Central Projections of Intrinsically Photosensitive Retinal Ganglion Cells in the Macaque Monkey

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ABSTRACT

Circadian rhythms generated by the suprachiasmatic nucleus (SCN) are entrained to the environmental light/dark cycle via intrinsically photosensitive retinal ganglion cells (ipRGCs) expressing the photopigment melanopsin and the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP). The ipRGCs regulate other nonimage-forming visual functions such as the pupillary light reflex, masking behavior, and light-induced melatonin suppression. To evaluate whether PACAP-immunoreactive retinal projections are useful as a marker for central projection of ipRGCs in the monkey brain, we characterized the occurrence of PACAP in melanopsin-expressing ipRGCs and in the retinal target areas in the brain visualized by the anterograde tracer cholera toxin subunit B (CtB) in combination with PACAP staining. In the retina, PACAP and melanopsin were found to be costored in 99% of melanopsin-expressing cells characterized as inner and outer stratifying melanopsin RGCs. Two macaque monkeys were anesthetized and received a unilateral intravitreal injection of CtB. Bilateral retinal projections containing colocalized CtB and PACAP immunostaining were identified in the SCN, the lateral geniculate complex including the pregeniculate nucleus, the pretectal olivary nucleus, the nucleus of the optic tract, the brachium of the superior colliculus, and the superior colliculus. In conclusion, PACAP-immunoreactive projections with colocalized CtB represent retinal projections of ipRGCs in the macaque monkey, supporting previous retrograde tracer studies demonstrating that melanopsin-containing retinal projections reach areas in the primate brain involved in both image- and nonimage-forming visual processing. J. Comp. Neurol. 522:2231–2248, 2014.

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INDEXING TERMS: neurotransmitter; circadian rhythms; RHT; melanopsin; PACAP; VIP; suprachiasmatic nucleus; LGN; PON

Mammalian eyes are equipped with two functionally and anatomically different light-detection systems. In addition to the classical image-forming system involving rods and cones, the eyes contain another system identified as an irradiance-detection system, which relays nonimage-forming visual information to the brain (Berson, 2003; Do and Yau, 2010). Nonimage-forming photic information synchronizes the circadian clocks to the light/dark (LD) cycle and regulates pineal melatonin secretion, sleep/wake, and pupillary constriction (Fu et al., 2005; Gamlin et al., 2007; Do and Yau, 2010). Nonimage-forming visual information reaches areas in the brain including the circadian clock located in the hypothalamic suprachiasmatic nucleus (SCN) via a monosynaptic neuronal pathway designated as the retinohypothalamic tract (RHT; Moore and Lenn, 1972; Moore et al., 1995). The RHT originates from a subset of retinal ganglion cells (RGCs), which are intrinsically photosensitive (ipRGCs) because of melanopsin and which in rodent and humans costore two neurotransmitters, glutamate and pituitary adenylate cyclase-

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activating polypeptide (PACAP; Hannibal et al., 2000, 2002, 2004). Central projections of ipRGCs have been demonstrated in mice using two different lines of melanopsin reporter mice, one line targeting the melanopsin gene by *tau-lacZ* under control of the melanopsin promoter and one a Cre-based line (Hattar et al., 2006; Ecker et al., 2010). For rat, hamster, and mouse, we have previously demonstrated melanopsin projections in the brain by staining for one of the RHT neurotransmitters, PACAP, in combination with the anterograde tracer cholera toxin B (CtB) injected into the eye (Bergström et al., 2003; Hannibal and Fahrenkrug, 2004, 2006). Knowledge of the circadian organization of behavior in humans is essential to understanding human adaptation to the environment both in health and in diseases that may disrupt or disturb the circadian system. Because humans cannot be examined by the anatomical techniques necessary for a detailed mapping of neuronal connectivity, studies in other primates are essential for such purposes. However, at the moment it is not possible to use genetically based reporter systems in monkeys, so classical retrograde tracing from the lateral geniculate complex and the pretectum has been used and has identified these areas as a targets for the ipRGCs (Dacey et al., 2005). A more detailed characterization of central melanopsin projections in monkeys, however, has not been performed. To investigate whether immunohistochemical staining of PACAP in combination with staining for the anterograde tracer CtB, delivered by an intraocular injection, can be used to identify central ipRGC projections in the macaque brain, we used this approach in two monkeys, one *Macaca fascicularis* (MF) and one *Macaca mulatta* (MM). This was performed after initially demonstrating that PACAP was costored with melanopsin in ~99% of melanopsin-expressing cells in the monkey eye.

MATERIALS AND METHODS

Animals

Two male macaque monkeys, one MF, aged 20 years, and one MM1, aged 5 years, were used for the tracer study. Two male MM, one aged 8 (MM2) and one aged 2.5 years (MM3), were used for the detailed retinal analyses. The animals were kept in the primate animal facility of the University of Alabama at Birmingham under 12:12-hour light:dark conditions. For the tracing study, MM1 and MF received a unilateral injection of 100 µl of 1% CtB (List Biological, Campbell, CA) in sterile water into the vitreous body with a 1-cc tuberculin syringe under isoflurane anesthesia. After a survival time of 7 days, the animals were deeply anesthetized with barbiturate and perfused through the aorta with 800 ml warm (~37°C) 1% sodium nitrite/0.9% sodium chloride, followed immediately by 4 L cold (~4°C) 4% paraformaldehyde (PFA in PBS, pH 7.2). Eyes and brain were removed, and the eyes were postfixed for 2 hours in PFA with the anterior chamber removed during the fixation. The retinas were removed and placed in PBS, followed by cryoprotectant (see below). The brains were placed in a solution of 30% sucrose in 0.1 M phosphate buffer (PBS) for 48–96 hours. The brains were subsequently frozen, coronally sectioned at 40 µm with an AO 860 sliding microtome, and placed in PBS, which was later replaced by cryoprotection solution (30% sucrose, 1% polyvinyl-pyrrolidone [PVP-40], 30% ethylene glycol, 0.05 M sodium phosphate buffer, pH 7.2) for better conservation and thereafter stored at –20°C until immunohistochemically processed (see below). The anesthesia used for MM2 and MM3 was initiated with Ketamine, followed by pentobarbital (200 mg/kg BW), and the animals were then perfused through the aorta with 2 L of 1% sodium nitrite/0.9% sodium chloride and fixed with 3 L Stefani fixative for 2 hours. Eyes were removed, the anterior chambers were removed, and the eyes were postfixed overnight in PFA/Stefanini fixative. Subsequently, the retinas were dissected and placed in PBS, followed by cryoprotectant. Experimental procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) and complied with the USPHS policy on humane care and use of laboratory animals.

Immunohistochemistry

Brain sections were treated with antigen retrieval solution for 16 hours at 40°C (Dako ChemMate, Glostrup, Denmark; code No. S 203120 in distilled water, pH 6) before processing for immunohistochemistry. Flat-mount retinas were treated with antigen retrieval solution for 1.5–2 hours at 80°C (Dako ChemMate; code No. S 203120 in distilled water, pH 6), followed by immunostaining for PACAP (see below). Subsequently, the retinas received another treatment in antigen retrieval solution for 1.5 hours at 80°C when using the C-terminal antimelanopsin antibody (see below) or no antigen retrieval when using the N-terminal antimelanopsin antibody (see below; Dako ChemMate; code No. S2367 in distilled water, pH 9).

Antibody characterization

The PACAP antibody (see Table 1) used in the present study was a mouse monoclonal anti-PACAP antibody described in detail previously (Hannibal et al., 1995). The antibody (code MabJHH1, diluted 1:2) was produced in mice immunized with PACAP38, and the antibody recognized an epitope located at PACAP6–16 (Hannibal et al.,

TABLE 1.
Primary Antibodies Used

Antibody	Immunogen	Manufacturer	Dilution used
PACAP (Code MabJHH1)	PACAP1–38	In house	1:5
Melanopsin C-terminal (Code No. 5J68)	C-terminal part of human melanopsin (amino acid sequence of the fusion protein; see methods)	In house	1:10,000
Melanopsin N-terminal (Code No. hNA)	Peptide consisting of 19 amino acid residues, MNPPSGPRVPPSPTQEPSC	In house by Dr. King-Wai Yau, John Hopkins University	1:500
Anti-CtB antibody (code No. 703, lot 7032E)	aB subunit pentamer of cholera toxin (choleragenoid)	List Biologicals, Campbell, CA	1:1,000
VIP (code E291E)	VIP1-28	In house	1:1,000

1995). Preabsorption with PACAP38, PACAP27, and PACAP6–27 but not PACAP16–38 eliminated all staining on brain tissue (Hannibal et al., 1995). No specific staining with the anti-PACAP antibody is seen in mice lacking the PACAP gene (our unpublished observation).

A commercially available anti-CtB antibody (code No. 703, lot 7032E; List Biological; diluted 1:1,000) raised in goat was used for tracer visualization. According to the manufacturer, the antigen was a B subunit pentamer of cholera toxin (choleragenoid). Omission of the primary antibody abolished all staining (not shown). The C-terminal anti-human melanopsin antibody was raised in house in rabbit against a fusion protein representing the C-terminal part of human melanopsin (amino acid sequence of the fusion protein was MRGSHHHHHHGMA SMTGGQQMGRDLYDDDDKDHPFTHPKYRVAIAQHLPCLGV LLGVSRRHSRPYPSYRSTHRSTLISHTSNLSWISIRRRQESLG SESEVGWTHMEAAAVWGAAQQANGRSLYQGLEDLEAKAPP RPQGHEAETPGKTKGLIPSQDPRM) as described in detail previously (Hannibal et al., 2004; Jusuf et al., 2007). The C-terminal anti-melanopsin antibody (from rabbit No. 5J68, diluted 10,000) was characterized as described previously (Hannibal et al., 2004; Jusuf et al., 2007) by preabsorption of the antibody with the immunization material dialyzed against PBS with 0.1% Tween, which abolished all staining. The rabbit anti-human melanopsin antiserum recognized human and monkey but not rodent melanopsin. An N-terminal anti-melanopsin antibody (a kind gift from Dr. King-Wai Yau, John Hopkins University; code hNA diluted 1:500) raised in rabbit against a peptide consisting of 19 amino acid residues, MNPPSGPRVPPSPTQEPSC, from the N-terminus of the conceptually translated human melanopsin protein (NCBI accession No. AAF24978; Dacey et al., 2005) was used together with either PACAP or the C-terminal anti-melanopsin antibody. The C- and the N-terminal antibodies were used to clarify whether the C-terminal antibody showed different staining of melanopsin ipRGCs (see Results). The antibody against vasoactive intestinal polypeptide (VIP) was in house rabbit anti-VIP antibody (code No. 291E, diluted 1:1,000) raised against human VIP1–28 described previously (Fahrenkrug et al., 1995). Preabsorption with VIP, but not PACAP, eliminated all staining.

Immunohistochemistry

Immunohistochemical detection of PACAP/CtB/VIP and PACAP/melanopsin was performed as described previously (Hannibal et al., 2001, 2004; Juhl et al., 2007), except that brain sections were incubated with primary antibodies for 72–96 hours at 4°C. For immunohistochemistry on flat mounts a 72–84-hour incubation period was used. For double and triple labeling, a mixture of biotinylated donkey anti-mouse antiserum (code No. 715-065-151; Jackson Immunoresearch, West Grove, PA; diluted 1:800), Texas red-conjugated donkey anti-goat antiserum (code No. 705-075-147; Jackson Immunoresearch; diluted 1:200), and Cy5-conjugated donkey anti rabbit antiserum (code No. 706-175-134; Jackson Immunoresearch; diluted 1:100) or Cy2/Alexa488-conjugated donkey anti-rabbit antiserum (code No.711-226-152 [Jackson Immunoresearch], diluted 1:100; code No. A21206 [Molecular Probes, Eugene, OR]) was used in combination with streptavidin biotin-horseradish peroxidase complex (Vector, Burlingame, CA), biotinylated tyramide (tyramide system amplification; DuPont NEN, Boston, MA), and streptavidin-Cy2 (Amersham, Birkerød, Denmark). When using two primary antibodies raised in the same species, we employed the tyramide amplification system (Hundahl et al., 2012) originally described by Berghorn et al. (1994). Briefly, C-terminal melanopsin (diluted 1:10,000, 5 days at 4°C) was visualized with Envision (code K4002, diluted 1:2, Dako) and tyramide-conjugated Alexa594 (Molecular Probes), followed by blocking in 1% H_2O_2 to quench horseradish peroxidase, followed by incubation in N-terminal anti-melanopsin antibody (diluted 1:500, 5 days at 4°C) and visualized with an Alexa488 conjugated donkey anti-rabbit antibody (code A21206; Molecular Probes). Images were obtained using an iMIC confocal microscope

(Till Photonics/FEI, Munich, Germany) equipped with appropriate filter settings for detecting DAPI, Cy2/Alexa488, Texas red/Alexa561/594, and Cy5. Colocalization was determined by using the colocalization plugin module in ImageJ/Fiji software (v. 1.47q, NIH) on digital images representing the retinal target areas in the brain. This plugin highlights the colocalized points of two eight-bit images, and colocalized points appear white (we used default value = 255). Pixels are considered colocalized if their intensities are higher than the threshold of their channels (we use threshold set at 50–100 depending on the background noise) and if the ratio of their intensity is higher than the ratio-setting value (we use the default set at 50%). Melanopsin/PACAP cell counts were performed from pieces of retina photographed by the iMIC confocal microscope (Till Photonics) using the wide-field camera and a ×10 objective. Each piece of retina was photographed, and images covering the entire piece were stitched together in the LA Stitch plugin in Fiji software (v. 1.47q, NIH). Each of these images was then analyzed using the cell counter plugin also in Fiji to mark and count the cells containing PACAP and melanopsin, only melanopsin, or only PACAP. Cell counts were performed in areas in which cells were well stained with both antigens, PACAP and melanopsin. Areas in which either one or both immunoreactions were insufficient were excluded. Furthermore, the evaluation of cell density was performed in central and more peripheral parts of the retina in which both the inner and the outer melanopsin processes could be identified. In some areas, the inner stratification plexus was not stained or only weekly stained, and these areas were not used in the evaluation. For a more detailed visualization of the melanopsin-immunoreactive network of outer and inner stratifying processes, Z-stacks with a focal depth of 30–40 μm were generated, and the Z-stacks of images were stitched as described above. For 3D analysis at the higher magnification, images were obtained by the spinning disk confocal part of the microscope. Z-stacks of typically 60–80 images separated in the Z-level by 0.5 μm were deconvolved in AutoQuant X (v. 3.02; Media Cybernetics, Rockville, MD), and the localizations of dendritic processes and cell bodies were further analyzed in Imaris (v. 7.6.4; Bitplane Scientific Software). Finally, images were corrected for brightness and contrast in Adobe Photoshop CS5 Extended and mounted into plates in Adobe Illustrator CS5.

RESULTS

Melanopsin immunoreactivity demonstrated with N- and C-terminal-directed antimelanopsin antibodies

In mouse, melanopsin has been found in two isoforms, OPNL (long) and OPNS (short), differentiated by the length of the C-terminal of the protein (Pires et al., 2009). In humans and in monkeys, such isoforms have so far not been identified. To ensure that our in-house C-terminal-directed anti-melanopsin antibody detects all forms of melanopsin, double staining on the same piece of retina was performed with both N- and C-terminal-directed antibodies. Both antibodies labeled the same population of cells, although the N-terminal antibody label appeared more robust (Fig. 1). The following results were obtained using either the C- or the N-terminal antibody.

Melanopsin-containing RGCs costore PACAP immunoreactivity

Melanopsin-immunoreactive RGCs were found throughout the entire macaque retina as described in detail previously (Dacey et al., 2005; Jusuf et al., 2007). Melanopsin immunoreactivity is located in the soma, axon, and dendritic membrane (Figs. 1–3). Melanopsin-immunoreactive cells form two distinct dendritic mosaics, one with dendrites stratified in the inner plexiform layer (IPL) close to the border of the ganglion cell layer (GCL; Figs. 1–3) and one with dendrites stratified in the outer IPL close to the border of the inner nuclear layer (INL; Figs. 1–3). Inner stratifying cells have cell bodies located exclusively in the GCL, but outer stratifying cell bodies were often displaced to the INL as previously described (Dacey et al., 2005; Jusuf et al., 2007; Figs. 1–3). In both types of melanopsin cells, PACAP was found to be costored (Figs. 1–3). In the melanopsin-expressing RGCs, the amount of melanopsin immunoreactivity varies in intensity (Figs. 1–3), and melanopsin cells with weak and strong intensity staining could be found among both inner and outer stratifying cells, although outer cells generally stain more strongly than inner stratifying cells (Figs. 2, 3). Similarly, PACAP immunostaining in the two cell types varied in intensity but did not follow the intensity of the melanopsin staining (Figs. 2, 3). In several cases, melanopsin-containing inner stratifying cells were often identified by using PACAP immunostaining because of the weak staining of melanopsin (Figs. 2, 3). Total cell counts were performed in pieces of retina in which immunostaining from both antigens was well preserved. We counted a total of 464 cells that contained both PACAP and melanopsin immunoreactivity. Three cells contained only melanopsin, and 26 cells located in the GCL contained only PACAP immunoreactivity. Thus, 99% of the melanopsin labeled cells costored PACAP, whereas 95% of the PACAP-labeled cells costored melanopsin. PACAP/melanopsin cell density varied with the lowest density of PACAP/melanopsin cells in the peripheral retina (3–5 cells/mm^2) and the highest

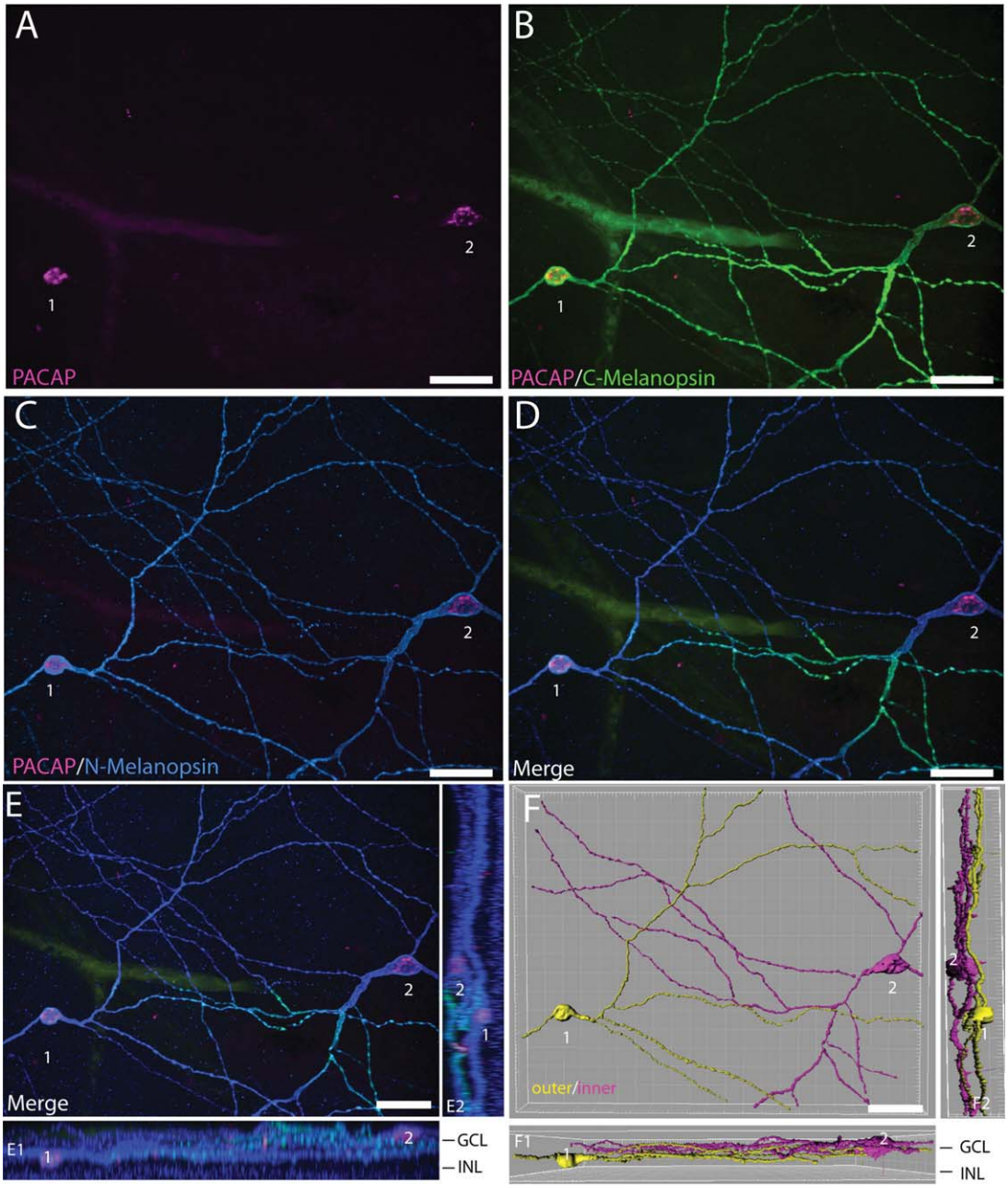

Figure 1. Melanopsin and PACAP immunoreactivity is found in inner and outer stratifying RGCs. **A–D:** Outer (cell 1) and inner (cell 2) stratifying RGCs expressing PACAP in magenta (A) and melanopsin identified with both a C-terminal antibody (B, green) and an N-terminal antibody (C, blue) and with the two antibodies shown merged (D). **E:** Extended view representing the same Z-stack of a total of 55 images of the cells shown in A–D presented in XY (E), XZ (E1), and ZY (E2) planes to illustrate dendritic stratification. **F:** The same cells shown in A–E analyzed for the localization of the dendritic processes using the Imaris filament tracer module (see Material and Methods). The majority of dendritic processes of cell 2 stratify in the most inner part of the inner plexiform layer (IPL; magenta, F1–2). The dendrites of cell 1 stratify exclusively at the border of the INL/IPL (yellow, F1–2) and the cell body is displaced to the INL (F1–2). Scale bars = 50 μm.

density in the more central retina (13–23 cells/mm^2) as reported previously (Dacey et al., 2005).

Retinal target areas in the brain innervated by PACAP

Because PACAP-IR was found in approximately 99% of the melanopsin-containing RGCs of the macaque retina, PACAP immunoreactivity when costored with CtB was used to demonstrate central retinal melanopsin projections in the macaque brain. We were, however, unable to evaluate whether all PACAP/melanopsin-immunoreactive RGCs express detectable PACAP-positive signals in their axon terminals in the brain. A similar approach has, however, been found to be useful

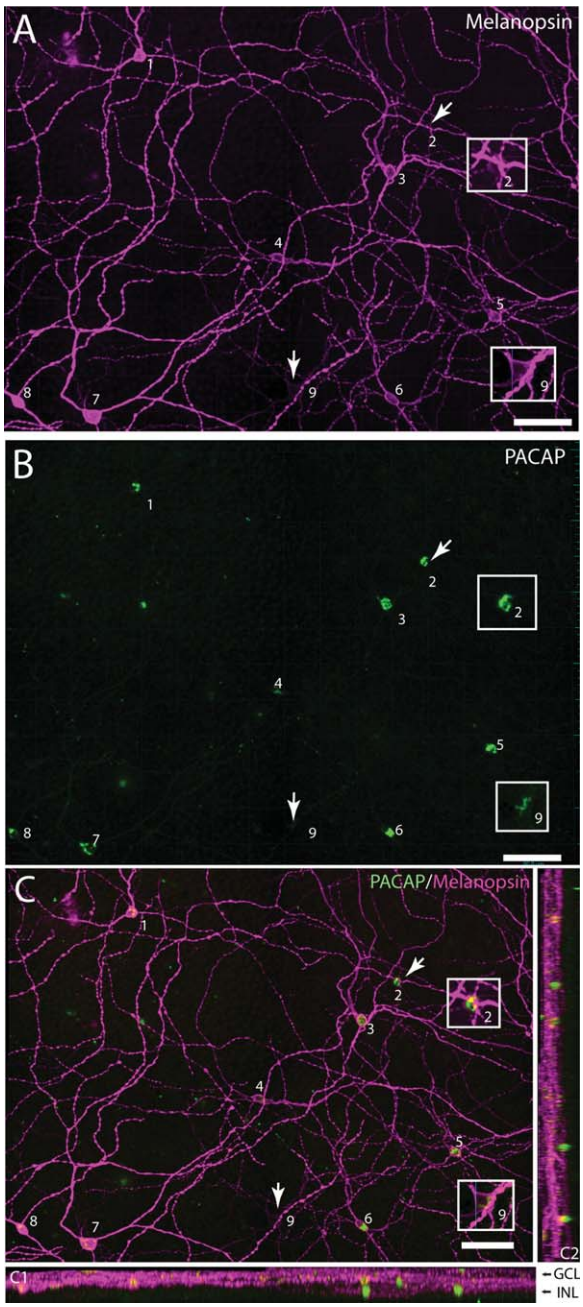

Figure 2. PACAP is found in melanopsin-immunoreactive RGCs of the macaque. **A:** Melanopsin immunoreactivity (magenta; visualized using the C-terminal-directed antimelanopsin antibody) shown in a flat-mount preparation of the *Macaca mulatta* (MM1) retina. The image represents a montage of four images each representing a Z-stack of 35 sections covering the depth required to ensure that both inner and outer stratifying melanopsin processes are visible. The melanopsin cells are numbered 1–9. Note the weak staining of two inner stratifying cells (2 and 9). **B:** PACAP (green) staining of the same piece of retina as shown in A with cells numbered as in A (1–9). Note the low level of PACAP found in cell 9. **C:** Extended view representing the same Z-stack of a total of 35 images of the cells shown in A,B presented in XY (C), XZ (C1), and ZY (C2) planes to illustrate dendritic stratification. Melanopsin (magenta) and PACAP (green) costored in the same RGCs numbered 1–9. GCL, ganglion cell layer; INL, inner nuclear cell layer. Scale bars = 80 μm.

for the rat brain (Hannibal and Fahrenkrug, 2004), and our findings for the monkey are comparable to those for the rat (Hannibal and Fahrenkrug, 2004). VIP, a neuropeptide that is densely expressed in ventrally located neurons of the retinorecipient part of the mammalian SCN, was used as a marker for the SCN (Moore, 1993). The CtB-containing retinal nerve fibers show equal distribution and staining intensity in the two animals. PACAP-IR retina projections were demonstrated in the SCN, lateral geniculate nucleus (LGN), pregeniculate complex (PrGC), the pretectum, the brachium of the superior colliculus (BSC), and the superior colliculus (SC).

SCN

The monkey SCN was densely innervated by CtB-immunoreactive fibers, which occurred with a slight ipsilateral dominance (Fig. 4B,E,H,K) ascending into the ventral SCN from the optic chiasm. As previously reported for monkey (Moore, 1993), the middle part of the SCN received the most intensively stained part of the retinohypothalamic tract (RHT) projection (Fig. 4H), whereas the rostral (Fig. 4B) and caudal (Fig. 4K) SCN were less intensely innervated from the eye. Corresponding to the unilateral injections of CtB, retinal fibers innervating the ventrolateral area of the rostral and mid-SCN also displayed PACAP immunoreactivity (Figs. 4A,D,G, 5A–D). In the caudal part of the SCN, retinal projections largely lacked PACAP immunoreactivity (Figs. 4J,K, 5E–H). These PACAP-negative fibers most likely represent retinal projections of a nonmelanopsin origin, as found in several rodent species (Gooley et al., 2003; Morin et al., 2003; Sollars et al., 2003; Hannibal and Fahrenkrug, 2004). PACAP-immunoreactive fibers lacking CtB were observed in the SCN in both animals, illustrating the occurrence of innervation from the noninjected eye and most likely also representing a minor group of PACAP nerve fibers originating in the brain, as found in rodents (Hannibal and Fahrenkrug, 2004; see also Hannibal et al., 1997).

LGN

Retinal projections to the LGN in the macaque are shown in Figure 6. Distinct autofluorescence was found in cell bodies of the LGN in both animals using filters for the detection of both CtB and PACAP and verified by UV illumination. The autofluorescence appears as colocalization in different parts of the LGN but can be distinguished from the delicate nerve endings of the retinal projections (Fig. 6). CtB projections were found in the rostrocaudal axis of the LGN. The retinal projections from the two eyes are segregated; crossed projections innervating layers I, IV, and VI (Fig. 6B), and

uncrossed retinal projections innervating layers II, III, and V are visible in both the MM1 (Fig. 6A) and the MF. PACAP immunoreactivity was located throughout the LGN (Fig. 6), representing both retinal and nonretinal innervation. PACAP-containing retinal fibers appeared to innervate both the parvocellular and the

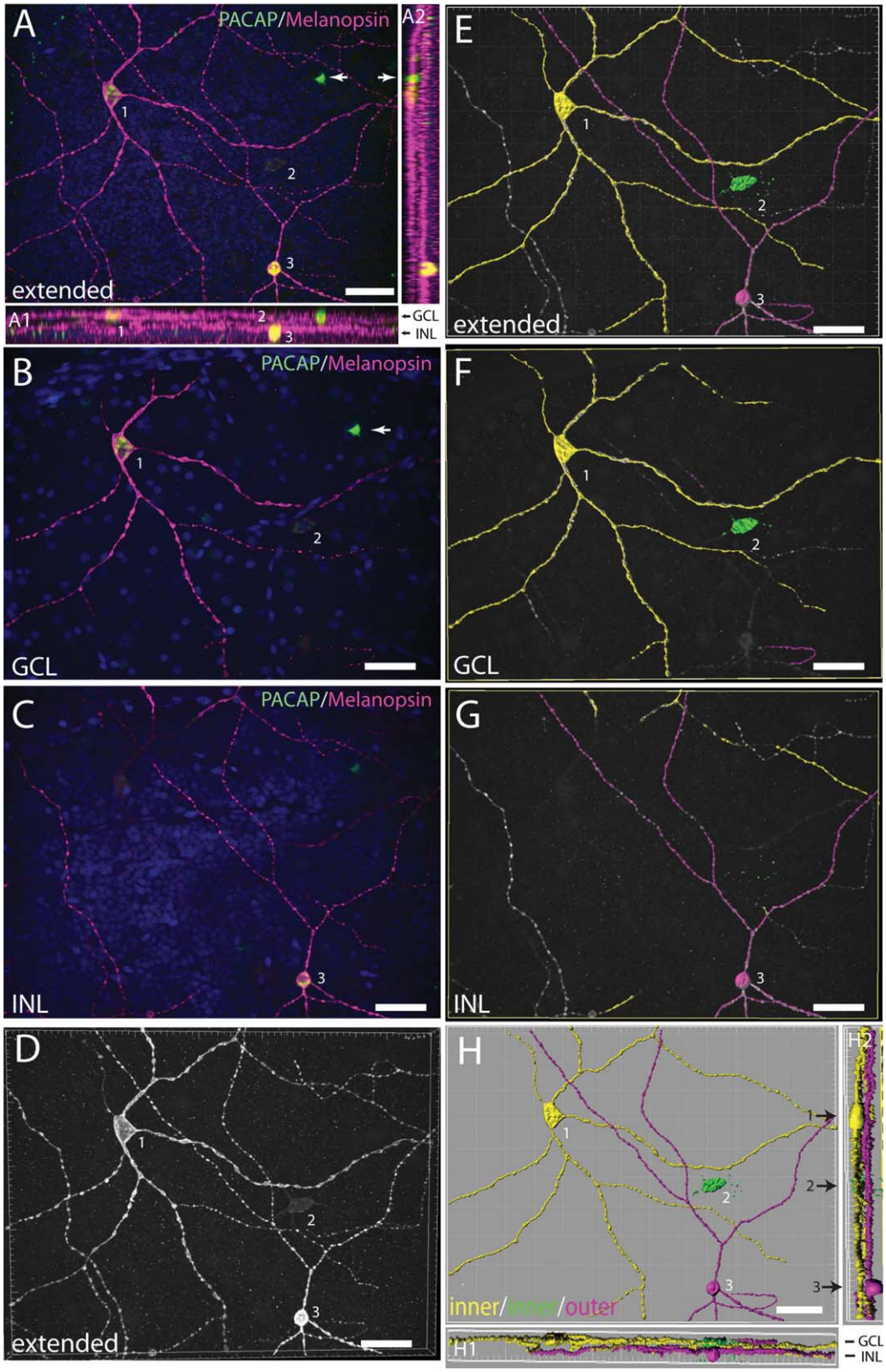

Figure 3.

magnocellular parts of the LGN, whereas we found no innervations of the koniocellular layers. PACAP fibers lacking CtB immunoreactivity may represent innervation from the contralateral eye or may originate from other areas in the brain. PACAP-immunoreactive fibers costoring CtB were found in layers V, III, and II on the ipsilateral side (Fig. 6C,D,F,I), and on the contralateral side in nerve fibers in layer VI, most intensely in the lateral part (Fig. 6O,P), and in layer IV and layer I (Fig. 6K,N). The delicate retinal projections containing both CtB and PACAP immunoreactivity are shown at high magnification in layer III in Figure 6I and in layer VI in Figure 6P.

PrGC

Retinal projections were found in the PrGC corresponding to the rostral part of the LGN complex with a similar distribution on the contra- and ipsilateral sides (Fig. 7A). PACAP immunostaining was found in the same area, and some PACAP fibers costored CtB immunoreactivity (Fig. 7E,H). PACAP-positive fibers not containing CtB were also found in this area of the PrGC as well as dorsal to these retinal projections (Fig. 7A,B). CtB-immunoreactive nerve fibers not costoring PACAP were also found in the PrGC (Fig. 7F–H). No PACAP-immunoreactive cell bodies were found in the PrGC.

The pretectum and SC

Retinal projections were found as bilateral projections to several pretectal nuclei as described previously (Gamlin, 2006). Dense innervation was observed in the pretectal olivary nucleus (PON; Fig. 8) and in the nucleus of the optic tract (NOT; Fig. 9), whereas the medial and posterior pretectal nuclei (PPN) receive sparse retinal innervation (not shown). Retinal projections were also found in the brachium of the superior colliculus (BSC; Fig. 10) and within the SC (Fig. 11). In the PON, a minor part of the retinal projections costored PACAP immunoreactivity (Fig. 8E,H). However, PACAP seems also to occur in a small group of cell bodies scattered in the PON (Fig. 8B,D), giving rise to some of the nonretinal PACAP fibers found in the nucleus. In the NOT, which also received relatively dense innervation of CtB-immunoreactive nerve fibers, the number of fibers also costoring PACAP was limited, and they were found mainly in delicate nerve fiber varicosities most likely passing the NOT toward the SC (Fig. 9E). As found in other pretectal nuclei, PACAP fibers of nonretinal origin also occur in the NOT (Fig. 9B,D). Single fibers immunoreactive for both CtB and PACAP could be found in the medial and posterior pretectal nucleus (not shown). In the BSC, a relatively large contingent of retinal projections costored PACAP (Fig. 10). Again, PACAP was found in delicate nerve fiber varicosities (Fig. 10E,H). Dense retinal innervation visualized by CtB immunoreactivity was found in the superficial layers of the SC, with a significant contralateral dominance (Fig. 11). PACAP, on the other hand, was found in nerve fibers in the superficial layer and in fibers and cell bodies of the deeper layers of the SC (Fig. 11C,D,I,L), and colocalization between CtB and PACAP was found in both the contra- and the ipsilateral retinal projections (Fig. 11G,J,M).

DISCUSSION

The present study uses a combination of immunohistochemistry for one of the RHT neurotransmitters, PACAP, and the anterograde tracer CtB to visualize central projections of melanopsin ipRGCs in retinal target areas of the macaque brain. PACAP, as in other mammalian species, is found in 99% of melanopsin-

Figure 3. Melanopsin and PACAP immunoreactivity is found in inner and outer stratifying RGCs. **A:** Three RGCs numbered 1–3 expressing both melanopsin (visualized with the N-terminal directed antimelanopsin antibody) and PACAP and one RGC stained only for PACAP (arrows), shown in an extended view representing a Z-stack of a total of 50 images presented in XY (A), XZ (A1) and ZY (A2) planes. Colocalization of melanopsin and PACAP appears yellow. Note the weak expression of both melanopsin and PACAP in cell 2. Inner and outer stratifying dendritic processes are shown in the XZ (A1) and YZ (A2) planes. **B:** Focus is in the inner IPL near the GCL border, showing PACAP (green) and melanopsin (magenta) labeling of cells 1 and 2 (same cells as shown in A). Arrow indicates the same PACAP-labeled cell indicated by the arrows in A. Nuclei from other ganglion cells are stained with DAPI (blue). **C:** Focus is in the outer IPL near the INL border showing PACAP (green) and melanopsin (magenta) labeling of cell 3 (same cell as shown in A). The cell body is displaced to the inner nuclear layer (INL). Colocalization of melanopsin and PACAP appears yellow. Nuclei from other cells in the INL stained with DAPI (blue). **D,E:** Extended view of the Z-stack of 50 images from A showing the melanopsin cells before (D) and after (E) being analyzed for the localization of dendritic processes using the Imaris filament tracer module (see Materials and Methods). **F:** Focus as in B after Imaris filament tracer analysis showing cell 1 (yellow) and cell 2 (green). The majority of dendrites of cell 1 are located in the most inner part of the IPL. Cell 2 expressed low levels of melanopsin, and the weakly stained proximal dendrites are also in the inner IPL. The axon of cell 3 (magenta) can be seen descending through the IPL toward the GCL. **G:** Focus as in C after Imaris filament tracer analysis showing the cell body and outer stratifying dendrites of cell 3 (magenta). Several dendrites from cell 1 (yellow) can be seen ascending to the outer IPL. **H:** Summary of the traced cells shown in E–G. H1 shows an extended view in the XZ plane, and H2 shows an extended view in the YZ plane. The dendrites of cell 3 are located exclusively in the outer IPL. GCL, ganglion cell layer; INL, inner nuclear layer. Scale bars = 50 μm.

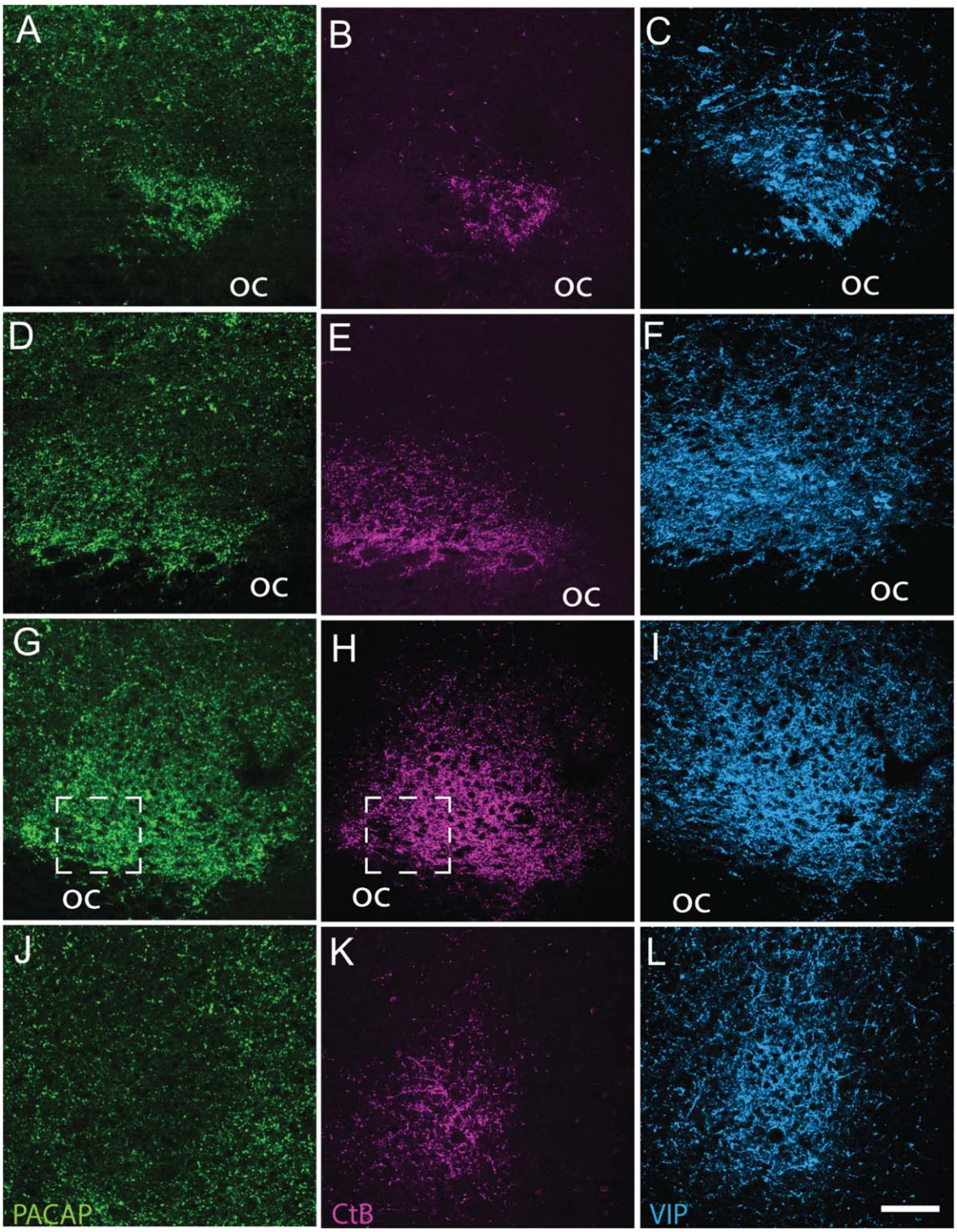

Figure 4. Retinal innervation of the macaque rostral (**A–C**), rostral-middle (**D–F**), middle (**G–I**), and caudal (**J–L**) SCN. The SCN was identified by immunostaining for VIP (C,F,I,L, blue). Retinal projections were visualized by cholera toxin subunit B immunoreactivity (B,E,H,K, magenta), and the melanopsin projections were demonstrated by staining for PACAP (A,D,G,J, green). Boxed areas in G,H are shown at higher magnification in Figure 5. OC, optic chiasm. Scale bar = 100 μm.

expressing RGCs, including both inner and outer stratifying populations. This was verified by using two well-characterized antimelanopsin antibodies one directed against the N- and one directed against the C-terminals of the protein, and identical results were found with each of the antibodies in combination with the PACAP

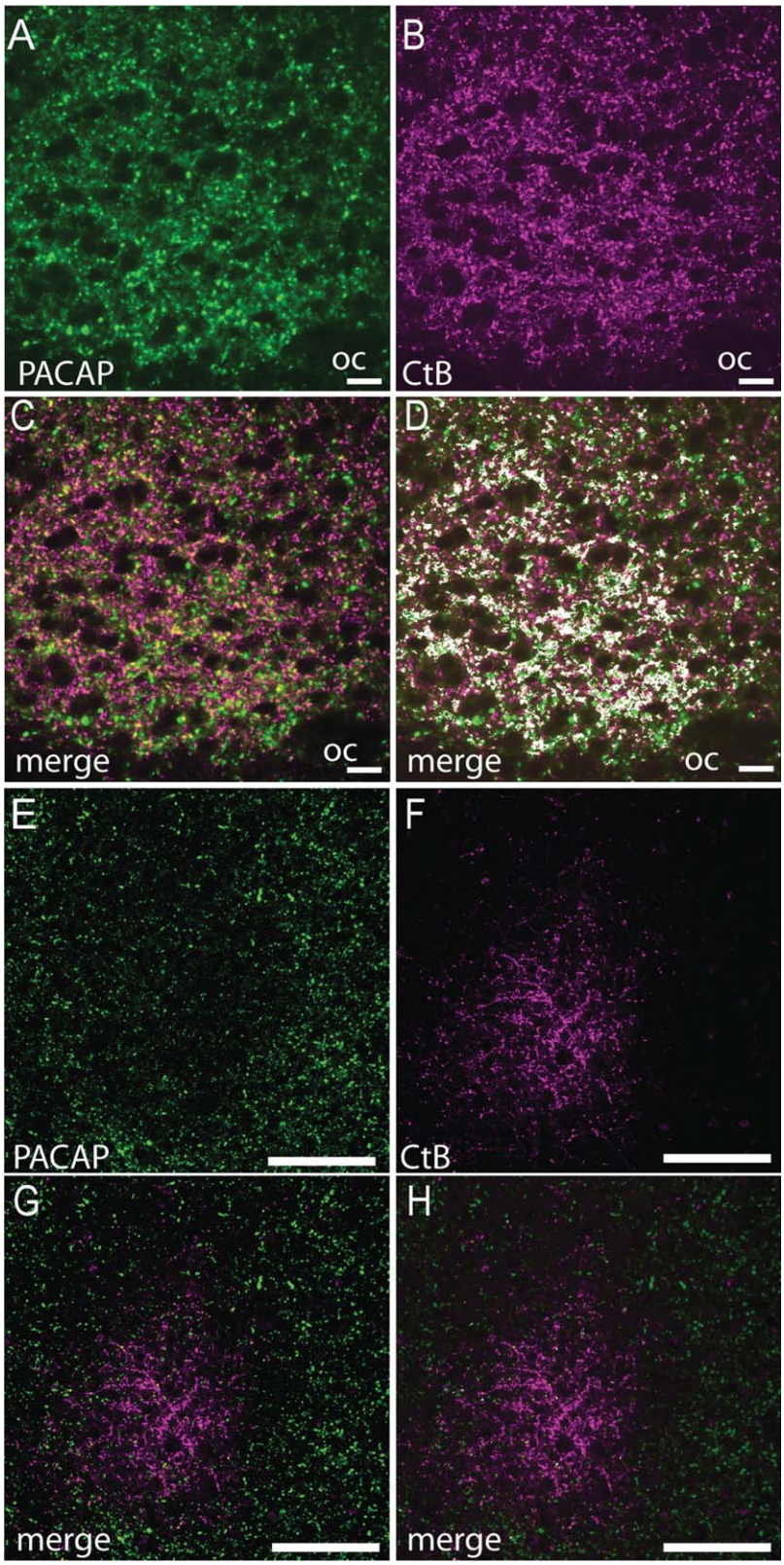

Figure 5. Retinal innervation of the macaque mid- and caudal SCN. **A–D:** Higher magnification view of retinal projections representing the melanopsin ipRGCs in the mid-SCN shown in the boxed area in Figure 4G,H, demonstrating colocalization between cholera toxin subunit B (CtB; B, magenta) and PACAP (A, green) immunoreactivity as visualized in C (yellow) and by white color representing 100% overlap in D (calculated using the colocalization plugin in Fiji). Note that the PACAP fibers not costoring CtB may originate from the contralateral eye and to a minor extent from the brain. **E–H:** Same area of the caudal SCN shown in Figure 4J–K. Colocalization between PACAP (E, green) and CtB (F, magenta) was very sparse, as visualized in G (yellow) and by the few white dots representing 100% overlap in H (calculated as in D). OC, optic chiasm. Scale bars in A–D = 20 μm, in E–H = 175 μm.

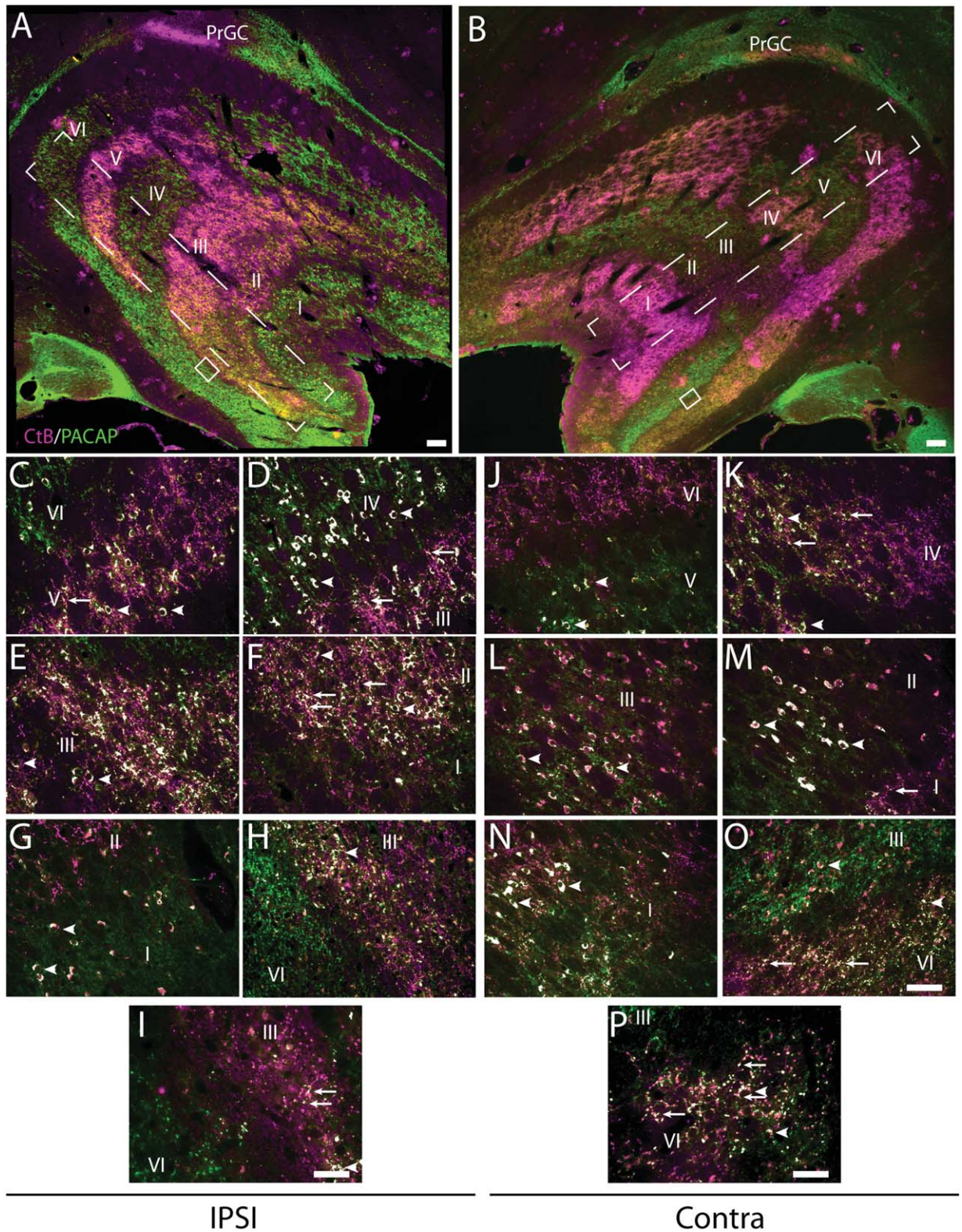

Figure 6. PACAP-containing retinal projections innervate the lateral geniculate nucleus (LGN) of the macaque monkey. **A,B:** Retinal projections visualized by CtB and PACAP immunostaining of the midipsilateral (A) and contralateral (B) LGN innervating layers II, III, and V on the ipsilateral side and layers I, IV, and VI on the contralateral side (the images in A and B consist of 6 × 8 tiled images, which were stitched together). **C–H:** High-magnification images of the six sublayers on the ipsilateral side taken within the region indicated by the boxed area in A. Colocalization between PACAP and CtB was determined by using the colocalization plugin in Fiji (see Materials and Methods) and is shown in white (arrows in C–P). **I:** PACAP and CtB colocalization in the ventral lateral area of layer VI indicated by the boxed area in A shown at a higher magnification. **J–O:** Images of the six sublayers on the contralateral side taken within the region indicated by the boxed area in B. **P:** A higher magnification image from the ventral lateral area of layer VI indicated by the boxed area in B. Note that autofluorescent cell bodies (arrowheads in 6C–P) found throughout the LGN appear as colocalization becsause they are seen with both filter settings. Scale bars = 200 μm in A,B; 25 μm in I,P; 50 μm in O (applies to C–H,J–O).

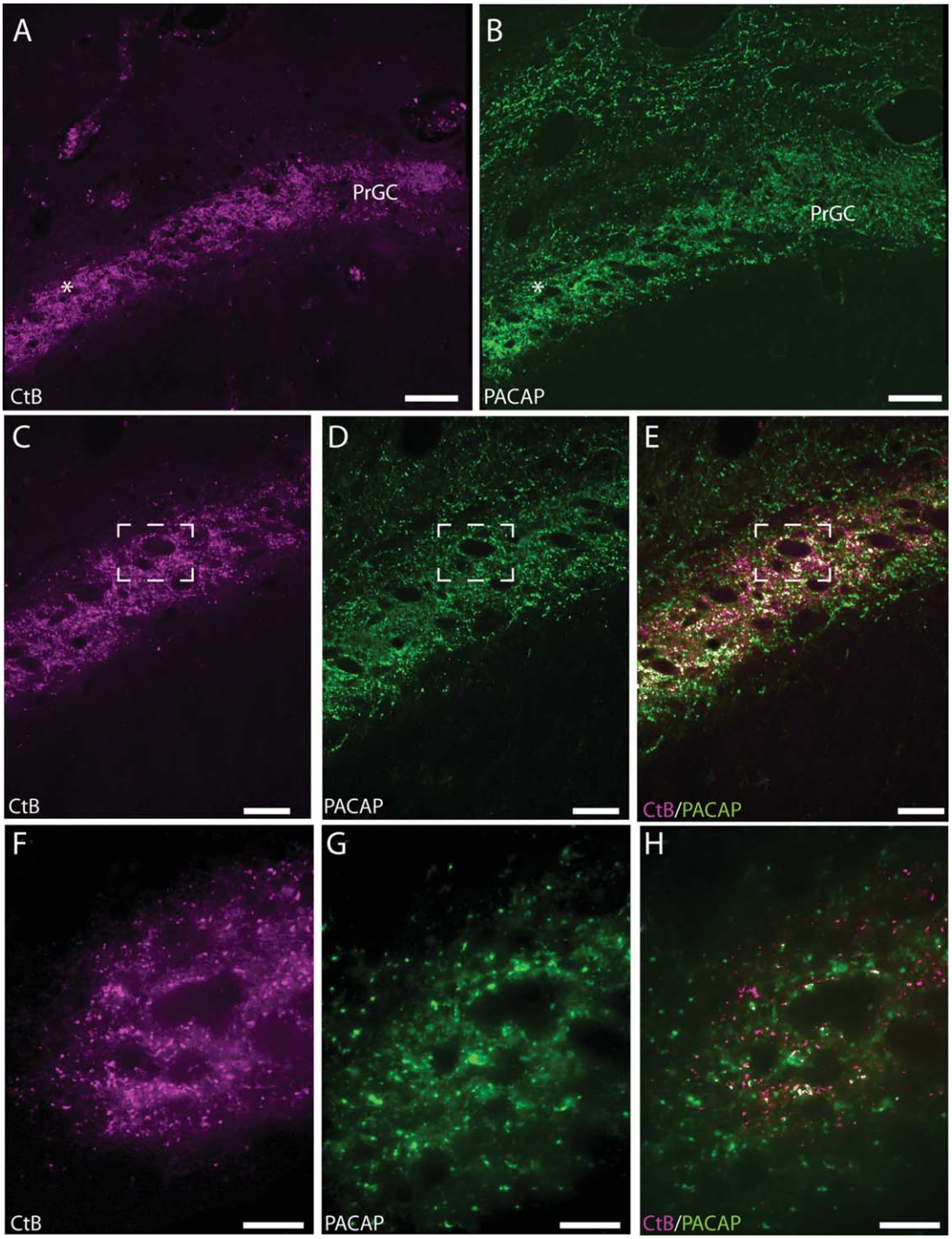

Figure 7. PACAP-containing retinal projections innervate the pregeniculate complex (PrGC) of the macaque. **A:** Retinal projections visualized by CtB immunostaining of rostral contralateral PrGC. The image consists of 3 × 3 tiled wide-field images that were stitched together. **B:** Same section as in A costained for PACAP, showing strong immunoreactivity in the PrGC. **C–E:** Higher magnification images from the area indicated by the asterisks in A,B. **F–H:** Ultrahigh magnification of the area indicated by the boxed areas in C–E. PACAP is found in retinal and nonretinal projections to the PrGC. Colocalization between CtB- and PACAP-containing retinal projections is shown in white in E,H (determined using the colocalization module in ImageJ; see Material and Methods). Scale bars = 100 μm in A,B; 50 μm in C–E, 20 μm for F–H.

antibody. As in rodents, melanopsin ipRGCs project to areas involved in nonimage-forming processes such as the SCN, the PrGC (which corresponds to the IGL in rodents), and the PON, the primary site for the regulation of the pupillary light reflex. Furthermore, this study confirms and extends a previous study demonstrating

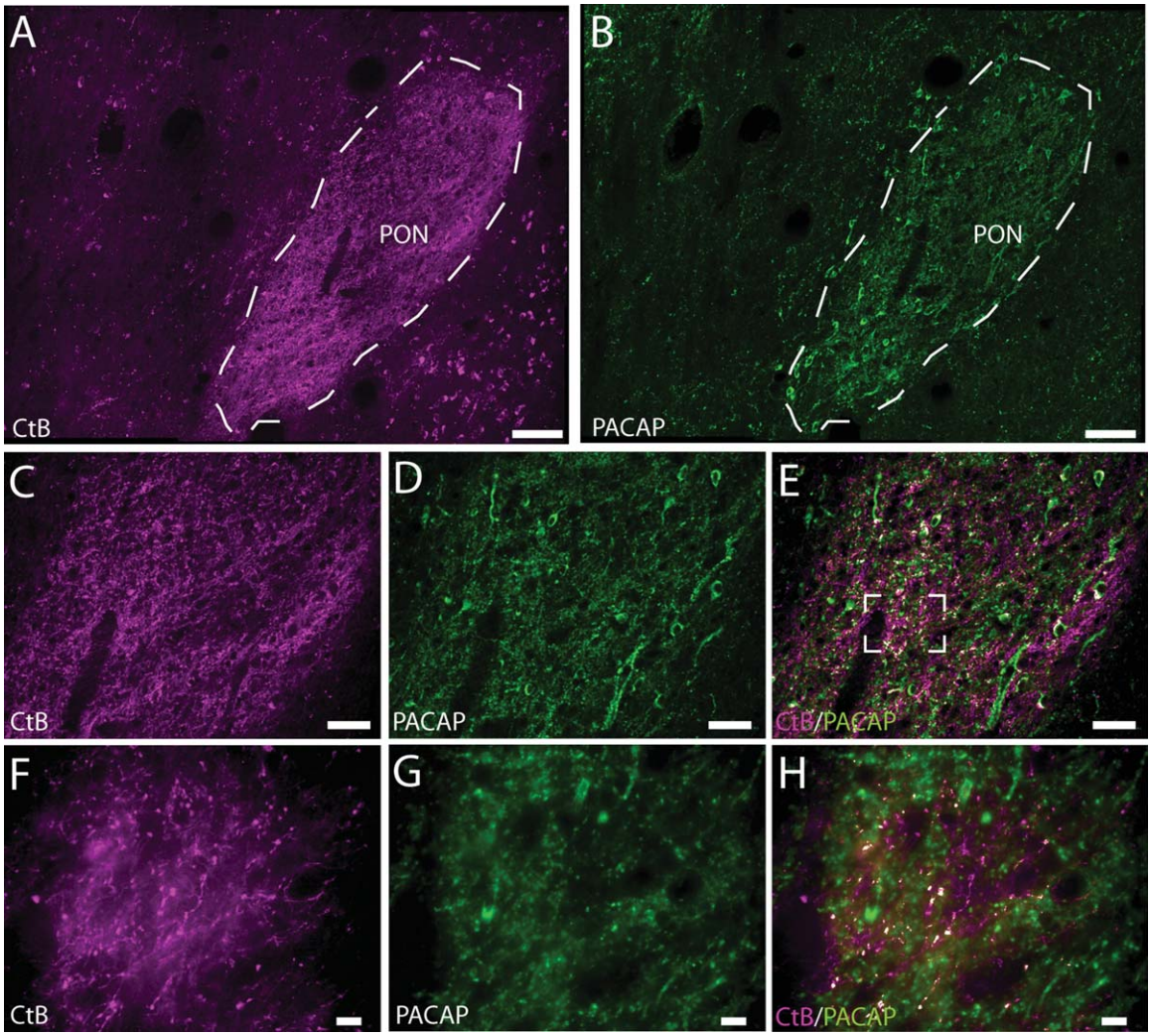

Figure 8. PACAP-containing retinal projections representing the melanopsin ipRGCs innervating the olivary pretectal nucleus (PON). **A:** Retinal projections visualized by CtB immunostaining in the PON. The image consists of 3 × 3 tiled confocal images stitched together for a better overview. **B:** Same section as in A costained for PACAP showing that PACAP was found both in retinal projections and in cell bodies located within the PON as seen at higher magnification (**C–E**) and ultrahigh magnification (**F–H**) representing the region indicated by the boxed area in E. Colocalization between CtB and PACAP containing retinal projections is shown in white (determined using the colocalization module in ImageJ; see Material and Methods). Scale bars = 100 μm in A,B; 50 μm in C–E; 10 μm for F–H.

that ipRGCs project to structures in the brain involved in image-forming visual processing such as the LGN (Dacey et al., 2005) and the SC.

Visualization of central melanopsin projections has until now been conducted in rodent species. In mice, melanopsin projections to the brain have been demonstrated using two different genetic approaches, one in which the melanopsin gene was targeted by using *tau-lacZ* driven by the melanopsin promoter in combination with CtB injections (Hattar et al., 2006) and one in which melanopsin projections were identified by using a Cre-based melanopsin reporter mouse line (Ecker et al., 2010; Brown et al., 2010). Both methods reveal melanopsin projections in the sleep-active area of the ventrolateral preoptic area, in the SCN and in the subparaventricular area, and in the lateral geniculate nucleus including the IGL and the ventral part of the complex. Melanopsin projections were also found in the pretectum, mainly in the PON and in the NOT, whereas single fibers could be found in the SC (Hattar et al., 2006). The Cre-based studies also demonstrate strong retinal projection to image-forming visual processing areas in the LGN and SC (Ecker et al., 2010; Brown et al., 2010). The slightly different results with these two approaches seems at least in part to be explained by different sensitivity of the two systems, in which the *tau-lacZ*-labeled melanopsin ipRGCs primarily are of the M1 subtype of ipRGCs (Ecker et al., 2010; Brown et al.,

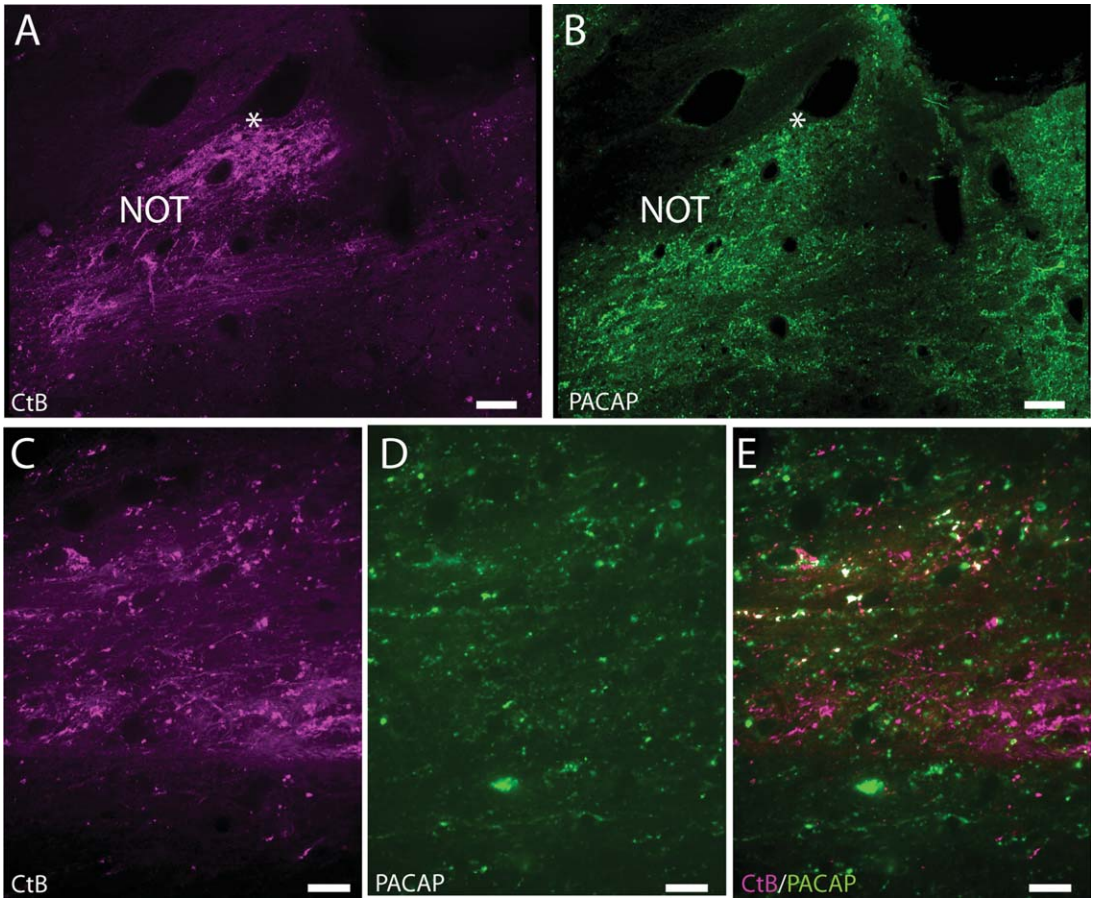

Figure 9. PACAP-containing retinal projections representing the melanopsin ipRGCs innervating the nucleus of the optic tract (NOT). **A:** Retinal projections visualized by CtB immunostaining in the NOT. The image consists of 4 × 4 tiled confocal images stitched together for a better overview. **B:** Same section as in A costained for PACAP showing that PACAP was found in both retinal and nonretinal projections located within the NOT. **C–E:** High-magnification images of the area indicated by the asterisks in A,B. Colocalization between CtB- and PACAP-containing retinal projections is shown in white in E (determined using the colocalization module in ImageJ; see Material and Methods). Scale bars = 200 μm in A,B; 20 μm in C–E.

2010). These cells express a higher level of melanopsin resulting from a specific isoform (short) of melanopsin. The Cre-based identified ipRGCs show both M1 and M2 cells, which express exclusively another (long) isoform of melanopsin (Pires et al., 2009). A third cell type, M3, which contains both isoforms and is melanopsin immunoreactive, and a fourth and a fifth cell type, M4 and M5, which lack detectable melanopsin immunoreactivity and have weak intrinsic light responses, were also identified by using this technique (Ecker et al, 2010).

We have used another approach in rat, hamster, and mouse to demonstrate melanopsin retinal fiber projections in the brain. By costaining for PACAP and CtB, we found very similar distributions of retinal projections in the albino rat, hamster, and mouse except in the dorsal geniculate nucleus, which contains few retinal projections (Bergström et al., 2003; Hannibal and Fahrenkrug, 2004, 2006; Engelund et al., 2012) compared with the studies by Ecker et al. (2010) and Brown et al. (2010). These studies indicate that the combination of double immunostaining for CtB and PACAP can be a useful tool for the study of central melanopsin projections in the mammalian brain. The present study demonstrates the usefulness of this approach in the primate brain. By using two antimelanopsin antibodies, each recognizing either the N- or the C-terminal part of melanopsin, we first confirmed that PACAP is found in 99% of all melanopsin-expressing RGCs in the monkey independent of whether they were inner or outer stratifying cells. We found only three melanopsin cells lacking PACAP immunoreactivity. The 0.6% of cells that only labeled for melanopsin represents a very small number and could be explained by a level of PACAP too low to be detected. We found that 95% of the PACAP cells costore melanopsin. The 5% of PACAP-positive/melanopsin-negative cells had cell bodies located in the GCL

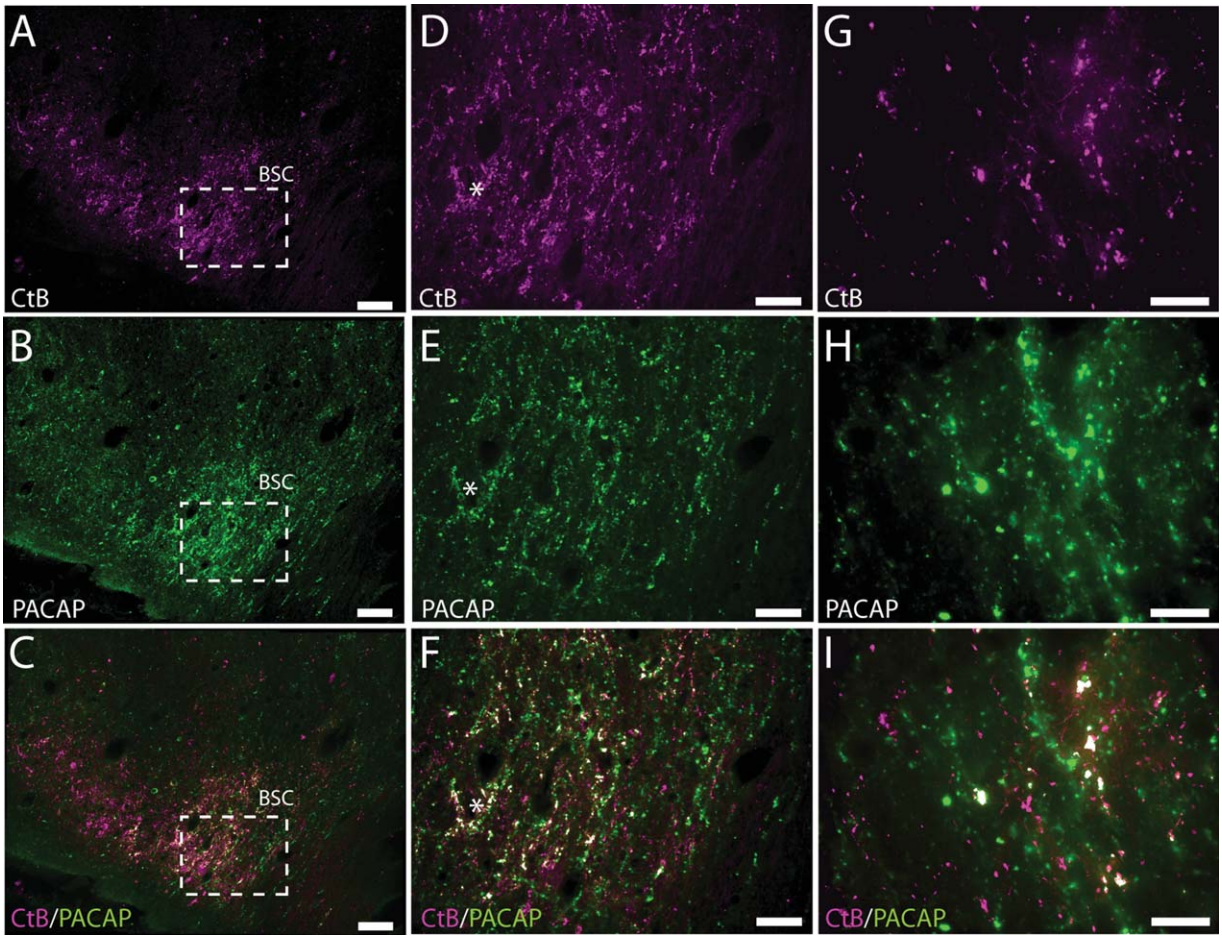

Figure 10. PACAP-containing retinal projections representing the melanopsin ipRGCs innervating the brachium of the superior colliculus (BSC). **A:** Retinal projections visualized by CtB immunostaining in the BSC. The image consists of 3 × 3 tiled confocal images stitched together for a better overview. **B,C:** Same section as in A costained for PACAP (B) and showing that PACAP was found in both retinal and nonretinal projections located within the BSC (C). **D–F:** Higher magnification images of the region indicated by the boxed area in A–C. **G–I:** Ultrahigh magnification of the area indicated by the asterisks in D–F. Colocalization between CtB- and PACAP-containing retinal projections is shown in white in C,F,I (determined using the colocalization module in ImageJ; see Material and Methods). Scale bars = 100 μm in A–C; 50 μm in D–F; 20 μm for G–I.

and were randomly distributed throughout the retina. Most likely, these cells represent melanopsin RGCs with a level of melanopsin too low to be detected by the antisera that we used in the current animals.

We then demonstrated the occurrence of retinal PACAP innervation in both nonimage-forming and image-forming visual processing areas of the brain. In the ventral and mid-SCN, dense PACAP innervation corresponding to the retinal input was found, whereas the caudal part of the SCN received retinal innervation that seems to be of non-PACAP/melanopsin origin from the lack of PACAP immunoreactivity in these retinal nerve terminals. Lack of PACAP in CtB-containing retinal projections was also found in a minor number of retinal nerve terminals in the mid-SCN. It may be possible that these non-PACAP-containing retinal fibers represent a low amount of PACAP in some retinal projections (because PACAP expression varies in the ipRGCs). However, it is more likely that these CtB-positive/PACAP-negative nerve fibers represent nonmelanopsin projecting retinal fibers as have been found in the rat and golden hamster, for which retrograde and anterograde tracer studies have indicated nonmelanopsin RGCs projecting to the SCN (Gooley et al., 2003; Morin et al., 2003; Sollars et al., 2003; Hannibal and Fahrenkrug, 2004). This is different from the case in the mouse, in which apparently almost all RGCs innervating the SCN express melanopsin (Baver et al., 2008). The role of these nonmelanopsin projections remains to be established, but they may represent input from the classical ganglion cells. Recent studies in mice in which the melanopsin-expressing RGCs are ablated demonstrate

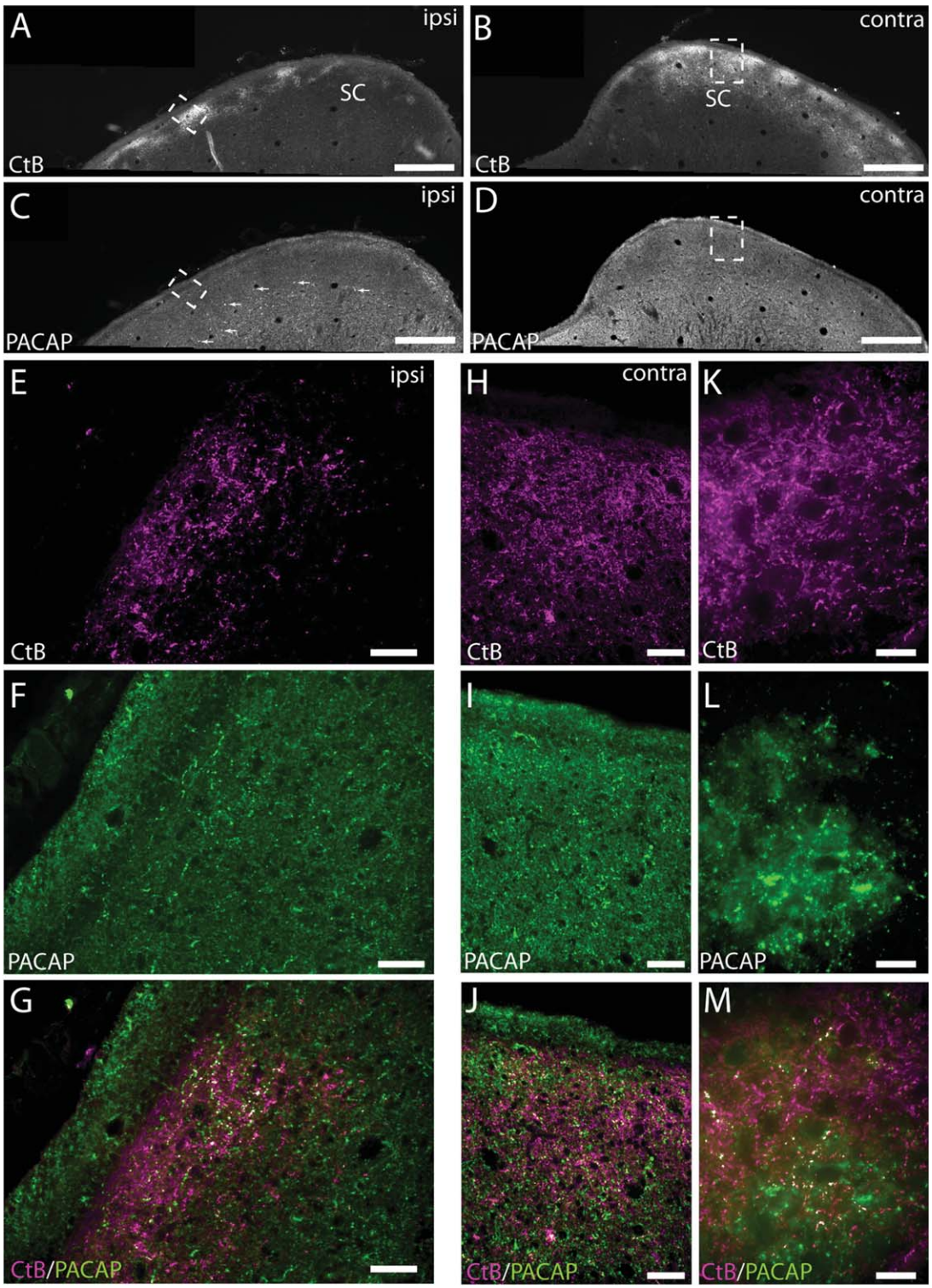

Figure 11. PACAP-containing retinal projections representing the melanopsin ipRGCs innervating the superior colliculus (SC). **A,B:** Retinal projections visualized by CtB immunostaining in the ipsi- and contralateral SC. The image consists of 2 × 8 tiled confocal images stitched together for a better overview. **C,D:** Same section as in A,B costained for PACAP showing that PACAP was found in cell bodies of deeper layers of the SC (arrows). High magnification demonstrates PACAP in retinal projections of both the ipsilateral (**E–G**) and the contralateral (**H–M**) sides. Colocalization between CtB- and PACAP-containing retinal projections is shown in white in G,J,M (determined using the colocalization module in ImageJ; see Material and Methods). Scale bars = 500 μm in A–D; 50 μm in E–G; 20 μm in H–M.

that the melanopsin-containing ipRGCs are essential for photoentrainment, masking behavior, and the pupillary light reflex in mammals (Hatori et al., 2008; Guler et al., 2008), suggesting that these dorsally located retinal projections may participate in other retina-driven functions that remain to be determined.

The role of the IGL in rodent is well established and mediates both indirect photic information as well as nonphotic information to the SCN (Harrington, 1997). Double immunohistochemistry for PACAP and CtB demonstrates that the most medial part of the rostral PrGC in the monkey is a target area of the PACAP/melanopsin projections. It is likely that this area of the PrGC has a circadian function similar to the IGL of rodents (Harrington, 1997), i.e., sending indirect light information from the melanopsin-containing ipRGCs to the monkey SCN. In a previous study, melanopsin-expressing RGCs were identified based on retrograde tracing from the LGN (Dacey et al., 2005). The present study demonstrates PACAP-containing retinal projections in all six layers of the LGN, linking melanopsin-expressing retinal projections (and PACAP) not only to nonimage-forming functions but to higher visual perception, as recently described for mice (Ecker et al., 2010; Brown et al., 2010).

The demonstration of PACAP in retinal projections to the PON supports a previous study of melanopsin projection to this part of the pretectum (Dacey et al., 2005) and provides additional anatomical support for a study in monkey that indicates a central role for melanopsin in regulation and control of the pupillary light reflex (Gamlin et al., 2007). In both monkey and humans, melanopsin signalling seems to participate in the pupillary light reflex, with a significant contribution giving rise to a characteristic component of the reflex (Gamlin et al., 2007).

Sparse melanopsin projections have been found in the SC in mouse, rat, and hamster (Morin et al., 2003; Hannibal et al., 2004; Hattar et al., 2006). The present study demonstrates PACAP/CtB projections in the SC, and double-labeled projections were also found in the BSC, indicating that PACAP/melanopsin ipRGCs project to the SC in monkey.

In conclusion, PACAP-immunoreactive projections costoring CtB represent ipRGC projections to visual targets in the macaque monkey. The present study supports and extends previous retrograde tracing studies demonstrating that melanopsin-containing retinal projections reach areas in the brain involved in both nonimage-forming and image-forming visual processing.

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CONFLICT OF INTEREST STATEMENT

The authors report no conflicts of interest.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: JH, BBP, DD, PDG. Acquisition of data: JH, LK, CES, PDG. Analysis and interpretation of data: JH, BBP, DD, PDG. Drafting of the manuscript: JH, BBP, PDG. Critical revision of the manuscript for important intellectual content: JH, BBP, DD, PDG. Statistical analysis: JH. Obtained funding: JH, PDG, DD.

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