R E P O R T S

The primary circadian pacemaker, in the suprachiasmatic nucleus (SCN) of the mammalian brain, is photoentrained by light signals from the eyes through the retinohypothalamic tract. Retinal rod and cone cells are not required for photoentrainment. Recent evidence suggests that the entraining photoreceptors are retinal ganglion cells (RGCs) that project to the SCN. The visual pigment for this photoreceptor may be melanopsin, an opsin-like protein whose coding messenger RNA is found in a subset of mammalian RGCs. By cloning rat melanopsin and generating specific antibodies, we show that melanopsin is present in cell bodies, dendrites, and proximal axonal segments of a subset of rat RGCs. In mice heterozygous for tau-lacZ targeted to the melanopsin gene locus, β-galactosidase–positive RGC axons projected to the SCN and other brain nuclei involved in circadian photoentrainment or the pupillary light reflex. Rat RGCs that exhibited intrinsic photosensitivity invariably expressed melanopsin. Hence, melanopsin is most likely the visual pigment of phototransducing RGCs that set the circadian clock and initiate other non–image-forming visual functions.

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Fig. 1. Immunocytochemistry of melanopsin-containing RGCs in the flat-mounted rat retina. (A) Confocal images at the level of the ganglion cell layer showing labeling with the melanopsin NH₂-terminal specific antibody. The fluorescent immunolabeling is in green, and the nuclei are stained by red fluorescent propidium iodide. Arrows 1 and 2 indicate axons associated with the indicated RGC cell bodies heading toward the optic disc. Note the beaded appearance of the dendrites. Because the image is at a particular focal plane, some dendrites and axons are not visible. (B) Nonstacked (1) and stacked (2) confocal images of the same retinal field from another preparation, but without nuclear counterstaining. The stacked picture combined all focal planes containing labeled processes. Note the peripheral localization of the melanopsin-labeling in the cell bodies in (B1). Because the stacking increased background, the sensitivity of the camera was reduced, making some faint processes not clearly visible. (C) Camera-lucida drawings of several melanopsin-positive RGCs, obtained from stacked images. The beaded appearance of the dendrites is not shown. The left and right panels show nondisplaced and displaced RGCs, respectively. The displaced cells have smaller and apparently more sparse dendritic fields. Arrows indicate axons. (D) Soma-size distribution of (a sample of) nondisplaced melanopsin-positive RGCs, which account for >95% of all labeled RGCs. (E) Overall distribution of melanopsin-positive RGCs on the flat-mounted right and left retinas of the same rat. Dots of local dark-field images were taken separately at low magnification, and the montage was assembled with Adobe Photoshop. Each cell body is represented by a dot of about the appropriate size. Note the higher cell density in the superior and temporal quadrants. Only nondisplaced RGCs (>95% of total) are included. S, superior; I, inferior; N, nasal; T, temporal.

Fig. 2. Melanopsin-positive RGCs in cross sections of rat retina. All are single (nonstacked) confocal images from 30-μm retinal sections, with a depth of field of only a few micrometers. Melanopsin fluorescent immunolabeling is in green and nuclear counterstaining is in blue. In all images, the brightness of the nuclear staining has been reduced to show the processes in or around the INL; as a result, the nuclear staining in the GCL is quite faint. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. (A1 to A4) Nondisplaced RGCs. Arrows in (A3) and (A4) indicate processes in the INL; the contrast and brightness of these two images have been enhanced to show these processes. (B1 and B2) Displaced RGCs. Axons and brightness in (B2) have also been enhanced to show the axon (arrow). (C1 and C2) Double immunostaining of melanopsin and the presynaptic protein synaptophysin (red Cy3 fluorescence). The punctate melanopsin labeling of the processes (see arrows) did not colocalize or juxtapose with synaptophysin labeling. The cell in (C2) is a displaced RGC. (D) Preadsorption of antibody with the peptide-BSA conjugate abolished all immunostaining.
pression of locomotor behavior (3), melatonin release (4), and others (5–7). Surprisingly, this non–image-forming system does not appear to originate from rods and cones. For example, rods and cones are not required for phototrainment of circadian rhythms (8), a function mediated by the retinohypothalamic tract (9, 10) and its target, the SCN, the brain’s circadian pacemaker (11). Nor are rods and cones necessary for the pupillary light reflex, mediated by the retinal projection to the pretectal region of the brainstem (2). At present, the best candidate for a photopigment is an opsin-like protein called melanopsin, which is expressed by a subset of mouse and human RGCs (11). The accompanying report (12) shows that RGCs projecting to the SCN are directly sensitive to light. Thus, melanopsin may be the photopigment responsible for this intrinsic photosensitivity, and it may also trigger other non–image-forming visual functions.

We cloned the full-length cDNA for rat melanopsin (13), on the basis of homology to mouse melanopsin (11). The predicted amino acid sequence lacks the last 43 residues of mouse melanopsin but otherwise shows 92% identity (14). Polyclonal antibodies were generated against its NH2- and COOH-terminal sequences (15). Fluorescent immunocytochemistry (16) of flat-mounted rat retina with the antibody to melanopsin labeled a small percentage of RGCs, including cell bodies, dendrites, and axons (Fig. 1A). Somatic immunoreactivity appeared mainly at the cell surface (Fig. 1B1), suggestive of melanopsin being targeted to the plasma membrane. Every labeled retinal cell was a ganglion cell, on the basis of the presence of an axon coursing into the optic fiber layer and toward the optic disc. Axonal labeling disappeared beyond the optic disc and was not visible in the innervated targets (see below). More than 95% of labeled cell bodies were in the ganglion cell layer, the remainder being displaced to the inner nuclear layer. Dendrites from adjacent cells overlapped extensively, forming a reticular network (Fig. 1B2). The stained dendrites and proximal axons had a beaded appearance, showing punctate, dense labeling. The complete dendritic fields of labeled cells, visualized from stacked confocal images (e.g., Fig. 1B2), had varied sizes and shapes (Fig. 1C). Labeled displaced RGCs (Fig. 1C, right three cells) had similar soma sizes but less extensive dendritic arborizations than nondisplaced cells (Fig. 1C, left three cells). The mean somatic diameter of labeled nondisplaced RGCs was 16 μm (Fig. 1D), but the limited sample of dendritic-field measurements precluded any statistics. Morphologically, these neurons fit within the type III group of rat RGCs (17), especially those shown to be intrinsically photosensitive (12). The density of melanopsin-positive cells was slightly higher in the superior and temporal quadrants of the rat retina (Fig. 1E). A complete count of these cells in the two retinas of Fig. 1E gave 2320 and 2590, respectively, although some faintly labeled cells could

**Fig. 3.** Targeting of tau-lacZ into the mouse melanopsin gene locus. (A) (Left) Targeting strategy. In the wild-type (WT) schema, the boxes represent partial fragments of exons 1 and 9 of the melanopsin gene, with ATG indicating the start site of the melanopsin protein. The ATG in the targeting vector (TV) corresponds to the start site of the tau-lacZ fusion protein. The pgk-neo is flanked by loxp sites (open, inverted triangles) for specific cre-recombinase removal. Upon homologous recombination, the tau-lacZ fusion protein in the targeted locus (TL) retains its own start site, whereas the melanopsin start site together with the rest of the gene was eliminated. The dark bar underneath wild-type schema represents the outer probe used for screening ES cells and subsequently generated animals, by Southern typing of heterozygous animals. (Right) Genomic DNA from wild-type and +/- mice digested with Bam HI and Pac I and hybridized with the outer probe, producing a 4.4-kb fragment in the wild-type locus and a 3.7-kb fragment in the targeted locus, as expected. (B) Colocalization of β-galactosidase and melanopsin immunoreactivities in a flat-mounted rat retina from a +/- mouse. (Left) β-Galactosidase labeling. (Middle) Melanopsin labeling. (Right) Merged image. (C) Flat-mount view of a +/- mouse retina stained with X-gal. Labeled axons (blue), which converge to the optic disc, are visible. The brown coloration near the optic disc is from remnants of the retinal pigment epithelium overlying the retina. (D) Magnified view of an X-gal-labeled RGC. (E) Ventral view of a +/- mouse brain stained with X-gal, showing bilateral blue staining of the SCN (arrow). (F) Magnified view of the blue-labeled axons in an optic nerve. (G) Coronal section of the +/- brain showing blue optic nerve fibers converging to, and innervating, the SCN bilaterally. (H) Coronal section of the +/- brain showing uniform blue-labeled innervation of the left IGL and scattered innervation of the VLG. The DLG shows no labeling. (I) Coronal section of the +/- brain showing innervation of the pretectal region. The blue staining corresponds approximately to the OPN, demarcated in red on the right.

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have been missed. Assuming 100,000 RGCs in the rat retina (18), these numbers represent about 2.5% of the total. The corresponding numbers for mouse melanopsin-immunoreactive RGCs were 680 and 780 from two eyes of one animal, or about 1% of the total [assuming 60,000 ganglion cells in a mouse retina (19)].

To locate more precisely the cell bodies and dendritic arborizations of the melanopsin-positive RGCs, we examined the rat retina in cross section (Fig. 2). Whether in the ganglion cell layer (Fig. 2A) or displaced to the inner nuclear layer (Fig. 2B), the melanopsin-expressing RGCs extended dendrites into the inner plexiform layer, where they arborized most extensively at the border with the inner nuclear layer. Some arborizations invaded and often terminated in the inner nuclear layer (arrows in Fig. 2A and 2A4). The displaced RGCs had dendritic arborizations that were more planar and sparse (Figs. 2B1 and 1C, right). Melanopsin-immunoreactive puncta were present throughout the dendrites, and they showed no correlation with the retinal laminae and no colocalization or juxtaposition with the presynaptic protein synaptophysin (Fig. 2C), suggesting that the puncta did not correspond to synaptic sites.

To examine the axonal projections of the melanopsin-positive RGCs, we targeted tau-lacZ into the melanopsin gene locus in mouse (20) (Fig. 3A). Tau-lacZ codes for a protein composed of the β-galactosidase enzyme fused to a signal sequence from tau (an actin-associated protein), which allows the fusion protein to be preferentially transported down the axon to the presynaptic terminal (21). In retinas from heterozygous animals, which have one copy each of the melanopsin and tau-lacZ genes, β-galactosidase and melanopsin immunoreactivities colocalized in the ganglion cells (Fig. 3B). The morphology of the melanopsin-immunopositive cells in mouse was similar to that in rat. In X-gal labeling (22) of β-galactosidase activity (Fig. 3, C and D; blue color), the axons, cell bodies, and proximal dendrites were well labeled. In one heterozygous mouse retina, the total number of labeled cells was about 600, similar to the number for melanopsin-immunopositive cells in the wild-type mouse retina mentioned above. A ventral view of the brain from a heterozygous mouse showed blue-labeled axons coursing in the optic nerves and targeting the SCN bilaterally (Fig. 3, E and F). A coronal section of the brain at the SCN showed dense axonal terminations in the paired nuclei (Fig. 3G). Labeled axons continued caudally in the optic tract to the lateral geniculate complex, terminating throughout the intergeniculate leaflet (IGL) (Fig. 3H). Stained fibers also sparsely invaded the ventral lateral geniculate (VLG) but did not invade the dorsal lateral geniculate (DLG). Labeled axons also formed a terminal field in the pretectum, in the vicinity of the olivary pretectal nucleus (OPN) (Fig. 3I). Neither melanopsin immunoreactivity nor β-galactosidase staining labeled neuronal somata in these or other brain regions, indicating that the axons containing melanopsin all originated from the retina.

To address whether melanopsin could be the photopigment responsible for the intrinsic light sensitivity of rat RGCs that project to the SCN (12), we injected these photosensitive RGCs intracellulary with Lucifer Yellow (LY) (23) and then stained them for melanopsin immunoreactivity. Intrinsically photosensitive ganglion cells were invariably melanopsin-positive (n = 18; Fig. 4), whereas conventional ganglion cells lacking intrinsic light responses were melanopsin-negative (n = 4). Thus, melanopsin is most likely the photopigment that confers the intrinsic light sensitivity to this subset of RGCs. In some photosensitive ganglion cells, individual dendrites were smoothly filled with LY but still exhibited punctate melanopsin immunoreac-

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**Fig. 4.** Presence of melanopsin in intrinsically light-responsive RGCs that innervate the SCN. Sample data from three cells (A to C) all identified by retrograde transport of fluorescent rhodamine beads from the SCN before whole-cell recording from the flat-mounted retina. In each panel, the voltage trace at left shows the cell’s response to a light signal indicated by the step: Broad-band tungsten light source; irradiance at retina of 6 × 10¹⁵ photons s⁻¹ cm⁻² at 500 nm. Bathing Ames medium contained agents to block Ca²⁺ and synaptic transmission: either (A and B) 2-mM CoCl₂ alone or (C) in combination with pharmacological blockers of the glutamatergic signaling between conventional photoreceptors and RGCs [100-μM (−)-2-amino-4-phosphonobutyric acid (APB), 20-μM 6,7-dinitroquinoxaline-2,3-dione (DNQX), and 50-μM DL-2-amino-5-phosphonovaleric acid (APV)] (12). Large-amplitude, fast depolarizing events are action potentials. Fluorescence images of the recorded cells are shown at right. (Left) Intracellular staining (green) with LY introduced from the whole-cell recording pipette. (Middle) Antibody to melanopsin immunofluorescence (Cy-3 fluorophore, red, in A and B; Alexa Fluor 647, white, in C). (Right) Superimposed fluorescence images. Far left in (C), fluorescence of rhodamine latex beads (red), the retrograde tracer. All micrographs are stacked, pseudocolored confocal images, except for the LY image in (C), which is a montage of optimized epifluorescence (nonconfocal, hence somewhat blurred) micrographs at different focal planes. Arrow in (A) marks the melanopsin-positive dendrite of a second ganglion cell neighboring the one recorded. In (B) and (C), other strongly melanopsin-immunopositive cells are visible near the recorded cell; these cells were not recorded from and hence showed no LY labeling.
tivity, suggesting that there may be local clusters of the opsins.

Taken together, our findings suggest that melanopsin may indeed be a photopigment, consistent with an opsin-based action spectrum demonstrated for the SCN-projecting RGCs (12). The presence of melanopsin throughout the dendritic arbors of these cells may permit spatial integration of retinal irradiance and is consistent with the extended receptive fields of these cells (12). It is unclear whether these photosensitive dendrites simultaneously serve the more conventional function of receiving synaptic inputs from rod- and cone-driven networks. There is evidence for rod and cone influences on neurons in both the SCN (24) and the OPN (2), but whether these reflect convergence at the photosensitive RGCs or convergence within the brain remains unclear. In both distribution and morphology, the melanopsin-positive RGCs described here broadly match those identified in rat and mouse retina that project to the SCN (25–28); see also (29, 30), although most of these other studies labeled presumably only a fraction of the cells.

Although the innervation of the SCN is the densest, the IGL, VLG, and OPN were also innervated by β-galactosidase–positive RGC axons in heterozygous tau-lacZ mice. Some of the fibers innervating the IGL and OPN could be collaterals of axons in the retinohypothalamic tract (31). Neurons in both IGL and OPN encode ambient light levels (32–34), a property almost certainly conferred by the photosensitive RGCs, which faithfully report retinal irradiance (12). Like the SCN, the IGL and the VLG are implicated in circadian photoentrainment (32), whereas the OPN is a key node in the circuit mediating the pupillary light reflex (33, 34), another non–image-forming visual function. Indeed, rodless and coneless mice retain a pupillary light reflex (35), whereas whole-cell current clamp recordings were made with patch pipettes (3 to 7 megaohms) containing 125 mM KCl, 5 mM MgCl2, and 5 mM HEPES, pH 7.4. The COOH-terminal peptide was used as the template in a second PCR with the same primer pairs as before, except that the polymerase chain reaction was extended at 72°C for 15 min. The PCR product was ligated to the vector pGEM-T (Promega, Madison, WI) and transformed into Escherichia coli DH5α. The resulting plasmid was sequenced (accession number Y072689). On the basis of the sequence, two new forward primers (5′-GCTATTACATACGCGTCC-3′ and 5′-GCTATTACATACGCGTCC-3′) were reverse primer (5′-GCTATTACATACGCGTCC-3′) and a reverse primer (5′-GCTATTACATACGCGTCC-3′) were used. The 3′ portion of the target sequence was nested 3′-RACE with the same PCR conditions as above. Several independent PCR products were sequenced, and the resulting DNA sequences were compared in order to reach consensus into amino acids.

14. See supplementary information available on Science online at www.sciencemag.org/cgi/content/full/295/5575/1065/DCl.

The NH2-terminal peptide was KMMPSPEVPPSSLTQDSPS [29], with the lysine added for linking purposes. The COOH-terminal peptide was EQKSTK- PTKTRHLPSLDRM [30]. They were conjugated to thyroglobulin and used to immunize rabbits (Covance, Denver, PA). The resulting antisera were purified by a peptide–bovine serum albumin (BSA)–conjugated affinity column. The specificity of the antibodies was confirmed in immunoblot experiments with melanopsin expressed in HEK 293 cells (31). In all experiments described in this paper, the NH2- terminal antisemur was used, but identical results were obtained with the COOH-terminal antisemur.

Whole retina or frozen 30–40-μm retinal sections were used as the template in a first PCR with a set of primers designed to amplify a 92-bp melanopsin sequence for a tau protein signal peptide fused to the COOH-terminus of a His-tagged α-tubulin. The resulting sequence was then used as the template in a second PCR with the same primer pairs as before, except that the polymerase chain reaction was extended at 72°C for 15 min. The PCR product was ligated to the vector pGEM-T (Promega, Madison, WI) and transformed into E. coli DH5α. The resulting plasmid was sequenced (accession number Y072689). On the basis of the sequence, two new forward primers (5′-GCTATTACATACGCGTCC-3′ and 5′-GCTATTACATACGCGTCC-3′) were reverse primer (5′-GCTATTACATACGCGTCC-3′) and a reverse primer (5′-GCTATTACATACGCGTCC-3′) were used. The 3′ portion of the target sequence was nested 3′-RACE with the same PCR conditions as above. Several independent PCR products were sequenced, and the resulting DNA sequences were compared in order to reach consensus into amino acids.

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### References and Notes

Phototransduction by Retinal Ganglion Cells That Set the Circadian Clock

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Light synchronizes mammalian circadian rhythms with environmental time by modulating retinal input to the circadian pacemaker—the suprachiasmatic nucleus (SCN) of the hypothalamus. Such photic entrainment requires neither rods nor cones, the only known retinal photoreceptors. Here, we show that retinal ganglion cells innervating the SCN are intrinsically photosensitive. Unlike other ganglion cells, they depolarized in response to light even when all synaptic input from rods and cones was blocked. The sensitivity, spectral tuning, and slow kinetics of this light response matched those of the photic entrainment mechanism, suggesting that these ganglion cells may be the primary photoreceptors for this system.

The SCN is the circadian pacemaker of the mammalian brain, driving daily cycles in activity, hormonal levels, and other physiological variables. Light can phase-shift the endogenous oscillator in the SCN, synchronizing it with the environmental day-night cycle. This process, the photic entrainment of circadian rhythms, originates in the eye and involves a direct axonal pathway from a small fraction of retinal ganglion cells to the SCN (1–3). A striking feature of this neural circuit is its apparent independence from conventional retinal phototransduction. In functionally blind transgenic mice lacking virtually all known photoreceptors (rods and cones), photic entrainment persists with undiminished sensitivity (4). Candidate photoreceptors for this system are nonrod, noncone retinal neurons, including some ganglion cells, that contain novel opsins or cryptochromes (5–8).

To determine whether retinal ganglion cells innervating the SCN are capable of phototransduction, we labeled them in the rat retina by retrograde transport of fluorescent microspheres injected into the hypothalamus (9). In isolated retinas, whole-cell recordings were made of the responses of labeled ganglion cells to light (10) (Fig. 1, A to E). In most of these cells (n = 150), light evoked large depolarizations with superimposed fast action potentials (Fig. 1, E to G) (11). The light response persisted during bath application of 2 mM cobalt chloride (Fig. 1F; n = 42), which blocks calcium-mediated synaptic release from rods, cones, and other retinal neurons (12). In contrast, other ganglion cells prepared and recorded under identical conditions but not selectively labeled from the SCN (control cells) lacked detectable responses to light even without synaptic blockade (47/50 cells; Fig. 1, I and J) (13). This is presumably because rod and cone photopigments were extensively bleached (10). A few control cells (3/50) exhibited weak, evanescent responses to light, but these were abolished by bath-applied cobalt (n = 2).

To ensure blockade of conventional synaptic influences from rods and cones, we supplemented cobalt with a mixture of drugs that independently disrupted both the glutamatergic synapses crucial to vertical signal transfer through the retina and the ionotropic receptors responsible for fast excitatory influences on ganglion cells (14). Robust light responses persisted in SCN-projecting ganglion cells under these conditions (Fig. 1G; n = 7). Furthermore, the somata of these ganglion cells exhibited photosensitivity even when completely detached from the retina by microdissection (Fig. 1H; n = 3). These light responses were not an artifact of photic excitation of either of the intracellular fluorophores we used, as the action spectrum of the light response (Fig. 2C) differed from the absorption spectra of both the retrograde tracer and Lucifer Yellow (LY) used for intracellular staining. Also, light-evoked increases in spike frequency were detectable in extracellular recordings, before patch rupture and LY dye filling (n = 5). Whole-cell recordings revealed normal light responses when LY was omitted from the internal solution (n = 8). In contrast, control cells lacked cobalt-resistant light responses even when labeled with both fluorescent beads and LY (n = 12; Fig. 1I). These data indicate that retinal ganglion cells innervating the SCN are intrinsically photosensitive.

To determine if these cells could serve as the primary photoreceptors for circadian entrainment, we assessed congruence between their photic properties and those of the entrainment mechanism. The responses of a single cell to narrow-band stimuli of various intensities showed that at each wavelength, peak depolarization increased with stimulus energy (Fig. 2, A and B). Intensity-response curves exhibited a consistent slope when plotted in semilogarithmic coordinates (Fig. 2B), as expected for responses mediated by a single phototopigment (principle of univariance). The horizontal displacements of the curves from one another reflect the spectral dependence of the pigment’s quantum efficiency and yield the spectral sensitivity function shown in Fig. 2C (red curve). Other cells exhibited similar action spectra (Fig. 2C, green curve) (15). These action spectra closely matched that predicted for a retinal-based pigment with peak sensitivity at 484 nm (Fig. 2C, black). They also resemble action spectra derived behaviorally for circadian entrainment in rodents (16, 17), as expected if these ganglion cells function as primary circadian photoreceptors (18).

From available spectral evidence, the phototopigment in these ganglion cells is more likely to be a retinaldehyde-based opsin such as melanopsin (5, 19, 20) than a flavin-based cryptochrome (21).