Neuroscience 202 (2012) 117–130

EXPRESSION AND LOCALIZATION OF THE CANNABINOID RECEPTOR TYPE 1 AND THE ENZYME FATTY ACID AMIDE HYDROLASE IN THE RETINA OF VERVET MONKEYS

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Abstract—The presence of a widespread endocannabinoid (eCB) system within the nervous system, including the retina, has been demonstrated in recent years. Expression patterns of the cannabinoid receptor type 1 (CB1R) and enzyme fatty acid amide hydrolase (FAAH) are available for rodents, but data for humans and monkeys are scarce. We, therefore, thoroughly examined the distribution pattern of CB1R and FAAH throughout the retina of the vervet monkey (*Chlorocebus sabeus*) using confocal microscopy. Our results demonstrate that CB1R and FAAH are expressed throughout the retina, from the foveal pit to the far periphery. CB1R and FAAH are present in the photoreceptor, outer plexiform, inner nuclear, inner plexiform, and retinal ganglion cell layers (PRL, OPL, INL, IPL, and RGCL, respectively). More specifically, in PRL, CB1R and FAAH are preferentially expressed in cones of the central retina. In OPL, these two components of the eCB system are concentrated not only in the cone pedicles but also in rod spherules with, however, a less intense staining pattern. Triple-labeling immunofluorescence revealed that both cone and rod bipolar cells express CB1R and FAAH. Heavy staining is detected in RGC somas and axons. Neither CB1R nor FAAH are found in the retinal glia, the Müller cells. These data indicate that the eCB system is present throughout the primate retina and is ideally positioned to modulate central and peripheral retinal functions.

Key words: endocannabinoids, cannabinoid receptor CB1, fatty acid amide hydrolase, monkey retina, immunohistochemistry, confocal microscopy.

The *cannabis sativa* plant contains a group of substances termed cannabinoids that modulate neuronal activity by activating two G protein-coupled receptors, the cannabinoid receptors CB1 (CB1R) and CB2 (CB2R) (Piomelli, 2003; Atwood and Mackie, 2010). These receptors exert their action through distinct signal transduction mechanisms and are activated physiologically by endogenous ligands called endocannabinoids (eCBs), such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Gómez-Ruiz et al., 2007). The activation of the CB1R inhibits the transmembrane enzyme adenylyl cyclase and modulates calcium and potassium ion channels through $G_{i/O}$ (Freund et al., 2003; Rodríguez de Fonseca et al., 2005; Turu and Hunyady, 2010). Anatomically, CB1R is prominently present on GABAergic and glutamatergic terminals (Tsou et al., 1998; Katona et al., 1999; Monory et al., 2006) and is widely expressed in CNS (hippocampus, cortex, basal ganglia, and cerebellum) and peripheral nervous system (Herkenham et al., 1991a,b; Egertová and Elphick, 2000). The presynaptic location of CB1R plays a key role in synaptic transmission allowing the eCB system to act as a modulatory system that regulates learning, memory, motor coordination, neuroprotection (Di Marzo et al., 1998), and visual processing (Straiker et al., 1999a, b).

Fatty acid amide hydrolase (FAAH), an intracellular enzyme that is attached to the membrane by the N-terminal domain, is mainly responsible for degrading AEA, one of the two chief eCBs, into arachidonic acid and ethanolamine (Deutsch and Chin, 1993; Elphick and Egertová, 2001 for review). FAAH has already been localized in selected areas of the human CNS but not in human visual structures (Romero et al., 2002). The CB1R ligand AEA is also considered as a candidate endogenous TRPV1 receptor ligand that colocalizes with some FAAH-positive amacrine cells (Zimov and Yazulla, 2007). Other lipids that are not CB1R ligands are also broken down by FAAH, such as oleamide (Cravatt et al., 1996). The expression pattern of FAAH in the retina has been demonstrated in photoreceptors, cone bipolar cells, ganglion cells, and some amacrine cells of rodents (Yazulla et al., 1999; Hu et al., 2010; Zabouri et al., 2011b), but data are not available for the primate retina.

The organization of the retinal mosaic has an incidence on visual functions, the center being mainly involved in visual acuity, color coding, and photopic sensitivity (cone vision), whereas the periphery is more concerned with scotopic functions (rod vision) (Wässle et al., 1995; Jacobs, 2008). If eCBs and cannabinoid receptors are mainly expressed in cones of the central retina, then visual functions associated with the foveal cones should be affected by cannabis consumption. Indeed, several case reports in the 1970s mentioned some visual effects after cannabis consumption, such as an increase in glare recovery at low contrast (Adams et al., 1978), a reduction in Vernier and Snellen acuities (Adams et al., 1975; Kiplinger et al.,

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Abbreviations: AEA, anandamide; CB1R, cannabinoid receptor CB1; eCB, endocannabinoid; EDTA, ethylenediaminetetraacetic acid; FAAH, fatty acid amide hydrolase; GCL, ganglion cell layer; GS, glutamine synthetase; HFL, Henle fiber layer; INL, inner nuclear layer; IPL, inner plexiform layer; IR, immunoreactivity; NFL, nerve fiber layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PBS, phosphate-buffered saline; PKCα, protein kinase C alpha; RGC, retinal ganglion cells; 2-AG, 2-arachidonoylglycerol.

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doi:10.1016/j.neuroscience.2011.11.041

Table 1. Primary antibodies used in this study

Antibody	Immunogen	Source[a]	Working dilution
CB	Purified bovine kidney calbindin-D28K	Sigma-Aldrich, St. Louis, MO, C9848, mouse monoclonal, clone CB-955	1:500
CHX10	Peptide containing the aa 44–61 of human CHX10	Santa Cruz Biotechnology, Santa Cruz, CA, sc-21690, goat polyclonal	1:100
PKCα	Peptide mapping the aa 296–317 of human PKCα	Santa Cruz Biotechnology, Santa Cruz, CA, sc-8393, mouse monoclonal, clone H-7	1:500
Brn3a	Fusion protein containing aa 186–224 of Brn3a protein	Chemicon, Temecula, CA, MAB1585, mouse monoclonal	1:100
Syntaxin	Synaptosomal plasma fraction of rat hippocampus (Barnstable et al., 1985)	Sigma-Aldrich, St. Louis, MO, S0664, mouse monoclonal, clone HPC-1	1:500
GS	Full protein purified from sheep brain	Chemicon, Temecula, CA, MAB302, mouse monoclonal, clone GS-6	1:500
CB1R	Fusion protein containing aa 1–77 of rat CB1R	Sigma-Aldrich, St. Louis, MO, C1233, rabbit polyclonal	1:150
FAAH	Synthetic peptide aa 561–579 of rat FAAH	Cayman Chemical, Ann Arbor, MI, 101600, rabbit polyclonal	1:100

Abbreviations: CB, calbindin; PKCα, protein kinase C (α isoform); GS, glutamine synthetase; CB1R, cannabinoid receptor type 1; FAAH, fatty acid amide hydrolase; aa, amino acids.

[a] The source column indicates the commercial company, catalog reference, and origin. The clone designation is given for monoclonal antibodies.

1971), blurred vision (Noyes et al., 1975), change in color discrimination, and increased photosensitivity (Dawson et al., 1977). There has also been evidence for central effects of cannabinoid use in vision by binocular depth inversion technique and electroencephalogram recordings of the occipital cortex (Semple et al., 2003; Skosnik et al., 2006).

The presence of CB1R in the retina of many species suggested that eCB signaling system is phylogenetically preserved and could play an important role in retinal functions (Straiker et al., 1999a; Argaw et al., 2011). Studies have reported the presence of the eCB system in various retinal cell types (cones, bipolar, ganglion, horizontal, and amacrine cells) (see Yazulla, 2008 for review). The modulatory effects of cannabinoids at all stages of retinal processing have also been described (Yazulla, 2008). Moreover, critical proteins defining cannabinoid circuitry like diacylglycerol lipase-α and -β, monoacylglycerol lipase, α/β-hydrolase domain 6, cannabinoid receptor-interacting protein 1a, FAAH, and *N*-acylethanolamine-hydrolizing acid amidase, have been localized in the adult mouse retina (Hu et al., 2010). CB1R and FAAH are expressed in cones, amacrine cells, and ganglion cells and have been both localized in the rat retina essentially in horizontal and rod bipolar cells (Yazulla et al., 1999).

The distribution of receptors and the organization of the retina in humans and primates vary from the center to the periphery. Indeed, there is a monotonous decrease in the number of cones from the *fovea centralis* (that exclusively contains cones) to the far periphery that contains mainly rods (Osterberg, 1935). Moreover, the density of ganglion cells is much higher in the fovea compared with the periphery (Herbin et al., 1997). Therefore, the expression of the eCB system should be different from the center of the retina to the far periphery, a difference that would also be evident in central retinal targets such as the dorsal lateral geniculate nucleus and the superior colliculus and their cortical recipient areas, the striate and extra-striate cortices.

To our knowledge, there has been only one comparative study that showed the presence of CB1R in the monkey retina (Straiker et al., 1999a). However, in that study the authors do not mention where in the retina the sample was taken and the complete specific retinal cell types expressing CB1R have not been entirely described. The present study, therefore, aims to extend previous data on the monkey retina by thoroughly characterizing the expression and cellular localization of CB1R and FAAH.

EXPERIMENTAL PROCEDURES

Animal preparation

One female and two male vervet monkeys (*Chlorocebus sabaeus*) at 42 months of age were used for this study. The number of specimen used was restricted to three in order to minimize animal use. The animals were born and raised in enriched environments in the laboratories of the Behavioral Sciences Foundation (St-Kitts, West Indies). The animals were fed with primate chow (Harlan Teklad High Protein Monkey Diet; Harlan Teklad, Madison, WI, USA) and fresh local fruits, with water available *ad libitum*. The monkey eyes were kindly provided by Professor Roberta Palmour. The monkeys were part of a developmental study approved by the McGill University Animal Care and Use Committee.

Antibody characterization

All the primary antibodies used in this work, their sources, and working dilutions, are summarized in Table 1. These antibodies were successfully used in previous studies and are well characterized by us and other authors in regards to the specific primate retinal cell type immunostaining, as described later for each antibody.

Calbindin. The mouse monoclonal (IgG1) to calbindin (CB) (Sigma-Aldrich, St. Louis, MO, USA) was obtained by using as an immunogen-purified bovine kidney calbindin-D-28K. This antibody recognizes a 28-kDa band on Western blots. Immunostaining against calbindin is known to label cones outside the foveal region, cone bipolar cells, and a subset of horizontal cells on human and monkey retinal sections (Fischer et al., 2001; Chiquet et al., 2002; Kolb et al., 2002; Martínez-Navarrete et al., 2007, 2008).

CHX10. The goat polyclonal (IgG) to CHX10 from Santa Cruz Biotechnology (Santa Cruz, CA, USA) was raised by using as an immunogen, a peptide containing the amino acids 44–61 of

human CHX10 (sequence PPSSHPRAALDGLAPGHL). According to the manufacturer, this antibody gives a single band of 46 kDa on Western blots of mouse eye extracts. This transcription factor targets the nuclei of all bipolar cells in mammals, including monkeys (Martínez-Navarrete et al., 2008).

PKC. The mouse monoclonal (IgG2a) to protein kinase C (PKC) was developed by Santa Cruz Biotechnology (Santa Cruz, CA, USA) by using as an immunogen-purified bovine PKC, and its epitope was mapped to its hinge region (amino acids 296–317). It detects the PKCα isoform, a well-known specific marker for rod bipolar retinal cells (Mills and Massey, 1999). As stated by the manufacturer, this antibody gives a single band of 80 kDa on Western blots of human cell lines, and has been previously used for immunohistochemistry on rodent (Zabouri et al., 2011a) and monkey (Cuenca et al., 2005; Martínez-Navarrete et al., 2008) retinas.

Brn3a The mouse monoclonal to Brn3a was developed by Chemicon International (Temecula, CA, USA) by using as an immunogen amino acids 186–224 of Brn3a fused to the T7 gene 10 protein. We used the POU-domain transcription factor Brn3a to label the nuclei of retinal ganglion cells (RGCs). The Brn3a antibody shows no reactivity to Brn3b or Brn3c by Western blot and no reactivity to Brn3a knockout mice (manufacturer's technical information, MAB1585). Its specificity for monkey (Xiang et al., 1995) and rodent (Nadal-Nicolás et al., 2009) RGCs has been documented.

Syntaxin. The mouse anti-syntaxin monoclonal clone HPC-1 was used to target retinal amacrine and horizontal cells and RGC axons. It was developed by Barnstable et al. (1985) and is produced by Sigma-Aldrich (St. Louis, MO, USA). The syntaxin antibody recognizes syntaxin-1, a 35-kDa protein, from hippocampal, retinal, and cortical neurons (Inoue et al., 1992). This antibody labels interneurons, horizontal and amacrine cells, in the developing and adult human retina (Nag and Wadhwa, 2001). We have successfully used this antibody to label monkey retinal amacrine and horizontal cells. The staining pattern obtained in the current study was similar to that found in human retina (Nag and Wadhwa, 2001).

GS. The mouse anti-glutamine synthetase (GS) monoclonal antibody was obtained from Chemicon International (Temecula, CA, USA) by using as an immunogen, the GS purified from sheep brain. This antibody generates a single 45-kDa protein in adult retinal tissue (Chang et al., 2007). It labels Müller cells in rat retina (Riepe and Norenburg, 1977) and across the monkey retina (Nishikawa and Tamai, 2001).

CB1R. The rabbit anti-CB1R (Sigma-Aldrich, St. Louis, MO, USA) recognizes a major band of 60 kDa and less intense bands of 23, 72, and 180 kDa (manufacturer's data sheet, C1233). This antibody targets the rat CB1R but specifically recognizes the CB1R (60 kDa) from many species, including monkey tissue (technical sheet). This antibody was shown to be specific using retinal tissue from CB1R knockout mouse (Zabouri et al., 2011a).

FAAH. The anti-FAAH was developed by Cayman Chemical (Ann Arbor, MI, USA) by using a synthetic peptide corresponding to 561–579 amino acid fragment of rat fatty acid amine hydrolase conjugated to KLH as an immunogen (manufacturer's data sheet). The rabbit anti-FAAH yields a dense band at about 66 kDa and a very light one below 37 kDa, and its specificity for rat FAAH-positive cells has been demonstrated (Suárez et al., 2008; Zabouri et al., 2011a).

Tissue preparation

The retina was dissected free from the eyecup in a phosphate-buffered saline (PBS) bath. The retina was laid flat so that the vitreous body could be removed by blotting with filter paper and gentle brushing (Burke et al., 2009). Samples of retina (4 mm^2) were taken at 2, 6, and 10 mm from the center of the optic disk in the temporal, nasal, dorsal, and ventral eccentricities along with the fovea. Each sample was then cryoprotected in 30% sucrose overnight and embedded in Shandon embedding media at −65 °C. Retinal samples were then sectioned in a cryostat (16

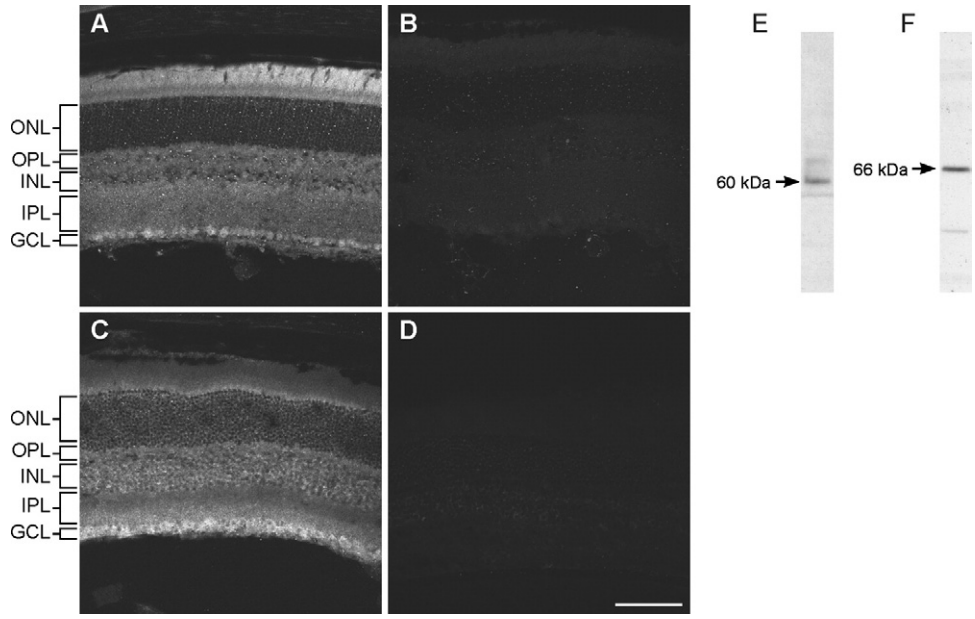


Fig. 1. Single-label immunofluorescence showing the specificity of the antibodies targeting the cannabinoid receptor CB1 (CB1R) and fatty acid amide hydrolase (FAAH). CB1R immunoreactivity in a wild-type mouse retina (A). CB1R labeling is not evident in the *cnr1*$^{-/-}$ mouse (B). FAAH enzyme immunoreactivity in a wild-type mouse retina (C). Lack of FAAH immunofluorescence in the *faah* knockout mouse (D). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar=75 μm. Immunoblots of CB1R-IR and FAAH-IR in the monkey retina (E, F). Specific recognition of CB1R was seen at 60 kDa (E) and of FAAH at 66 kDa (F).

μm) and mounted onto gelatinized glass microscope slides, air dried, and stored at −20 °C for further processing.

Western blotting

A fresh dissected sample of retina was homogenized by hand using a sterile pestle in RIPA buffer (150 mM NaCl, 20 mM Tris, pH 8.0, 1% NP-40 (USB Corporation, Cleveland, OH, USA), 0.1% SDS, 1 mM EDTA), supplemented with a protease inhibitor mixture (aprotinin (1:1000), leupeptin (1:1000), pepstatin (1:1000), and phenylmethylsulfonyl fluoride (0.2 mg/ml; Roche Applied Science, Laval, QC, Canada). Samples were then centrifuged at 4 °C for 10 min, and the supernatant was extracted and stored at −20 °C until further processing. Protein content was equalized using a Thermo Scientific Pierce BCA Protein Assay Kit (Fischer Scientific, Ottawa, ON, Canada). Thirty micrograms of protein/sample of the homogenate was resolved with 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane filter (BioTrace NT, Life Sciences, Pall, Pensacola, FL, USA), blocked for 1 h in 5% skim milk (Carnation, Markham, ON, Canada) in TBST (0.15 M NaCl, 25 mM Tris–HCl, 25 mM Tris, 0.5% Tween-20), and incubated overnight with primary antibodies, namely rabbit anti-CB1R (1:1000) and rabbit anti-FAAH (1:500), in blocking solution. The following day, the blot was exposed to a secondary antibody conjugated to horseradish peroxidase (1:5000; Jackson ImmunoResearch, West Grove, PA, USA) in blocking solution for 2 h. Detection was carried out by using home-made ECL Western blotting detection reagents. The membrane was then stripped, reblocked, and exposed to a second primary antibody until all proteins of interest were tested. Densitometric analysis was performed using Scion Image software (version 4.03) (Frederick, MD, USA).

Immunohistochemistry

Single, double, and triple labelings of the retina were performed according to previously published methods (Zabouri et al., 2011a). Briefly, sections were postfixed for 5 min in 70% ethanol, rinsed 3×5 min in Tris 0.1 M buffer, pH 7.4/Triton 0.03%, and blocked for 90 min in 10% normal goat serum (NDS) in Tris 0.1 M buffer/0.5% Triton. Sections were incubated overnight at room temperature with primary antibody in blocking solution. The CB1R or FAAH antibody was used conjointly with a known retinal cell type marker: calbindin, CHX10, PKCα, syntaxin, Brn3a, or GS (Table 1). The next day, sections were washed for 10 min and 2×5 min in Tris 0.1 M/Triton 0.03%, blocked in 10% NDS, Tris 0.1 M/0.5% Triton for 30 min, and incubated with a secondary antibody for 1 h: Alexa 488 donkey anti-mouse, Alexa 488 donkey anti-goat, Alexa 555 donkey anti-rabbit, or Alexa 647 donkey anti-mouse, (1:200) all in blocking solution as described previously. Sections were washed again in Tris buffer, counterstained with bisbenzimide (Hoechst 33258, Sigma-Aldrich (St. Louis, MO, USA); 2.5 μg/ml), a fluorescent nuclear marker, and coverslipped with GelTol Mounting Medium (Thermo Electron Corporation, Nepean, ON, Canada). To test the specificity of our antibodies directed either against CB1R or FAAH, immunolabelings were performed on mice retinal tissue, one where the *cnr1* gene has been deleted (generously provided by Dr. Beat Lutz) (*cnr1*$^{-/-}$—Marsicano et al., 2002) and the other where the *FAAH* gene has been deleted (generously provided by Dr. Gabriella Gobbi, McGill University) (*faah*$^{-/-}$—Cravatt et al., 2001).

Sequential labeling of CB1R and FAAH

The CB1R and FAAH antibodies that we selected came from the same host, making the use of simultaneous double-labeling

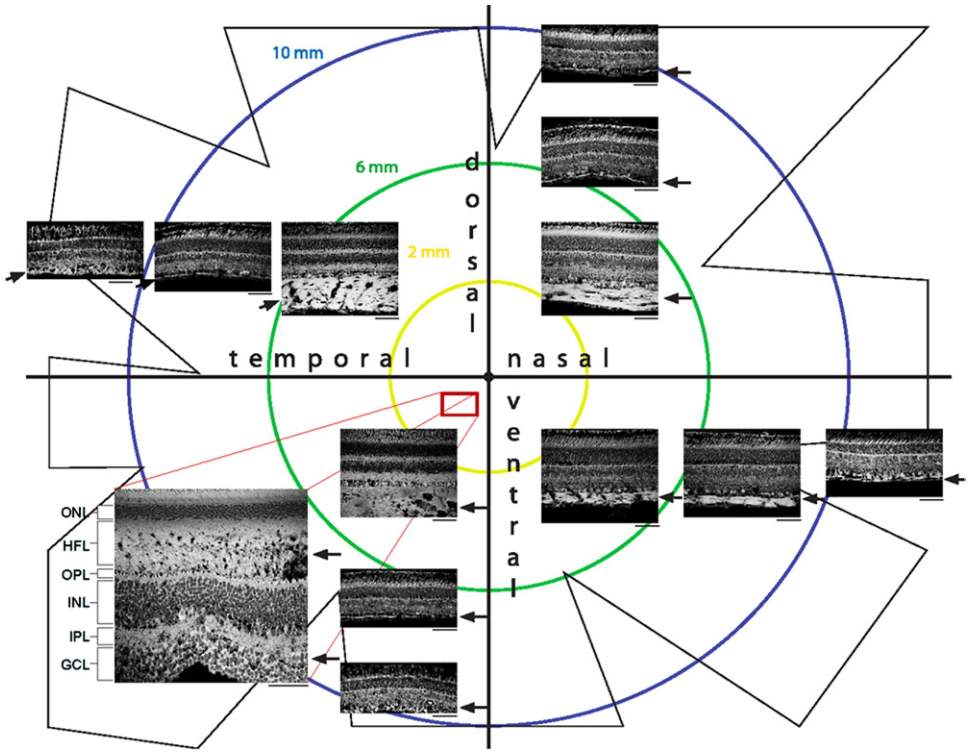


Fig. 2. Schematic illustration of the labeling pattern of CB1R-IR throughout the monkey retina. Note that the most prominent staining of CB1R is located in the nerve fiber layer and central retinal ganglion cell layer (indicated by the arrows). ONL, outer nuclear layer; NFL, nerve fiber layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar=75 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

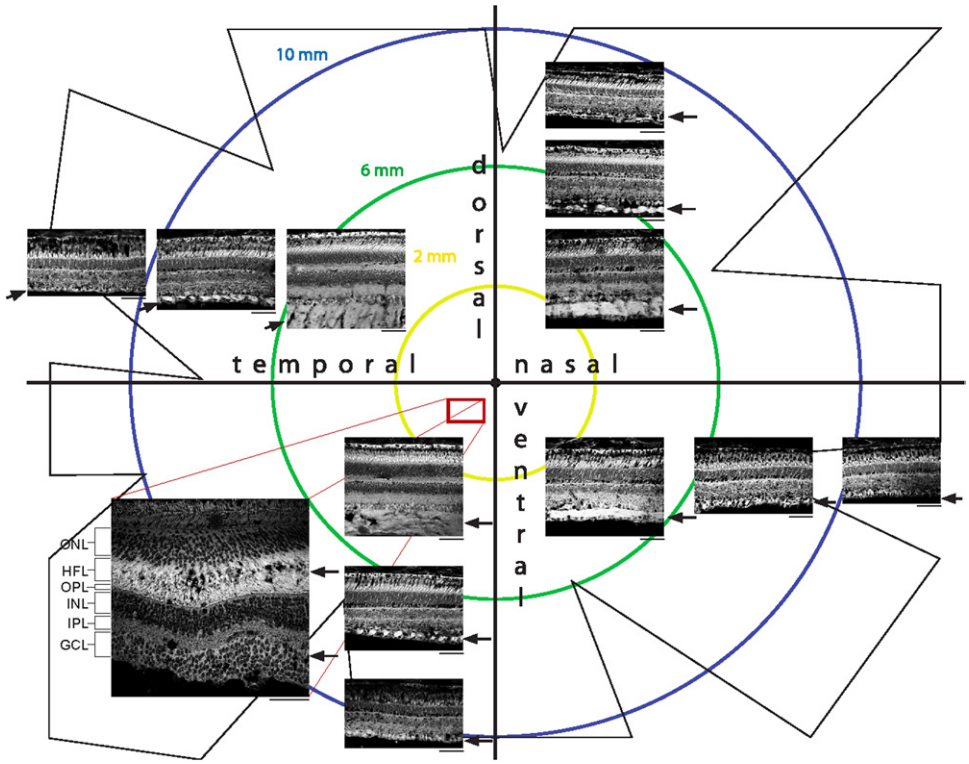


Fig. 3. Schematic illustration of the labeling pattern of FAAH-IR throughout the monkey retina. Note that the most prominent staining of FAAH is located in the nerve fiber layer and retinal ganglion cell layer of the central retina (indicated by the arrows). ONL, outer nuclear layer; NFL, nerve fiber layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar=75 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

protocol not adequate. To circumvent this problem, we used a sequential protocol previously described by our research group (Zabouri et al., 2011a, b). Briefly, the sections were labeled in a serial manner. The exposition to the first primary antibody was conducted as described previously, followed by incubation of a goat anti-Fab fragment solution (Jackson ImmunoRe-

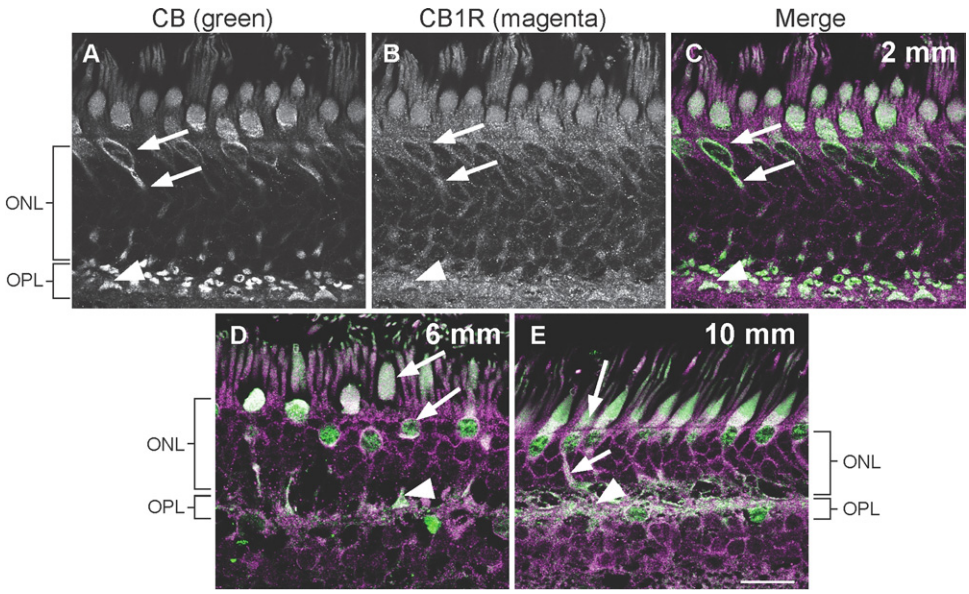


Fig. 4. Double-label immunofluorescence illustrating colocalization of CB1R-IR with calbindin-IR. Confocal micrographs of retinas coimmunolabeled for CB1R (magenta) and calbindin (green), a specific marker for cones in the primate, at different retinal eccentricities (A–C: 2 mm; D: 6 mm; E: 10 mm). Arrows indicate CB1R-positive cones and arrowheads-positive cone pedicles. ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bar=25 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

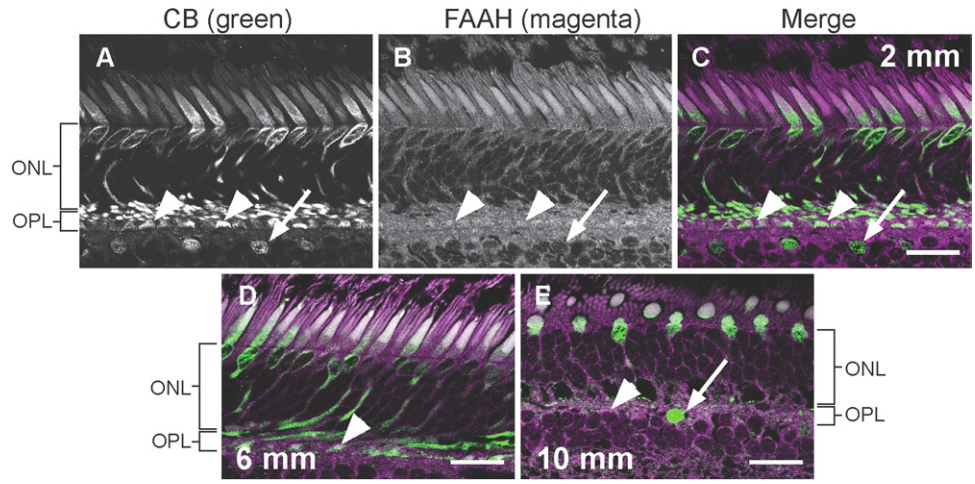


Fig. 5. Double-label immunofluorescence illustrating colocalization of FAAH-IR with calbindin. Confocal micrographs of retinas coimmunolabeled for FAAH (magenta) and calbindin (green), a specific marker for cones in the primate, at different retinal eccentricities (A–C: 2 mm; D: 6 mm; E: 10 mm). Arrowheads indicate FAAH-positive cone pedicles. Arrows point at calbindin-positive horizontal cells that express FAAH. ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bar=18.75 μm for A–D and 25 μm for E. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

search Laboratories; Brandon, 1985). This allowed for the tagging of the first primary antibody as goat rather than rabbit. The sections were revealed with a secondary Alexa donkey anti-goat 488. Thereafter, they were exposed to a second primary antibody overnight and revealed the following day with an Alexa donkey anti-rabbit 647. The validity of the sequential staining was then verified for FAAH/CB1R colabeling with the following two controls: (1) omission of the second primary antibody resulted in a strong staining with the goat secondary 488 but no staining with rabbit secondary 647; (2) omission of the first secondary and second primary antibodies revealed no signal for the goat secondary 488 and faint signal for the rabbit secondary 647.

Confocal microscopy

Fluorescence was detected with a Leica TCS SP2 confocal laser-scanning microscope (Leica Microsystems, Exton, PA, USA), using a 40× or a 100× objective. Images were obtained sequentially from the green, red, and far-red channels on optical slices of less than 0.9 μm of thickness. Throughout the results section, images taken from the green channel correspond to the retinal cell markers, and those from the red channel correspond to the CB1R or the FAAH; for the triple labeling, the far-red channel relates to an additional cell marker.

RESULTS

Single-label immunocytochemistry

Single-label immunohistochemistry was performed to test the specificity of the CB1R or FAAH antibodies in *cnr1* or *faah* knockout mouse retinas, and no staining was found in *cnr1* or *faah* knockout mouse retinas (Fig. 1A–D). Immunoblot analysis of vervet monkey retinal tissue for anti-CB1R and anti-FAAH was very similar to that previously reported for rodent retinas (Yazulla et al., 1999; Zabouri et al., 2011a). For CB1R-immunoreactivty (IR) (Fig. 1E), a single band was detected at 60 kDa, and for FAAH-IR (Fig.

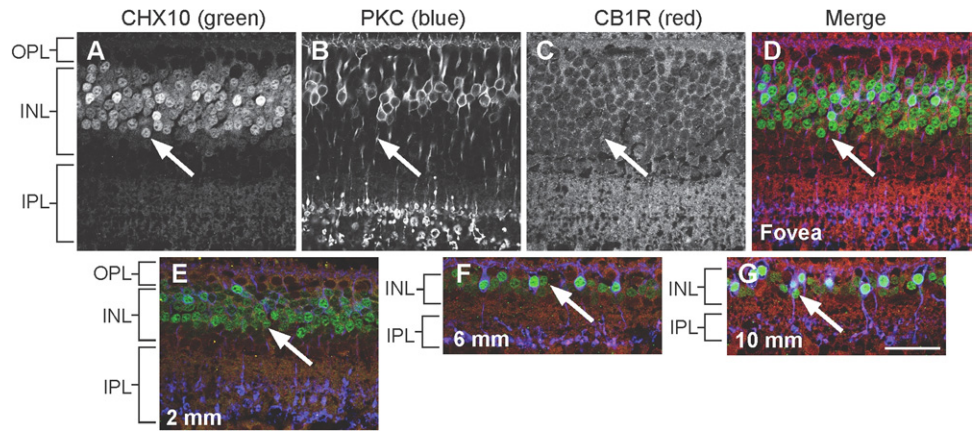


Fig. 6. CB1R is present in all bipolar cells (CHX10 positive), but is preferentially expressed in rod bipolar cells (CHX10 and PKC positive). Confocal micrographs illustrating single or triple labeling in the foveal region (A–D), at 2 mm (E), at 6 mm (F), and at 10 mm (G) of eccentricity. Arrows indicate, in each panel, one of the rod bipolar cells that are CB1R immunoreactive. OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer. Scale bar=25 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

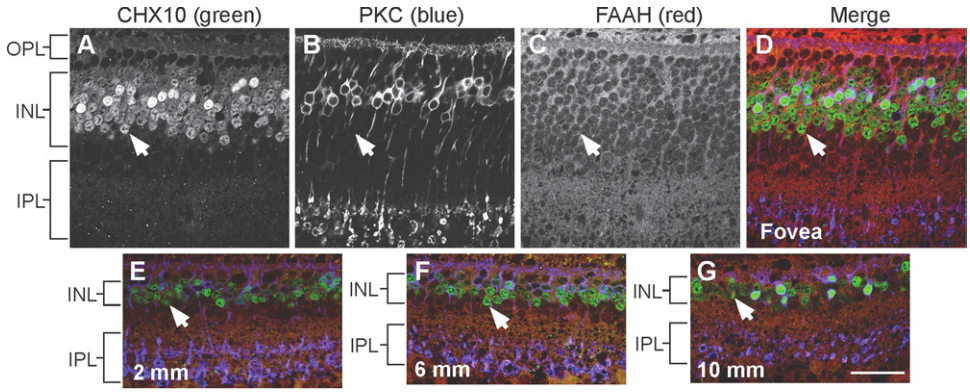


Fig. 7. FAAH is present in all bipolar cells (CHX10 positive). Confocal micrographs illustrating single or triple labeling in the foveal region (A–D), at 2 mm (E), at 6 mm (F), and at 10 mm (G) of eccentricity. Arrows indicate, in each panel, an example of the rod bipolar cells that are FAAH immunoreactive. OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer. Scale bar=25 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

1F), a single band was observed at 66 kDa. Even though the CB1R and FAAH antibodies were targeting rat protein sequences, they generated robust and specific staining in the vervet monkey retina. Control sections in which primary antibodies were omitted were also processed in parallel and did not show any specific IR. CB1R and FAAH were found throughout the retinal layers (photoreceptor layer; outer plexiform layer, OPL; inner nuclear layer, INL; inner plexiform layer, IPL; and ganglion cell layer, GCL) and at all eccentricities studied from the *fovea centralis* to the far periphery. However, the intensity of the IR decreases with retinal eccentricity (Figs. 2 and 3).

Cellular distribution of CB1R and FAAH

To verify the retinal cell type expression, double immunostaining was carried out for CB1R or FAAH and a specific molecular marker for primate retinal cells. A consistent staining pattern across all three monkey retinas was found for each double staining. Although labeling was located in all layers of the retina, from the photoreceptor to the GCLs, CB1R IR was most prominent in the plexiform layers and the retinal GCL within the central retina (Fig. 2). FAAH distribution was similar to the CB1R distribution and densely expressed in the photoreceptor and GCLs (Fig. 3). The CB1R and FAAH distribution profile showed a consistent expression pattern across the retina, as illustrated in the low-magnification (40×) views of immunostained retinal sections shown in Figs. 2 and 3.

CB1R-IR in the photoreceptor layer was found throughout the cones, with positive staining in the membrane and cytosol (Fig. 4). CB1R is present in the outer and inner segments, in the cell body, and in the pedicles. It is preferentially expressed in cones with little evidence of staining in the inner segments and spherules of rods. FAAH, in contrast, was more prominent in the Henle fiber layer (HFL) and cone pedicles (Fig. 5). CB1R and FAAH are expressed in cones both in the central and peripheral retina (Figs. 4 and 5).

The INL comprises bipolar, horizontal, amacrine, and Müller cells. To distinguish the cone and rod bipolar cells from the other cell types, a triple immunolabeling was performed. The antibodies targeting the homeobox transcription factor CHX10 present in all bipolar cells nuclei, and the PKC present in rod bipolar cells and a subset of amacrine cells, were used to identify the cell type localization of the eCB components. Both cone and rod bipolar cells were CB1R and FAAH immunoreactive (Figs. 6 and 7). No differences in staining were observed between central versus peripheral retina.

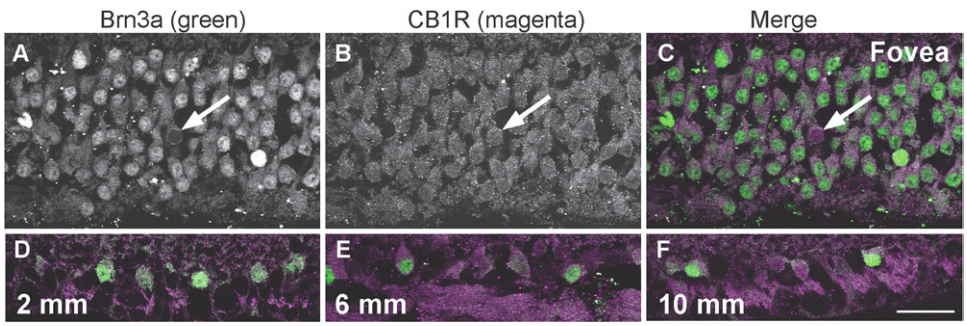


Fig. 8. Double-label immunofluorescence illustrating colocalization of CB1R-IR (magenta) with Brn3a-IR (green) at different eccentricities (A–C: fovea; D: 2 mm; E: 6 mm; F: 10 mm). The antibody against Brn3a labels the nucleus of ganglion cells in the monkey retina, and these cells were also all CB1R immunoreactive. The intense labeling of CB1R in the ganglion cells was localized in the ganglion cells cytosol. Arrows point at non-Brn3a–positive cells that are CB1R immunoreactive. Scale bar=30 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

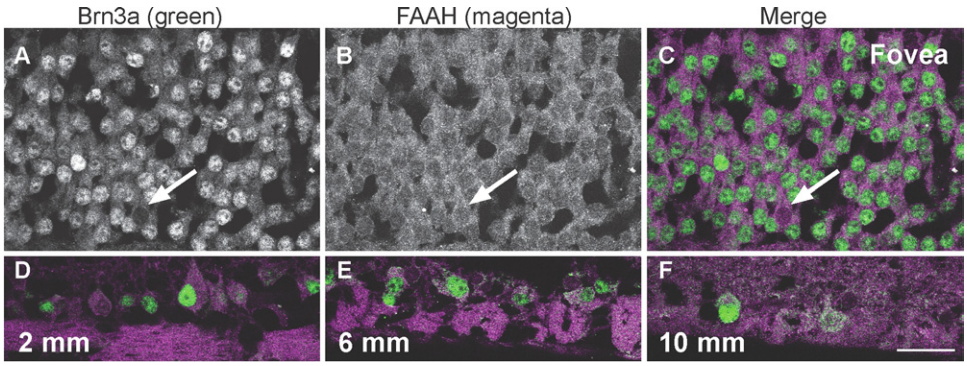


Fig. 9. Double-label immunofluorescence illustrating colocalization of FAAH (magenta) with Brn3a-IR (green) at different eccentricities (A–C: fovea; D: 2 mm; E: 6 mm; F: 10 mm). All Brn3a-positive ganglion cells in the monkey retina were also FAAH immunoreactive. The intense labeling of FAAH in the ganglion cells was localized in the ganglion cells cytosol. Arrows point at non-Brn3a–positive cells that are FAAH immunoreactive. Scale bar=30 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

For targeting the RGC population, we used the Brn3a-IR that specifically labels RGC nuclei. CB1R staining was detected in the GCL, present in the RGC soma (Fig. 8) and axons (Fig. 10). Axon fiber staining was obtained with syntaxin (Wiedenmann and Franke, 1985; Nag and Wadhwa, 2001). Double-labeling Brn3a/CB1R and syntaxin/CB1R indicated that CB1R is expressed throughout the ganglion cells including their axons. This distribution pattern is similar for FAAH (Figs. 9 and 10). Non-Brn3a–positive cells that are CB1R or FAAH immunoreactive were found and are presumably displaced amacrine cells (Fig. 8 and Fig. 9).

The monoclonal antibody HPC-1 that recognizes syntaxin was also used to label the retinal interneurons, horizontal and amacrine cells. Those lateral projecting neurons show little expression of CB1R and FAAH. Their staining in horizontal and amacrine cells was limited to the membrane of the soma as well as the cytosol. Large amacrine cell bodies were slightly more labeled than others. No notable differences were found in relation to eccentricity (Figs. 11 and 12).

To assess if retinal glia express the eCB components, the antibody against GS was used to identify Müller cells throughout the retina. Müller cells did not show any expression of CB1R or FAAH (Figs. 13 and 14, respectively). In all three pairs of retinas, we found the same staining pattern. No differences in the expression of CB1R and FAAH with regard to eccentricity were observed.

Coexpression of FAAH and CB1R in all retinal cells is presented in Fig. 15A–C. There is a large overlap in the expression of these two proteins in the OPL, INL, IPL, GCL, and nerve fiber layer (NFL). Detailed analysis of the expression of both proteins for each cell type is found throughout Figs. 4–14. These data are summarized in Fig. 15J for all retinal cell types. Note that for the most part, CB1R and FAAH expression overlap at different eccentricities and in all neuronal cell types with the exception of the rod outer segments and somas and Müller cells. In the fovea, however, the signal intensity is higher as expected from the cone (Osterberg, 1935) and ganglion cells distributions (Herbin et al., 1997).

DISCUSSION

The present study reports that the distribution of CB1R and FAAH is widespread throughout the vervet monkey retina. These eCB components are present in different retinal cell types, namely cones, bipolar, ganglion, horizontal, and amacrine cells, and are consistent with that found in the rodent retina (Yazulla et al., 1999; Yazulla, 2008, for review; Zabouri et al., 2011a). The cellular expression pattern of CB1R labeling in the vervet monkey retina resembles that found in other vertebrates, particularly rhesus monkeys (Straiker et al., 1999a) and humans (Straiker et al., 1999b). We provide here a comprehensive set of results that further extends the

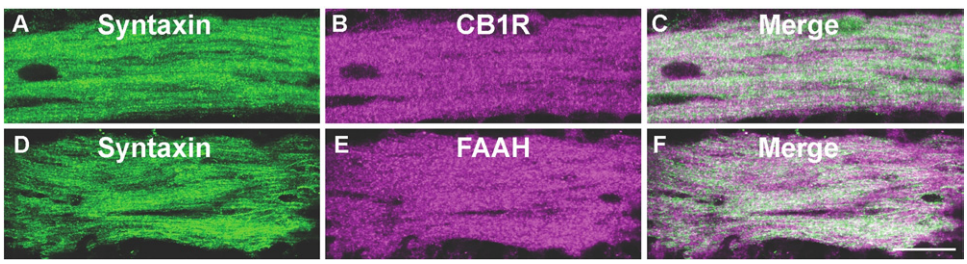


Fig. 10. Double-label immunofluorescence illustrating colocalization of CB1R-IR (A–C) and FAAH-IR (D–F) with syntaxin-IR in a parafoveal region taken at 2 mm of eccentricity where the retinal ganglion cell axons are dense. High magnification confocal micrographs of retinas coimmunolabeled for CB1R or FAAH (magenta) and syntaxin (green), a marker of RGC axons. Scale bar=18.75 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

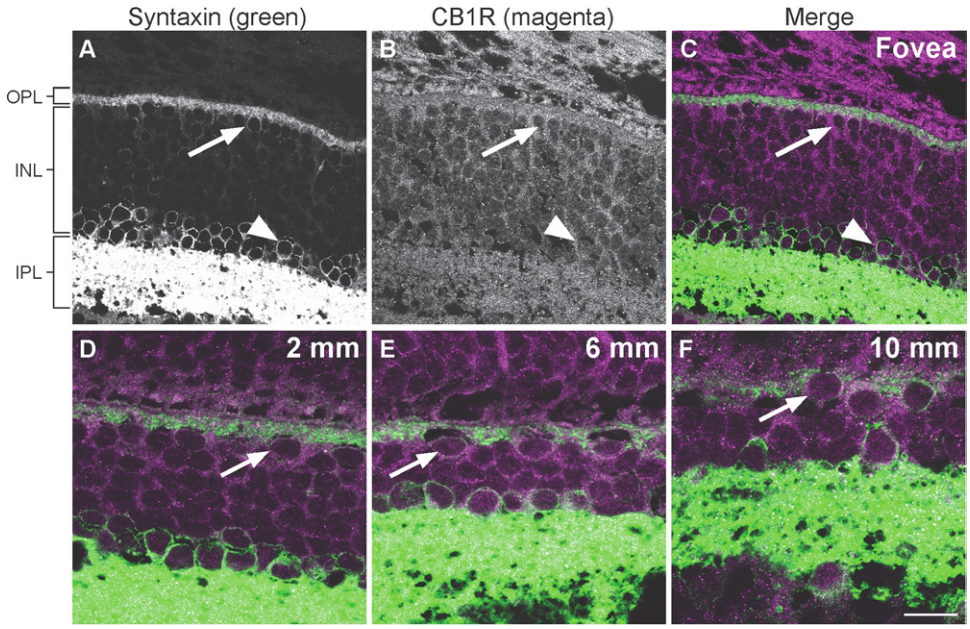


Fig. 11. Double-label immunofluorescence illustrating colocalization of CB1R-IR (magenta) with syntaxin-IR (green) near the *fovea centralis* (fovea) (A–C) and at 2 mm (D), at 6 mm (E), and at 10 mm (F) of eccentricity. Syntaxin-immunoreactive horizontal (arrows) and amacrine cells (arrowheads) were double labeled for CB1R. Syntaxin-IR labeled heavily the membrane of horizontal cells and OPL but lightly their cytosol. Syntaxin-IR labeled heavily the membrane of amacrine cells and IPL but lightly their cytosol. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar=18.75 μm for A–D and 15 μm for E, F. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

data obtained by Straiker et al. (1999a) by showing the retinal specific cell types expressing CB1R at various eccentricities. We also demonstrate the expression and cellular localization of FAAH. Although CB1R is clearly present in cones, their pedicles revealed a more prominent labeling. CB1R-IR was not detected in rod inner segments. Heavy CB1R staining was observed in the cone pedicles, not only in the foveal pit but also throughout the retina. However, FAAH was present throughout the cone with a more intense staining in the inner seg-

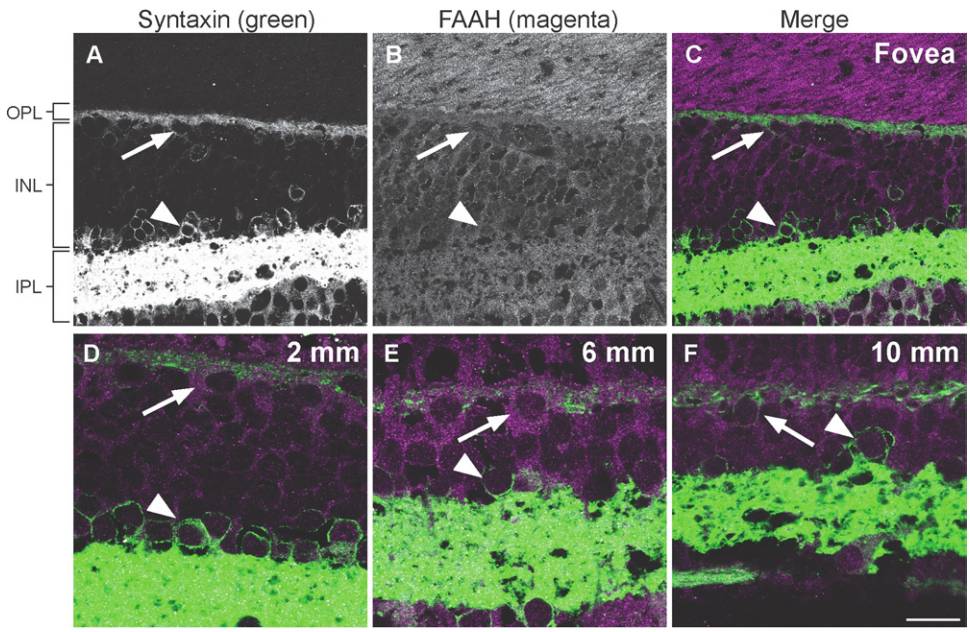


Fig. 12. Double-label immunofluorescence illustrating colocalization of FAAH-IR (magenta) with syntaxin-IR (green) near the *fovea centralis* (fovea) (A–C) and at 2 mm (D), at 6 mm (E), and at 10 mm (F) of eccentricity. Syntaxin-immunoreactive horizontal (arrows) and amacrine cells (arrowheads) were double labeled for FAAH. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar=30 μm for A–C and 15 μm for D–F. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

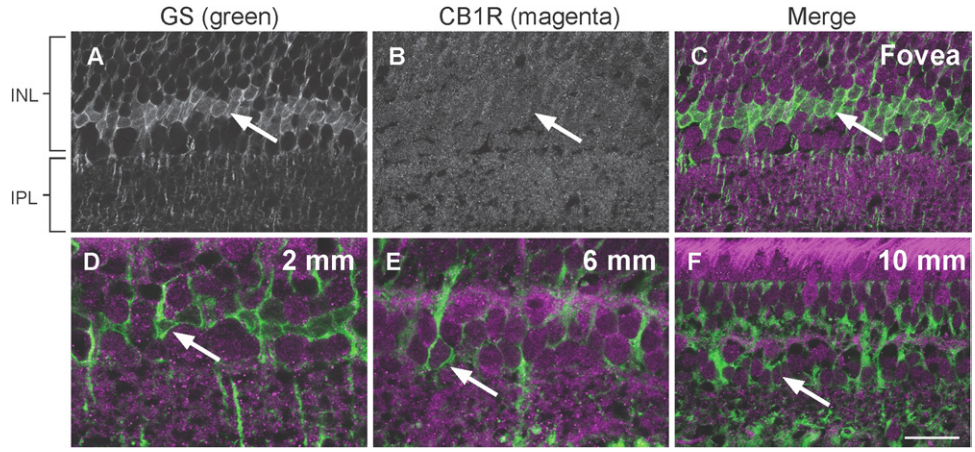


Fig. 13. CB1R immunoreactivity in Müller cells. (A–F) Vertical sections taken near the fovea (A–C) and at 2 mm (D), at 6 mm (E), and at 10 mm (F) of eccentricity. Confocal micrographs of retinas coimmunolabeled for CB1R and a cell type specific marker for Müller cells, glutamine synthetase (GS). Each protein is presented alone in gray scale in the first columns. The merge image is presented in the last column (CB1R in magenta and GS in green). Arrows point at Müller cells that do not express CB1R. INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar=30 μm for A–C, F and 18.75 μm for D, E. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

ments including the pedicles. Globally, the expression of CB1R and FAAH throughout the retina relates to cell density.

Like the photoreceptors, the bipolar cells within the INL (cone and rod bipolar cells) express both CB1R and FAAH. This suggests that eCBs might modulate cone (photopic) and rod (scotopic) vision. Indeed, there is evidence that marijuana use alters color discrimination (Dawson et al., 1977) and increases the glare recovery at low contrast (Adams et al., 1978). Our results are consistent with the suggestion that the vertical cone-bipolar-RGC pathway that prominently expresses CB1R and FAAH plays an important role in glutamate release in each one of the retinal cell types (Wässle, 2004; Yazulla, 2008). However, the low expression of both CB1R and FAAH in horizontal and amacrine cells (the lateral pathway) in this study and in lower mammals (Yazulla et al., 1999) reinforces the pivotal role exerted by the vertical retinal pathway. The presence of the eCB system within the plexiform layers suggests an autoregulatory mechanism in horizontal and amacrine inhibitory neurons. The different expression of the eCB components within the vertical and lateral retinal pathways could result in a modulation of the synaptic gain in the plexiform layers by the inhibition of neurotransmitter release in cone pedicles (Yazulla et al., 1999).

Our understanding of the role of the eCB system in visual processing stems primarily from studies conducted in lower mammals and vertebrates, namely mouse, rat, goldfish, and the tiger salamander (Yazulla et al., 1999; Straiker et al., 1999a; Yazulla, 2008 for review). As such, some retinal circuits have a species-specific function that could be acted upon by the eCB system. For example, the

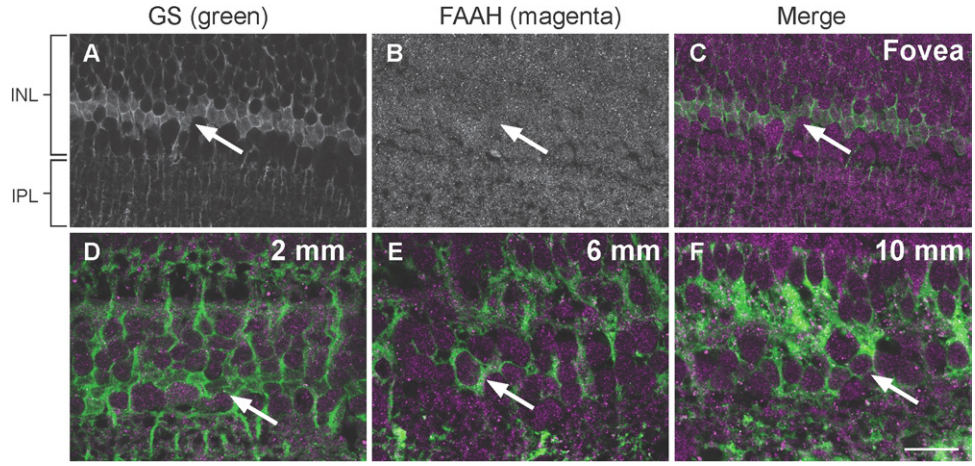
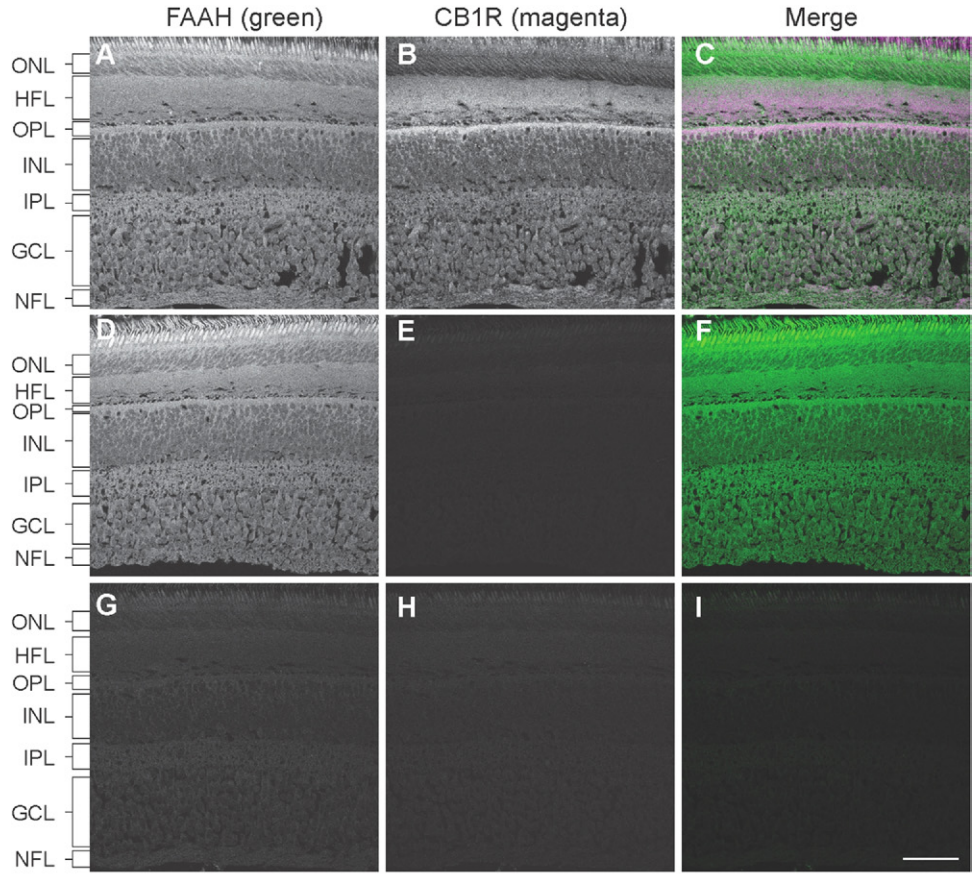


Fig. 14. FAAH immunoreactivity in Müller cells. (A–F) Vertical sections taken near the fovea (A–C) and at 2 mm (D), at 6 mm (E), and at 10 mm (F) of eccentricity. Confocal micrographs of retinas coimmunolabeled for FAAH and GS. Each protein is presented alone in gray scale in the first columns. The merge image is presented in the last column (FAAH in magenta and GS in green). Arrows point at Müller cells that do not express FAAH. INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar=30 μm for A–C and 18.75 μm for D–F. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.



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Cell type \ Retinal location	Fovea	2 mm	6 mm	10 mm
Cone outer segments	+++ / +	+++ / +	++ / +	++ / +
Cone somas	+++ / +++	++ / +++	++ / +++	++ / +++
Cone pedicles	+++ / +++	+++ / +++	+++ / +++	+++ / +++
Rod outer segments	- / -	- / -	- / -	- / -
Rod somas	- / -	- / -	- / -	- / -
Rod spherules	- / -	+ / +	+ / +	+ / +
Horizontal cells	+ / +	+ / +	+ / +	+ / +
Cone bipolar cells	+ / +	+ / +	+ / +	+ / +
Rod bipolar cells	++ / ++	++ / ++	++ / ++	++ / ++
Amacrine cells	+ / +	+ / +	+ / +	+ / +
Ganglion cells	+++ / +++	+++ / +++	+++ / +++	+++ / +++
Müller cells	- / -	- / -	- / -	- / -

Fig. 15. Comparison of CB1R and FAAH expression. Confocal micrographs of retinas coimmunolabeled for CB1R (green) and FAAH (magenta). FAAH (A, D, G) and CB1R (B, E, H) signals, and their overlay (C, F, I) for the complete sequential protocol (A–C) in the monkey central retina. (D–F) The second primary antibody was omitted; (G–I) the first secondary and second primary antibodies were lacking. Scale bar=75 μm. (J) Table summarizing CB1R (green) and FAAH (magenta) distribution at different retinal eccentricities. The staining intensity was scored as − (no signal), + (weak), ++ (medium), +++ (high). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

rod pathway is highly preserved across mammals, whereas trichromatic color processing is found primarily in humans and most old world monkeys (Ptito et al., 1973; Rosenberg and Talebi, 2009). Indeed, in these species, the *fovea centralis,* made exclusively of cones, is responsible for color vision and optimal visual acuity (Osterberg, 1935). This biological adaptation optimizes the visual information available in the environment (Herbin et al., 1997). The eCB signaling system may be restricted in its phylogenetic distribution because to date its existence has only been firmly established in vertebrate species (Elphick and Egertová, 2001).

In the CNS, the activation of CB1R modulates the neuronal membrane permeability to Ca^{2+} and K^{+} ions and the activity of adenylyl cyclase, thereby affecting neurotransmitter release and action (Di Marzo et al., 1998). Electrophysiological research carried out in the mammalian retina to find a specific eCB neuromodulatory action has not been conclusive (Straiker et al., 1999a; Yazulla, 2008 for review). For example, recordings in goldfish cones following the application of WIN 55,212-2 (a CB1R agonist) showed that the photocurrent recovered to baseline more quickly than in controls. It was, therefore, concluded that the functional consequence of this effect was to increase the photosensitivity to bright flashes (Yazulla, 2008). On the contrary, Adams et al. (1978) reported an increase time in the glare recovery in photopic conditions under the influence of marijuana in humans. These two results appear contradictory, but given that the expression of CB1R is more widely expressed throughout the monkey cones than the goldfish ones (Yazulla et al., 2000), it would be plausible that the sites of action of tetrahydrocannabinol (THC, the active compound in marijuana) are broader in primates.

The most prominent CB1R and FAAH expression was found in cone synaptic terminals and in the GCL. This suggests that cannabinoids act not only on photoreceptors as previously reported (Yazulla, 2008 for review) but also directly on ganglion cells. Indeed, at the level of RGCs, CB1R and FAAH are strongly expressed in the cell body and axons. This appears to be unusual because in the mammalian CNS, it has been thought that CB1R is not present in large neurons, like pyramidal and Purkinje cells, but instead in smaller presynaptic neurons (Elphick and Egertová, 2001). However, recent research suggests that the eCB system is also found in large caliber axons (Marsicano and Lutz, 1999; Hill et al., 2007). RGCs do not have large caliber axons, yet they strongly express CB1R and FAAH providing anatomical evidence that eCBs serve an autoregulatory function to modulate the output of ganglion cells as proposed by Yazulla (2008).

Our results also showed that Müller cells in monkeys do not express CB1R or FAAH, which is in agreement with some studies conducted on the retina of other adult mammals (Yazulla, 2008; Zabouri et al., 2011a). CB1R is transiently expressed in rat Müller cells between postnatal day 3 and day 9 (Zabouri et al., 2011a). However, only one study (Yazulla et al., 2000) reported the presence of CB1R in Müller cells of the goldfish retina. The existence of the eCB system within the retinal glia is largely unexplored, and further research is needed to establish its presence across species and its specific functions.

Although the literature on the distribution of the molecular components of the eCB system in the rodent retina has evolved, little is still known about the expression of this signaling system in the retina of species more closely related to humans. In the rodent retina, CB1R has been reported in photoreceptors, bipolar cells, GABAergic amacrine cells, horizontal cells, and the IPL (Yazulla et al., 1999; Hu et al., 2010; Zabouri et al., 2011a). Similarly, in the human retina, the eCB system is expressed in the outer segments of photoreceptors, the IPL and OPL, the INL, and the GCL (Straiker et al., 1999b). This overall pattern of CB1R distribution is also found in the rhesus monkey, indicating that the eCB system is similarly expressed in the retina across species (Yazulla et al., 1999; Hu et al., 2010). Our results support and extend this notion by showing that the eCB system is not only present in the monkey retina but is also more salient in the foveal region compared with the periphery. This different center-periphery distribution of the eCB system suggests an additional role of this system in central retinal functions.

The eCB system has also been observed in the CNS. The patterns of expression of CB1R and FAAH have been assigned to different types of distributions: complementary, overlapping, or unrelated (Egertová et al., 2003; Yazulla, 2008). In the complementary pattern, in brain regions like the cerebellar cortex, hippocampus, and neocortex, FAAH-positive neurons are postsynaptic to processes expressing CB1R. This expression pattern proposes a retrograde presynaptic regulation of transmitter release by eCBs (Tsou et al., 1998; Egertová et al., 1998, 2003). In the overlapping pattern, neurons express both CB1R and FAAH (Marsicano and Lutz, 1999; Hill et al., 2007), with FAAH located in neurons that are proximal to CB1R expressing axon fibers. Here, FAAH may influence eCB signaling but more remotely (Egertová et al., 2003). Finally, in the unrelated pattern, neurons express only one of these two components, suggesting that the spatial impact and/or duration of eCB signaling may be less restricted than in regions enriched with FAAH (Egertová et al., 2003). In the present study, CB1R and FAAH in the retina are generally expressed in an overlapping pattern (Fig. 15), suggesting that the eCB system might be responsible for an autofeedback control of neurotransmitter release. We also show that FAAH is targeted to the axonal and somatodendritic compartments of the retinal ganglions cells; hence, supporting the notion that FAAH is located both presynaptically (at the photoreceptor level) and postsynaptically (at the bipolar and ganglion cell level). This result is consistent with what has been previously shown in the mouse olfactory bulb, where FAAH is also expressed pre- (olfactory-receptor neuron terminals) and postsynaptically (mitral cells) (Egertová et al., 2003). Not surprisingly and in agreement with our previous assumption, eCBs are also expressed in the visual cortex of the developing rodent brain (Jiang et al., 2010), indicating that the whole visual

pathway from retina to cortex is influenced by the eCB system.

CONCLUSION

To our knowledge, this is the first report that CB1R and FAAH have been localized in specific cell types in the old world monkey retina at all eccentricities of tissue sampling. The distribution of the eCB system throughout the retina might explain the deleterious effects of marijuana consumption on visual functions. Because CB1R and FAAH are highly expressed in central cones, the administration of exogenous cannabinoids may alter several retinal functions, such as visual acuity, color discrimination, and photosensitivity.

Acknowledgments—This work was supported in part by the Natural Science and Engineering Research Council of Canada (M.P. and C.C.) and the Canadian Institutes of Health Research (MOP-86495, J.-F.B.). J.B. was supported by a CIHR studentship and J.-F.B. by a Chercheur-Boursier Junior 2 from the Fonds de la recherche en santé du Québec. We thank Sophie Charron, Florence Dotigny and Ikiel Ptito for excellent technical assistance. We are grateful to Dr. Frank Ervin and Dr. Roberta Palmour of the Behavioral Sciences Foundation Laboratories of St Kitts, West Indies, for supplying the eyes and the fresh retinas.

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(Accepted 17 November 2011)
(Available online 28 November 2011)