N-ethylmaleomide (NEM, Calbiochem), and DL-AP5, NF023, KT5720 and thapsigargin (all Tocris Cookson). Drugs used in superfusion experiments were applied for 15–20 min before light stimulation.

Light stimuli were generated using a Cairn Optoscan Xenon arc source comprising a slit monochromator. Unless otherwise stated, all stimuli were 10 s in duration with a 20-nm half-bandwidth. Irradiance was measured using an optical powermeter (Macam Photometrics) and converted to photon flux. In view of the sustained nature of light responses, analysis was restricted to a single light response from each cell for all of the data except that depicted in Fig. 2b–d and 3b, c, in which paired responses for each cell were recorded. Although subsequent stimuli were presented before full recovery in these instances, we did not find a significant effect on the response (Fig. 2d). The magnitude of the responses was defined by the peak sustained current, measured using Clampfit (Axon Instruments). It has been suggested that NMDA receptors may include a light sensitive moiety[30]. The nature of glutamate receptor expression in Neuro-2a cells remains ambiguous, nonetheless we excluded the possibility that this might be the origin of the melanopsin light response by application of the selective NMDA antagonist DL-AP5 (100 μM), which had no effect on the light sensitive current (data not shown).

Opsin expression in Neuro-2a cells by RT–PCR

Cells were collected both before and after differentiation and after transfection with pCMS-EGFP vector alone or the human melanopsin–EGFP vector. RNA was extracted using Tri reagent (Sigma) and treated with DNaseI (Promega) before reverse transcription. Single-stranded cDNA was synthesized using a SuperScript kit (Invitrogen). Specific primers that amplify both mouse and human melanopsin were used, as well as species-specific primers for mouse rod opsin, UVS/MWS-cone opsins, RGR-opsin, peropsin and rod/cone transducin alpha subunits (Gnat1 and Gnat2). The absence of vector DNA carry-over in both of the transfected Neuro-2a cDNA samples was confirmed using primers designed to the CMV promoter (data not shown).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in PBS buffer and exposed to a primary Tetra-His antibody (Qiagen) at 1:100 dilution. Visualization was achieved by fluorescence microscopy (Nikon Eclipse E600; MetaMorph, Universal Imaging Corp) after incubation with a tetramethylrhodamine goat anti-mouse secondary antibody at 1:400.

Received 21 October 2004; accepted 12 January 2005; doi:10.1038/nature03344.
Published online 26 January 2005.

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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements This work was supported by a Wellcome Trust Showcase Award (M.W.H. and R.J.L.) and in part by the BBSRC (R.J.L.) and NSBRI through NASA NCC 9-58 (R.J.L. and R. Foster). We are grateful to R.K. Crouch for the gift of 11-*cis* retinaldehyde, to R. Douglas for scientific discussions and comments on the manuscript, and to K. Wells for help and advice with cell culture.

Competing interests statement The authors declare that they have no competing financial interests.

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Induction of photosensitivity by heterologous expression of melanopsin

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Melanopsin[1–8] has been proposed to be the photopigment of the intrinsically photosensitive retinal ganglion cells (ipRGCs)[7–15]; these photoreceptors of the mammalian eye drive circadian and pupillary adjustments through direct projections to the brain[5,6,8–14,16–18]. Their action spectrum ($\lambda_{max} \approx 480$ nm) implicates an opsin[10] and melanopsin is the only opsin known to exist in these cells. Melanopsin is required for ipRGC photosensitivity[13] and for behavioural photoresponses that survive disrupted rod and cone function[14,17]. Heterologously expressed melanopsin apparently binds retinaldehyde and mediates photic activation of G proteins[19]. However, its amino-acid sequence differs from vertebrate photosensory opsins[1,20] and some have suggested that melanopsin may be a photoisomerase, providing retinoid chromophore to an unidentified opsin[3,20]. To determine whether melanopsin is a functional sensory photopigment, here we transiently expressed it in HEK293 cells that stably expressed TRPC3 channels. Light triggered a membrane depolarization in these cells and increased intracellular calcium. The light

response resembled that of ipRGCs, with almost identical spectral sensitivity ($\lambda_{max} \approx 479$ nm). The phototransduction pathway included Gq or a related G protein, phospholipase C and TRPC3 channels. We conclude that mammalian melanopsin is a functional sensory photopigment, that it is the photopigment of ganglion-cell photoreceptors, and that these photoreceptors may use an invertebrate-like phototransduction cascade.

To test melanopsin's capacity to form a functional sensory photopigment, we heterologously expressed mouse *melanopsin* (*Opn4*) complementary DNA in human embryonic kidney cells (HEK293) stably expressing TRPC3 receptor-operated non-specific cation channels[21]. The mouse melanopsin open reading frame was cloned into a bicistronic expression vector (pIRES2–EGFP). HEK293-TRPC3 cells transfected with this vector faithfully co-expressed the enhanced green fluorescent protein (EGFP) reporter protein (Fig. 1a, c) and mouse melanopsin (Fig. 1b, c). Melanopsin was localized predominantly in the cell membrane (Fig. 1b), as it is in ipRGCs[22].

Many of these cells were photosensitive. They exhibited large light-evoked changes in transmembrane voltage that were sluggish and sustained (Figs 1d, e). Light responses were never observed among neighbouring untransfected, EGFP-negative cells ($n = 11$), nor in cells transiently transfected with empty pIRES2–EGFP, which expressed EGFP but not melanopsin ($n = 10$; Fig. 1f). Increasing stimulus irradiance augmented response amplitude (Fig. 1e; see also Fig. 4a) and decreased onset latency from >10 s to a minimum of ~500 ms. Maximal responses were typically >15 mV. Thresholds were approximately $(5.6 \pm 1.7) \times 10^{12}$ photons s^{-1} cm^{-2} at 480 nm (mean ± standard error of the mean, s.e.m.; $n = 12$), about one log unit higher than for ipRGCs[10]. Although there was no retinaldehyde in the bath, responsiveness persisted for hours.

With voltage clamped at negative potentials, light triggered an inward current (Fig. 1g) exhibiting strong outward rectification and reversing near 0 mV (4.0 ± 3.8 mV, mean ± s.e.m., $n = 9$; Fig. 1h). Maximal white-light stimuli evoked peak inward currents averaging 30 pA (±13 pA standard deviation, s.d.; $n = 5$; $V_{hold} \approx -44$ mV). Light-evoked inward currents were never observed in untransfected cells (Fig. 1h, bottom) or in cells expressing EGFP but not melanopsin.

Light also triggered increases in intracellular free calcium in these cells. Only transfected cells exhibited light-evoked increases in fluorescence of the calcium indicator Rhod-2-AM (Fig. 2). Calcium responses were also detectable in melanopsin-transfected cells lacking the EGFP reporter (Supplementary Fig. 1).

Melanopsin is presumably the photopigment in these cells

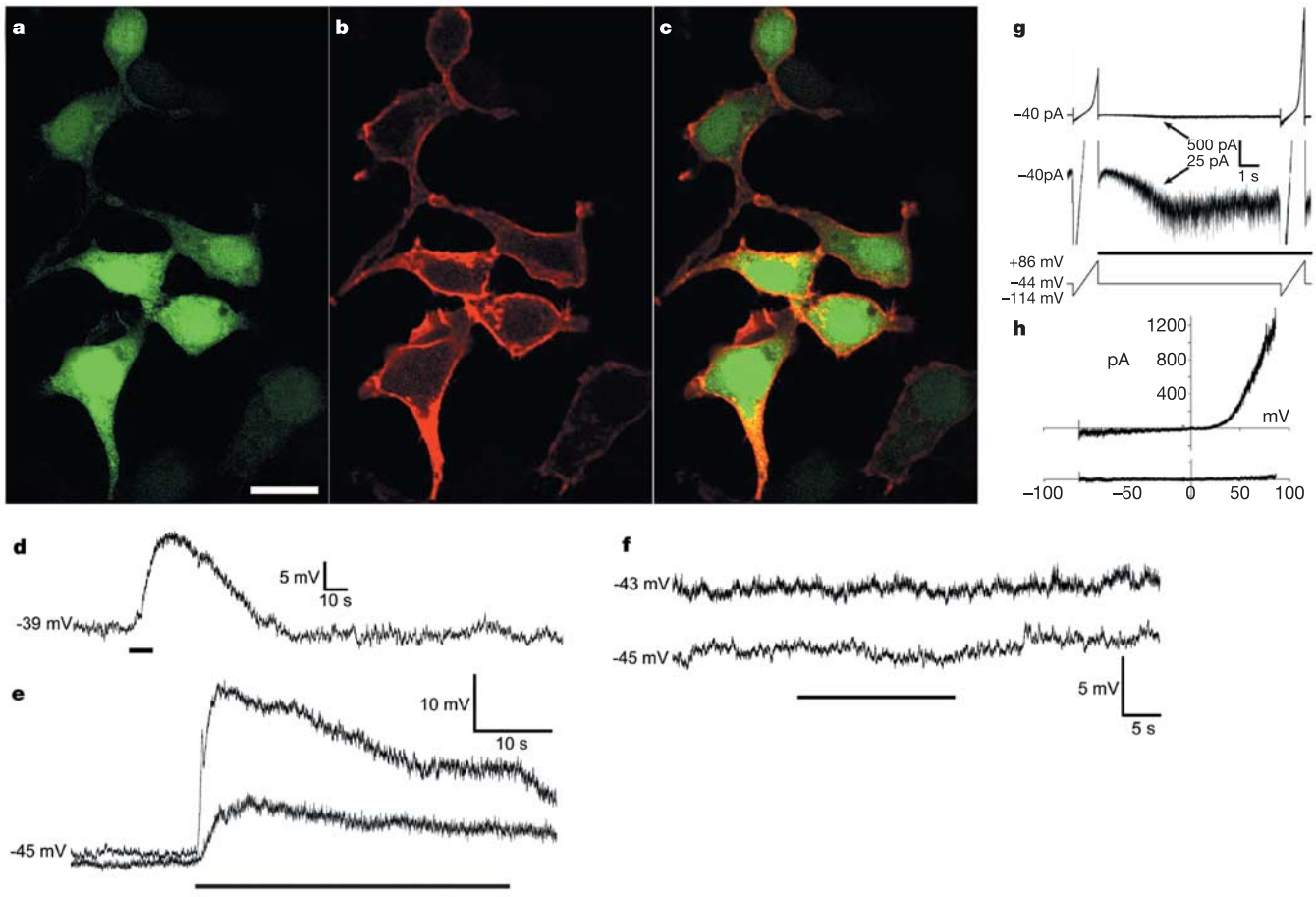

Figure 1 Light-evoked responses of melanopsin-expressing HEK293-TRPC3 cells. **a**–**c**, Melanopsin expression. Confocal photomicrographs showing EGFP (**a**, green), melanopsin immunofluorescence (**b**, red) and merge (**c**). Scale bar, 20 μm. **d**, **e**, Current-clamp recordings of light-evoked depolarization in transfected cells. **d**, Sluggish response to 10-s flash (bar marks stimulus; 480 nm, 9×10^{14} photons s^{-1} cm^{-2}). **e**, Intensity coding. The white-light stimulus for top trace was one log unit more intense than for bottom trace. **f**, Absence of photosensitivity in untransfected cells (top trace) and in cells expressing EGFP but not melanopsin (bottom trace). **g**, **h**, Voltage-clamp recordings. **g**, The light stimulus (horizontal bar) elicits tonic inward current and increased current noise (middle trace). The same trace at lower gain (top trace) shows responses to voltage ramps (bottom trace) imposed before and during light response. **h**, Current–voltage relations of light-evoked current in **g** (stimulated minus dark current) (top trace). Absence of light-evoked current in untransfected cell (bottom trace).

because it was required for the observed photosensitivity. To test the hypothesis that melanopsin, like other opsin photopigments, triggers a G-protein signalling cascade, we infused guanosine 5′-O-(2-thiodiphosphate) (GDPβS). Light-evoked depolarizations were reduced by more than half their initial amplitude within 30 min (Fig. 3a; in 3 of 4 cells tested). This was a specific effect, not an artefact of cell dialysis, because cells recorded with the control internal solution maintained stable response amplitudes for ≥70 min (data not shown). Gq or a related protein seems most likely to be the cognate G protein for melanopsin. The light response was effectively abolished within 20 min by internal application of GPant-2a, a competitive inhibitor of Gq, and was suppressed 75–100% by bath-application of U73122, an antagonist of phospholipase C (PLC; $n = 3$; Fig. 3c), the effector enzyme for Gq-like G proteins[23]. An inactive analogue of the PLC antagonist (U73343; $n = 3$; Supplementary Fig. 2a) and pertussis toxin (a Gi inactivator; 250 ng ml^{-1}, 2 h) were ineffective. TRPC3 channels apparently carry the light-activated current in these cells (see Supplementary Data and Supplementary Figs 2b and c).

Relative sensitivity at different wavelengths was assessed for individual cells as shown in Fig. 4a. The optimal wavelength (λ_{max}) for each cell was estimated from the best-fitting retinaldehyde template function[24] (see Methods). These values, plotted in the histogram of Fig. 4b, had a mean value of 479.2 nm (±1.5 nm s.e.m.; $n = 12$), very close to the optimal wavelength for isolated rat ipRGCs[10] (484 nm). Mean estimates of relative sensitivity at each tested wavelength (Fig. 4b, points) adhered closely to the best-fitting retinaldehyde template function (Fig. 4b, curve), supporting the assumption that the action spectrum in this system adheres to the standard form for opsin-mediated responses.

This study provides the first direct physiological evidence that melanopsin is a functional sensory photopigment and that it can activate a signalling cascade that gates an ionic conductance. Melanopsin is an opsin, with the canonical retinaldehyde binding site[1,20], and most cells expressing it are known or presumed to be directly photosensitive[1,2,6–8,10–14]. Its overexpression increases photosensitivity in dermal melanophores[25], and its deletion abolishes the light response in ganglion-cell photoreceptors[13]. Heterologously expressed melanopsin reportedly mediates photic activation of a G protein[19], again supporting a photosensory role.

Our findings contradict the idea that melanopsin could be exclusively a photoisomerase instead of a photosensory pigment[3,20]. Introducing melanopsin into HEK293-TRPC3 cells transformed them into photoreceptors. The action spectrum of the induced photoresponse peaked at 479 nm, closely matching the spectral tuning of isolated rat ipRGCs[10] ($\lambda_{max} \approx 484$ nm) and of melanopsin-dependent behavioural light responses of rodless/coneless

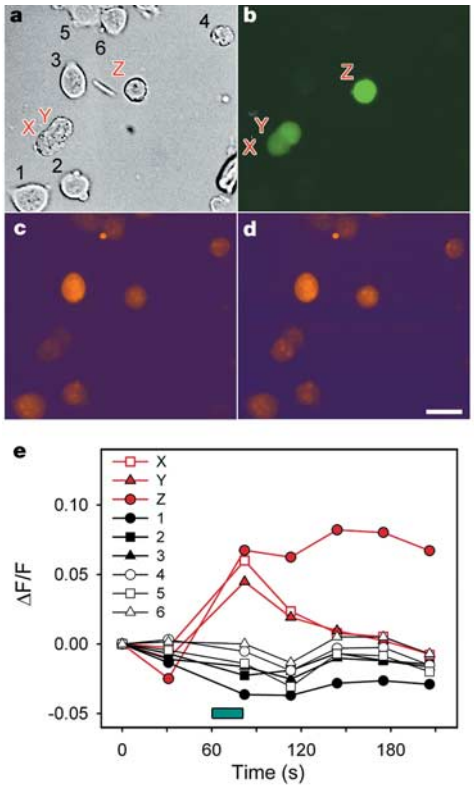

Figure 2 Light evokes calcium responses. Brightfield image (**a**) and EGFP fluorescence (**b**) of live cells. Of nine cells, three (X, Y and Z) were transfected (EGFP+). **c**, **d**, Pseudocolour images of Rhod-2-AM fluorescence before (**c**) and after (**d**) exposure to a light stimulus (500 nm, 10^{15} photons s^{-1} cm^{-2}). Only transfected cells exhibited increased calcium signals. Scale bar, 20 μm. **e**, Normalized change in fluorescence over time for transfected cells (red lines) and untransfected cells (black lines). The green bar marks the stimulus.

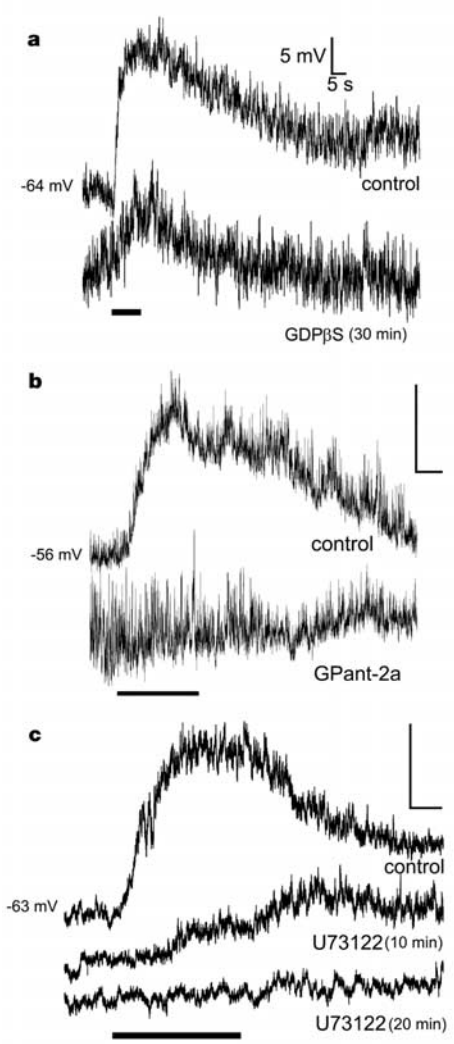

Figure 3 Phototransduction cascade. Voltage responses to bright white-light stimuli (horizontal bars) before and during drug application. Photoresponses were reduced or blocked by internal application of GDPβS (**a**; 2 mM), a non-specific antagonist of G proteins, or of GPant-2a (40 μM), a Gq/11 antagonist (**b**). **c**, Bath application of the PLC antagonist U73122 (10 μM) eliminated the light response (**c**); the inactive analogue U73343 had no effect (Supplementary Fig. 2a). Calibration bars, 5 mV, 5 s.

rodents (pupillary light reflex: $\lambda_{max} \approx 479$ nm, ref. 16; circadian phase shifting: $\lambda_{max} \approx 480$–481 nm, refs 17 and 26). Such correspondence was lacking in an earlier study of heterologously expressed melanopsin, which reported maximal absorption at 421 nm (ref. 20). The discrepancy with our spectral results could stem from the earlier study's use of a different expression system, protein modifications for epitope tagging, effects of opsin solubilization and immunopurification, or the use of chemical rather than photic bleaching to generate the difference spectrum.

Though melanopsin is now firmly established as a photosensory pigment, it may also possess photoisomerase activity. In bistable invertebrate photopigments, the chromophore–opsin linkage is non-dissociating and light can trigger photoreversal of metarhodopsin to rhodopsin. Support for possible melanopsin bistability comes from its invertebrate-like aromatic amino acid at the 'counter-ion' position[1], a key determinant of chromophore binding, and, as shown here, the persistence for hours of melanopsin-based light responses without supplementary retinaldehyde despite repeated exposure to bright illumination. Still, there is no direct evidence that melanopsin is bistable. In photoreceptors using bistable pigments, appropriate narrow-band stimuli can terminate persistent post-stimulus responses and augment sensitivity to subsequent stimuli by converting metarhodopsin to rhodopsin. We have not detected such behaviour in transfected HEK293-TRPC3 cells (see Supplementary Data). If melanopsin instead dissociates from its chromophore like other vertebrate photopigments, its regeneration in HEK293 cells may be attributable to the intrinsic retinoid processing capacity of these cells[27].

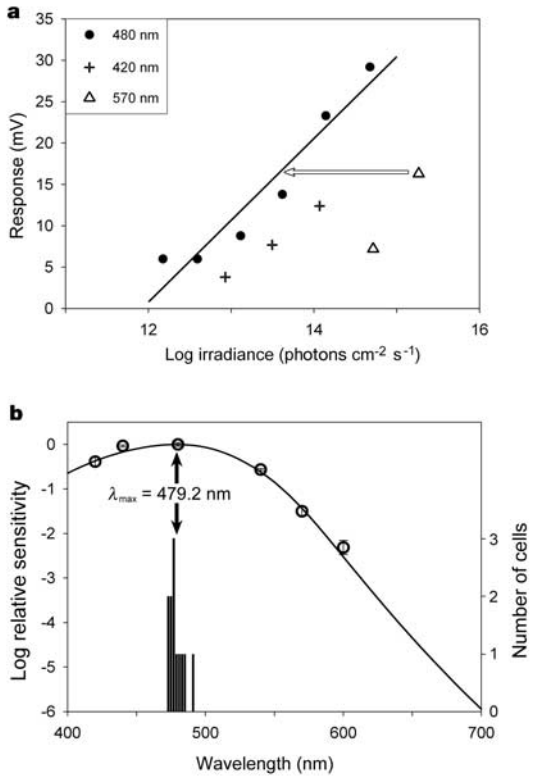

Figure 4 Spectral sensitivity. **a**, Plot of peak depolarization evoked in one cell by stimuli of various wavelengths. The solid line shows the least-squares linear fit to 480-nm data (solid circles). The horizontal displacement of other points from this line (open arrow) estimates sensitivity at corresponding wavelength relative to 480 nm. **b**, The histogram shows the distribution of the estimated optimal wavelength (λ_{max}), determined for each cell from the best-fitting retinaldehyde template. Mean $\lambda_{max} = 479.2$ nm $\pm$ 1.5 nm ($n = 12$). Above the histogram, each point represents the mean value of relative sensitivity at that wavelength among all cells (error bars, barely visible, are s.e.m.). The curve is the least-squares best-fitting retinaldehyde template function; λ_{max} (479.2 nm) has an ordinate value of 0. Medium supplemented with 11-*cis*-retinaldehyde.

By demonstrating that melanopsin is activated maximally near 480 nm and can indirectly gate cation channels in the plasma membrane, our findings provide nearly all of the missing links in the chain of evidence for melanopsin as the photopigment of ipRGCs[8,10,20]. There are striking similarities between the photoresponses of melanopsin-expressing HEK293 cells and those of ipRGCs including response polarity, low sensitivity, sluggish onset, tonic response to continuous illumination, and slow post-stimulus recovery. It seems probable that some of these properties, like the spectral tuning, derive from features of melanopsin itself, while others are shaped by the downstream signalling cascade.

In HEK293-TRPC3 cells, this cascade involves Gq or a related pertussis toxin-insensitive G protein. Some G-protein-coupled receptors (GPCRs) couple promiscuously to multiple G-protein families[28] and melanopsin itself reportedly interacts with highly concentrated rod transducin (Gt) in a biochemical assay[19]. Under physiological conditions, however, most GPCRs couple mainly to only one of the four G-protein families[28], so the cognate G protein for melanopsin in ganglion cells is probably a member of the Gq family[23] (Gq, G11, G14, G15 and/or G16). In HEK293-TRPC3 cells, the G protein activated by melanopsin stimulates PLC to open TRPC3 channels and depolarize the plasma membrane[21,23,29]. *Drosophila* photoreceptors apparently use a similar phototransduction cascade[30], but it is not yet known whether ipRGCs use (or even express) these specific downstream components.

Note added in proof: While in proof we became aware of three other papers to be published that concurrently support our conclusion that melanopsin is a functional photopigment, signalling through G proteins[31–33]. □

Methods

Cell culture and melanopsin expression

Cells were cultured conventionally. The mouse melanopsin (*Opn4*) open reading frame (GenBank accession number NM_013887) was amplified by polymerase chain reaction (PCR) from a vector containing the open reading frame. PCR primers were generated from the melanopsin sequence with restriction enzyme sequences appended to the 5′ end to facilitate directional cloning. Two different PCR products were generated for two expression vectors: the plasmid pcDNA 3.1(+) (Invitrogen) and the bicistronic pIRES2–EGFP (BD Biosciences). PCR products were digested with restriction enzymes and ligated into the multiple cloning site of the vectors, also digested with restriction enzymes. Expression vector sequences were confirmed by dideoxy terminator sequencing. Cells were transfected with the calcium phosphate method and seeded on coverslips. From transfection until recording, cells were provided with retinaldehyde (0.5 μM 11-*cis*-retinaldehyde for spectral studies, 2 μM all-*trans*-retinaldehyde for all other experiments) and were kept in darkness, except for brief exposures to laboratory lighting. Omitting these retinoids did not preclude photosensitivity (see Supplementary Data).

Melanopsin immunohistochemistry

Cells were fixed overnight (4% paraformaldehyde in phosphate-buffered saline, PBS), incubated in polyclonal rabbit anti-melanopsin antiserum (UF006, ref. 7, 1:2,500; 24 h, 4 °C), then in Cy-3-conjugated anti-rabbit IgG (1:500; Jackson ImmunoResearch; 1 h; 21 °C). Photomicrographs were obtained on a Zeiss Pascal confocal microscope using a 40 × objective.

Electrophysiology

Cells transfected 1–2 days earlier were mounted in a chamber perfused with Tyrodes solution containing no retinaldehyde (1–2 ml min^{-1}, 21 °C). Although incubated in darkness, cells were exposed to normal laboratory lighting during preparation for recording except in spectral studies, which were conducted in darkness. Cells were visualized with a 40 × water-immersion objective on an upright epifluorescence microscope equipped with a cooled charge-coupled device (CCD) camera, integrating frame grabbers and video monitor. Except for brief blue epifluorescence to identify EGFP-positive cells (Chroma no. 41001), only infrared light was used to view cells.

Whole-cell patch recordings were made conventionally. Micropipettes (3–5 MΩ) contained 120 mM K-gluconate, 5 mM NaCl, 4 mM KCl, 0.5–2 mM EGTA, 10 mM HEPES, 0.5–4 mM ATP-Mg, 7 mM phosphocreatine, 0.05–0.3 mM GTP-Tris; (280–300 mOsm, pH 7.3). Liquid junction potentials (−14 mV) were corrected. In darkness, most cells had series resistance <10 MΩ, $V_m \approx$ −35 to −45 mV and input resistance of 0.5–1.5 GΩ. Evoked currents or voltages are baseline-subtracted.

Photic stimulation and spectral analysis

Light stimuli from a 100-W tungsten source were filtered (neutral density and narrow-band interference filters;10 nm width; Oriel), gated by a shutter (Uniblitz VS35;Vincent Associates) and calibrated by a radiometer (S370, UDT Instruments). The irradiance of the unfiltered ('white') stimulus was (in photons s^{-1} cm^{-2}): 4×10^{12} at 400 nm, 6×10^{13} at 500 nm and 1×10^{14} at 600 nm.

For spectral analysis, the culture medium contained 11-*cis*-retinaldehyde. Current injection held cells near −44 mV. Stable sensitivity, confirmed by retesting with a standard stimulus, required long interstimulus intervals (7 min). Stimuli of 480 nm were interleaved with those of two to three other wavelengths (420, 440, 540, 570 or 600 nm). Each response to one of these other wavelengths yielded an estimate of relative sensitivity normalized to that at 480 nm (see Fig. 4a), and these were averaged for each wavelength. For each cell, we determined the retinaldehyde template function[24] that best fitted these data (least-squares method). The two free parameters for the fit were λ_{max} and the vertical offset. Relative sensitivities were then re-normalized to that at the theoretical optimum (λ_{max}).

Calcium imaging

Cells loaded with the long-wavelength Ca^{2+} indicator Rhod-2-AM (4–6 μM in Tyrodes, 30 min, 37 °C) were imaged at 30 Hz using green excitation (525–550 nm; emission 580–650 nm; Chroma 31002a) attenuated 128- to 512-fold by neutral density filters. Integration time was fixed within trials (typically 32 frames) to optimize sensitivity and avoid saturation. Responses, analysed by ImageJ software (W. Rasband; http://rsb.info.nih.gov/ij/; 2004), were expressed as post-stimulus change in fluorescence intensity above baseline divided by the baseline intensity ($\Delta F/F$).

Further methodological details are provided in the Supplementary Methods.

Received 5 November 2004; accepted 10 January 2005; doi:10.1038/nature03345.
Published online 26 January 2005.

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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank V. Maine for technical support; M. Zhu for donating the HEK293-TRPC3 cells; T. Helton, K. Richard, D. Lipscombe, J. Bai and X. Wang for guidance with cell culture; E. Newman and D. O'Malley for advice on calcium imaging; and M. Rollag and J. McIlwain for discussions and comments on the manuscript. This work was supported by NIH grants to D.M.B. and I.P.

Competing interests statement The authors declare that they have no competing financial interests.

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Melanopsin-expressing ganglion cells in primate retina signal colour and irradiance and project to the LGN

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Human vision starts with the activation of rod photoreceptors in dim light and short (S)-, medium (M)-, and long (L)- wavelength-sensitive cone photoreceptors in daylight. Recently a parallel, non-rod, non-cone photoreceptive pathway, arising from a population of retinal ganglion cells, was discovered in nocturnal rodents[1]. These ganglion cells express the putative photopigment melanopsin and by signalling gross changes in light intensity serve the subconscious, 'non-image-forming' functions of circadian photoentrainment and pupil constriction[1–7]. Here we show an anatomically distinct population of 'giant', melanopsin-expressing ganglion cells in the primate retina that, in addition to being intrinsically photosensitive, are strongly activated by rods and cones, and display a rare, S-Off, (L + M)-On type of colour-opponent receptive field. The intrinsic, rod and (L + M) cone-derived light responses combine in these giant cells to signal irradiance over the full dynamic range of human vision. In accordance with cone-based colour opponency, the giant cells project to the lateral geniculate nucleus, the thalamic relay to primary visual cortex. Thus, in the diurnal trichromatic primate, 'non-image-forming' and conventional 'image-forming' retinal