Immunohistochemical Evidence of a Melanopsin Cone in Human Retina

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Purpose. Melanopsin, expressed in a subset of intrinsically photosensitive ganglion cells that project to the suprachiasmatic nucleus (SCN), is involved in the photic entrainment of circadian rhythms and other non–image-forming functions (pupil light reflex, masking, acute heart rate response, and alertness). Melanopsin has recently been shown to be a "bireactive" photopigment that functions as a photosensory opsin using 11-*cis* retinaldehyde as a chromophore and has intrinsic photoisomerase activity. Melanopsin is widely distributed in the retina of vertebrates and, depending on the species, is expressed in ganglion, amacrine, horizontal, and photoreceptor cells. The present study was conducted to determine the distribution of this opsin in the human retina.

Methods. Human donor eyes were obtained from donors and fixed shortly after death. Immunohistochemistry was used to determine melanopsin expression in the retinas of three donors. The possible coexpression of this photopigment with other opsins was studied by double-labeling immunocytochemistry and confocal analysis.

Results. In addition to the expected labeling in ganglion cells of the inner retinal layers, an unexpected finding showed melanopsin-immunopositive label in the outer segments of cones that did not coexpress other known opsins. These melanopsin-expressing cones are extremely sparse (5–25 cones/mm^2; 0.1%–0.5% of the entire cone population) and are located in the peripheral retina.

Conclusions. The presence of melanopsin in human cones suggests image and non–image-forming roles in visual responses at both the cone input and ganglion cell output stages and their involvement in a broad spectrum of irradiance detection functions in the visual system. (*Invest Ophthalmol Vis Sci.* 2006;47:1636–1641) DOI:10.1167/iovs.05-1459

Several novel opsins mediating nonvisual responses have been identified in cells and tissues other than the classic retinal photoreceptors in a several vertebrate species. These photopigments include pinopsin in chicken, vertebrate ancient opsin in salmon and zebrafish, encephalopsin, neuropsin in mice and humans, and melanopsin in *Xenopus* and mammals. Among these photopigments, only neuropsin and melanopsin have been described in the retina of mammals, including humans.[1]

Melanopsin (*Opn4*), an opsin-based photopigment originally cloned from amphibian melanophores,[2] is expressed in the retina of many vertebrate species. In rodents and primates, melanopsin is expressed in a subset of retinal ganglion cells that are intrinsically photosensitive.[3,4] The action spectrum of these ganglion cells is typical of an opsin-based photopigment peaking near 480 nm. In rodents, melanopsin-positive cells are uniformly distributed throughout the ganglion cell layer with a few displaced ganglion or amacrine cells in the inner nuclear layer.[5–7] Most of the ganglion cells that express melanopsin project to the suprachiasmatic nucleus (SCN) and also express the neuropeptide pituitary adenylate cyclase activating peptide (PACAP).[7] Melanopsin participates significantly in several circadian and nonvisual responses to light, including photic entrainment of the circadian system, pupillary light reflex, masking responses, and photic induction of FOS in the suprachiasmatic nucleus (SCN).[8–11] In mice without melanopsin, the amplitude of a light-induced phase shift, masking, and pupillary responses are reduced.[8,9,11] Triple-knockout mice with targeted deletion of the melanopsin gene and a nonfunctional rod–cone transduction pathway display a complete loss of photic entrainment, pupillary light response, masking and arylalkylamine-*N*-acetyltransferase suppression by light,[12,13] demonstrating that other photopigments are not involved in these responses. Electrophysiological and ultrastructural evidence in rodents show that melanopsin-expressing ganglion cells receive excitatory bipolar and inhibitory amacrine cell inputs, suggesting that their intrinsic photic response can be modified by rod and/or cone signals.[3,14] In primates, a recent electrophysiological study shows that short-wavelength (SW) cones provide an inhibitory input to melanopsin-containing ganglion cells, whereas rods and medium/long-wavelength (MW/LW) cones supply an excitatory input.[4]

In humans, melanopsin has been cloned, and the messenger RNA is highly expressed in the retina with trace amounts detected in the retinal pigment epithelium.[15] A recent study using in situ hybridization and immunohistochemistry demonstrated that the subset of retinal ganglion cells that express melanopsin is uniformly distributed[16] and coexpress PACAP.[17]

There has been considerable debate concerning whether melanopsin has an intrinsic photoisomerase activity or acts as a photosensory opsin using 11-*cis* retinaldehyde as a chromophore. Recent evidence from several groups using different heterologous expression systems suggests that melanopsin is bi-reactive and can use both biochemical mechanisms.[18–20] When incubated with 11-*cis* retinaldehyde, the action spectrum shows an opsin-based nomogram with a peak around 480 nm, which is similar to that obtained from studies of pupillary reflex, light entrainment of circadian rhythms and more specifically, for electrophysiological recordings from intrinsically melanopsin-expressing ganglion cells.[3,12,21]

In the present study, we show that the human retina contains cones that express melanopsin, that are distinct from

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known SW, MW, and LW cones which form the basis of human trichromatic vision. Together with recent results of biochemical studies showing that this opsin can act as a classical photopigment, the localization of melanopsin in outer segments supports the idea that these cones constitute functional photoreceptors in humans.

Materials and Methods

Tissue Samples

All material used in the present study was obtained in compliance with the Declaration of Helsinki for research involving human tissue. Eyes were obtained from three donors (Department of Anatomy, University of Lyon, Université Claude Bernard-Lyon 1), under approval of the Institutional Human Subjects Committee. Donors were of both sexes, were aged from 40 to 65 years, and had no previous history of eye disease. The eyes, collected 6 to 18 hours after death, were placed overnight in Zamboni's fixative (4% paraformaldehyde with 15% picric acid in phosphate buffer 0.1 M [pH 7.4]) at 4°C and rinsed in phosphate buffer (PBA 0.1 M and sodium azide 0.1%, [pH 7.4]) the next day. Fixed eyes were cryoprotected by immersion in 30% sucrose (in PBA) overnight, embedded in a solution of 2.5% agar with 30% sucrose and cut on a microtome (Reichert-Jung, Vienna, Austria) used for frozen sections at a thickness of 15 μm. Free-floating retinal sections were placed in titration wells containing PBA and stored until use.

Immunohistochemistry

Single immunolabeling using antiserum against mouse and human melanopsin[5,16] and an immunoperoxidase technique were used to identify melanopsin-expressing cells in the human retina. Endogenous peroxidase was first suppressed with a solution of 50% ethanol in saline with 0.03% H_2O_2. Free-floating sections were briefly rinsed in PBS (0.01 M, pH 7.2) containing 0.3% Triton and 0.1% sodium azide (PBSTA) and blocked with 1% BSA. Sections were incubated in the anti-melanopsin primary antibody (the kind gift of Ignacio Provencio, University of Virginia, Charlottesville, VA; 1:1200) for 2 days at 4°C. Immunoreactivity was visualized with an avidin-biotin complex (ABC) kit (Vectastain Elite kit PK-6100; Vector Laboratories, Burlingame, CA), followed by incubation in 0.2% 3,3′-diaminobenzidine, 0.5% ammonium nickel sulfate, and 0.003% H_2O_2 in Tris buffer (0.05 M; pH 7.6).

Multiple immunostaining using combinations of anti-opsin antibodies and/or lectin were used to identify melanopsin-positive photoreceptors and to study coexpression of melanopsin with other cone opsins. To characterize melanopsin-positive photoreceptors, we use a biotinylated peanut agglutinin lectin (PNA; Vector Laboratories) that specifically binds to the extracellular matrix of all cones. In this case, retinal sections were washed twice in PBST and incubated at room temperature for 2 hours in biotinylated PNA (100 mg/mL), which was revealed using FITC avidin D (100 mg/mL; Invitrogen). To identify SW and MW/LW cone opsins in human retinal sections, we used, respectively, the mouse monoclonal antibody OS-2 (1:2500) produced against chicken photoreceptor membranes and the mouse monoclonal antibody COS-1 (1;100) generated to chick opsins.[22] Free-floating sections were preincubated in PBSTA with 1% BSA to block nonspecific binding sites. Retinal sections were then incubated in two mixtures of antibodies for melanopsin and either SW or MW/LW opsins. After 4 days of incubation at 4°C, the binding sites of primary antibodies were demonstrated by using a mixture of fluorescent probes (i.e., goat anti-rabbit Alexa 594, 100 mg/mL, and goat anti-mouse Alexa 488, 100 mg/mL; both from Invitrogen) for 2 hours. Control experiments were performed by omitting primary antibodies or by preadsorption of the primary melanopsin antiserum with the immunizing peptide, which blocked all labeling. When a mixture of two antibodies or two fluorescent probes was used, a control was performed by using each antibody singly and by applying them in different sequences. For example, the patterns of melanopsin and opsin staining were examined in retinal sections when their respective antibodies were used, both simultaneously or in succession in different sequences.

Digitized images for single labeling of melanopsin-positive neurons were captured with a digital camera (Spot II; Diagnostic Instruments, Sterling Heights, MI) with 40× and 63× objectives. Confocal microscopy (TCS SP; Leica, Deerfield, IL) was used to verify the presence of double-fluorescent label in cones with melanopsin and anti-opsin antibodies. Laser lines and emission filters were optimized with the software (PowerScan Leica). Confocal images obtained as stacks of images (0.2 μm thickness) were analyzed with the confocal software (Leica). Image processing was performed electronically (Photoshop; Adobe Systems, Mountain View, CA).

Results

Single immunolabeling using antiserum against both human and mouse melanopsin and an immunoperoxidase technique revealed immunopositive ganglion cells and cones in the human retina. In the inner retina, melanopsin was expressed in neurons in the ganglion cell layer (GCL; Fig. 1A) and in the inner nuclear layer (INL; Fig. 1B) as recently shown in macaques and humans.[4,17] The latter population has been suggested to be composed of displaced ganglion cells or possibly amacrine cells. These two populations of melanopsin-positive cells present a large soma (15–20 μm diameter) with dendritic processes localized at the inner plexiform layer (IPL)–INL and IPL–GCL borders.

Melanopsin immunopositive photoreceptors were observed when using both mouse and human antibodies, with distinct label in the outer segment (Figs. 1C, 1D). These photoreceptors have a typical conelike morphology and share common features with the other cone types in the human retina. Similar to all other human cone opsins—SW, MW, and LW— the photopigment melanopsin was expressed only in the outer segment. Second, the use of the marker lectin PNA, which is specific to the extracellular matrix of cones, stained all cones including those expressing melanopsin (Fig. 2). These melanopsin-positive photoreceptors were observed in the retina of all three donor eyes.

The spatial distribution of the labeled cones differs from that of other cone types, since melanopsin-positive cones are sparse, evenly distributed, and observed only in the peripheral region of the retina, from the parafoveal region (estimated as approximately 20° from the foveal area) up to approximately 2 mm from the ora serrata. Retinas from the three subjects were used to quantify the density of melanopsin-containing cones. In the 110 retinal sections examined we counted a total of 40 immunopositive melanopsin cones (immunoperoxidase and immunofluorescence). This corresponded to a total section length of 98.2 mm and, given the 20-μm section thickness, a total retinal surface area of 1.96 mm^2. The melanopsin cone density can thus be estimated as 20.4 cones per square millimeter of retina. To further confirm this density, the relative number of SW to melanopsin-expressing cones was compared in sections stained with fluorescent probes for both opsins (40 sections). The counts show a ratio of one to five melanopsin-positive cones per 100 SW cones. In the peripheral regions of the human retina,[23] SW cones show an even distribution of 500 cones/mm^2, yielding 5 to 25 melanopsin cones/mm^2, which is close to the spatial density estimated herein. Curcio et al.[24] has estimated that the mean cone density in the human retina is approximately 4500 cones/mm^2, and therefore melanopsin immunopositive cones equal 0.11% to 0.55% of the entire cone population. This percentage is equivalent to the 0.25% estimated for melanopsin-expressing ganglion cells versus total ganglion cells in the primate retina.[4]

Because single mammalian cones can express more than one opsin, either during development or in the adult

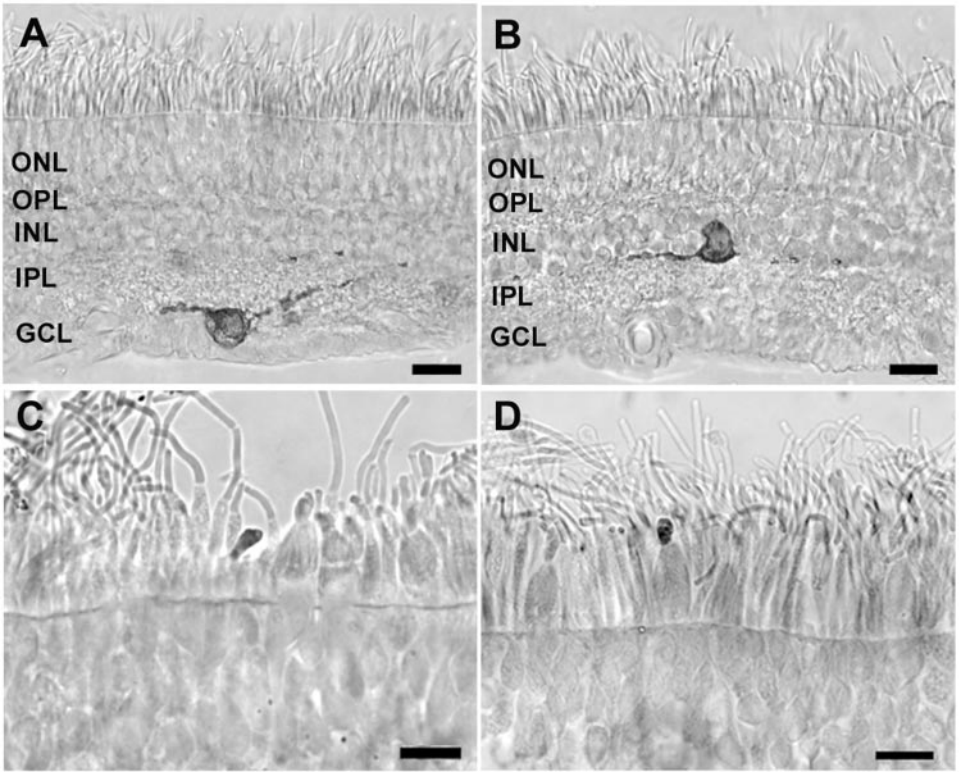

FIGURE 1. Melanopsin expression in the human retina. (**A**) Melanopsin was found in a subset of cells located in the GCL and (**B**) in the INL (anti-human melanopsin). These melanopsin-expressing cells had large somas (15–20 μm) and showed strongly stained dendrites located at the INL–IPL and IPL–GCL borders. Melanopsin-expressing cones were detected with antibodies against both human (**C**) and mouse (**D**) melanopsin. These melanopsin-expressing cones were extremely rare and were observed only in peripheral regions of the retina, from the parafoveal region up to roughly 2 mm of the ora serrata. The density of melanopsin containing cones is estimated at 5 to 25 cones/mm^2, corresponding to approximately 5% of the SW cone population density. Scale bar, 20 μm.

stages,[25,26] it was important to determine whether melanopsin is expressed uniquely or is coexpressed with other opsins in cone outer segments. We thus used different combinations of double-label immunohistochemistry with specific, well-characterized monoclonal antibodies against cone photopigments and combined confocal microscopy. Several control experiments were first performed, to exclude any possible cross-reactions between opsin antibodies in human retina.[27] Figure 3A shows a sample section of a melanopsin-positive cone flanked by several different cones immunopositive for MW/LW opsins. Figure 3B shows a double-labeled retinal section with a single melanopsin and a single SW opsin–immunopositive cone. In all sections examined, confocal analysis clearly showed that the melanopsin-positive cones were distinct and did not contain either SW or MW/LW opsins. No occurrence of coexpression of melanopsin with other cone opsins was ever observed.

DISCUSSION

Humans and other diurnal primates typically possess three types of cones with sensitivity in the LW (556–562 nm), MW (530–550 nm) and SW-sensitive range (near 410–435 nm) which allow for trichromatic color vision. In this study we describe a new cone type in the human retina that stains immunopositive for melanopsin and which lacks other known opsins.

This result adds to a wide pattern of neuronal distribution of melanopsin in different retinal cell types among vertebrates. In amphibians, melanopsin is found in ganglion and horizontal cells of the inner retina, as well as in iris and retinal pigment epithelium. In the retina of teleosts (zebrafish, Atlantic cod, and roach), melanopsin is expressed in horizontal, amacrine, and ganglion cells.[28,29] In the Atlantic cod, two melanopsin genes (*Opn4a*, *Opn4b*) have been identified, both of which are expressed in cells located in the inner nuclear and ganglion cell layers, whereas only *Opn4a* is expressed in horizontal cells. In chicken retina, melanopsin mRNA is expressed in a few dispersed ganglion cells, in cells located in the outer (bipolar and horizontal cells) and the inner (amacrine cells) parts of the inner nuclear layer and in the photoreceptors.[30]

In mammals, melanopsin has been described in rodents and primates, including humans. In rodents, melanopsin is expressed in a subpopulation of neurons in the ganglion and inner nuclear cell layers and in the retinal pigment epithelium.[5,6,31] In primates (monkey and human), melanopsin mRNA is expressed in cells within the ganglion cell and the inner nuclear layers.[15,17] In humans, trace expression of melanopsin had been detected in the retinal pigment epithelium, which was considered at that time to represent contamination from the retina.[15] Our results, however, suggest that the small amount of melanopsin mRNA could originate from cone photoreceptor outer segments that remain in the pigment epithelium during processing. Because of the difficulty in obtaining good-quality retinal tissue, studies in humans are scarce, and none have provided adequate staining of the photoreceptor layer in humans. In addition, the extremely low density coupled with the localization of label in the outer segment, melanopsin-positive cones could have been overlooked in previous studies in which flatmounted retinas or human retinas were used that were of pathologic origin and in which almost all the photoreceptors lacked outer segments.[4,17] Identification of the immunopositive outer segment requires histologic retinal sections of high quality which preserves the outer segments intact.

An important finding of our study is the unique expression of melanopsin in human cone outer segments, since coexpression of opsins in other cell types is a common feature in vertebrates including humans. Melanopsin is coexpressed with rod-opsin in *Xenopus* melanophores[32] and possibly, with pinopsin, in chick pinealocytes[33] or with VA opsin in zebrafish horizontal cells.[34] It is not known whether the chicken photoreceptors that express melanopsin also contain other cone or rod opsins. The cell type and spatial distribution of these chick

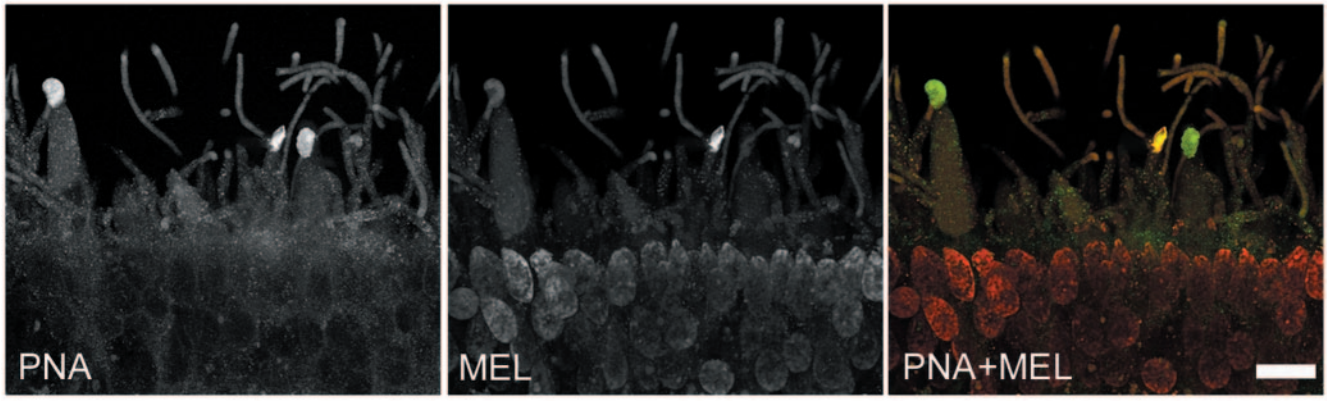

FIGURE 2. Confocal images of a transverse immunostained human retinal section showing localization of fluorescent PNA-lectin (*left*) and anti-melanopsin label (MEL, *middle*). The separate images (*left* and *middle*) and the overlayed color image (*right*; melanopsin in *yellow* and PNA in *green* fluorescence) clearly show that melanopsin-positive photoreceptors are labeled by PNA, demonstrating that melanopsin-positive photoreceptors are cones. Scale bar, 20 μm.

photoreceptors is also unknown.[30] In rodents, colocalization of two opsin types in single cones is widespread in either part of[35–38] or in the entire[26] cone population. In human fetal retinas, cones containing both SW and MW/LW opsins are found early during development, and although the number decreases after birth, some coexpression in cones is still present in the adult.[25] Confocal analysis in our study clearly shows the lack of coexpression of melanopsin with other human cone opsins. The finding by Cornish et al.[39] of a small population of cones that do not express SW or MW/LW opsins in human fetal retina may correspond to the immunopositive melanopsin cones described in our study.

The vertebrate opsins are proteins that use a retinaldehyde chromophore in their photosensory or photoisomerase activity in the visual/irradiance detection cycle. In the photosensory opsins (rod and cone opsins), light is absorbed by the chromophore 11-*cis* retinal, which is converted to all-*trans* retinal. Opsins that act as photoisomerases (e.g., RGR-opsin and peropsin) bind all-*trans* retinal and use light for conversion to 11-*cis* retinal, thus supplying photosensory opsins with chromophore. Understanding the biochemical light-transduction pathway of melanopsin has been a major challenge, because this opsin-based photopigment had been difficult to express in vitro[40] and had been thought to use an invertebrate transduction signaling pathway. This view was also based on the fact that melanopsin is expressed in neuronal cell types located at a distance from the pigment epithelium and thus would not have access to the chromophore retinaldehyde. Recent evi-

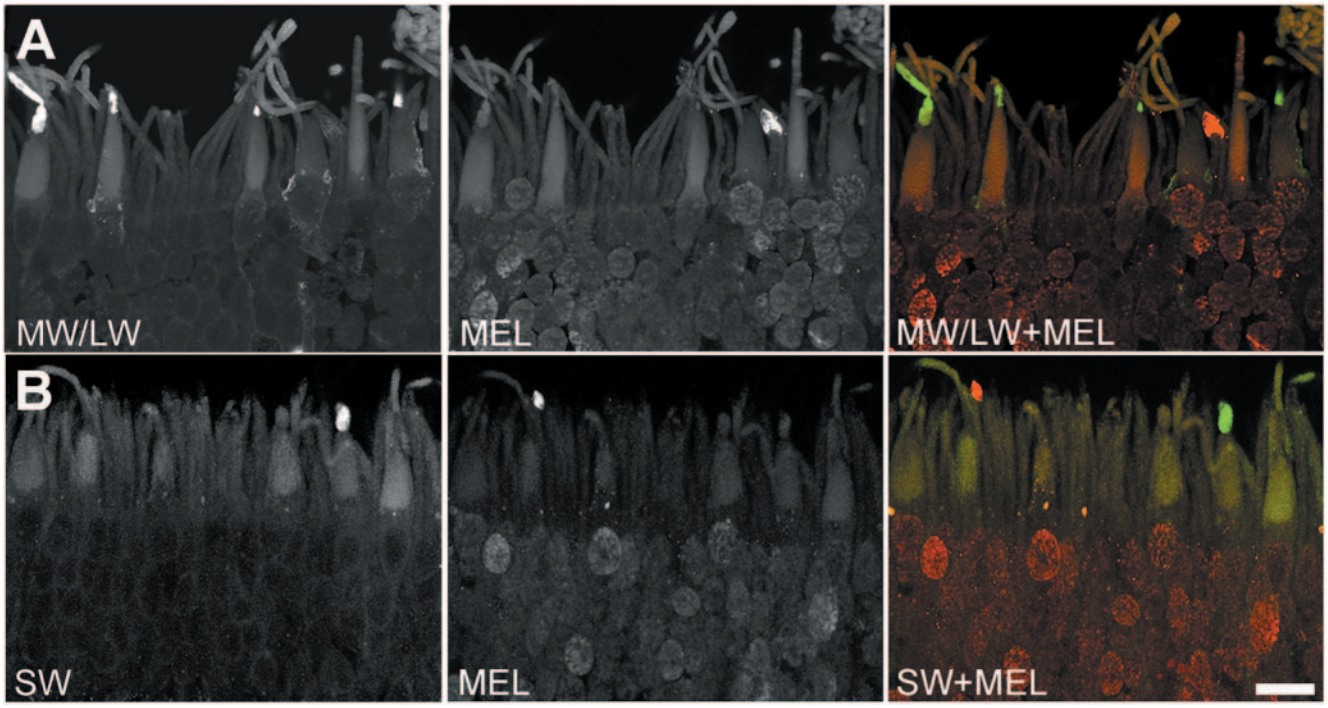

FIGURE 3. Characterization of melanopsin-positive cones showing the absence of coexpression with other cone opsins. (**A**) A series of confocal images of cones immunostained for MW/LW opsins (*left*), melanopsin (MEL, *middle*), and the overlayed color image (*right*). (**B**) Confocal images of cones immunostained for SW opsin (*left*), melanopsin (MEL, *middle*) and the overlayed color image (*right*). In the color overlays in (**A**) and (**B**) the melanopsin cones (*red* fluorescence) are distinct from the SW or MW/LW cones shown in *green* fluorescence. Scale bar, 20 μm.

dence from several groups shows that heterologously expressed melanopsin confers photosensitivity to nonphotosensitive cell lines.[18–20,41] In addition, melanopsin is a bireactive photopigment with intrinsic photoisomerase activity or functions as a photosensory opsin that uses 11-*cis* retinaldehyde as a chromophore. The latter pathway, confirms the possibility that melanopsin can form a functional photopigment in the photoreceptor outer segments of both chickens and humans.

Melanopsin contributes to a wide variety of non–visual-irradiance detection processes including circadian entrainment, suppression of pineal melatonin synthesis, inhibition of locomotor activity, pupillary constriction, and modulation of sleep–wake states.[8,9,11–13,42] These functions are related to the intrinsic photosensitivity of melanopsin-expressing ganglion cells and their projections to structures mediating these processes (SCN, pretectum, intergeniculate leaflet, ventrolateral preoptic nucleus, and ventral subparaventricular zone) in rodents. In humans, both melanopsin-containing ganglion cells and cones may be involved in mediating these non–image-forming responses, since results of action spectrum and phase-shift studies suggest the involvement of a non-rod non-cone photopigment (Cooper HM, et al. *IOVS* 2004;45:ARVO E-Abstract 4345).[43–45] In humans, the possible role of melanopsin in nonvisual functions has recently been expanded to include functions such as changes in alertness, aspects of autonomic control (thermoregulation and heart rate[46]), and conceivably modulation of a large-scale network of cortical areas involved in attention processes.[47] In the primate, Dacey et al.[4] have also suggested that melanopsin expressing ganglion cells that project to the lateral geniculate nucleus may perhaps convey irradiance information to the visual cortex, a function that could also involve melanopsin-containing cones in humans. Furthermore, the suggested non-rod/non-cone mechanism that regulates the latency of the b-wave ERG cone response in humans, shows an action spectrum (λ_{max} = 483 nm)[48] similar to that of melanopsin-containing cells.[3] This response, which has been demonstrated only in humans is suggested to occur presynaptic to second-order neurons, and thus the melanopsin-expressing cone is ideally situated to exert such a modulation of the activity of other cones, possibly through horizontal cell pathways.

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