Pergamon

www.neuroscience-ibro.com

PII: S0306-4522(02)00327-5

Neuroscience Vol. 115, No. 4, pp. 1323–1333, 2002

0306-4522/02 $22.00+0.00

CHARACTERIZATION OF CALBINDIN-POSITIVE CONES IN PRIMATES

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Abstract—The aim of this study is to characterize calbindin-positive photoreceptors and their opsin content in the retina of nocturnal prosimians (*Microcebus murinus*), New World monkeys (*Callithrix jacchus)*, Old World monkeys (*Macaca fascicularis*), and humans. To identify the calbindin and opsin content of cones, combined multiple labeling with different fluorescent probes, antibodies directed against calbindin, short, and mid–long wavelength opsins, and lectin peanut agglutinin cytochemistry were used. With the exception of *Microcebus*, calbindin is present in the cones of all primates but is absent from rods. The distribution of calbindin is similar in human and macaque cones, with dense label in the inner segment, cell body, axon and cone pedicle. Cones in marmoset also show dense staining in the cell body, axon and pedicle but only light label in the inner segment. Primate cone outer segments do not contain calbindin. In the primates studied, three patterns of calbindin and opsin localization are observed. In macaque and marmoset all short and mid–long wavelength cones contain calbindin. In humans, all mid–long wavelength cones contain calbindin whereas all short wavelength cones are devoid of calbindin as confirmed by confocal microscopy. In the nocturnal prosimian *Microcebus* none of the mid–long or short wavelength cones contain calbindin. In addition to primates, calbindin is absent in cones of other nocturnal species but is present in cones of diurnal species suggesting a difference in the role of calbindin possibly related to the adaptational states or other photoreceptor properties.

Key words: retina, photoreceptor, calcium-binding protein, opsin, immunocytochemistry, prosimian.

Most primate species possess mid (MW) and/or long wavelength (LW) cones sensitive to medium or long wavelength light, and short wavelength (SW) cones sensitive to short wavelength light (Bowmaker et al., 1991; Jacobs, 1993; Tovee, 1994; Jacobs, 1996). Normal trichromacy, found in humans and Old World monkeys, arises from the encoding of the three photopigments by two or more X-chromosome pigment genes and an autosomal pigment gene. Differences in color vision between Old and New World monkeys result from different arrangements of the pigment genes on the X chromosome (Jacobs et al., 1996a). The males of most New World monkey species are dichromate, and only some females have X-linked polymorphisms which make them trichromate (Jacobs et al., 1993b). In contrast, certain nocturnal primates, including a New World monkey (the owl monkey, *Aotus trivirgatus*) and prosimians (*Galago garnetti, Otolemur crassicaudatus*), are reported to be monochromates since they lack SW cones based on anatomical (Wikler and Rakic, 1990), psychophysical, and electrophysiological studies (Jacobs et al., 1993a). However, this does not appear to be the case for all nocturnal primates since two recent studies in *Tarsius* (Hendrickson et al., 2000) and the gray mouse lemur (*Microcebus murinus*; Dkhissi-Benyahya et al., 2001) have provided evidence that SW opsin is expressed in a distinct cone population.

Studies aimed at understanding the anatomical identification and distribution of primate cone types have progressed in recent years through the use of antibodies directed against specific cone opsins (Wang et al., 1992; Szel et al., 1996; Vissers and DeGrip, 1996). In addition to opsin, several other proteins which are part of the transduction pathway (G protein transducin, arrestin, phosducin, recoverin; Lee et al., 1992; Nir and Ransom, 1992; von Schantz et al., 1994; Sakuma et al., 1996; Sears et al., 2000) have also been used as cellular markers for identification of cones. Other cellular components such as calbindin are observed in primate cones and to some extent in the cones of non-primate

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Abbreviations: DAB, 3,3′-diaminobenzidine; INL, inner nuclear layer; LW, long wavelength; MW, mid wavelength; ONL, outer nuclear layer; OPL, outer plexiform layer; PBA, phosphate buffer; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with Triton; PNA, peanut agglutinin lectin; SW, short wavelength.

mammals (Verstappen et al., 1986; Röhrenbeck et al., 1989; Pochet et al., 1991), but are absent from rods in all species. Calbindin, the 28-kDa vitamin D-induced calcium-binding protein, is a member of the protein family which also includes calretinin, calmodulin, parvalbumin and recoverin (Baimbridge et al., 1992). Together they constitute a group of homologous proteins with a specific structure, consisting of pouches for the acceptance of Ca^{2+}. Calbindin possesses an 'EF-hand' motif, which is defined by an amino acid sequence with a characteristic three-dimensional structure. The EF-hand binds Ca^{2+} with a high affinity at physiological concentrations of the intracellular compartments. Calcium-binding proteins have a restricted distribution in neurons, although their precise function, particularly in the retina, remains unclear. However, it has been suggested that calbindin could contribute to the Ca^{2+}-buffering capacity of neurons to maintain the intracellular Ca^{2+} concentration at appropriate levels and regulate the effects of Ca^{2+} on intracellular metabolism (Baimbridge et al., 1992).

In addition to cones, calbindin has been widely used as a neuroanatomical marker of different cell types in the retina and its distribution depends on the species (Peichl and Gonzalez-Soriano, 1994) and stage of development (Nag and Wadhwa, 1997; Yan, 1997). The expression of calbindin in retinal neurons has been useful for studying neural pathways, synaptic connections, and specific retinal cell types. For example, calbindin is characteristic of the horizontal cells in rabbit (Röhrenbeck et al., 1987; Scheibe et al., 1995), cat (Röhrenbeck et al., 1987), rodent (Rabie et al., 1985; Peichl and Gonzalez-Soriano, 1994) and may be localized in a subset of horizontal cells (H2 type) in monkey (Röhrenbeck et al., 1987, 1989; Wässle et al., 2000) as well as specialized groups of cone bipolar cells (DB3) in the macaque and marmoset (Martin and Grünert, 1992; Grünert et al., 1994; Luo et al., 1999; Jacoby and Marshak, 2000; Jacoby et al., 2000). In primates, calbindin is widely detected in various neuronal cell types from the photoreceptor to the ganglion cell layer (Verstappen et al., 1986; Hamano et al., 1990; Pochet et al., 1991).

In primates, the presence of calbindin in cones is reported to vary according to retinal location (Haley et al., 1995; Yan, 1997). For example, calbindin-positive cones are reported to be absent from the foveola in macaque and humans (Pasteels et al., 1990; Haley et al., 1995), a region also considered to contain sparse or no SW cones (de Monasterio et al., 1985; Szel et al., 1988; Wikler and Rakic, 1990; Martin and Grünert, 1996; Bumsted and Hendrickson, 1999). Although calbindin had been considered to be present in all cones of all primates (Röhrenbeck et al., 1989; Haley et al., 1995; Luo et al., 1999), a recent study has reported the absence of calbindin in cones of two nocturnal species (Hendrickson et al., 2000). In addition, a single study which has addressed the question of calbindin localization in relation to specific cone types found no difference according to opsin content (Haley et al., 1995).

The present study aims to characterize the distribution of calbindin in relation to cone type in the main haplorhine and strepsirhine primate lineages, represented by four primate species, including a nocturnal prosimian, the gray mouse lemur (*Microcebus murinus*), New World (*Callithrix jacchus)* and Old World (*Macaca fascicularis*) monkeys, and humans. The study of nocturnal primates is of particular interest since the absence or reduced numbers of SW cones is associated with nocturnality in some primates (Wikler and Rakic, 1990; Jacobs et al., 1993a), rodents, and carnivores (Jacobs and Deegan, 1992; von Schantz et al., 1997; Peichl and Moutairou, 1998).

EXPERIMENTAL PROCEDURES

Tissue samples

Three human eyes were obtained from donors and fixed within 16 h after death from the Department of Anatomy (University of Lyon, UCBL1), under approval of the Institutional Human Subjects Committee. Donors were of both sexes and varied in age from 40 to 65 years, and had no previous history of eye disease. The eyes were placed in Zamboni's fixative (4% paraformaldehyde with 15% saturated picric acid in phosphate buffer; 0.1 M, pH 7.4) overnight at 4°C and rinsed in phosphate buffer (PBA; 0.1 M; sodium azide 0.1%, pH 7.4) the next day.

Retinae were obtained from four macaques (*Macaca fascicularis)*, two marmosets (*Callithrix jacchus*) and three prosimians (*Microcebus murinus*). The mouse lemurs were obtained from the Laboratory of General Ecology in Brunoy, France, where the breeding colony is located (licence approval No. A91.114.1). The other primates were part of the colony in INSERM (licence approval No. B 69-685). All animals were maintained and treated according to current national and international standards. Most animals were used as subjects in other anatomical or physiological studies at the end of which they were killed by a lethal dose of sodium pentobarbital and subsequently perfused transcardially with Zamboni's fixative. The cornea and lens were removed and after one night in fixative, the eyes were transferred to PBA at 4°C.

Fixed eyes were cryoprotected by immersion in 30% sucrose (in PBA) overnight, embedded in a solution of agar (2.5%) with 30% sucrose and subsequently cut on a freezing microtome (Polycut, Reicher-Jung) at a thickness of 15 µm. Free-floating retinal sections were placed in titration wells containing PBA and stored until use.

Immunohistochemical procedure

Antibodies. Anti-calbindin immunochemistry (Sigma C-8666, 1/500, mAb) was performed on free-floating sections to identify different cell types in the retina. Several antibodies directed against SW or MW/LW cone opsin were used to distinguish different cone types with multiple labeling. The anti-SW cone opsin antibodies included a mouse monoclonal antibody (OS-2, dilution: 1/10 000; Szel et al., 1988) and a rabbit polyclonal antibody (JH455, 1/5000, kindly provided by J. Nathans; Wang et al., 1992). The anti-MW/LW cone opsin antibodies consisted of a mouse monoclonal antibody (COS-1, 1/100; Rohlich and Szel, 1993) and a rabbit polyclonal antibody (CERN956, 1/1000; Vissers and DeGrip, 1996). Peanut agglutinin lectin (PNA) was used as a general cone marker, since it binds specifically to the extracellular matrix of all cones (biotinylated PNA, Vector ref. # B-1075, 1/20).

General procedure. For all processing, free-floating retinal sections were placed in filtered wells (72-µm mesh, Costar®). All rinses and incubations were carried out under gentle agitation. During the first series, immunostaining with each antibody alone (calbindin or anti-opsin) was carried out. Calbindin-positive immunoreactivity was revealed using avidin-biotin complex

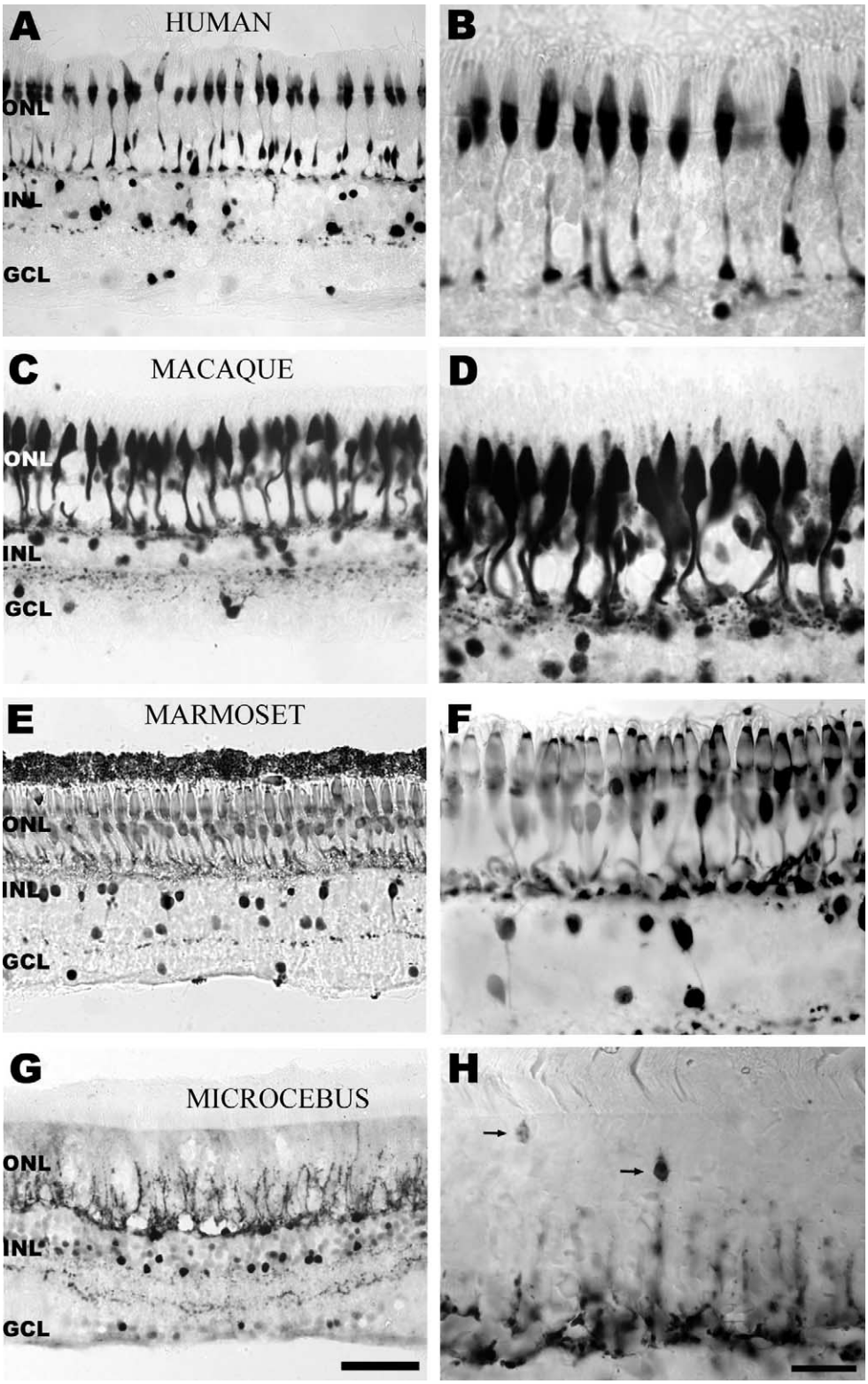

Fig. 1. Distribution of calbindin label in the retinas of human (A, B), macaque (C, D), marmoset (E, F) and *Microcebus* (G, H) at low and high magnification. In all species calbindin is expressed in bipolar cells, amacrine cells, and a few neurons in the ganglion cell layer. All haplorhine primates (human, macaque, marmoset) show various patterns of calbindin distribution within cones. In contrast, no photoreceptors are labeled in the prosimian (strepsirhine) *Microcebus*, although a few unidentified cell bodies (arrows) are detected in the ONL. Calbindin expression is absent from rods in all species. A schematic drawing of the calbindin labeling of primate cones is shown in Fig. 7. Scale bars = 50 μm (in G), 20 μm (in H).

and 3,3′-diaminobenzidine (DAB) reaction. Free-floating sections were first incubated in a solution of alcohol–saline–H_2O_2 (30 min, absolute alcohol 50%, saline solution 50%, H_2O_2 0.05%) and then rinsed twice in phosphate-buffered saline (PBS 0.01 M, 0.9% NaCl, pH 7.4, 10 min). Retinal sections were incubated in normal horse serum (Vector ref. # S-2000, 1/100, 1 h) and then in anti-calbindin antibody at 4°C for 48 h. Sections were then rinsed twice in PBST (PBS with 0.3% Triton) and incubated in the secondary biotinylated antibody (anti-mouse IgG, Vector ref. # BA-2000, dilution 1/100, 2 h) followed by two rinses in PBST, and an incubation in avidin–biotin complex (Vectastain ABC Rabbit IgG; ref. # PK-6100) for 2 h. Retinal sections were rinsed once in PBST and twice in Tris solution (0.05 M, pH 7.6). The sections were pre-incubated for 10 min in a mixture of DAB (Sigma, ref. # D5637) and nickel ammonium sulfate (0.5%) after which 0.001% H_2O_2 was added for 5–10 min. When the DAB reaction was used in combination with fluorescent label (see below) the incubation time in DAB was reduced to allow simultaneous observation of the fluorescent probe.

Multiple labeling experiments were performed using a mixture of antibodies directed against SW or MW/LW cone opsin after incubation in bovine serum albumin (Sigma, ref. # A-7030, 1/100, 1 h). The antibody incubation (48 h, 4°C) was carried out simultaneously with the two following mixtures: (1) OS-2 and CERN956 or (2) JH455 and COS-1. A mixture of the following secondary fluorescent probes was used for visualization at a dilution of 1/200 (2 h): Alexa 546 (Molecular Probes, ref. # A-11003) or Alexa 488 (ref. # A-11001) goat anti-mouse for monoclonal antibodies (OS-2 and COS-1), and Alexa 568 goat anti-rabbit (ref. # A-11011) for polyclonal antibodies (CERN956 and JH455).

Subsequently, retinal sections were washed in PBST and incubated in biotinylated PNA (1/20, 2 h). In order to avoid non-specific binding of avidin-D AMCA (Vector ref. # A-2008, 1/20) on the biotinylated antibody, a blocking solution (Vector, ref. # SP-2001) was used prior to incubation in biotinylated PNA. The sections were mounted on gelatinized slides and cover-slipped with PB 0.1 M. The coverslip was sealed with varnish and the slides were kept at 4°C.

Negative controls were performed using the same technique but omitting each primary antibody. When a mixture of two antibodies or two fluorescent probes was used, a control was done using each antibody singly and also applying them in different sequential order.

Digitized images were captured using a Spot II camera (Diagnostic Instruments) with 40, 63 and 100× immersion objectives, and several filters for AMCA (UV, emission at 450 nm), rhodamine (emission at 569 nm for Alexa 546 or 596 nm for Alexa 568), and fluorescein (emission at 515 nm for Alexa 488). The color images were converted to grayscale for illustration. In some cases, confocal microscopy (Leica TCS SP) was used to verify the presence of double fluorescent label in cones with anti-calbindin and anti-opsin antibodies. Laser lines and emission filters were optimized with the Leica PowerScan software. Image processing was carried out with Adobe Photoshop software.

RESULTS

In all primates the general pattern of distribution of calbindin-immunopositive neurons in the inner (INL) and outer nuclear layers (ONL) of the retina was similar.

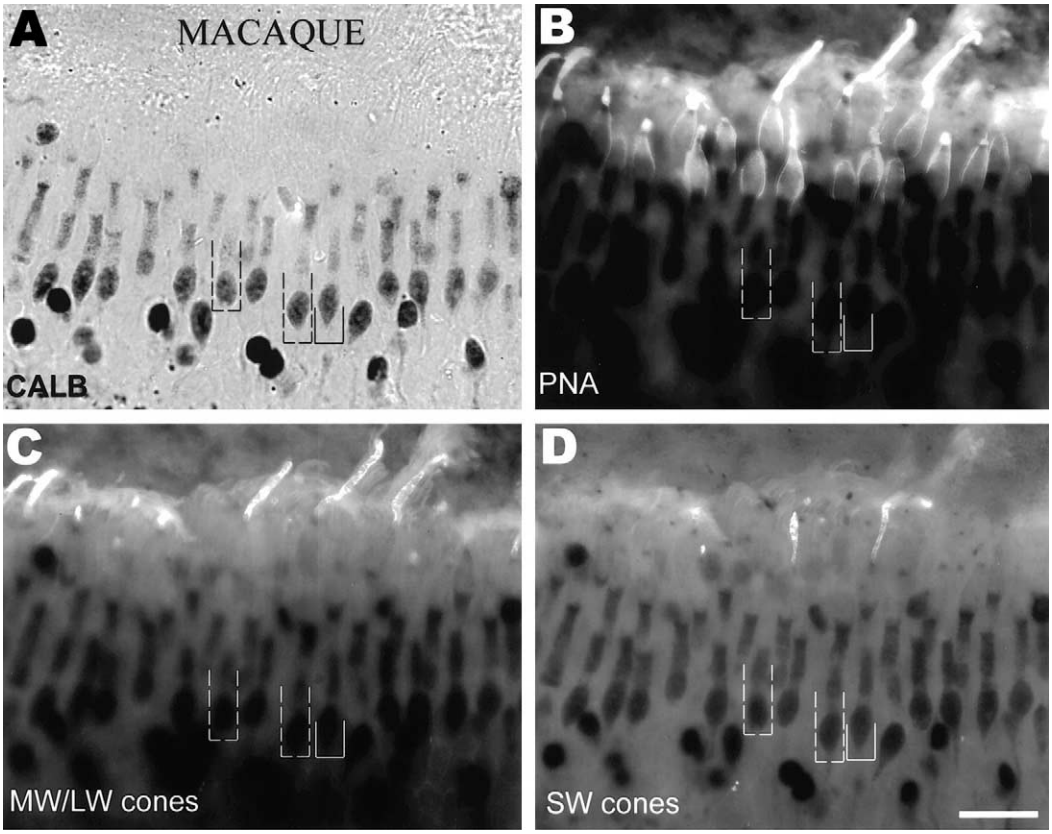

Fig. 2. Section of a macaque retina illustrating multiple label in cones with anti-calbindin (A; DAB label), and fluorescent label of PNA (B), anti-MW/LW opsin (C; CERN956), and anti-SW opsin (OS-2). Calbindin-positive cones were strongly stained from the inner segment to the cone pedicle (A). All calbindin- and PNA-positive cones (A, B) were identified as MW/LW cones (C, see dashed line brackets) or SW cones (D, see solid line bracket). Scale bar = 20 μm. (Photoreceptors appear long due to angle of section.)

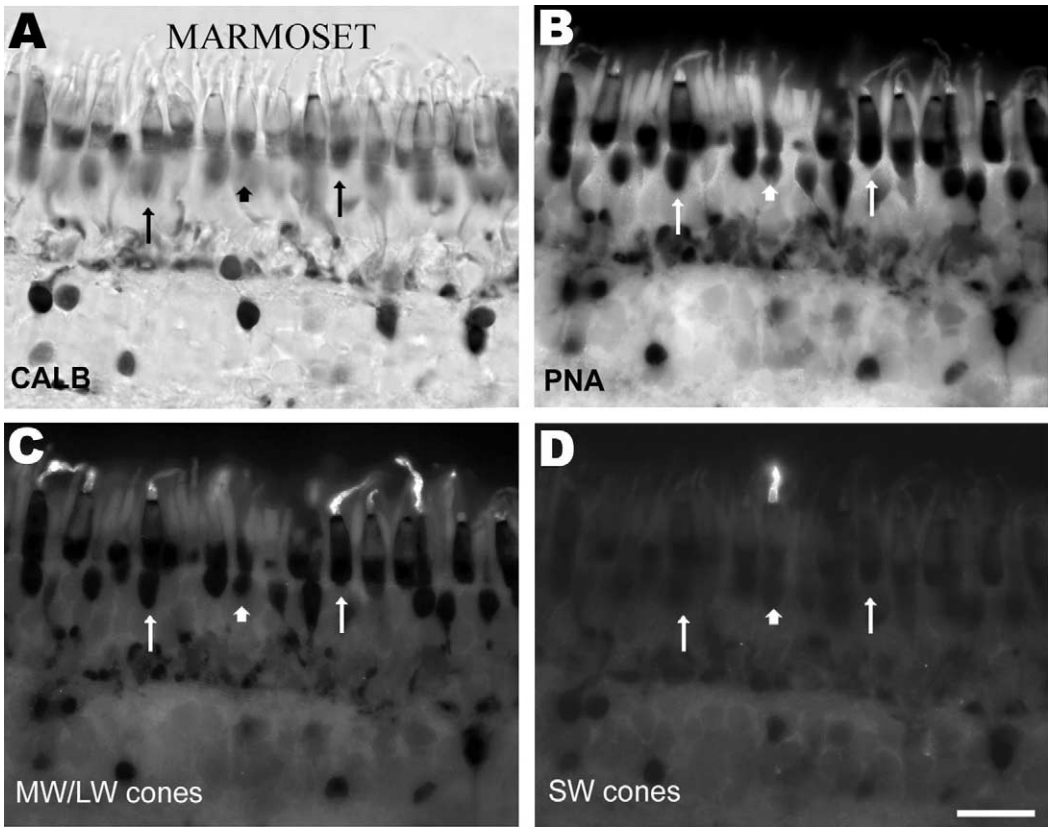

Fig. 3. Section of a marmoset retina illustrating multiple label in cones with DAB label of calbindin (A), fluorescent label of PNA (B), anti-MW/LW opsin (C; COS-1), and anti-SW opsin (D, JH455). Calbindin is present in the cell body, pedicle and axon. (A) MW/LW (long, thin arrows) and SW cones (short, thick arrow) are clearly identified using COS-1 and JH455 antibodies, respectively. Scale bar = 20 μm.

The INL contained a scattered population of neurons, mainly well stained bipolar cells and less intensely labeled amacrine cells (Fig. 1). In the macaque and *Microcebus* INL adjacent to the outer plexiform layer (OPL), scattered neurons with processes in the ONL showed a morphology typical of horizontal cells (Fig. 1C, G). In contrast, no neurons with a horizontal cell morphology were identified in humans and marmosets (Fig. 1A, E). The ganglion cell layer of all primates contained a few sparsely distributed calbindin-positive neurons.

Calbindin expression in primate cones

All calbindin-positive photoreceptors in primates appear to have a cone-like morphology whereas no rod-like photoreceptors are calbindin-positive (Fig. 1). The cone outer segment in all primates is devoid of calbindin-positive label, although the distribution of label within the cone inner segment and cell body differs between species.

In the human retina, the base of the inner segment and the cell body are densely labeled (Fig. 1B). In most photoreceptors the axons and especially the cone pedicles are also evident. In the macaque, calbindin-positive cones are strongly stained from the tip of the inner segment to the cone pedicle (Fig. 1D). Relatively thick axons and pedicles are apparent in practically all cones. In the marmoset retina calbindin is present in the cell body, pedicle and axon (Fig. 1F). In contrast to macaque and humans, the inner segment of cones is lightly stained, except for a dense ring-like band of label in the distal part of the inner segment corresponding to the cilium base. The photoreceptor layer of *Microcebus* was immunonegative for calbindin except for a few rare cell bodies, which were impossible to identify as either rods or cones (Fig. 1H). At the base of the ONL vertically oriented nerve processes apparently corresponding to Müller cells were seen. Horizontal cells and their processes were also densely labeled in the OPL.

Identification of calbindin-immunopositive cones

In macaque and marmoset retinas, multiple labeling showed that calbindin-positive cones are consistently labeled by PNA and, inversely, all cones identified by PNA contained calbindin (Figs. 2 and 3A, B). In human retinas, all calbindin-positive cones are labeled by PNA (Fig. 4A, B). However, a small population of PNA-positive cones are devoid of calbindin reactivity. In marmoset, macaque, and humans, the external matrix of both inner and outer segments, as well as the cell body were uniformly stained by PNA. The outer synaptic layer and cone pedicles showed weak PNA labeling. This fluo-

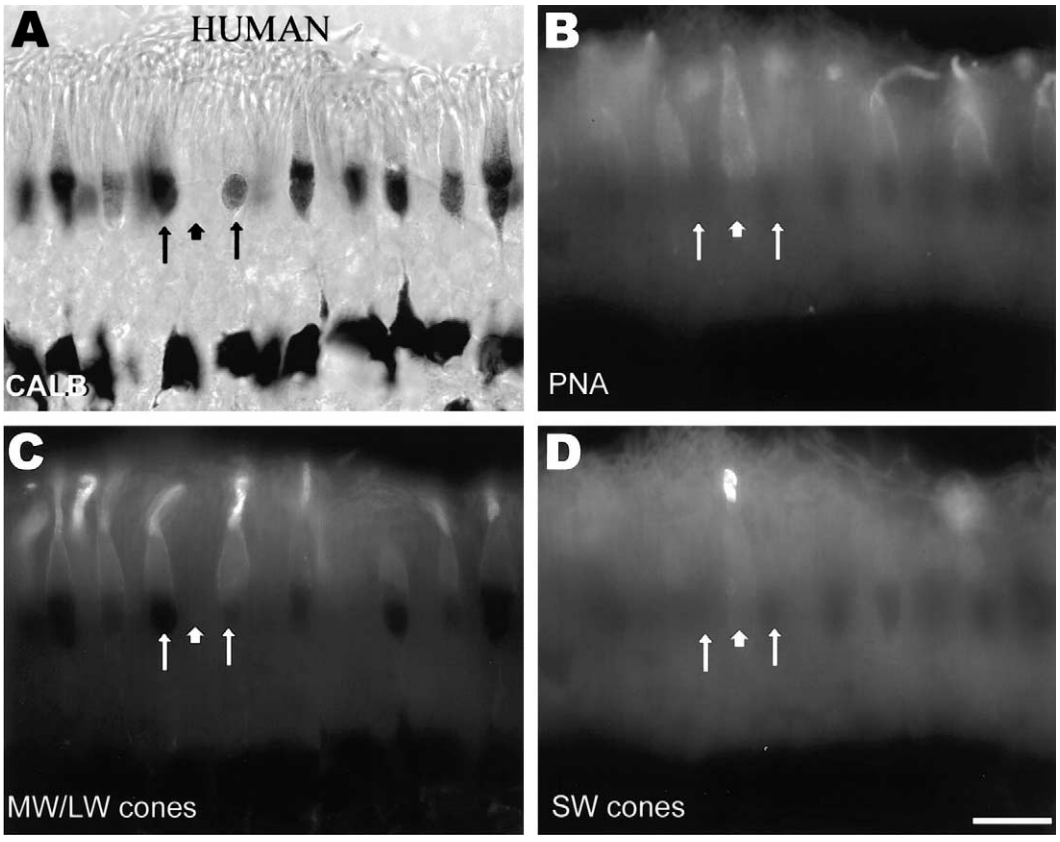

Fig. 4. Section of a human retina illustrating multiple label in cones with calbindin (A; DAB label), and fluorescent label of PNA (B), anti-MW/LW opsin (C; CERN956), and anti-SW opsin (D, OS-2). All calbindin- and PNA-positive photoreceptors are MW/LW cones (C, long, thin arrows) whereas all SW cones (D, short, thick arrow) are devoid of calbindin labeling. Scale bar = 20 μm.

rescent staining was evident when PNA was used alone, but was found to be less pronounced when used in combination with calbindin DAB label (especially in the digitized black and white images). Rods were never stained with PNA in any species.

In *Microcebus* retina, the outer segment was strongly labeled by PNA and showed a distinct swelling in the mid-region that is never observed in the other primate species (Fig. 5B, see Dkhissi-Benyahya et al., 2001). The inner segment and cell body were only lightly stained by PNA as compared to the other primates. In contrast to other species, none of the PNA-labeled cones were calbindin-positive (Fig. 5A, B). A few rare calbindin-positive cell bodies are seen in the ONL (Fig. 1H) but it was impossible to determine whether these corresponded to PNA-positive cones or to a different cell type.

Opsin content of calbindin-immunopositive cones

Prior to studying calbindin and opsin colocalization, we examined the pattern of opsin staining in retinal sections of the SW and MW/LW cone antibodies individually and in various combinations. For example, the two pairs of monoclonal/polyclonal antibodies, COS-1/JH455 and OS-2/CERN956, were used both simultaneously and in succession in different sequence in order to exclude any colocalization or cross-reactions.

In macaque, marmoset and humans the CERN956 (MW/LW), COS-1(MW/LW) or OS-2 (SW) antibodies label the cone outer segments while the inner segment remains unstained. The JH455 antibody (SW) labels the entire photoreceptor from the outer segment to the cone pedicle in these species (Fig. 6B). In *Microcebus* the anti-MW/LW opsin antibodies label the outer segment from the limit of the inner segment to the region containing a distinct swelling (Fig. 5C), but label is absent distal to the swelling. In contrast to the other primates, no SW cone opsin immunoreactivity was detected with OS-2 antibody in *Microcebus*, whereas the polyclonal JH455 antibody recognizes a very small scattered population of SW cones (Fig. 5D). The shape of the outer segment of SW cones was similar to the MW/LW cones.

When two anti-opsin antibodies are used simultaneously, colocalization of SW and MW/LW cone opsins is never observed in prosimian, macaque, and human retinas. Cones recognized by anti-MW/LW opsin antibodies are unstained by anti-SW opsin antibodies, and vice versa. When only one of the opsin antibodies is combined with PNA, unstained cones, corresponding to the other spectral class, are always observed.

The pattern in the marmoset differs since when OS-2 and CERN956 are used in combination, all OS-2-positive cones (SW) are also CERN956-positive (MW/LW), whereas MW/LW cones are not stained with OS-2. This double immunostaining was initially observed when the retina was incubated simultaneously with both antibod-

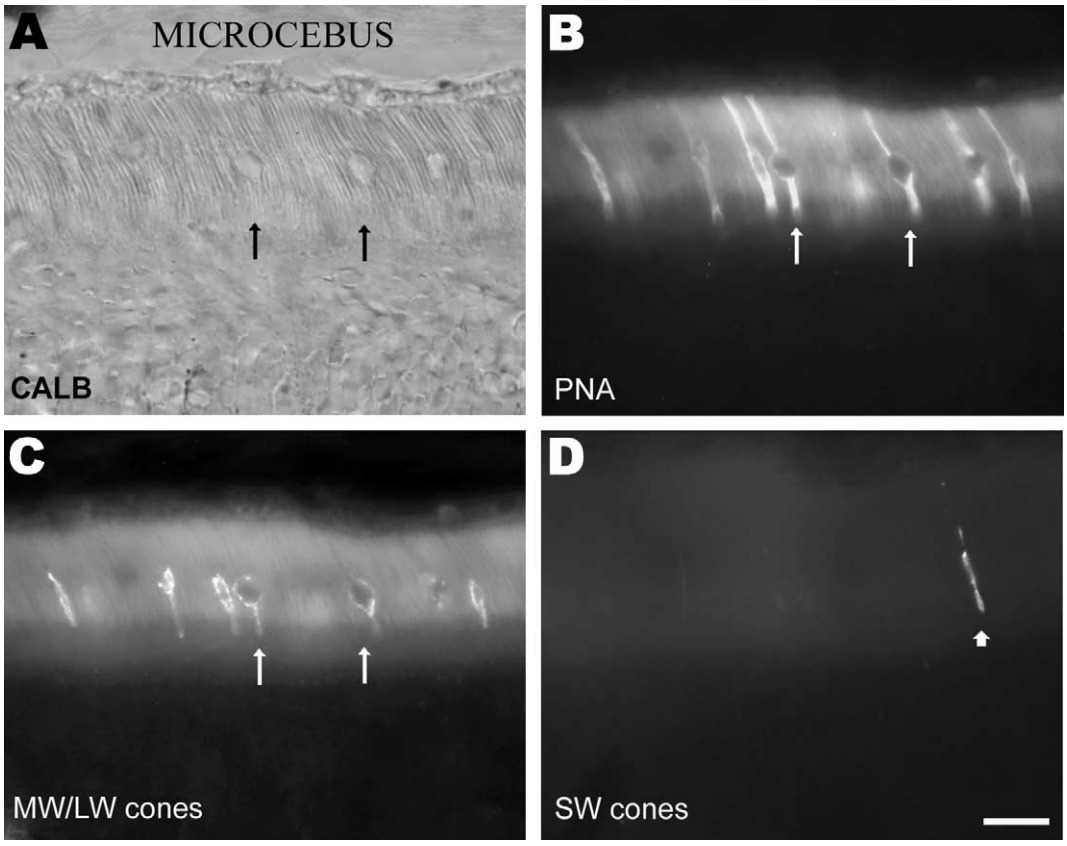

Fig. 5. Section of a *Microcebus* retina illustrating multiple label with calbindin (A; DAB label), and fluorescent label of PNA (B), anti-MW/LW opsin (C; COS-1, long, thin arrows), and anti-SW opsin (D, JH455, short, thick arrow). In *Microcebus* cones are calbindin-negative. The morphology of the outer segment shows a distinct swelling in the mid-region of the outer segment (B). Panel D (SW cone) is taken from a different region of the retina, since SW cones are rare in this species. Scale bar = 20 μm.

ies. However, when the antibodies were used separately and consecutively, double staining depended on the order of incubation. When the monoclonal OS-2 (SW) was used prior to incubation in the polyclonal CERN956 (MW/LW), all SW cones also showed label by CERN956, whereas when OS-2 was used after CERN956 no colocalization of label was observed in any cones. In addition, to test the possible interference between the secondary fluorescent probes, we incubated each primary antibody simultaneously with both anti-rabbit and anti-mouse fluorescent probes. In this case, no cross-reaction between the secondary antibody probes was observed. In contrast, no colocalization of both opsins is found in the marmoset when the anti-MW/LW cone opsin (COS-1) and the anti-SW opsin antibody (JH455) are used (again in combination or separately in any order), similar to the other primate species.

In the primates studied, three patterns of calbindin and opsin colocalization are observed. In macaque and marmoset all cones types contain calbindin (Figs. 2 and 3). This result is not unexpected since all cones labeled with PNA also contain calbindin. A second pattern of labeling is seen in humans, in whom only the MW/LW cones contain calbindin. We never observed any cones labeled with the anti-MW/LW opsin antibody which were devoid of calbindin. A surprising result was the observation that the SW cones in humans lack calbindin (Fig. 4D). The use of confocal microscopy clearly shows that the cones labeled with the anti-SW opsin antibody do not contain calbindin label (Fig. 6). This result also corresponds to the absence of calbindin observed in a subset of the PNA-labeled cones. In contrast to all other species of primates studied, none of the cones in *Microcebus* were calbindin-positive (Fig. 5).

DISCUSSION

Calbindin expression in primate cones

In the primate retina, the presence of calbindin differs according to species and cone type. With the exception of *Microcebus* and other prosimians (Hendrickson et al., 2000), calbindin expression is observed in cones of all diurnal primates including humans (Hamano et al., 1990; Haley et al., 1995), macaque (Röhrenbeck et al., 1989; Pochet et al., 1991; Grünert et al., 1994), and marmoset (Luo et al., 1999). Calbindin is never observed in primate rod photoreceptors. In contrast to anti-opsin staining which is restricted to cone outer segments, calbindin immunoreactivity shows a species-specific distribution in the inner segment and cell body (see summary Fig. 7).

Consistent with previous studies in humans

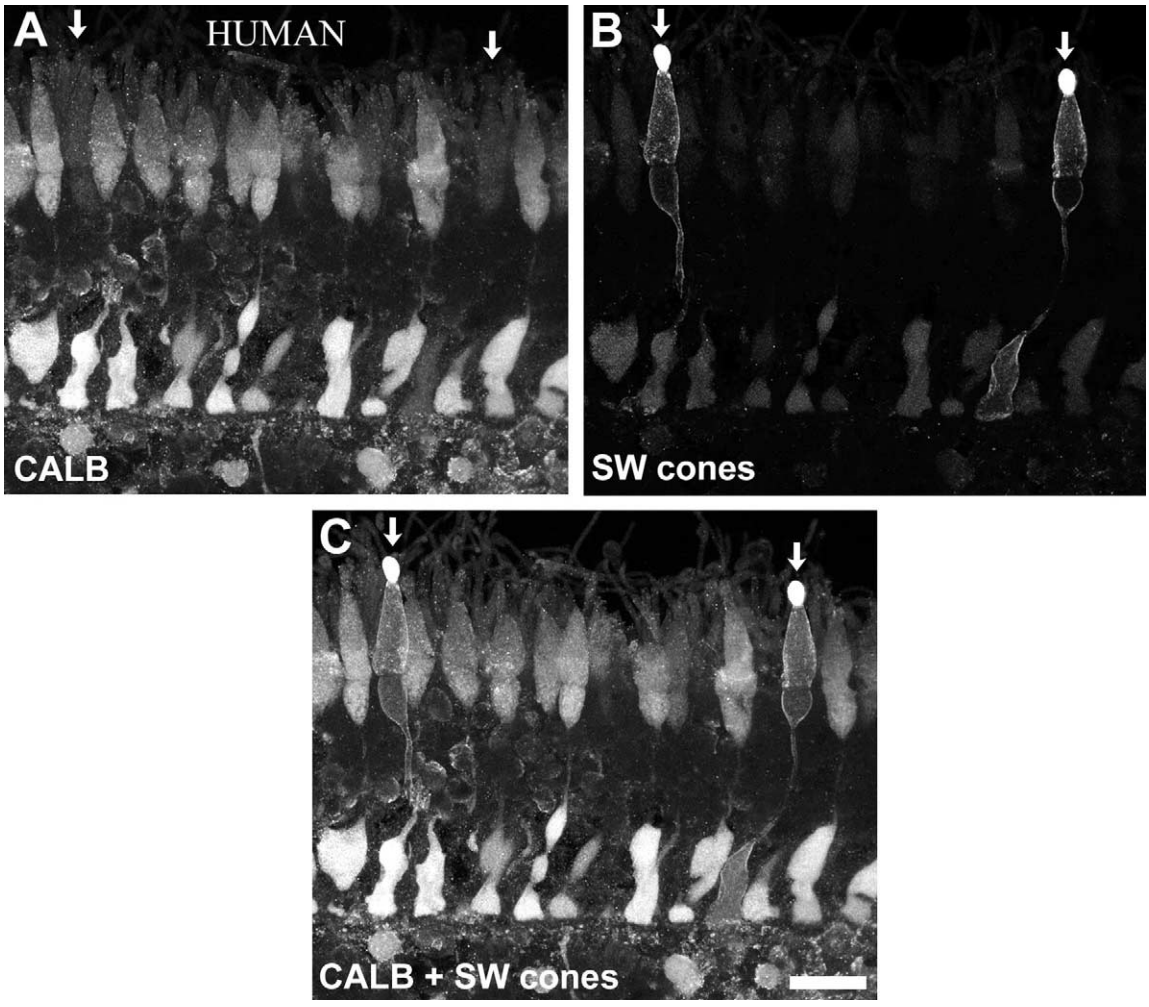

Fig. 6. Confocal image of fluorescent anti-calbindin (A) and anti-SW opsin (B) antibodies in human retina. The separate images (A, B) and the combined image (C) clearly show that SW cones (arrows) are devoid of calbindin. Scale bar = 20 μm.

(Verstappen et al., 1986; Hamano et al., 1990; Haley et al., 1995), calbindin labeling of cones was restricted to the cell body, the inner segment, axons and cone pedicles. When PNA lectin was used to identify all cones, a minority of cones were found to be devoid of calbindin label. In contrast with previous studies in the human retina (Verstappen et al., 1986; Hamano et al., 1990; Haley et al., 1995), we show that all SW cones are devoid of calbindin labeling. This result is unexpected since SW cones in other diurnal primates contain calbindin and a previous study which specifically addressed this question (Haley et al., 1995) found no difference according to opsin content. This discrepancy probably arises from differences in methodology. The use of confocal microscopy in our study allowed unambiguous identification of the lack of calbindin label in SW cones.

All cone types in the macaque and marmoset contain calbindin. In the macaque, the distribution of calbindin immunoreactivity in cones is similar to that reported in previous studies (Röhrenbeck et al., 1989; Pochet et al., 1991; Grünert et al., 1994). In comparison, cones in the marmoset retina exhibit a particular pattern of anti-calbindin staining with two areas of denser staining (the cell body and the distal region of the inner segment corresponding to the cilium base). In previous studies of the marmoset, although calbindin was also reported to be present in cones (Ghosh et al., 1997; Luo et al., 1999), the dense band of label in the distal part of the inner segment was not observed.

In the nocturnal primates including the prosimians *Microcebus*, *Tarsius*, and the nocturnal owl monkey, *Aotus* (Hendrickson et al., 2000), no cones contain calbindin. This result is surprising since the MW/LW cones which represent the majority of the cone population in these nocturnal species are calbindin-immunoreactive in diurnal primates.

Calbindin expression in cones of diurnal and nocturnal species

A possible difference in the role of calbindin in the cones of diurnal versus nocturnal species has not yet been considered. In addition to diurnal primates, calbindin is also present in the cones of certain non-primate mammals such as the cat (Goebel and Pourcho, 1997), sheep (Pasteels et al., 1990) and pig (Pasteels et al., 1990), as well as in several non-mammals (chick, frog, turtle, salamander; Hamano et al., 1990; Pasteels et al., 1990;

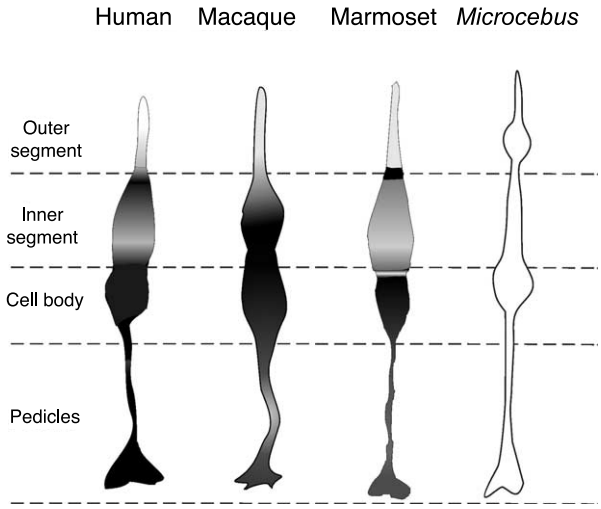

Fig. 7. Schematic drawing summarizing the differences in distribution of calbindin within cones of different primates: human (A) and macaque (B) cones are densely labeled in the inner segment, cell body and pedicle. (C) Marmoset cones are characterized by a ring-like band in the distal part of the inner segment. (D) *Microcebus* cones are immunonegative for calbindin.

Pochet et al., 1991). All the above species are partly (crepuscular) or completely diurnal suggesting that calbindin in cones is related to some aspect of photopic vision. In contrast, calbindin has not been observed in cones of mainly nocturnal animals (rat, mouse, rabbit, guinea-pig; Hamano et al., 1990; Pasteels et al., 1990; Pochet et al., 1991) including the nocturnal prosimians and *Aotus* (Hendrickson et al., 2000). It has been suggested that the high affinity Ca^{2+}-binding sites of calbindin might be related to different adaptational properties of cones (Haley et al., 1995). Calcium is thought to act as a modulator of light adaptation (Pugh and Lamb, 1990) and the absence of calbindin in cones of nocturnal species may be related to a different mode of light adaptation. Calbindin belongs to the group of buffer proteins which may limit a stimulated rise in intracellular free Ca^{2+} concentration (Airaksinen et al., 1997). Calbindin could either modulate or mediate the actions of Ca^{2+} ions which play a key role in transmembrane signaling and intracellular signal transmission.

The absence of calbindin in *Microcebus* and other nocturnal primates might be compensated by the presence of other calcium-binding proteins. For example, the calcium-binding proteins recoverin, parvalbumin, visinin and calretinin are present in cones of other primates (Yamagata et al., 1990; McGinnis et al., 1997). Indeed, recoverin is present in *Microcebus* cones, although calretinin and parvalbumin are absent (C. Chiquet, unpublished observations). By comparison, all cones in the cat retina contain calretinin (outer segment of cones) and calbindin (cell body and axons of cones), whereas parvalbumin is only seen in SW cones (Goebel and Pourcho, 1997). Various functional roles have been described for the different calcium-binding proteins (Koch, 1995). Recoverin mediates adaptation to light by activating rhodopsin kinase (Polans et al., 1996), arrestin provides Ca^{2+}-buffering capacity in photoreceptors and is involved in the cascade of the light-induced signal, whereas calmodulin after binding of calcium ions influences the activity of various proteins, including the cGMP-dependent cation channel (Kaupp, 1995). The related Ca^{2+}-binding proteins GCAP-1 and GCAP-2 are also located in the outer segment of photoreceptors and may play a crucial role in Ca^{2+}-mediated feedback loop of phototransduction (Muller and Koch, 1998; Kachi et al., 1999). However, in a calbindin-null mutant mouse (Wässle et al., 1998) no immunocytochemically detectable up-regulation of parvalbumin, calretinin or calmodulin was observed and the functional consequences of the absence of calbindin in this model remain unknown.

Comments on multiple anti-opsin immunostaining

Our results on anti-opsin immunoreactivity of cones in marmoset demonstrate the limits and precautions necessary in the use of multiple labeling with two anti-opsin antibodies, even when directed against different regions of the antigen and produced in different hosts. For example, we found that in the marmoset, when two of the antibodies (OS-2 and CERN956) are incubated either simultaneously or subsequently (depending on the order), SW cones show immunopositive label for both antibodies. Double staining is also observed if OS-2 incubation precedes that of CERN956, but not in the inverse order. The possible colocalization of two antibodies in the same cone was perplexing since there is no evidence for colocalization of two opsins in individual cones from microspectrophotometric studies in the marmoset (Tovee et al., 1992). Additional controls in the marmoset using a different combination of anti-opsin antibodies, each against SW (JH455) and MW/LW cones (COS-1), confirmed that there is no colocalization of MW/LW and SW opsins in a single cone. It is difficult to explain the observed affinity of the CERN956 antibody for OS-2 label in SW cones in light of the lack of a similar double label in the other primates. Perhaps the complex of the first antibody CERN956 with the pigment presents a substrate for the second antibody OS-2 with increased affinity compared to the pigment alone. The fact that it is only observed in marmoset may be due to differences in sequence and/or epitope location as compared to other species. In human, macaque and *Microcebus*, the use of two combinations of anti-opsin antibodies, i.e. OS-2/CERN956 or JH455/COS-1, always resulted in complementary staining patterns of the cone population. The only other case of ambiguous double immunostaining of photoreceptors using opsin antibody combinations was reported in the ground squirrel (Kryger et al., 1998). In this study, the polyclonal JH492, which labels only MW/LW cones in other species, labels all cones in the squirrel, and thus leads to double staining if used in combination with another opsin antibody. In addition, the polyclonal JH455 anti-SW antibody labels rods in the squirrel only if applied after incubation with an anti-rod antibody (Rho4D2) but not if used alone. In non-primates however, opsin colocalization is not unusual and has been described in cones located in certain regions of

the rabbit, mouse and guinea-pig retina (Rohlich et al., 1994; Szel et al., 1994). In some rodent species all cones contain both SW and MW/LW opsins (Lukats et al., 2002). In human fetal retinas, cones containing both SW and MW/LW opsins are found early during development around the fovea and then in the peripheral retina, decreasing in number after birth, although some are still present in adult temporal retina (Xiao and Hendrickson, 2000).

The results concerning the identification of SW cones in the prosimian *Microcebus* are also perplexing. It is unclear why SW cones are present in nocturnal primates such as *Microcebus* and *Tarsius* (Hendrickson et al., 2000) but absent in others (Galago, *Aotus*; Wikler and Rakic, 1990; Jacobs et al., 1993a, 1996b). Surprisingly, SW cones in *Microcebus* were only recognized by the polyclonal anti-SW opsin (JH455) and not by the monoclonal anti-SW opsin OS-2. The absence of OS-2-positive cones is remarkable since this is a C-terminal-specific antibody and the C-terminus is a relatively highly conserved domain of visual pigment molecules (Rohlich and Szel, 1993). Whereas the two antibodies are considered robust markers of mammalian SW cone opsins, use of both markers for recognition of the SW cone opsin may be necessary in certain species. The difference in detection may be indicative of some unknown differences in the primary or tertiary structure of the SW opsin of *Microcebus* as compared to other primates (and possibly non-primates). However, no functional (microspectrophotometry, electrophysiology) or genetic studies are presently available concerning the spectral properties of the cones in this species.

Acknowledgements—Grant sponsor: Human Frontiers; Grant number: RG95/68; Grant Sponsor: Biomed2; Grant number: BMH4-CT972327; Grant sponsor: INSERM Est-West; Fondation de France (Grant Fouassier), Grant sponsor: Hungarian OTKA; Grant number: T29048. We would like to thank J. Nathans for the JH455 antiserum and P. Morin who generously provided human eyes. We thank M. Perret (MNHN, Brunoy, France) for making the prosimians available for this study.

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(*Accepted* 15 *May* 2002)