immunotoxin (saporin linked to a nonspecific mouse immunoglobulin G) injections, saline injections into subplate, and kainate injections into layer 4 have no effect on ODCs (*5*, *16*) (fig. S3). Furthermore, control immunotoxin and saline injections do not alter synaptic physiology as assessed in slices (fig. S5). Layer 4 injections of kainate or saline injections into subplate, although right at the site of the disconnection effects and causing mechanical damage from the injection needle, do not disrupt any known aspect of cortical structure or gene expression that have been monitored (*5*, *16*) (fig. S6).

19. D. Cai, G. C. DeAngelis, R. D. Freeman, *J. Neurophysiol.* **78**, 1045 (1997).
20. G. G. Turrigiano, S. B. Nelson, *Curr. Opin. Neurobiol.* **10**, 358 (2000).
21. J. T. Isaac, M. C. Crair, R. A. Nicoll, R. C. Malenka, *Neuron* **18**, 269 (1997).
22. E. M. Finney, J. R. Stone, C. J. Shatz, *J. Comp. Neurol.* **398**, 105 (1998).
23. E. Friauf, C. J. Shatz, *J. Neurophysiol.* **66**, 2059 (1991).
24. M. C. Crair, R. C. Malenka, *Nature* **375**, 325 (1995).
25. D. E. Feldman, R. A. Nicoll, R. C. Malenka, J. T. Isaac, *Neuron* **21**, 347 (1998).
26. J. T. Trachtenberg, C. Trepel, M. P. Stryker, *Science* **287**, 2029 (2000).
27. J. C. Crowley, L. C. Katz, *Nature Neurosci.* **2**, 1125 (1999).
28. M. Weliky, L. C. Katz, *Science* **285**, 599 (1999).
29. P. O. Kanold, P. Kara, R. C. Reid, C. J. Shatz, unpublished data.
30. E. S. Lein, A. Hohn, C. J. Shatz, *J. Comp. Neurol.* **420**, 1 (2000).
31. We thank R. Born for helpful discussions; M. Marcotrigiano, B. Printseva, and S. Yurgensen for surgical, histological, and technical assistance; and D. Butts, M. Majdan, J. Syken, and Y. Tagawa for helpful comments on the manuscript. Supported by NIH R01 EY02858 (C.J.S.), F32 EY1352 (P.O.K.), R01 EY10115 (R.C.R.), and P30 EY12196 (R.C.R.).

Supporting Online Material

www.sciencemag.org/cgi/content/full/301/5632/521/DC1
Materials and Methods
SOM Text
Figs. S1 to S6

4 March 2003; accepted 13 June 2003

Melanopsin Is Required for Non–Image-Forming Photic Responses in Blind Mice

Satchidananda Panda,[1,2]* Ignacio Provencio,[4]* Daniel C. Tu,[5]* Susana S. Pires,[1] Mark D. Rollag,[4] Ana Maria Castrucci,[4,7] Mathew T. Pletcher,[1,2] Trey K. Sato,[1,2] Tim Wiltshire,[1] Mary Andahazy,[1] Steve A. Kay,[2] Russell N. Van Gelder,[5,6] John B. Hogenesch[1,3]†

Although mice lacking rod and cone photoreceptors are blind, they retain many eye-mediated responses to light, possibly through photosensitive retinal ganglion cells. These cells express melanopsin, a photopigment that confers this photosensitivity. Mice lacking melanopsin still retain nonvisual photoreception, suggesting that rods and cones could operate in this capacity. We observed that mice with both outer-retinal degeneration and a deficiency in melanopsin exhibited complete loss of photoentrainment of the circadian oscillator, pupillary light responses, photic suppression of arylalkylamine-*N*-acetyltransferase transcript, and acute suppression of locomotor activity by light. This indicates the importance of both nonvisual and classical visual photoreceptor systems for nonvisual photic responses in mammals.

The eye is the principal mediator of light input to the central nervous system in mammals. In addition to vision, the eye mediates several nonvisual responses to light, including photoentrainment of the circadian oscillator, constriction of the pupil, acute suppression of pineal melatonin, acute suppression of activity (masking) in nocturnal mammals, and regulation of sleep latency. Many of these responses persist in mice that are visually blind from outer-retinal degeneration but are abolished by bilateral enucleation of the eyes (*1*). Here, we demonstrate the presence of inner-retinal, nonvisual ocular photoreceptors that specifically subserve these nonvisual photic responses.

Intrinsically photosensitive retinal ganglion cells (ipRGCs) (*2*, *3*) project to brain sites that mediate many of these ocular, yet nonvisual, responses to light, including the suprachiasmatic nucleus (SCN), the intergeniculate leaflet, and the olivary pretectal nucleus, which mediates pupillary light reflexes (PLR) (*1*). The photosensitivity of these cells ex vivo depends on the presence of melanopsin (Opn4), a member of the opsin family of photopigment proteins (*4*). Whereas melanopsin-deficient (*Opn4*$^{-/-}$) mice exhibit moderate attenuation in light-induced phase resetting of the circadian oscillator (*5*, *6*) and reduced PLR under high irradiance levels (*4*), most nonvisual photic responses in these mice remain largely intact. This suggests either the presence of additional inner-retinal photoreceptors, or contributions from the outer-retinal classical photoreceptors to nonvisual photoresponses. To test the latter hypothesis, we generated mice that were deficient in both melanopsin and classical photoreceptors by breeding *Opn4*$^{-/-}$ mice (*5*) with the C3H/HeJ mouse strain that carries the retinal degeneration (*rd*) mutation (*7*). Mice homozygous for the *rd* allele are visually blind as a result of a primary degeneration of the rods and a secondary loss of cones, but they retain melanopsin-containing RGCs (fig. S1). The *Opn4*$^{-/-}$; *rd*/*rd* mice were healthy and viable with intact optic nerves. Outer retinal degeneration was indistinguishable between *rd*/*rd* and *Opn4*$^{-/-}$; *rd*/*rd* mice (fig. S1).

To assess the circadian photoentrainment and acute light suppression of activity, we subjected the *Opn4*$^{-/-}$; *rd*/*rd* mice, littermate wild-type, *rd*/*rd*, and *Opn4*$^{-/-}$ mice to a 24-hour light:dark (LD) cycle (8L:16D) (*7*). Under conditions of constant darkness (DD), mice have a free-running circadian locomotor period of less than 24 hours. However, in a 24-hour LD cycle, photic input to the oscillator makes a small phase adjustment in each cycle and synchronizes the clock to an exact 24-hour period (photoentrainment). Wild-type mice and the single *Opn4*$^{-/-}$ and *rd*/*rd* mutants entrained normally and consolidated their wheel-running activity to the dark period of the LD cycle (Fig. 1) as has been previously reported (*5*, *6*, *8*). In contrast, the *Opn4*$^{-/-}$; *rd*/*rd* mice failed to entrain to the external lighting cycle and continued to exhibit free-running rhythms (Fig. 1 and Table 1). In addition, increasing the light intensity to 800 lux during the photoperiod and increasing the photoperiod to 12 hours failed to entrain these mice (Fig. 1 and Table 1; fig. S2).

All four genotypes exhibited free-running DD periods of <24 hours (Table 1). Under constant light (LL) conditions, most nocturnal

[1]Genomics Institute of the Novartis Research Foundation, 10675 John J. Hopkins Drive, San Diego, CA 92121, USA. [2]Department of Cell Biology, [3]Department of Neuropharmacology, Scripps Research Institute, 10550 North Torrey Pines Road, San Diego, CA 92037, USA. [4]Department of Anatomy, Physiology, and Genetics, Uniformed Services University, 4301 Jones Bridge Road, Bethesda, MD 20814, USA. [5]Department of Ophthalmology and Visual Sciences, [6]Department of Molecular Biology and Pharmacology, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, USA. [7]Department of Physiology, Biosciences Institute, University of São Paulo, Rua do Matão, travessa 14, 05508-900, São Paulo, Brazil.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: hogenesch@gnf.org

rodents demonstrate lengthening of their free-running periods. In LL, wild-type and *rd/rd* mice free ran with comparable periods of more than 24 hrs. As has been previously observed (*5*, *6*), *Opn4*$^{-/-}$ mice exhibited a slightly shorter (albeit >24-hour) period length in LL. The *Opn4*$^{-/-}$; *rd/rd* mice, however, continued to free run with an unchanged, <24-hour period length (Table 1; fig. S2), comparable to the period of free run in DD.

The entrainment phenotype of *Opn4*$^{-/-}$; *rd/rd* mice is thus comparable to that of bilaterally enucleated mice, which also free run in LD conditions (*9*), suggesting that the entrainment deficiency in these mice is a result of a complete loss of photic input to a functioning oscillator in the SCN. Loss of photic entrainment has also been reported in *math5*$^{-/-}$ (*10*), a mutant that fails to develop most RGCs, and in anophthalmic mice (*11*). However, the intrinsic period length of the oscillator in these mice is lengthened. We suspect that the *Opn4*$^{-/-}$; *rd/rd* mouse does not mimic these severe developmental mutants owing to the presence of an intact optic nerve and, presumably, retinohypothalamic tract. Most likely, a light-independent interaction between the inner retina and the SCN neurons is necessary to finely determine the free-running period of the SCN oscillator.

Acute suppression of activity (masking) was tested by a brief pulse of light during the dark phase of the LD cycle (*7*). *Opn4*$^{-/-}$ mice showed reduced masking responses to a 2-hour pulse of white light (100 or 800 lux) administered during the first half of the dark phase (Fig. 2). However, considerable variability was seen in this response; masking was found to be further reduced in the presence of an unlinked genetic locus (fig. S3). The *rd/rd* littermate animals also exhibited reduced masking under low irradiance. In contrast, *Opn4*$^{-/-}$; *rd/rd* showed no masking responses under any irradiance conditions tested (Fig. 2 and Table 1). Because *Opn4*$^{-/-}$ and *rd/rd* mice each exhibit partial deficiency in masking under these lighting conditions, complete absence of masking in the double mutant *Opn4*$^{-/-}$; *rd/rd* mice demonstrates the partially redundant role of these light-signaling pathways.

The PLR to 470-nm blue light was compared among wild-type, *Opn4*$^{-/-}$, *rd/rd*, and *Opn4*$^{-/-}$; *rd/rd* mice (*7*). The PLR of *Opn4*$^{-/-}$ and wild-type littermate control mice were comparable, although at high irradiance levels the maximal pupillary constriction of *Opn4*$^{-/-}$ mice was less than that of the wild type, as has been previously reported (*4*). Whereas *rd/rd* mice showed a ~1 logarithmic unit decrease in sensitivity compared with wild-type animals (*12*, *13*), *Opn4*$^{-/-}$; *rd/rd* mice showed no pupillary constriction at any intensity tested (Fig. 3; movie S1). These mice showed normal pupillary constriction after topical application of pilocarpine, suggesting no defect in pupillary motor function (*14*). Thus, melanopsin is absolutely required for PLR in *rd/rd* mice.

The synthesis of the pineal hormone melatonin is acutely suppressed by light (*15*). Photic suppression of melatonin also persists in the absence of rods and cones (*16*), suggesting a possible role of melanopsin-expressing ipRGCs in this photoresponse. Arylalkylamine *N*-acetyltransferase (AA-NAT) (E.C. 2.3.1.87) is the rate-limiting enzyme of the melatonin biosynthetic pathway. The nocturnal rise in AA-NAT mRNA is acutely inhibited by light (*17*). Photoinhibition of AA-NAT mRNA was measured with quantitative re-

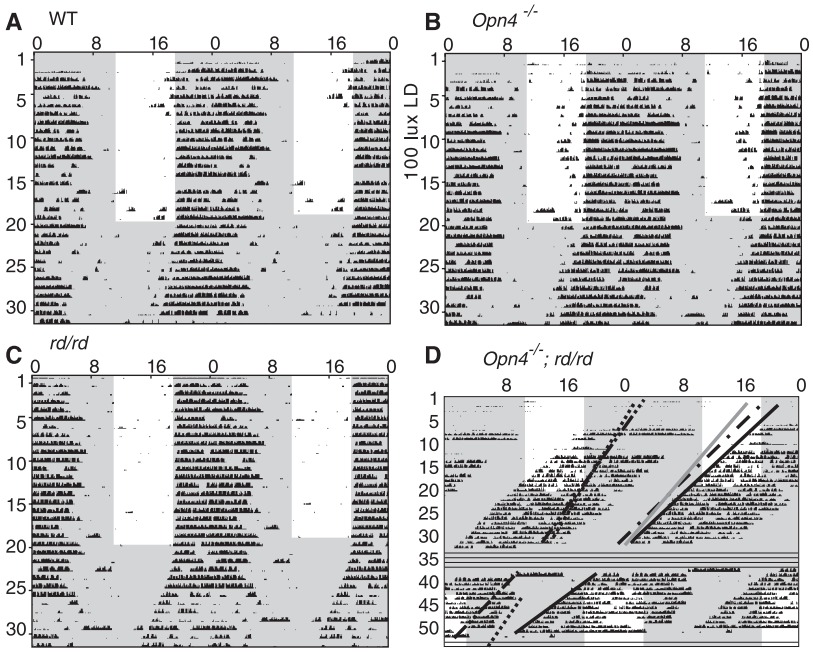

Fig. 1. Entrainment deficiency in *Opn4*$^{-/-}$; *rd/rd* mice. Representative double-plotted wheel-running activity records of mice during photoentrainment and free run in DD. (**A**) Wild-type (WT) mice, (**B**) *Opn4*$^{-/-}$ mice, and (**C**) *rd/rd* mice entrained to a LD cycle of 8 hours of 100-lux white light and 16 hours of darkness (LD::8:16), whereas (**D**) *Opn4*$^{-/-}$; *rd/rd* mice did not entrain. The slope of activity onset is shown with a solid line. The slope of activity onset of four additional *Opn4*$^{-/-}$; *rd/rd* mice is shown with broken or gray lines. After 2 weeks of constant darkness, three *Opn4*$^{-/-}$; *rd/rd* mice were again subjected to entraining conditions of LD::8:16 with 800-lux white light. Local time is indicated at the top, and the light and dark periods are indicated by white and gray backgrounds, respectively.

Table 1. Period length estimates and acute suppression of activity by light. Average period length estimates or percent activity suppression ± SEM (number of animals) are shown. Average values that are significantly different from those of the wild type (Student's *t* test, two tailed, equal variance, $P < 0.005$) are in bold letters. Corresponding *P*-values are shown below the averages.

	Wild type	*Opn4*$^{-/-}$	*rd/rd*	*Opn4*$^{-/-}$; *rd/rd*
		Period length (hours)		
8 hours 100 lux: 16 hours dark	24.00 + 0.003 (8)	23.99 + 0.015 (15)	24.02 + 0.009 (9)	**23.44 + 0.087 (5)**
		0.1603	0.3541	2.96×10^{-14}
8 hours 800 lux: 16 hours dark	24.00 + 0.01 (17)	23.97 + 0.01 (8)	23.98 + 0.02 (16)	**23.49 + 0.06 (11)**
		0.0466	0.2451	2.2×10^{-11}
12 hours 800 lux: 12 hours dark	24.00 + 0.01 (7)	23.97 + 0.03 (3)	24.00 + 0.003 (13)	**23.52 + 0.06 (3)**
		0.1523	0.5974	1.62×10^{-6}
Constant darkness	23.55 + 0.1 (5)	23.64 + 0.06 (7)	23.6 + 0.05 (7)	23.28 + 0.06 (4)
		0.6037	0.4062	0.0587
Constant light (100 lux)	25.28 + 0.14 (17)	**24.33 + 0.23 (6)**	25.65 + 0.27 (7)	**23.32 + 0.16 (8)**
		0.001	0.092	6.73×10^{-9}
		Light suppression of activity (%)		
2 hours 100 lux	75.00 + 3.16 (10)	**38.91 + 9.79 (5)**	56.24 + 7.82 (11)	**2.35 + 9.79 (5)**
		0.0033	0.0838	2.34×10^{-5}
2 hours 800 lux	93.25 + 3.87 (15)	**64.95 + 13.40 (6)**	90.45 + 4.85 (10)	**0.98 + 2.56 (8)**
		0.0023	0.2795	1.7884×10^{-14}

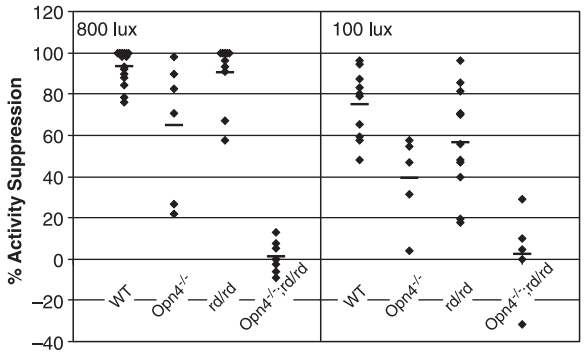

Fig. 2. Acute suppression of locomotor activity by a 2-hour pulse of white light during the early night. The activity suppression is estimated by comparing the percent of daily activity during the time of light pulse (target activity) with the average percent daily activity during a comparable time over three previous nights (control activity) and is calculated as 100 × [(control activity – target activity)/control activity]. Values from individual animals (diamonds) as well as group average values (horizontal bars) are shown.

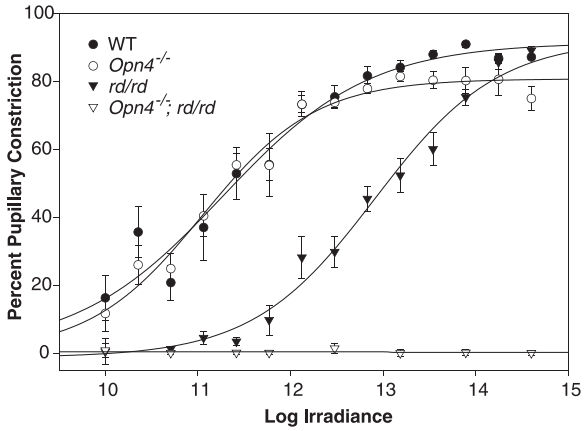

Fig. 3. Irradiance-response curves for pupillary constriction after exposure to 30 seconds of monochromatic 470-nm light. The percent pupillary constriction is calculated as 100 × [1 – (minimum pupil area during the 30-s light pulse/dark-adapted pupil area)]. The mean ± SEM is displayed. *x* axis, log irradiance (photons cm^{-2} s^{-1}).

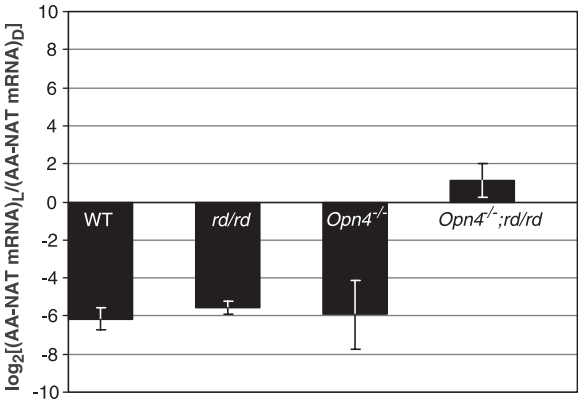

Fig. 4. Photoinhibition of the nocturnal AA-NAT mRNA levels by extension of light (800-lux white light) into the anticipated dark phase. Values represent the mean (±SEM) for the log-transformed ratio of AA-NAT mRNA in mice exposed to light compared with that of genotype-paired, dark-exposed controls.

verse transcription polymerase chain reaction (*7*). Wild-type, *rd*/*rd*, and *Opn4*$^{-/-}$ mice demonstrated photic inhibition of AA-NAT mRNA transcription. In contrast, *Opn4*$^{-/-}$; *rd*/*rd* mice showed no photic inhibition of AA-NAT transcription (Fig. 4).

The most parsimonious explanation of the severe deficiency in nonvisual photic responses in the *Opn4*$^{-/-}$; *rd*/*rd* mice is that either the melanopsin-containing ipRGCs or the classical outer-retinal photoreceptors are sufficient for transducing photic information to critical brain areas. At least partial functional redundancy, thus, exists between rods and/or cones and melanopsin-containing ipRGCs for nonvisual photoreception. Whether the classical photoreceptors function by signaling "through" the ipRGCs (by way of synaptic input to these cells) is not known, but it is suggested by the synaptic contacts of bipolar and amacrine cells onto melanopsin-expressing ipRGCs (*18*). Differences in the neurocircuitry and downstream signaling pathways may underlie the observed differences in the relative contributions of classical rod and cone photoreceptors and of melanopsin-containing ipRGCs to nonvisual photophysiology.

The complete loss of photic responses in *Opn4*$^{-/-}$; *rd*/*rd* mice also demonstrates that no additional photopigments are required for nonvisual photic signaling. Cryptochromes, which function as circadian photopigments in *Arabidopsis* (*19*) and *Drosophila* (*20*), are also expressed in the mammalian eye (*21*). However, a subset of *rd*/*rd* mice lacking cryptochromes still shows masking responses (*22*), and pupillary responses are intact under very bright light (*13*). Melanopsin appears to be expressed normally in eyes of cryptochrome-deficient mice (*22*). It would thus appear that the primary photopigment in nonvisual photoreception is melanopsin dependent, but not cryptochrome dependent.

Multiple photoreceptor systems thus subserve ocular nonvisual photic responses. The integration of photic input from multiple photoreceptors appears to be common to circadian systems across phylogeny and has been noted in *Neurospora*, *Arabidopsis,* and *Drosophila* (*23*, *24*). Presumably, the unique characteristics of each photopigment system and photoreceptor cell type contribute to this integrated response.

References and Notes

1. R. G. Foster, M. W. Hankins, *Prog. Retinal Eye Res.* **21**, 507 (2002).
2. S. Hattar, H. W. Liao, M. Takao, D. M. Berson, K. W. Yau, *Science* **295**, 1065 (2002).
3. D. M. Berson, F. A. Dunn, M. Takao, *Science* **295**, 1070 (2002).
4. R. J. Lucas *et al*., *Science* **299**, 245 (2003).
5. S. Panda *et al*., *Science* **298**, 2213 (2002).
6. N. F. Ruby *et al*., *Science* **298**, 2211 (2002).
7. Materials and methods are available as supporting material on *Science* Online.
8. R. G. Foster *et al*., *J. Comp. Physiol. A* **169**, 39 (1991).
9. R. J. Nelson, I. Zucker, *Comp. Biochem. Physiol. A* **69**, 145 (1981).
10. R. Wee, A. M. Castrucci, I. Provencio, L. Gan, R. N. Van Gelder, *J. Neurosci.* **22**, 10427 (2002).
11. H. Faradji, R. Cespuglio, G. Rondot, L. Paut, M. Jouvet, *Brain Res.* **202**, 41 (1980).
12. R. J. Lucas, R. H. Douglas, R. G. Foster, *Nature Neurosci.* **4**, 621 (2001).
13. R. N. Van Gelder, R. Wee, J. A. Lee, D. C. Tu, *Science* **299**, 222 (2003).
14. S. Panda, J. B. Hogenesch, unpublished observation.
15. D. C. Klein, J. L. Weller, *Science* **177**, 532 (1972).
16. R. J. Lucas, M. S. Freedman, M. Munoz, J. M. Garcia-Fernandez, R. G. Foster, *Science* **284**, 505 (1999).
17. P. H. Roseboom *et al*., *Endocrinology* **137**, 3033 (1996).
18. M. A. Belenky, C. A. Smeraski, I. Provencio, P. J. Sollars, G. E. Pickard, *J. Comp. Neurol.* **460**, 380 (2003).
19. D. E. Somers, P. F. Devlin, S. A. Kay, *Science* **282**, 1488 (1998).
20. P. Emery, W. V. So, M. Kaneko, J. C. Hall, M. Rosbash, *Cell* **95**, 669 (1998).
21. Y. Miyamoto, A. Sancar, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6097 (1998).
22. R. N. Van Gelder *et al*., *J. Neurogenet.* **16**, 181 (2002).
23. P. F. Devlin, S. A. Kay, *Annu. Rev. Physiol.* **63**, 677 (2001).
24. C. Helfrich-Forster, C. Winter, A. Hofbauer, J. C. Hall, R. Stanewsky, *Neuron* **30**, 249 (2001).
25. Supported by the Novartis Research Foundation (to S.P., M.T.P., T.W., M.A., and J.B.H.), the National Institute of Mental Health (MH 62405 to I.P., MH51573 to S.A.K.), grants from Research to Prevent Blindness (to R.N.V.G. and D.C.T.), Becker/Association of University Professors of Ophthalmology/Research to Prevent Blindness Clinician-Scientist Award, the Culpepper Medical Scientist Award (to R.N.V.G.), National Eye Institute (K08-EY00403 and R01-EY14988 to R.N.V.G.), the American Cancer Society (PF-02-108-01-DDC to T.K.S.), the Fundacáo de Amparo à Pesquisa do Estado de São Paulo (01102460-1 to A.M.C.), and Icep, Portugal (to S.S.P.). We thank M. Morrison, J. Watson, and K. Spencer for help with histology and microscopy and R. McDowell and R. M. Allan for animal care.

Supporting Online Material
www.sciencemag.org/cgi/content/full/1086179/DC1
Materials and Methods
Figs. S1 to S3
References
Movie S1

28 April 2003; accepted 19 June 2003
Published online 26 June 2003;
10.1126/science.1086179
Include this information when citing this paper.