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Immunocytochemical Localization of Cannabinoid CB1 Receptor and Fatty Acid Amide Hydrolase in Rat Retina

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ABSTRACT

Cannabinoids have major effects on central nervous system function. Recent studies indicate that cannabinoid effects on the visual system have a retinal component. Immunocytochemical methods were used to localize cannabinoid CB1 receptor immunoreactivity (CB1R-IR) and an endocannabinoid (anandamide and 2-arachidonylglycerol) degradative enzyme, fatty acid amide hydrolase (FAAH)-IR, in the rat retina. Double labeling with neuron-specific markers permitted identification of cells that were labeled with CB1R-IR and FAAH-IR. CB1R-IR was observed in all cells that were protein kinase C-immunoreactive (rod bipolar cells and a subtype of GABA-amacrine cell) as well as horizontal cells (identified by calbindin-IR). There was also punctate CB1R-IR in the distal one-third of the inner plexiform layer (IPL) that could not be assigned to a cell type. FAAH-IR was most prominent in large ganglion cells, whose dendrites projected to a narrow band in the proximal IPL. Weaker FAAH-IR was observed in the soma of horizontal cells (identified by calbindin-IR); the soma of large, but not small, dopamine amacrine cells (identified by tyrosine hydroxylase-IR); and dendrites of orthotopic- and displaced-starburst amacrine cells (identified by choline acetyltransferase-IR) but in less than 50% of the starburst amacrine cell somata. The extensive distribution of CB1R-IR on horizontal cells and rod bipolar cells indicates a role of endocannabinoids in scotopic vision, whereas the more widespread distribution of FAAH-IR indicates a complex control of endocannabinoid release and degradation in the retina. J. Comp. Neurol. 415:80–90, 1999. © 1999 Wiley-Liss, Inc.

Indexing terms: dopamine; eicanosoids; FAAH; bipolar cells; endocannabinoid

There is substantial evidence that cannabinoids, the psychoactive components of the marijuana plant, act through two families of inhibitory G protein-coupled receptors, CB1R and CB2R (see Axelrod and Felder, 1998; Howlett, 1998, for reviews). CB1 receptors are distributed primarily in neural tissue (Devane et al., 1988), whereas CB2 receptors are found predominately in immune cells (Munro et al., 1993). The localization of CB1 receptors in the mammalian central nervous system (CNS) has been studied by in vitro autoradiography, in situ hybridization, and immunocytochemistry; all these studies show enrichment of CB1 receptors in the hippocampus, basal ganglia, cerebellum, and pyriform and cerebral cortices (Herkenham et al., 1991; Mailleux and Vanderhaeghen, 1992; Westlake et al., 1994; Pettit et al., 1998; Tsou et al., 1998a). Although there have been numerous studies on the therapeutic use of cannabinoids in the treatment of glaucoma, pain, motor deficits, and chemotherapy-induced nausea (see Voth and Schwartz, 1997, for review), interest in cannabinoid research has increased following the isolation of an endogenous ligand for cannabinoid receptors from porcine brain (Devane et al., 1992). This endogenous ligand, anandamide (arachidonylethanolamide), inhibits

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adenylate cyclase (Vogel et al., 1993) and is hydrolyzed by fatty acid amide hydrolase (FAAH), a membrane-bound enzyme (Hillard et al., 1995), to arachidonic acid and ethanolamine (Deutsch and Chin, 1993; Cravatt et al., 1996). Other endogenous ligands, collectively now referred to as *endocannabinoids* have been described. For example, 2-arachidonoylglycerol (2-AG) is also a substrate for FAAH (see Mechoulam et al., 1998, for review).

Cannabinoids are known to have profound effects on neural function, including dopaminergic (Schlicker et al., 1996; Glass and Felder, 1997; Gessa et al., 1998), GABAergic (Manueuf et al., 1996; Romero et al., 1998; Chan et al., 1998), and glutamatergic mechanisms (Shen et al., 1996). Regarding the eye, in addition to the effects of cannabinoids on intraocular pressure and ocular blood vessels (see, e.g., Green, 1979, 1998, for review), recent studies indicate the presence of cannabinoid function in the neural retina. For example, CB1-receptor agonists stimulate dopamine release from the guinea pig retina (Schlicker et al., 1996). CB1-receptor expression has been detected in the rat retina by in situ hybridization and RT-PCR (Buckley et al., 1998; Porcella et al., 1998). Also, hydrolysis of anandamide, measured in porcine ocular tissues and brain, occurs at twice the rate in the retina as in the brain (Matsuda et al., 1997). Furthermore, there are reports that cannabinoids may increase photosensitivity (Dawson et al., 1977; Reese, 1991; West, 1991; Consroe et al., 1997), which in view of recent data may have a retinal component.

The availability of specific antisera against CB1 receptors and FAAH has facilitated investigation of the distribution of cannabinergic transmission in the mammalian CNS (McIntosh et al., 1998; Patricelli et al., 1998). Here, these antisera have been used to study, in detail, the cellular localization of cannabinergic transmission in the rat retina.

MATERIALS AND METHODS

Subjects

Albino rats (Sprague-Dawley) were obtained from commercial suppliers and maintained at 22°C on a 12/12-hour light/dark cycle. Animals were treated according to the guidelines of the National Institutes of Health and the Association for Research in Vision and Ophthalmology. The animals were adults (over 3 months old) at the time of experimentation.

Antibodies

Rabbit polyclonal antibodies were generated against amino acids 1–14 of the rat CB1 receptor (CB1R). Details of the characterization of this antiserum by ELISAs and immunoblots in rat and human CNS can be found in McIntosh et al. (1998). Affinity-purified rabbit polyclonal antibodies against FAAH were raised against an FAAH-glutathione-S-transferase (GST) fusion protein (including amino acids 38–579) of the rat FAAH protein. The reactive affinity chromatography-purified FAAH-GST antibody (provided by Dr. B. Cravatt) was isolated from rabbit serum, after being depleted of GST cross-reactive antibodies. Details of the characterization can be found in Patricelli et al. (1998); characterization by immunoblot analysis in the rat CNS has appeared in Egertova et al. (1998). Mouse monoclonal antibodies against bovine-adrenal tyrosine hydroxylase (TOH) and rat polyclonal anticholine acetyltransferase (ChAT) were obtained from Boerhinger Mannheim (Indianapolis, IN). Mouse monoclonal antibodies against the α/β subunits of protein kinase C (PKC; clone MC5) were obtained from Amersham (Arlington Heights, IL). Mouse monoclonal antibodies against calbindin D (28 kD, isolated from chicken gut) were obtained from Sigma Chemical (St. Louis, MO). Goat anti-mouse fluorescein isothiocyanate (FITC) was obtained from Sigma Chemical; donkey anti-guinea pig FITC, donkey anti-rabbit carboxymethylindocyanine 3 (Cy3), and goat anti-rat FITC were obtained from Jackson Immunoresearch Laboratories (West Grove, PA).

Preparation

Rats were anesthetized with carbon dioxide and decapitated. Eyes were removed, the cornea and lens were cut off, and eyecups were fixed and processed for immunocytochemistry. The degree of fixation was reduced to preserve antigenicity of CB1R and FAAH. Eyecups were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4, 0.15 mM $CaCl_2$) at 4°C for 30–60 minutes. Longer fixation drastically reduced CB1R immunoreaction but had less effect on FAAH immunoreaction. Tissue was washed 3 × 15 minutes in PB (with 5% glucose, pH 7.4, 0.15 mM $CaCl_2$, 0.02% Na-azide) and cryoprotected in 30% sucrose, 0.1 M PB (pH 7.4) at 4°C overnight. Eyecups were embedded in Tissue Tek and quickly frozen in liquid nitrogen-cooled isopentane. Cryosections (12–14-μm-thick) were placed on slides that permitted electrostatic adherence without coating (Superfrost/Plus; Fisher Scientific, Pittsburgh, PA) or gelatin/chromium-coated slides, air dried, and stored at −20°C.

Gel electrophoresis and immunoblot analysis

Rats were anesthetized with carbon dioxide and decapitated. Isolated retinas and forebrain were removed and placed into ice-cold extraction buffer (10 mM Tris, pH 7.4, 0.3 mM sucrose, 1.0 mM ethylenedinitrilo tetraacetic acid [EDTA], 0.5 mM dithithreitol [DTT; Cleland's reagent], 1.0 mM benzamidine, 0.3 mM phenylmethysulfonyl flouride [PMSF], and 10 μg/ml each of trypsin inhibitors I and II). Samples were homogenized with a Teflon size A pestle and centrifuged at 200*g* for 10 minutes at 4°C. The supernatant was saved and the pellet was rehomogenized and spun again as before. The combined supernatants were spun at 48,000*g* for 30 minutes at 4°C. The pellet was resuspended in ice-cold 50 mM Tris buffer (pH 7.4) and stored at −70°C until use. The protein content of the tissue extracts was assessed using a BioRad Protein Assay Kit (BioRad, Hercules, CA).

Electrophoretic conditions were antibody-specific. For detection of FAAH immunoreactivity (IR), tissue extracts were diluted 1:1 in a denaturing buffer, final concentration (10 mM Tris HCl, pH 8.0, 4% sodium dodecyl sulfate [SDS], 5% EDTA, 5% β-mercaptoethanol, 20% glycerol). The diluted samples were boiled for 4 minutes, cooled, then spun in a microfuge at 14,600*g* for 10 minutes. For detection of CB1R-IR, tissue extracts were centrifuged at 48,000*g* for 30 minutes and the pellets were solubilized in a urea sample buffer (40 mM Tris HCl, pH 6.8, 5% SDS, 0.1 mM EDTA, 1% β-mercaptoethanol, 8 M urea; Blumer et al., 1988) The samples were then heated at 37°C for 10 minutes, cooled, and centrifuged in a microfuge at 19,000*g* for 10 minutes. Samples, 10–160 μg protein/lane, were subjected to SDS-PAGE. Samples were run through a 3%

polyacrylamide stacking gel at 10 mA for 1 hour and through a 10% polyacrylamide running gel at 15 mA for 3.75–4.0 hours. High-molecular-weight standards (Gibco BRL, Grand Island, NY) were run in adjacent lanes. Gels were electroblotted onto nitrocellulose (NC) overnight at 13 V for FAAH-IR detection. Polyvinylidene difluoride (PVDF; BioRad, Hercules, CA) membranes were used for detection of CB1R-IR because of the higher protein binding capacity. Transfers to PVDF were overnight at 20 V followed by 30 minutes at 30 V.

The membranes were processed for detection by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). After transfer, the membranes were placed in plastic trays and quickly rinsed in PBS and then in Blotto (0.1 M PBS, pH 7.4, 5% dry milk, 0.1% Tween-20) for 1 hour at room temperature on a shaker table. Normal goat serum (5%) was included in the Blotto when immunostaining for CB1R. Membranes were exposed to primary antibodies (anti-FAAH, 1:10,000; anti-CB1R, 1:1,000) or preabsorbed primary antibody for 1 hour at room temperature and secondary antibodies, goat anti-rabbit conjugated-horseradish peroxidase (HRP; Jackson Laboratories, Bar Harbor, ME), for 30 mnutes (FAAH) or 60 minutes (CB1R). All washes were with TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween-20). ECL substrate conversion was detected on Hyerperfilm-ECL (Amersham) with exposure times of 1 minute to greater than 1 hour.

Immunohistochemistry

A standard procedure was followed for conventional immunocytochemistry (see, e.g., Yazulla and Studholme, 1998). Sections were washed 3 × 10 minutes in PBS, pH 7.4, postfixed for 5 minutes (fixative as described above), rinsed 3 × 5 minutes, treated with 0.1% sodium borohydride (in PBS) for 1–2 minutes, rinsed 5 × 5 minutes (0.1 M PBS), and blocked in 5% normal goat serum albumin (NGS) in 0.1 M PBS/0.3 % Triton X-100 for 20 minutes. Sections were incubated overnight in either CB1R antiserum (1:400–1:800) or FAAH-antisera alone (1:250) or each of these antisera and antisera against either TOH (1:800), PKC (1:200), calbindin (1:500), or ChAT (1:100). After washing in PBS for 30 minutes, tissue was blocked again and incubated with secondary antisera: donkey anti-rabbit-Cy3 (1:1,000, for CB1R and FAAH), and goat anti-mouse FITC (1:150) or goat anti-rat FITC (1:100) for 35 minutes at 37°C. Following a 30-minute wash in PBS, slides were coverslipped with Vectashield (Vector Laboratories, Burlingham, CA) and stored at −20°C until they were viewed with an Olympus BH2 epifluorescence microscope. Sections were observed with filter sets that were optimized for FITC and Cy3 viewing. An additional FITC narrow-bandpass filter (D535; Chroma Technology Corp., Brattleboro, VT) was inserted when viewing FITC to ensure further that there was no crossover from the Cy3. Sections, labeled for either FITC or Cy3 alone, showed no evidence of crossover fluorescence when viewed with the alternate filter set.

RESULTS

Immunoblots

Immunoblot analysis of rat retina for anti-CB1R and anti-FAAH was very similar to that previously reported for brain, as would be expected insofar as the retina is part of the CNS. For CB1R-IR (Fig. 1), three bands were noted:

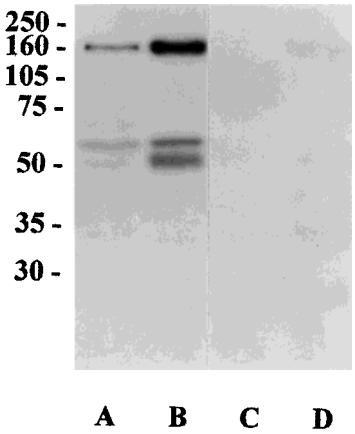

Fig. 1. Immunoblots of CB1 cannabinoid receptor immunoreactivity (CB1R-IR) in rat retina and rat brain. Proteins were solubilized in urea/SDS sample buffer, separated on a 10% polyacrylamide gel, transferred to PVDF, and immunostained for detection by ECL. Recognition by anti-CB1R(1–14) is demonstrated in **lane A:** 160 µg rat retinal membrane protein and **lane B:** 40 µg rat brain membrane protein. Anti-CB1R(1–14), preadsorbed by the antigenic peptide CB1R(1–14) failed to recognize the cognate protein, as shown in **lane C:** 160 µg rat retinal membrane protein and **lane D:** 80 µg rat brain membrane protein. Specific CB1R recognition was seen on a 53 kDa band (which is consistent with the unmodified receptor monomer), a 62 kDa band (which is consistent with the glycosylated monomer [Song and Howlett, 1995]), and a 160 kDa band (which is consistent with a disruption-resistant homotrimer or other unknown disruption-resistant protein complex containing the CB1R).

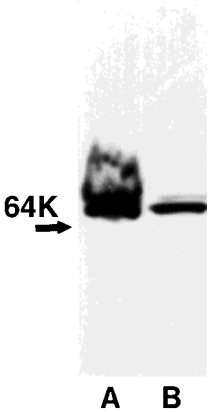

Fig. 2. Immunoblots of fatty acid amide hydroxylase (FAAH)-IR in rat retina and rat brain. Proteins were solubilized in an SDS sample buffer, separated on a 10% polyacrylamide gel, transferred to PVDF, and immunostained for detection by ECL. Recognition by anti-FAAH is demonstrated in **lane A:** 35 µg protein of rat brain membrane protein and **lane B:** 50 µg rat retinal membrane protein at 66 kDa.

160 kDa was the most intense, followed by a moderate band at 62 kDa and a band at 53 kDa that was of moderate density in the brain and very weak in the retina. All CB1R-immunoreactive bands were abolished after preabsorption with the peptide antigen. For FAAH-IR (Fig. 2), there was a single dense band in the retina at 66 kDa.

Single-label immunocytochemistry

CB1R-IR was present throughout the retina (Fig. 3) extending from the inner segments of the photoreceptors

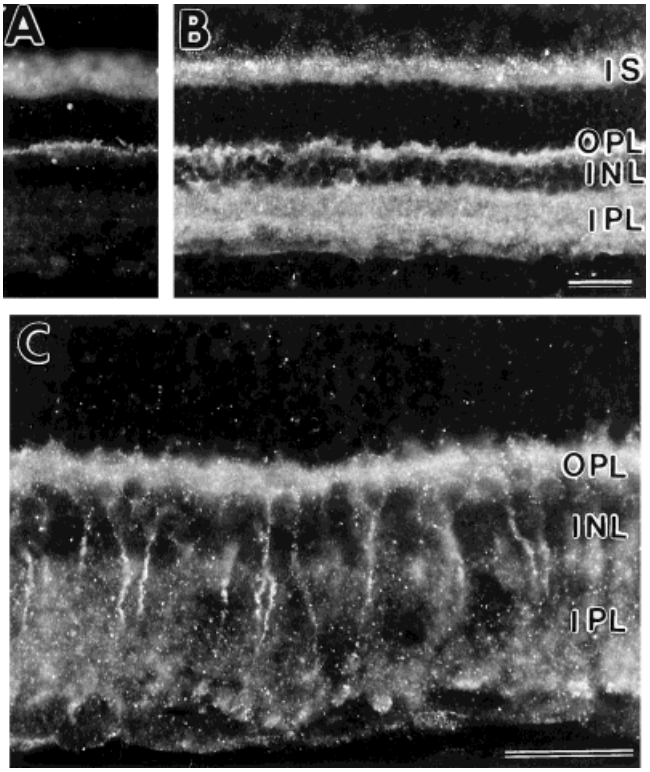

Fig. 3. Localization of CB1R-IR in the rat retina. At low magnification (**B**), CB1R-IR was observed over the photoreceptor inner segments (IS) and the outer plexiform layer (OPL), scattered cell bodies in the inner nuclear layer (INL), and two broad bands in the inner plexiform layer (IPL). At higher magnification (**C**), punctate CB1R-IR was observed over the OPL and cell bodies in the distal INL that gave rise to axons that entered the IPL. Clusters of CB1R-IR were observed in the most proximal IPL. **A:** Preabsorption of the primary antibody with peptide antigen abolished CB1R-IR throughout most of the retina, with the exception of moderate label over the inner segments and OPL, indicating that much of the labeling in these two regions was nonspecific. Scale bars = 25 μm.

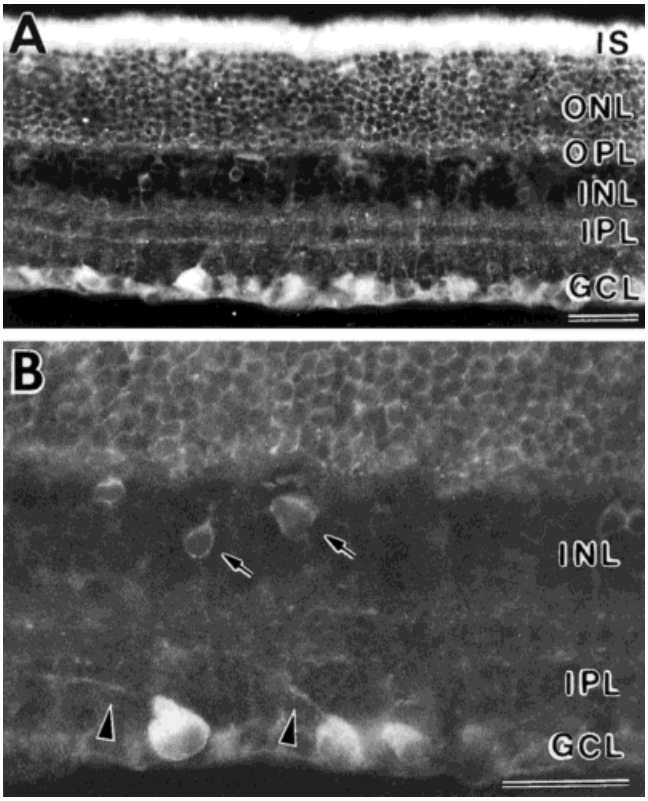

Fig. 4. Localization of FAAH-IR in the rat retina. At low magnification (**A**), FAAH-IR was observed over the photoreceptor inner segments (IS) and the outer nuclear layer (ONL), weakly in the outer plexiform layer (OPL), scattered cell bodies in the inner nuclear layer (INL [B], arrows), two narrow bands in the inner plexiform layer (IPL), and intensely in cells in the ganglion cell layer (GCL). At higher magnification (**B**), FAAH-immunoreactive processes from cells in the GCL entered the IPL, where they appeared to end in the more proximal of the two FAAH-IR laminae (arrowheads). Scale bars = 25 μm.

to the ganglion cell layer (GCL). CB1R-IR (Fig. 3B) was densest over the inner segments, the outer plexiform layer (OPL) and the inner plexiform layer (IPL), in which two laminae were visible, one at the distal margin of the IPL and the other occupying the proximal one-third of the IPL. The outer nuclear layer (ONL) was not labeled, and somatic labeling, including occasional large amacrine cells, was visible within the inner nuclear layer (INL). At higher magnification (Fig. 3C), CB1R-IR appeared as punctate label over the OPL and IPL and over cell bodies in the distal INL, which gave rise to axonal processes that entered the IPL. Cell bodies in the GCL were not CB1R-immunoreactive. After preabsorption of the primary antisera with peptide antigen, there was residual labeling over the inner segments and to a lesser extent over the OPL (Fig. 3A), indicating that labeling over the inner retina was due specifically to CB1R-IR.

FAAH-IR (Fig. 4A) was most intense over the inner segments and the ONL and over the cell bodies in the GCL. In addition, there were FAAH-immunoreactive cell bodies scattered throughout the INL and two distinct FAAH-immunoreactive laminae in the middle of the IPL. Higher magnification (Fig. 4B) clearly shows labeled cell bodies in the ONL and INL, which had a distinct ring-like appearance owing to the restriction of FAAH-IR to the plasma membrane. Cell bodies in the GCL had more extensive somatic labeling and often were observed to extend a large-caliber dendrite to the proximal FAAH-immunoreactive lamina in the IPL. Labeling at the inner limiting membrane, surrounding cell bodies in the ONL, and at the inner segments suggests that Müller cells were FAAH-immunoreactive. For the most part, radial streaks of FAAH-IR were not prominent in the IPL (but see Fig. 9A), which would be expected if all parts of the Müller cell were labeled.

Double-label immunocytochemistry

The numerous CB1R-immunoreactive cell bodies along the border of the distal INL were suggestive of rod bipolar cells. This notion was tested by double labeling CB1R-IR with PKC-IR, because PKC-IR labels rod bipolar cells and a subset of amacrine cells in the rat retina (Negishi et al., 1988; Greferath et al., 1990). Every cell that was PKC-immunoreactive also was double labeled for CB1R-IR (Fig. 5). This included the bipolar cell bodies and their axons and axon terminals. Occasional PKC-immunoreactive amacrine cells also were CB1R-immunoreactive, although this

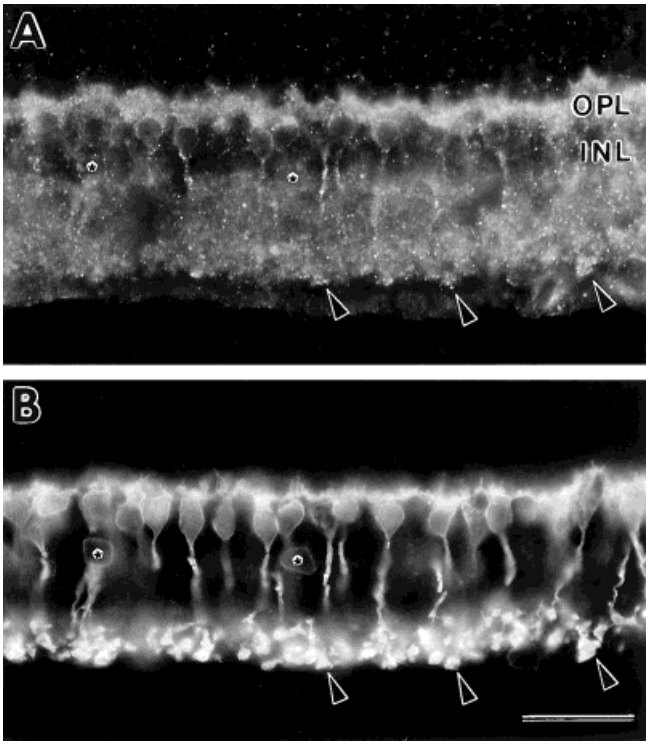

Fig. 5. Double-label immunofluorescence illustrating colocalization of CB1R-IR (**A**; FITC) with PKC-IR (**B**; Cy-3). Note that PKC-IR was uniform in appearance, whereas CB1R-IR was punctate. All PKC-immunoreactive cells were CB1R-immunoreactive. These were largely rod bipolar cells that were CB1R-immunoreactive on their processes in the OPL, cell bodies in the distal INL, axons, and axon terminals (arrowheads). PKC-immunoreactive amacrine cells were characterized by a fine halo of punctate CB1R-IR (asterisks). Scale bar = 25 μm.

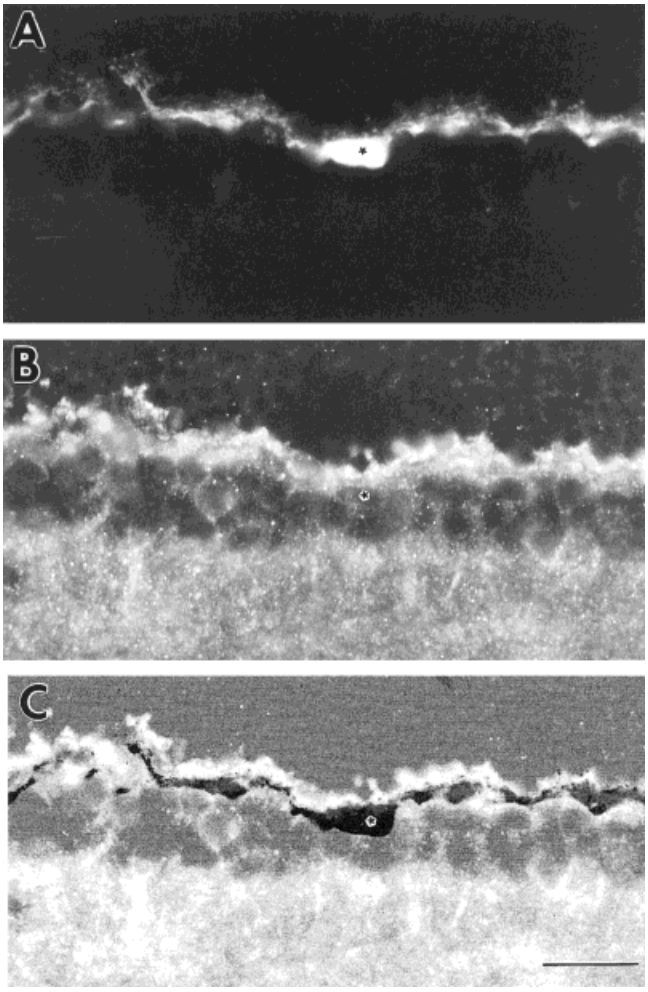

Fig. 6. Double-label immunofluorescence illustrating colocalization of CB1R-IR (**A**; FITC) with calbindin-IR (**B**; Cy-3). Calbindin-IR labeled horizontal cells in the rat retina, and these were CB1R-immunoreactive (asterisks). Note that CB1R-IR appeared weakly as a ring of puncta that outlined the calbindin-immunoreactive cell bodies. Note that CB1R-IR was not present on the calbindin-immunoreactive dendrites of the horizontal cells (**C**), as illustrated in the overlay of the two micrographs (A,B). This overlay clearly shows that the CB1R-immunoreactive dendrites (light) were adjacent to, but distal to, the calbindin-immunoreactive dendrites (dark) of the horizontal cells. Scale bar = 25 μm.

is difficult to see in Figure 5 because the finer punctate CB1R-IR is slightly out of the focal plane. Occasionally, there were CB1R-immunoreactive cell bodies in the distal INL that were not PKC-immunoreactive. Because these were in the position of horizontal cells, the tissue was double labeled with CB1R-IR and calbindin-IR, a calcium binding protein that specifically labels horizontal cells in the rat retina (Pasteels et al., 1990; Peichl and González-Soriano, 1994). As is indicated in Figure 6, calbindin-immunoreactive horizontal cells had a thin ring of CB1R-IR, apparently in the plasma membrane. However, the dendrites of the calbindin-immunoreactive horizontal cells were not CB1R-immunoreactive. Although the patterns in the OPL appeared very similar, it is clear from an overlay projection illustrated in Figure 6C that CB1R-IR was adjacent to but distal to the calbindin-immunoreactive horizontal cell dendrites. Thus, it appears that CB1R-IR in the OPL was due exclusively to dendrites of the rod bipolar cells and did not include horizontal cell dendrites.

A greater variety of cells in the inner retina appeared to be FAAH-immunoreactive. The first possibility was that the FAAH-immunoreactive cells in the most distal INL were horizontal cells. This was verified in that all calbindin-immunoreactive horizontal cells were also FAAH-immunoreactive (Fig. 7). Other FAAH-immunoreactive cell bodies in the distal half of the INL did not colocalize with calbindin-IR and were thus likely to be bipolar cells. However, FAAH-IR did not colocalize with PKC-IR (Fig. 8) indicating that rod bipolar cells were not FAAH-immunoreactive. Thus, CB1R-immunoreactive bipolar cells and FAAH-immunoreactive bipolar cells were mutually exclusive populations.

Occasionally, large amacrine cell bodies were FAAH-immunoreactive (Fig. 4A). Their size and low frequency suggested that they might correspond to the large dopamine amacrine cells (Nguyen-Legros et al., 1983). This proved to be the case in that all of the large TOH-immunoreactive amacrine cells were also FAAH-immunoreactive (Fig. 9A,B). Notice that FAAH-IR was restricted to the soma; there was no appearance of FAAH-IR in the large TOH-immunoreactive dendrites, even those just emerging from the soma. A second population of small, lightly labeled, more numerous TOH-immunoreactive ama-

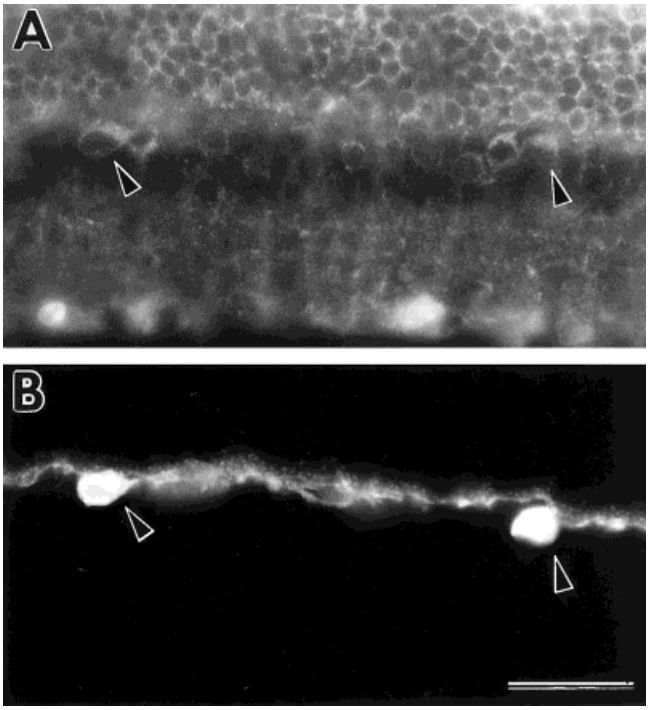

Fig. 7. Double-label immunofluorescence illustrating colocalization of FAAH-IR (**A**; FITC) with calbindin-IR (**B**; Cy-3). Calbindin-IR labeled horizontal cells in the rat retina, and these were FAAH-immunoreactive (arrowheads). Note that FAAH-IR was more prominent over the calbindin-immunoreactive cell bodies and was not apparent over the processes of the calbindin-immunoreactive horizontal cells. Scale bar = 25 μm.

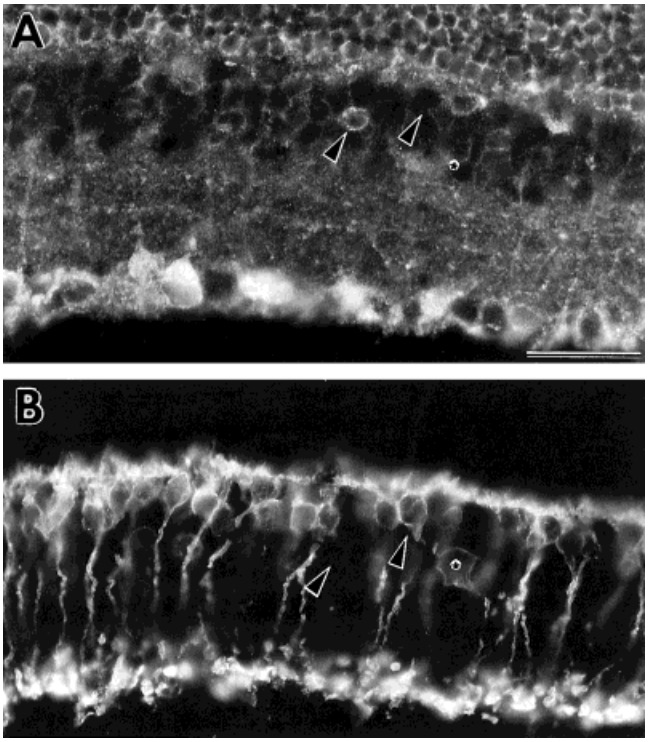

Fig. 8. Double-label immunofluorescence of FAAH-IR (**A**; FITC) and PKC-IR (**B**; Cy-3). FAAH-IR did not colocalize with either PKC-immunoreactive bipolar cells bodies (arrowheads) or amacrine cell bodies (asterisks). Scale bar = 25 μm.

crine cells also has been described in the albino rat (Nguyen-Legros et al., 1983); these were not FAAH-immunoreactive (Fig. 9C,D).

The two narrow FAAH-immunoreactive strata in the middle of the IPL immediately suggested the dendritic patterns of the orthotopic- and displaced-cholinergic starburst amacrine cells (Puro et al., 1982; Kondo et al., 1985; Voigt, 1986). Double labeling with FAAH-IR and ChAT-IR (Fig. 10) showed what appeared to be an exact correspondence between the two narrow strata in the IPL with each label. However, fewer than one-half of the ChAT-immunoreactive cell bodies were FAAH-immunoreactive. This discrepancy in somatic vs. dendritic labeling was observed for both the orthotopic and the displaced ChAT-immunoreactive cell bodies.

The mutually exclusive distributions of CB1R-IR and FAAH-IR in "dopamine" cells raised a problem. Negishi et al. (1988) reported that PKC-IR and TOH-IR were colocalized in large amacrine cells in a wide variety of species, including rat retina. We observed that CB1R-IR colocalized with PKC-immunoreactive amacrine cells, whereas FAAH-IR did not. Furthermore, FAAH-IR colocalized with the large TOH-immunoreactive amacrine cells, whereas CB1R-IR did not. These results contradict the report of Negishi et al. (1988), which predicts colocalization between FAAH-IR and PKC-IR in the large (i.e., TOH-immunoreactive) amacrine cells. We repeated the experiment of Negishi et al. (1988) and found that neither the large (Fig. 11) nor the small (not shown) TOH-immunoreactive amacrine cells in rat retina colocalized with PKC-IR. Furthermore, the PKC-immunoreactive amacrine cells were not TOH-immunoreactive either. Therefore, we did not replicate the work of Negishi et al. (1988), and, in our work, PKC-IR and TOH-IR labeled mutually exclusive populations of amacrine cells.

DISCUSSION

We have shown that there is a widespread distribution of cannabinoid CB1 receptor and the degradative enzyme FAAH in the rat retina. The distributions, however, with the exception of horizontal cells, are not overlapping but rather appear to be complimentary, as is illustrated in the schematic summary (Fig. 12). These findings extend the work of others, who have demonstrated by immunocytochemical techniques the presence of CB1R and FAAH in other regions of the rat CNS (Egertova et al., 1998; Pettit et al., 1998; Tsou et al., 1998a,b; Katona et al., 1999).

As would be expected, immunoblots of rat retina revealed patterns similar to those in other areas of the rat brain in which the antisera against CB1R and FAAH were characterized initially. The dense FAAH band at 66 kDa in the retina is comparable to the value of 63 kDa (Egertova et al., 1998) reported in various regions of the rat brain with this anti-FAAH. Regarding CB1R, all reports agree on a major band at about 64 kDa in rat brain (Pettit et al., 1998; McIntosh et al., 1998; Tsou et al., 1998a), comparable to the 62 kDa band reported here in the retina, that is consistent with the glycosylated monomer (Song and Howlett, 1995). In addition, multiple bands have been reported at 83 kDa, 123 kDa, and 180 kDa, explained as

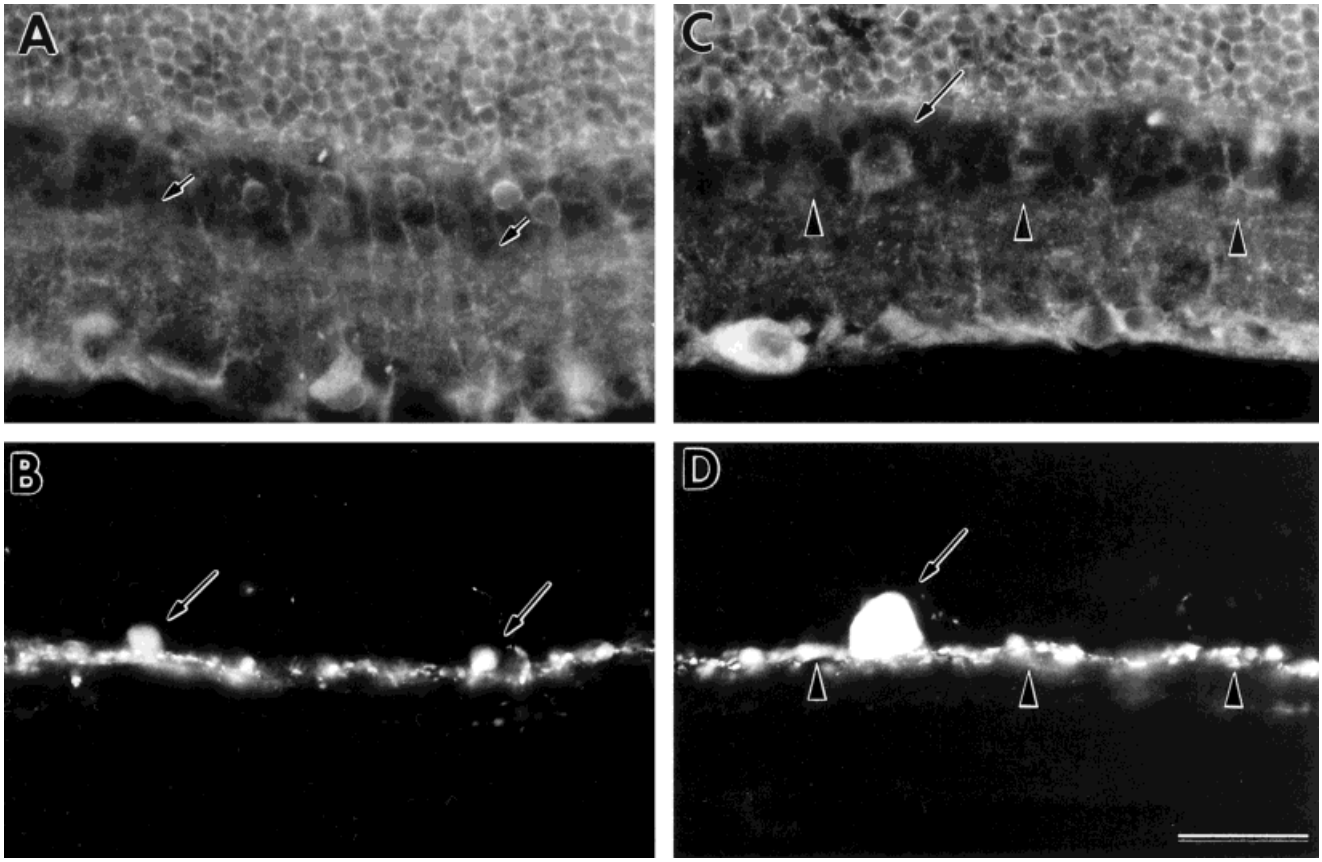

Fig. 9. Double-label immunofluorescence of FAAH-IR (**A,C**; FITC) and TOH-IR (**B,D**; Cy-3). The small TOH-immunoreactive cell bodies did not colocalize with FAAH-IR (A,B, arrows). The large TOH-immunoreactive cell bodies co-localized with FAAH-IR (C,D, arrow). However, their processes in the distal IPL did not appear to be FAAH-immunoreactive (C,D, arrowheads). Scale bar = 25 μm.

combinations of glycosylated and nonglycosylated dimers and trimers (Pettit et al., 1998). Similarly, we found in the rat retina a band at 53 kDa, likely to represent a nonglycosylated CB1R, and a band at 160 kDa, possibly due to a trimer of the nonglycosylated receptor.

The localization of CB1R-IR in the rat retina supports studies that have demonstrated CB1R expression in the rat retina by in situ hybridization and RT-PCR (Buckley et al., 1998; Porcella et al., 1998). Here we found that CB1R-IR was restricted largely to cells that were PKC-immunoreactive. These included the dendrites, soma, axon, and axon terminals of rod bipolar cells (Greferath et al., 1990) as well as a type of amacrine cell. We did not corroborate the findings of Negishi et al. (1988) demonstrating that PKC-immunoreactive amacrine cells were also TOH-immunoreactive. We found, in contrast, that TOH-IR and PKC-IR stained mutually exclusive populations of amacrine cells. Recently, Kim et al. (1998) showed that PKC-immunoreactive amacrine cells were GABA-immunoreactive. Therefore, we conclude that the CB1R-immunoreactive amacrine cells are not dopaminergic but, rather, are a subset of GABAergic amacrine cells. In addition, CB1R-IR was present in horizontal cells, but at a level that was less than in rod bipolar cells. CB1R-IR was restricted to the horizontal cell soma and did not include the dendrites, unlike the rod bipolar cells. The distribution of CB1R mRNA has been studied by in situ hybridization in the rat embryo (Buckley et al., 1998). CB1R expression was detected in the retina by E13, at which time presumed ganglion cells (which appear between E12 and E17; Stone, 1988) were labeled. By E20, a second row of cell bodies was labeled distal to the presumed ganglion cells. The resolution was insufficient to identify the labeled cells, but they could have been rod bipolar cells, which appear between E13 and P4 (Stone, 1988). The origin of the more diffuse labeling of CB1R-IR in the mid- and distal layers of the IPL is, at present, unknown.

Endocannabinoids, and other ligands for cannabinoid CB1 receptors, inhibit adenylate cyclase via a $G_{i/o}$ protein-coupled receptor (Howlett et al., 1986; Vogel et al., 1993; DiMarzo et al., 1998). Activation of CB1 receptors has a variety of effects on brain physiology, including modulation of dopamine function (Schlicker et al., 1996; Glass and Felder, 1997; Gessa et al., 1998), inhibition of GABAergic and glutamatergic neurons (Manueuf et al., 1996; Shen et al., 1996; Romero et al., 1998; Chan et al., 1998), and modulation of long-term potentiation (LTP; Collin et al., 1995; Stella et al., 1997). Schlicker et al. (1996) reported that CB1R agonists increased dopamine release from guinea pig retina. However, in rat, CB1R-IR was not present on TOH-immunoreactive amacrine cells, indicating either a species difference or an alternative mechanism for the CB1R-induced release of dopamine from the retina.

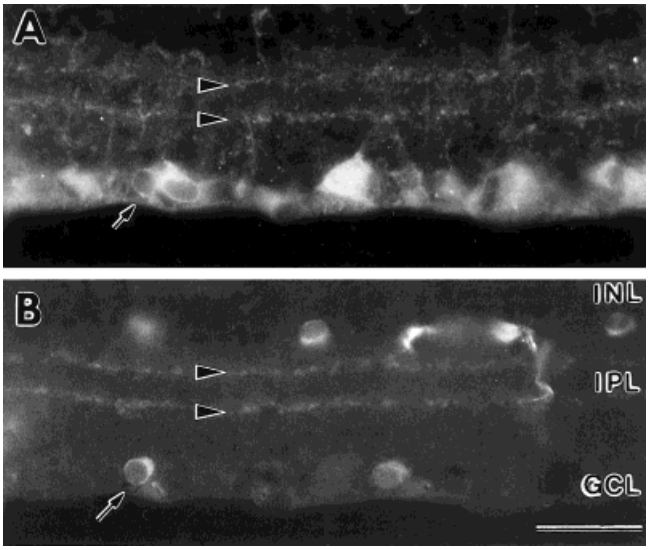

Fig. 10. Double-label immunofluorescence illustrating colocalization of FAAH-IR (**A**; FITC) with ChAT-IR (**B**; Cy-3). ChAT-IR labeled symmetrical groups of amacrine cells in the INL and GCL, with their narrow strata in the IPL. There appeared to be an exact match between FAAH-IR and ChAT-IR in the two laminae of the IPL (arrowheads). However, colocalization over the cell bodies was not complete. In this micrograph, the only clear example of colocalization over cell bodies was in one cell body in the GCL (arrow). Scale bar = 25 μm.

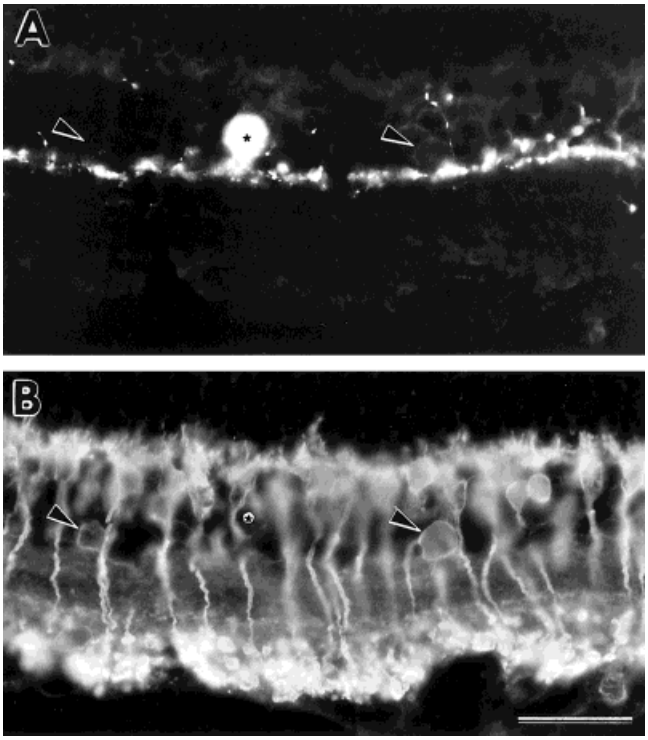

Fig. 11. Double-label immunofluorescence of TOH-IR (**A**; FITC) and PKC-IR (**B**; Cy-3). TOH-immunoreactive cell bodies in the INL (asterisk) were not double-labeled for PKC-IR, nor were PKC-immunoreactive amacrine cell bodies (B, arrowheads) double-labeled for PKC-IR. Scale bar = 25 μm.

The extensive distribution of CB1R-IR to rod bipolar cells suggests a role for endocannabinoids in scotopic vision. Indeed, there is evidence for increased photosensitivity following short- or long-term marijuana use (Dawson et al., 1977; Reese, 1991; West, 1991; Consroe et al., 1997). Rod bipolar cells are ON neurons that depolarize to increments of light intensity. They are subject to considerable GABAergic influence, dominated largely by $GABA_C$ receptors (Feigenspan et al., 1993; Pan and Lipton, 1995; Euler and Wässle, 1998), which in turn are modulated by agonists of metabotropic glutamate receptor mGluR1/5 (Euler and Wässle, 1998). The introduction of endocannabinoids into this circuit would result in a more complex system devoted to modulation of glutamate release from rod bipolar cells than is currently thought.

The presence of CB1R-IR on horizontal cells suggests a role of endocannabinoid receptors in modulating the synaptic gain in the OPL. This is complicated by the observation that horizontal cells also were FAAH-immunoreactive (see below), indicating that they are sites of degradation of endocannabinoids. If this is so, then an autoregulatory mechanism could be at work, somewhat analogous to that proposed for GABAergic horizontal cells in salamander and catfish retinae (Kamermans and Werblin, 1992; Dong et al., 1994).

The source and identity of the endocannabinoids are more problematic. Although FAAH hydrolyzes endocannabinoids, anandamide and 2-AG (Deutsch and Chin, 1993; Ueda et al., 1998), there are other substrates for FAAH such as the sleep factor oleamide (Cravatt et al., 1995, 1996). Also, a second enzyme, separated from anandamide amindohydrolase in porcine brain, hydrolyzes 2-AG but not anandamide (Goparaju et al., 1999). Furthermore, the biosynthetic pathway by which endocannabinoids are synthesized in vivo is still under contention (Mechoulam et al., 1998). Given these qualifications, it is thought that neurons that transport and degrade endocannabinoids are also likely to be the ones releasing them as transmitters. FAAH-IR was most prominent in ganglion cells, which is consistent with a recent study in rat brain showing that

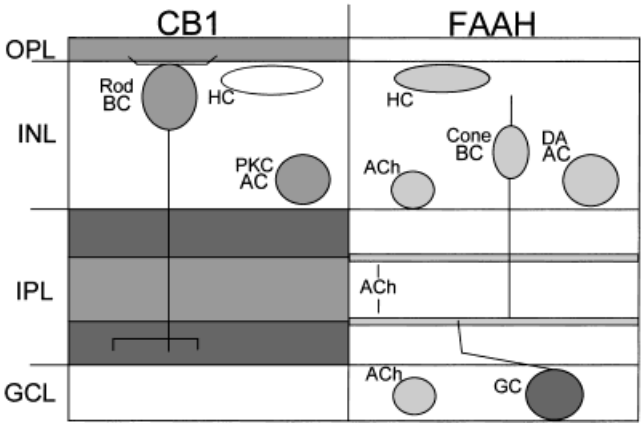

Fig. 12. Schematic illustration of the labeling patterns of CB1R-IR and FAAH-IR in the rat inner retina: OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; rod BC, rod bipolar cell; HC, horizontal cell; PKC AC, protein kinase C-immunoreactive amacrine cell; ACh, cholinergic amacrine cell bodies and strata in the IPL; Cone BC, cone bipolar cell; DA AC, dopaminergic amacrine cell; GC, ganglion cell.

FAAH-IR is most prominent in the principal output neurons, i.e., the Purkinje cells of the cerebellum, pyramidal cells of the hippocampus and cortex, and mitral cells of the olfactory bulb (Tsou et al., 1998b; Egertova et al., 1998). Considering that none of these output neurons is presynaptic in its region, the meaning of this correlation is unknown. Recently, Egertova et al. (1998) suggested that endocannabinoids were synthesized and released from postsynaptic neurons and inhibited transmitter release by acting on presynaptic CB1 receptors. Recent evidence in support of this idea has been obtained for presynaptic CB1R-modulated release of GABA in rat hippocampal interneurons (Katona et al., 1999). Although such a mechanism may work in other regions of the CNS, retinal ganglion cells are not postsynaptic to rod bipolar cells, and thus a paracrine mechanism of endocannabinoid transmission may operate in the inner retina.

Rod bipolar cells, horizontal cells, and a type of GABA amacrine cell appear to be the major targets, as identified by CB1R-IR, of endocannabinoid transmission in the rat retina. In addition to ganglion cells, endocannabinoids could be released from the cell types that were more weakly labeled by FAAH-IR. These included horizontal cells, large dopamine amacrine cells, cholinergic starburst amacrine cells, and some as yet unidentified cone bipolar cells. Euler and Wässle (1995) showed that antisera against GLT-1 labeled two types of cone bipolar cell (types 5 and 6), with two very narrow strata in the proximal half of the IPL, very close to the proximal stratum of starburst amacrine cells. However, Brandstätter et al. (1995) showed that the GLT-1-immunoreactive strata did not colocalize with ChAT-IR but rather flanked the proximal ChAT-immunoreactive stratum. Because we found that FAAH-IR colocalized with ChAT-IR in the IPL, we conclude that FAAH-IR does not colocalize with GLT-1-immunoreactive cone types 5 and 6 bipolar cells.

Rod bipolar cells in rat retina receive direct input from rods in the OPL. Although no direct synaptic inputs from horizontal cells to rod bipolar cells have been described, the dendrites of horizontal cells, together with those of rod bipolar cells, invaginate rod spherules (Chun et al., 1993) and therefore would be in a position to modulate bipolar cell responses. As was mentioned, horizontal cells also contain CB1R-IR, so there may be autoregulation of endocannabinoid release, which could affect gain across synapses in the OPL. Rod bipolar cells in rat retina receive direct synaptic input only from amacrine cells in the IPL. These amacrine cells include GABAergic and glycinergic amacrine cell types (Chun et al., 1993; Feigenspan et al., 1993; Greferath et al., 1995; Enz and Bormann, 1995; Enz et al., 1996; Fletcher et al., 1998). FAAH-IR was found in cholinergic and dopaminergic amacrine cells, both types of which colocalize with GABA in the rodent retina (Kosaka et al., 1987; Simon et al., 1989; Wulle and Wagner, 1990). However, there is no evidence that either dopaminergic or cholinergic amacrine cells synaptically contact rod bipolar cells.

There was a distinct band of CB1R-IR in the distal 25% of the IPL, a region within the arbor of dopaminergic amacrine cells and the lobular appendages of glycinergic AII amacrine cells. FAAH-IR was found only in the large TOH-immunoreactive amacrine cells, in which it was restricted to the soma. The intense TOH-immunoreactive dendrites were not FAAH-immunoreactive, suggesting that FAAH either was confined to the cell body or was maintained at a relatively low level in the dendrites. The major output of dopaminergic amacrine cells is onto other amacrine cells, one type of which is the glycinergic AII amacrine cell, a critical component in the rod bipolar cell pathway (see Wässle and Boycott, 1991; Djamgoz and Wagner, 1992, for reviews). Although we have not identified the cell type ramifying in the distal IPL that is CB1R-immunoreactive, there is a good possibility for cannabinergic modulation of the rod pathway through the AII amacrine cells as well as directly on the rod bipolar cells.

There was intense FAAH-IR at the inner limiting membrane and surrounding the cell bodies of rod photoreceptors that likely is a result of Müller cell labeling, despite the absence of prominent FAAH-immunoreactive radial streaks in the inner retina. Müller cells play a critical role in maintaining retinal homeostasis, for example, the maintenance of pH, the transport of K^+, glucose, glutamate, and GABA (see Reichenbach and Robinson, 1995, for review). Degradation of endocannabinoids would be consistent with this general role of Müller cells in the metabolism and recycling of some retinal neurotransmitters.

In summary, we have shown a distribution of cannabinoid CB1 receptors in the retina that is consistent with psychophysical demonstrations of enhanced visual sensitivity following use of marijuana. Although CB1 receptors are prevalent on rod bipolar cells, the mechanism by which any enhanced photosensitivity would be achieved remains to be determined. The potential sources of endocannabinoids, as determined by the distribution of FAAH-IR, are more widespread and do not fit neatly into a scheme of presynaptic/postsynaptic targets. There appears to be a strong possibility for more unconventional release and receptor mechanisms that include autoregulation, presynaptic inhibition, and paracrine transmission. It is clear that the widespread distribution of cannabinoid CB1 receptors and degradative enzyme in the retina indicates an important role for endocannabinoids as modulators of retinal activity.

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