Mammalian circadian rhythms generated in the hypothalamic suprachiasmatic nuclei are entrained to the environmental light/dark cycle via a monosynaptic pathway, the retinohypothalamic tract (RHT). We have shown previously that retinal ganglion cells containing pituitary adenylate cyclase-activating polypeptide (PACAP) constitute the RHT. Light activates the RHT via unknown photoreceptors different from the classical photoreceptors located in the outer retina. Two types of photopigments, melanopsin and the cryptochromes (CRY1 and CRY2), both of which are located in the inner retina, have been suggested as “circadian photopigments.” In the present study, we cloned rat melanopsin photopigment cDNA and produced a specific melanopsin antibody. Using in situ hybridization histochemistry combined with immunohistochemistry, we demonstrate that the distribution of melanopsin was identical to that of the PACAP-containing retinal ganglion cells. Colocalization studies using the specific melanopsin antibody and/or cRNA probes in combination with PACAP immunostaining revealed that melanopsin was found exclusively in the PACAP-containing retinal ganglion cells located at the surface of somata and dendrites. These data, in conjunction with published action spectra analyses and work in retinally degenerated (rd/rd/cl) mutant mice, suggest that melanopsin is a circadian photopigment located in retinal ganglion cells projecting to the biological clock.

Key words: colocalization; suprachiasmatic nucleus; circadian rhythm; rat; immunohistochemistry; melanopsin antibodies
Using PACAP as a marker for the RHT-projecting retinal ganglion cells and using in situ hybridization combined with immunohistochemistry, we show here that the distribution of melanopsin mRNA and protein in the rat retina is identical to that of the PACAP-containing retinal ganglion cells, and that melanopsin is exclusively present in PACAP-immunoreactive cells.

MATERIALS AND METHODS

Animals and tissue preparation
Male Wistar rats (180–220 gm; n = 20) housed under standard laboratory conditions in a 12 hr light/dark cycle with access to food and water ad libitum were used in the study. [Light was turned on at zeitgeber 0 (ZT0) and turned off at ZT12.] Experiments were performed according to the principles of laboratory animal care in Denmark (Publication No. 382; June 10, 1987). Because initial experiments showed the highest level of both PACAP and melanopsin at subjective day, animals were killed between ZT0 and ZT8. Animals were decapitated and eyes were rapidly removed after marking the most medial point of the cornea with ink to allow orientation of the retina. After removal of the cornea and the anterior chamber, the vitreous body was gently removed. The retinas located in situ in the posterior chamber were fixed in Stefanini’s fixative (2% paraformaldehyde and 0.2% picric acid in 0.1 m sodium phosphate buffer, pH 7.2) for 12–24 hr at 4°C, removed from the eyecup, transferred to cryopreservation media, and stored at −20°C until they were processed for in situ hybridization and immunohistochemistry as whole mounts according to procedures described below. Eyeballs from additional animals were sectioned rather than processed as whole mounts. In these cases, sections (12 μm/section) were cut perpendicular to the long axis of the eyeball to allow a determination of the laminar localization of melanopsin and/or PACAP gene-expressing cells.

Cloning of the rat melanopsin cDNA

A 498 bp cDNA fragment of rat melanopsin was obtained from purified total RNA extracted from rat retinas by reverse transcriptase (RT)-PCR using the following primers: 5'-GTCCGTAGGCTTCTGGCTTTATTT-3' and 5'-GTACCTGGGGTGAGTGATGGCGTA-3' (Sigma-Genosys Ltd., Cambridge, UK). The product was cloned into the PACAP gene-expressing cells.

In vitro hybridization histochemistry

In situ hybridization

In situ hybridization combined with immunohistochemistry, melanopsin and/or PACAP mRNA-containing cells. Positive cells with signals above threshold from each section were then compiled and the number of ganglion cells containing melanopsin and PACAP mRNA was determined.

Immunohistochemistry

Melanopsin antibodies. A cDNA fragment encoding the C-terminal predicted cytoplasmatic part of mouse melanopsin was generated by RT-PCR. The cDNA was made from total RNA prepared from mouse retinas using oligo(dT) primers and SuperScript (Life Technologies, Taastrup, Denmark). The PCR was done using the primers 5'-TCTCTAC-TGAGGCACCACATC-3' and 5'-TTCTCTGCTGAGGCCACATA-3' (Provencio et al., 2000) as well as platinum Taq polymerase (Life Technologies). The PCR fragment was cloned in pCRII-TOPO (Invitrogen). A clone containing 13 base mismatches was used as template for a second PCR using the primers 5'-CACCCCAAGTACAGGGTGCCCAT-3' and 5'-TCTCTCTGCTGAGGCCACATA-3'; the fragment from this second PCR was subcloned in the vector pCR1/NT-TOPO (Invitrogen) as described by the manufacturer. The sequence contained an open reading frame that represents 160 amino acid residues (underlined) of the C-terminal cytoplasmatic part of mouse melanopsin (MRRGSHHHHHHHGSMASMTGQMGKLRKVYDQDDDKDPTLHKYRPVAYOHALPHPLGVLGLVGSGSHPSLWSTRTHRTLSASSDLSWSSGRKRRQESLSGESEVGTDTETTAAGWGTAQAGGOSCNEDLGEDKASSPPVORSKTLPVIPDRFMRNSTOPSLRQGLAVGSLSEINGLSTFQPCFGGR). The recombinant fusion protein was expressed in the host strain BL21(DE3) pLyS5, expressed in Tris-equilibrated phenol, pH 8.0, and precipitated with ethanol. The protein pellet was solubilized in 6 m guanidinium chloride, 0.1 m DTT, and 50 mm Tris, pH 8.0. The solubilized protein was passed over Sephadex G25 column equilibrated with 5 m mercaptoethanol, 0.5 m urea, 0.5 m NaCl, and 50 mm Tris, pH 8.0 (MUNIT). Finally, the Histagged recombinant fusion protein was captured on a Ni-nitrilotriacetic acid Superflow column (Qiagen, Hilden, Germany); equilibrated with MUNIT; washed in 0.5 m NaCl and 50 mm Tris, pH 8.0; and eluted with 10 mm EDTA, 0.5 m NaCl, and 50 mm Tris, pH 8.0. Four rabbits were immunized with 50 μg of melanopsin fusion protein, dialyzed against 100 mm Tris and 0.15 m NaCl. The immunization material was emulsified in an equal volume of complete Freund’s adjuvant for the first immunization and incomplete Freund’s adjuvant for the subsequent immunizations at 10 intervals. Rabbit serum (code no. 41K9, diluted 1:1000) drawn by venipuncture 5 weeks after the second immunization reacted with purified recombinant protein (molecular mass of 28 kDa) in a Western blot performed as described previously (Hindersson et al., 1987). The Western blot antibody reactivity as well as immunostaining were removed by absorption of the anti-melanopsin antibody (code MabHHI) directed against an epitope (amino acids 6–16) that recognizes both PACAP-38 and PACAP-27 was used for PACAP immunostaining (Hannibal et al., 1997). A rabbit serum (code no. 41K9, diluted 1:1000) drawn by venipuncture 5 weeks after the second immunization reacted with purified recombinant protein (molecular mass of 28 kDa) in a Western blot performed as described previously (Hannibal et al., 1997). The PCR was done using the primers 5'-TCTCTAC-TGAGGCACCACATC-3' and 5'-TTCTCTGCTGAGGCCACATA-3' (Provencio et al., 2000) as well as platinum Taq polymerase (Life Technologies). The PCR fragment was cloned in pCRII-TOPO (Invitrogen). 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Photomicrographs

Images were obtained via a Leica DC200 camera using Leica DC200 software. For double immunohistochemistry, an OlympusIX70 confocal microscope equipped with Flouview v 2.1.39 (Olympus, Copenhagen, Denmark) was used. Image-editing software ( Adobe Photoshop and Adobe Illustrator; Adobe Systems, San Jose, CA) was used to combine the obtained images into plates, and figures were printed on a Tektronix (Wilsonville, OR) Phaser 480 dye sublimation printer.

RESULTS

Using in situ hybridization and immunohistochemistry, melanopsin mRNA and protein were detected in a subset of retinal
ganglion cells distributed throughout the retina and in a few seemingly displaced ganglion or displaced amacrine cells located between the ganglion cell layer and the inner nuclear cell layer (INL) (Figs. 1A,D,G; also see Fig. 4). However, the distribution pattern was not uniform, because the density of ganglion cells containing melanopsin was in the range of 36–39 cells/mm² in the superior half of the retina and 5–9 cells/mm² in the lower half of the retina (Fig. 1D,G). The distribution of melanopsin-containing cells was identical to the distribution of retinal ganglion cells expressing PACAP mRNA. The density of the cells containing PACAP mRNA ranged from 31 to 37 cells/mm² in the superior half of the retina and from 5 to 8 cells/mm² in the lower half of the retina (Fig. 1E,H). Because an identical distribution of cells containing PACAP immunoreactivity was observed (Fig. 1F,I), we investigated whether melanopsin and PACAP were present in the same ganglion cells using in situ hybridization histochemistry or the melanopsin antibody in combination with PACAP immunostaining. Melanopsin was demonstrated exclusively in the PACAP-containing retinal ganglion cells and in a few PACAP-expressing displaced ganglion or displaced amacrine cells (Figs. 2–4). The punctate melanopsin immunoreactivity was located at the surface of the ganglion cell.

Figure 1. Melanopsin and PACAP expression in the rat retina. In situ hybridization histochemistry on whole-mount rat retinas using 33P-UTP-labeled cRNA probes for melanopsin mRNA (A, D, G), PACAP mRNA (B, E, H), and PACAP immunoreactivity (C, F, I) demonstrate an identical distribution pattern for melanopsin and PACAP located to a subset of retinal ganglion cells. Higher magnification clearly showed a fourfold to fivefold higher density of melanopsin- and PACAP-expressing retinal ganglion cells in the superior half of the retina (D–F) compared with the inferior half (G–I). Scale bars: A–C, 1000 μm; D–I, 200 μm.
Melanopsin is colocalized with PACAP in retinal ganglion cells. A photomicrograph of melanopsin mRNA visualized by 33P-UTP labeled cRNA probes (A) and PACAP immunostaining visualized by CY2 (B) on the same whole-mount retina is shown. Individual retinal ganglion cells are numbered, and each number indicates the same cells in the two photomicrographs. Note that silver grains representing melanopsin mRNA are also present in the PACAP-containing retinal ganglion cells (B). Scale bars, 100 µm.

DISCUSSION

In mammals, light entrainment of the clock is dependent on ocular light perception (Nelson and Zucker, 1981). However, mice lacking the classical rod and cone photoreceptors (rda/rd mice) are still able to entrain to a light/dark cycle (Freedman et al., 1999; Lucas et al., 1999), suggesting that unidentified photoreceptors located in other parts of the retina are responsible for light entrainment of the circadian rhythm (Lucas and Foster, 1999). Our finding that melanopsin is located exclusively in the PACAP-containing retinal ganglion cells constituting the RHT (Hannibal et al., 2001a) supports the existence of a “non-image-forming” visual pathway to the circadian system and makes melanopsin a candidate as a circadian photopigment. Additional support for this notion comes from an electrophysiological study on a flat-mount preparation of rat retina in vitro that demonstrated that SCN-projecting ganglion cells, in contrast to conventional ganglion cells, responded to light regardless of chemical blocking of synapses (Berson et al., 2001). Furthermore, the light response was not attributable to electrical coupling to rods and cones because the SCN projecting ganglion cells were depolarizing, not hyperpolarizing as the in rods and cones, suggesting that the phototransduction occurs within these retinal ganglion cells themselves. Another interesting observation of the present study was that the localization of melanopsin immunoreactivity was restricted to the membrane of the cell soma and to processes within the retina but not to processes in the optic nerve or in the brain. This localization increases the light-perceiving surface of the ganglion cells projecting to the SCN and may increase their sensitivity to light stimulation.

The melanopsin photopigment is located in ganglion cells constituting the RHT

As reported for melanopsin mRNA in mice (Provencio et al., 2000), we found that melanopsin (mRNA and protein) in the rat was only present in a subset of retinal ganglion cells and in a few displaced amacrine or displaced ganglion cells; this finding agrees with the proposed localization in the inner retina of a specific “circadian” photoreceptor (Freedman et al., 1999; von Schantz et al., 2000). The expression of melanopsin in PACAP-containing retinal ganglion cells was demonstrated by a combination of in situ hybridization histochemistry/immunohistochemistry and by double immunohistochemistry. The number of retinal ganglion cells constituting the RHT is much higher in rats (Moore et al., 1995; Hannibal et al., 2001a) than in mice (Provencio et al., 1998a); however, of greater interest is the demonstration that the density of SCN-projecting cells was four- to fivefold higher in the superior than in the inferior retina. At present we have no explanation for this uneven distribution, but dopamine, which is a circadian transmitter of the retina, is located by a group of amacrine and displaced amacrine cells that also show a superior–inferior difference in density in the retina (Versaux-Butteri et al., 1986). Whether there is any relationship between these amacrine cells and the melanopsin/PACAP-containing cells remains to be investigated.

Action spectra analysis of possible circadian photopigments

Using action spectrum analysis for light entrainment of locomotor activity in mammals, it has been reported that the light-sensitive material responsible for circadian photoentrainment could be an opsin-based photopigment with an absorption peak of ~500 nm (Takahashi et al., 1984; Provencio and Foster, 1995). However, in a recent study in humans, a photopigment with an absorption maximum close to 480 nm seemed to be involved in light-induced suppression of melatonin at night (Brainard et al., 2001). In blind rd/rd/cl mice that are able to entrain to a light/dark cycle, a photopigment with an absorption peak of 480 nm has been shown to mediate the pupillary light reflex (Lucas et al., 2001). A possible explanation for the observation described above could be the existence of at least two photopigments, one mediating pupillary reflex and melatonin suppression and one that is responsible for light entrainment of locomotor activity. To further clarify the role of melanopsin in circadian photoentrainment, the absorption peak needs to be determined. In contrast to melanopsin, the vitamin B2-based CRY1 and CRY2, which are present in a large number of retinal ganglion cells and in unidentified cells of the inner nuclear cell layer (Miyamoto and Sancar, 1998; Sancar, 2000), have absorption maxima close to 420 nm (Miyamoto and Sancar, 1998), making them less likely as circadian photoreceptors. This notion is supported by studies in mCRY-deficient mice (mCRY1–/–; mCRY2–/–) demonstrating that although the mCRYs are important components of the molecular clock in the SCN, they are not essential for transmitting light information to...
the SCN (Vitaterna et al., 1999). However, a role for the CRYs in light perception to the clock has been suggested recently. Triple-mutant mice lacking both CRYs, rods, and cones (rd/rd, mCRY1−/−, mCRY2−/−) were found to have lost the ability to adjust their behavioral rhythm to a 12 hr light/dark cycle, in contrast to their littermate controls (rd/rd, mCRY1−/−, and mCRY2−/− mutant mice (Selby et al., 2000). Whether the CRYs are directly photosensitive or whether they participate in a signaling pathway downstream to another photoreceptor like melanopsin is an open question.

Light activation of the retinal ganglion cells of the RHT

The physiological properties of retinal ganglion cells of the RHT in response to light stimulation have been studied using extracellular recordings (Pu, 2000). Morphologically, cat retinal ganglion cells characterized as non-α, non-β cells (Pu, 1999) resemble the melanopsin/PACAP-containing retinal ganglion cells of the rat in terms of their size and number of dendritic processes (Hannibal et al., 1997, 2000, 2001a). The recordings are characterized by a sustained “on” response to light lasting as long as light is turned on and peaking at 500 nm (Pu, 2000). We have shown recently...
the surface of the cell body and the processes. The whole-mount retinas. Note the punctate melanopsin immunoreactivity on focal laser scanning photomicrograph showing double-immunostaining of fos (A–C) and in a single displaced retinal ganglion/amacrine cell. Confocal photomicrograph showing double-immunostaining of melanopsin on the PACAP-containing retinal ganglion cells (A, D), PACAP (B, E), and melanopsin/PACAP (C, F) in a retinal ganglion cell (A–C) and in a single displaced retinal ganglion/amacrine cell. G, Confocal laser scanning photomicrograph showing double-immunostaining of melanopsin (green) and PACAP (red) in the same ganglion cells of whole-mount retinas. Note the punctate melanopsin immunoreactivity on the surface of the cell body and the processes. The inset shows high magnification of a melanopsin/PACAP-containing dendrite. GCL, Ganglion cell layer. Scale bars: A–F, 50 μm; G, 20 μm.

that c-fos expression is induced by white light in the PACAP-containing retinal ganglion cells and that Fos immunoreactivity is sustained only in the PACAP-containing retinal ganglion cells as long as light is turned on (Hannibal et al., 2001a). The presence of melanopsin on the PACAP-containing retinal ganglion cells suggests that the light-on response is attributable to activation of the melanopsin photopigment, possibly via a signaling pathway coupled to G-protein, and is regulated by kinases (Provencio et al., 1998b).

Is PACAP an intraretinal transmitter?

In addition to innervation of the SCN, PACAP-immunoreactive processes branch to the inner plexiform layer and INL (Hannibal et al., 2000). Furthermore, the PACAP-specific PAC receptor is expressed in the INL of the rat retina (Seki et al., 1997). Thus, it is possible that light information received by the PACAP-containing retinal ganglion cells could be transmitted to other retinal cells, and it is tempting speculate that the peptide may participate in the entrainment of the “retinal circadian clock” expressing many of the recently identified clock genes (King and Takahashi, 2000). In contrast, the melanopsin/PACAP-containing cells may receive inputs from other retinal neurons.

In conclusion, our data, in conjunction with published action spectra analyses and work in retinally degenerated (rd/rd/crl) mutant mice, suggest that melanopsin is a circadian photoreceptor located in PACAP-containing retinal ganglion cells projecting to the biological clock.

REFERENCES


Hannibal et al. • Melanopsin in PACAP-Containing Retinal Ganglion Cells


