigma)$, where V_{max} is the amplitude of the *b*-wave, I is light intensity, and σ is a constant equal to the intensity at half-maximum amplitude.

For the paired-flash experiments, we first measured the ERG generated by the standard test flash followed by another 20 min of dark adaptation in a dark-adapted fish. Pairs of flashes (conditioning and test flashes) were repeatedly presented to the same fish with 10 min darkness between them. In any one fish, between 2 and 6 pairs were tested, limited by the stability of the preparation. Flash intensities, durations and interflash intervals were varied as described in the figure legends.

To isolate the *a*-wave, larvae were submerged in fish water containing 1 mM APB (Sigma), pH 7.0, for 5 min. The fish were then transferred onto the filter paper support in the ERG recording chamber, soaked in the same APB-containing solution. Light-adapted fish were first tested to ensure that the *b*-wave was completely blocked by the drug and were then allowed to dark adapt for 20 min before commencing test recordings. The same photostimulation and data recording protocols were used as above, but this time the peak amplitude of the negative signal (*a*-wave) was measured. The *a*-wave amplitude-intensity relationship was fitted to the Naka-Rushton equation (above).

Pharmacology. L-DOPA, melatonin and PTU were purchased from Sigma. DH-97 was from Tocris. Pharmacological agents were dissolved to their final concentration in water on the day of the experiment. Individual methylcellulose-mounted fish were immersed in a drop (100 μl) of the drug. For longer incubations, the liquid surrounding the fish was renewed every 60 min.

Accession Numbers. The GenBank accession number for zebrafish tyrosinase is NM_131013.

ACKNOWLEDGMENTS

We thank J. Kay, A. Wehman, S. Taha, and M. Orger for comments on the manuscript and all members of our laboratory for discussions. We also thank A. Churchland and L. Gitlin for assistance in the initial identification of zebrafish tyrosinase. This study was supported by a NARSAD Young Investigator award and the UCSF Neuroscience training grant (P.P.-M.), a B.I.F. fellowship (T.R.), the Packard Foundation, the Sloan Foundation and the NIH (H.B.).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 15 July; accepted 26 August 2004

Published online at <http://www.nature.com/natureneuroscience/>

1. Fain, G.L., Matthews, H.R., Cornwall, M.C. & Koutalos, Y. Adaptation in vertebrate photoreceptors. *Physiol. Rev.* **81**, 117–151 (2001).
2. Deary, A. & Burnside, B. Dopaminergic regulation of cone retinomotor movement in isolated teleost retinas: I. Induction of cone contraction is mediated by D2 receptors. *J. Neurochem.* **46**, 1006–1021 (1986).

3. Wagner, H.J. & Djamgoz, M.B. Spinules: a case for retinal synaptic plasticity. *Trends Neurosci.* **16**, 201–206 (1993).
4. Biehlmaier, O., Neuhauss, S.C. & Kohler, K. Synaptic plasticity and functionality at the cone terminal of the developing zebrafish retina. *J. Neurobiol.* **56**, 222–236 (2003).
5. Witkovsky, P. Dopamine and retinal function. *Doc. Ophthalmol.* **108**, 17–40 (2004).
6. Djamgoz, M.B. & Wagner, H.J. Localization and function of dopamine in the adult vertebrate retina. *Neurochem. Int.* **20**, 139–191 (1992).
7. Dowling, J.E. & Ehinger, B. Synaptic organization of the amine-containing interplexiform cells of the goldfish and Cebus monkey retinas. *Science* **188**, 270–273 (1975).
8. Douglas, R.H., Wagner, H.J., Zaunreiter, M., Behrens, U.D. & Djamgoz, M.B. The effect of dopamine depletion on light-evoked and circadian retinomotor movements in the teleost retina. *Vis. Neurosci.* **9**, 335–343 (1992).
9. Ball, A.K., Baldrige, W.H. & Fernback, T.C. Neuromodulation of pigment movement in the RPE of normal and 6-OHDA-lesioned goldfish retinas. *Vis. Neurosci.* **10**, 529–540 (1993).
10. Lin, Z.S. & Yazulla, S. Depletion of retinal dopamine does not affect the ERG b-wave increment threshold function in goldfish *in vivo*. *Vis. Neurosci.* **11**, 695–702 (1994).
11. Lin, Z.S. & Yazulla, S. Depletion of retinal dopamine increases brightness perception in goldfish. *Vis. Neurosci.* **11**, 683–693 (1994).
12. Li, L. & Dowling, J.E. Effects of dopamine depletion on visual sensitivity of zebrafish. *J. Neurosci.* **20**, 1893–1903 (2000).
13. Yang, X.L., Tornqvist, K. & Dowling, J.E. Modulation of cone horizontal cell activity in the teleost fish retina. II. Role of interplexiform cells and dopamine in regulating light responsiveness. *J. Neurosci.* **8**, 2269–2278 (1988).
14. Wagner, H.J., Behrens, U.D., Zaunreiter, M. & Douglas, R.H. The circadian component of spinule dynamics in teleost retinal horizontal cells is dependent on the dopaminergic system. *Vis. Neurosci.* **9**, 345–351 (1992).
15. Yazulla, S., Lin, Z.S. & Studholme, K.M. Dopaminergic control of light-adaptive synaptic plasticity and role in goldfish visual behavior. *Vision Res.* **36**, 4045–4057 (1996).
16. Haffter, P. *et al.* Mutations affecting pigmentation and shape of the adult zebrafish. *Dev. Genes Evol.* **206**, 260–276 (1996).
17. Kelsh, R.N. *et al.* Zebrafish pigmentation mutations and the processes of neural crest development. *Development* **123**, 369–389 (1996).
18. Neuhauss, S.C. *et al.* Genetic disorders of vision revealed by a behavioral screen of 400 essential loci in zebrafish. *J. Neurosci.* **19**, 8603–8615 (1999).
19. Jeffery, G. The albino retina: an abnormality that provides insight into normal retinal development. *Trends Neurosci.* **20**, 165–169 (1997).
20. Easter, S.S. Jr. & Nicola, G.N. The development of vision in the zebrafish (*Danio rerio*). *Dev. Biol.* **180**, 646–663 (1996).
21. Bilotta, J., Saszik, S. & Sutherland, S.E. Rod contributions to the electroretinogram of the dark-adapted developing zebrafish. *Dev. Dyn.* **222**, 564–570 (2001).
22. Brockerhoff, S.E. *et al.* A behavioral screen for isolating zebrafish mutants with visual system defects. *Proc. Natl. Acad. Sci. USA* **92**, 10545–10549 (1995).
23. Roeser, T. & Baier, H. Visuomotor behaviors in larval zebrafish after GFP-guided laser ablation of the optic tectum. *J. Neurosci.* **23**, 3726–3734 (2003).
24. Oetting, W.S. & King, R.A. Molecular basis of albinism: mutations and polymorphisms of pigmentation genes associated with albinism. *Hum. Mutat.* **13**, 99–115 (1999).
25. Opitz, S., Kasmann-Kellner, B., Kaufmann, M., Schwinger, E. & Zuhlke, C. Detection of 53 novel DNA variations within the tyrosinase gene and accumulation of mutations in 17 patients with albinism. *Hum. Mutat.* **23**, 630–631 (2004).
26. Inagaki, H., Koga, A., Bessho, Y. & Hori, H. The tyrosinase gene from medakafish: transgenic expression rescues albino mutation. *Pigment Cell Res.* **11**, 283–290 (1998).
27. Van Epps, H.A., Yim, C.M., Hurley, J.B. & Brockerhoff, S.E. Investigations of photoreceptor synaptic transmission and light adaptation in the zebrafish visual mutant nrc. *Invest. Ophthalmol. Vis. Sci.* **42**, 868–874 (2001).
28. Seeliger, M.W., Rilk, A. & Neuhauss, S.C. Ganzfeld ERG in zebrafish larvae. *Doc. Ophthalmol.* **104**, 57–68 (2002).
29. Behrens, U.D., Douglas, R.H., Sugden, D., Davies, D.J. & Wagner, H.J. Effect of melatonin agonists and antagonists on horizontal cell spinule formation and dopamine release in a fish retina. *Cell Tissue Res.* **299**, 299–306 (2000).
30. Cahill, G.M. & Besharse, J.C. Resetting the circadian clock in cultured *Xenopus* eyecups: regulation of retinal melatonin rhythms by light and D2 dopamine receptors. *J. Neurosci.* **11**, 2959–2971 (1991).
31. Manglapus, M.K., Iuvone, P.M., Underwood, H., Pierce, M.E. & Barlow, R.B. Dopamine mediates circadian rhythms of rod-cone dominance in the Japanese quail retina. *J. Neurosci.* **19**, 4132–4141 (1999).
32. Zaunreiter, M., Brandstatter, R. & Goldschmid, A. Evidence for an endogenous clock in the retina of rainbow trout: I. Retinomotor movements, dopamine and melatonin. *Neuroreport* **9**, 1205–1209 (1998).
33. Wu, J., Peachey, N.S. & Marmorstein, A.D. Light-evoked responses of the mouse retinal pigment epithelium. *J. Neurophysiol.* **91**, 1134–1142 (2004).
34. Peirson, S.N. *et al.* Expression of the candidate circadian photopigment melanopsin (Opn4) in the mouse retinal pigment epithelium. *Brain Res. Mol. Brain Res.* **123**, 132–135 (2004).
35. Tychsen, L. & Sitaram, N. Catecholamine depletion produces irrepressible saccadic eye movements in normal humans. *Ann. Neurol.* **25**, 444–449 (1989).
36. Salas, C., Navarro, F., Torres, B. & Delgado-Garcia, J.M. Effects of diazepam and D-amphetamine on rhythmic pattern of eye movements in goldfish. *Neuroreport* **3**, 131–134 (1992).
37. Rios, M. *et al.* Catecholamine synthesis is mediated by tyrosinase in the absence of tyrosine hydroxylase. *J. Neurosci.* **19**, 3519–3526 (1999).
38. Witkovsky, P. & Shi, X.P. Slow light and dark adaptation of horizontal cells in the *Xenopus* retina: a role for endogenous dopamine. *Vis. Neurosci.* **5**, 405–413 (1990).
39. Nir, I. *et al.* Dysfunctional light-evoked regulation of cAMP in photoreceptors and abnormal retinal adaptation in mice lacking dopamine D4 receptors. *J. Neurosci.* **22**, 2063–2073 (2002).
40. Libby, R.T. *et al.* Modification of ocular defects in mouse developmental glaucoma models by tyrosinase. *Science* **299**, 1578–1581 (2003).
41. Illia, M. & Jeffery, G. Retinal mitosis is regulated by dopa, a melanin precursor that may influence the time at which cells exit the cell cycle: analysis of patterns of cell production in pigmented and albino retinas. *J. Comp. Neurol.* **405**, 394–405 (1999).
42. Kubrusly, R.C. *et al.* L-DOPA supply to the neuro retina activates dopaminergic communication at the early stages of embryonic development. *J. Neurochem.* **86**, 45–54 (2003).
43. Geisler, R. *et al.* A radiation hybrid map of the zebrafish genome. *Nat. Genet.* **23**, 86–89 (1999).