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Architecture of cannabinoid signaling in mouse retina

Sherry Shu-Jung Hu¹, Andy Arnold¹, Jacqueline M. Hutchens¹, Josh Radicke¹, Benjamin F. Cravatt², Jim Wager-Miller¹, Ken Mackie¹, and Alex Straiker¹

¹The Gill Center for Biomolecular Science and the Department of Psychological and Brain Sciences, Indiana University, Bloomington, IN 47405, USA.

²Department of Chemical Physiology, the Scripps Research Institute, La Jolla, CA 92037, USA.

Abstract

Cannabinoid receptors and their ligands constitute an endogenous signaling system that is found throughout the body, including the eye. This system can be activated by Δ^9 -tetrahydrocannabinol, a major drug of abuse. Cannabinoids offer considerable therapeutic potential in modulating ocular immune and inflammatory responses and in regulating intraocular pressure. The location of cannabinoid receptors 1 (CB₁) in the retina is known, but recently a constellation of proteins has been identified that produce and break down endocannabinoids (eCBs) and modulate CB₁ function. Localization of these proteins is critical to defining specific cannabinoid signaling circuitry in the retina.

Here we show the localization of diacylglycerol lipase α and β (DGL α/β), implicated in the production of the eCB 2-arachidonoyl glycerol (2-AG); monoacylglycerol lipase (MGL) and α/β -hydrolase domain 6 (ABHD6), both implicated in the breakdown of 2-AG; cannabinoid receptor interacting protein 1a (CRIP1a), a protein that may modulate CB₁ function; Fatty acid amide hydrolase (FAAH) and *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) which have been shown to break down the eCB anandamide and related acyl amides. In our most prominent finding, DGL α is present in post-synaptic Type 1 OFF cone bipolar cells juxtaposed to CB₁-containing cone photoreceptor terminals. Interestingly, CRIP1a is reliably presynaptic to DGL α , consistent with a possible role in cannabinoid signaling, NAAA is restricted to retinal pigment epithelium (RPE), while DGL β is limited to retinal blood vessels. These results taken together with previous anatomic and functional studies define specific cannabinoid circuitry likely to modulate eCB signaling at the first synapse of the retina as well as in the inner plexiform layer (IPL).

Keywords

CB₁; cannabinoid; retina; ABHD6; CRIP1a; MGL; DGL; FAAH; NAAA

INTRODUCTION

Cannabinoid receptors and their ligands constitute an endogenous signaling system that is found throughout the body, and is particularly prominent in the nervous system (Devane et al., 1992; Herkenham et al., 1991; Stella et al., 1997). It is this system that is activated by exogenous cannabinoids such as Δ^9 -tetrahydrocannabinol (Δ^9 -THC). Significant strides have been made to advance our understanding of the cannabinoid signaling system since the identification of the two known cannabinoid receptors 1 and 2 – CB₁ and CB₂ (Matsuda et

al., 1990; Munro et al., 1993). Two important identified roles for CB₁, the major subtype found in the brain, are retrograde inhibition of neurotransmission and mediation of long-term depression of synaptic responses (Kreitzer and Regehr, 2001; Sjostrom et al., 2003; Wilson and Nicoll, 2001).

Reports of visual effects of cannabinoids

A variety of visual effects of cannabinoids have been reported in the literature (reviewed in Yazulla 2008). Taken together these studies cautiously suggest the existence of cannabinoid-mediated visual effects, particularly an enhancement of light sensitivity (a common theme), most marked at higher exposures.

Cannabinoid receptors and fatty acid amide hydrolase (FAAH) in retina

Matsuda et al. (Matsuda et al., 1997) found that the porcine retina possessed enzymatic activity capable of hydrolyzing the endocannabinoid (eCB) *N*-arachidonoyl ethanolamine (anandamide or AEA). In addition, homogenates of iris, choroid, optic nerve and lacrimal gland (but not lens) also hydrolyzed AEA. In 1996, Schlicker et al. (Schlicker et al., 1996) determined that application of cannabinoids reduced dopamine release in guinea pig retina. Shortly afterward, Buckley et al. (Buckley et al., 1998) published an in situ hybridization study of the expression of CB₁ mRNA in the retina of the embryonic rat. They detected two bands of expression appearing at different embryonic stages, but anatomical identification of these layers was not possible. In addition, Porcella et al. (Porcella et al., 1998; Porcella et al., 2000) using RT-PCR found that the mRNA for CB₁ (but not CB₂) was present in retinal homogenates. Localization of CB₁ receptors was investigated in several vertebrate species using immunocytochemical approaches (Straiker et al., 1999a; Yazulla et al., 1999). Both papers reported CB₁ labeling in subsets of amacrine cells and in horizontal cells as well as dense labeling in the inner plexiform layer (IPL). We also reported labeling in rod and cone photoreceptor terminals while Yazulla and colleagues (Yazulla et al., 1999) reported rod bipolar cell labeling. This latter group also described labeling with an antibody to fatty acid amide hydrolase (FAAH), an enzyme known to hydrolyze the candidate eCB anandamide (Cravatt et al., 1996). In the rat, they detected FAAH immunoreactivity in large ganglion cells with processes proximal to the ganglion cell layer, in the somata of horizontal cells, in large dopaminergic amacrine cells and in the dendrites of starburst amacrine cells. Expression of CB₁ receptors and FAAH has also been described in goldfish retina (Fan and Yazulla, 2004; Struik et al., 2006; Yazulla et al., 2000).

Endocannabinoids in retina

Bisogno (Bisogno et al., 1999) and Straiker (Straiker et al., 1999a) reported the presence of several candidate eCBs in bovine and rat retina respectively. Significantly, the disposition of endogenous cannabinoids – the conditions of their production, transport, and breakdown—remains poorly understood. In the case of AEA, even the receptor-pairing is uncertain since AEA also activates transient receptor potential channel 1 (TRPV1) receptors (Howlett et al., 2004; Qin et al., 2008). Meanwhile a variety of structural analogues of AEA and 2-arachidonoyl glycerol (2-AG) (e.g. palmitoyl ethanolamine (PEA)) have been investigated as candidate eCBs. For simplicity, we will refer to all of these molecules as ‘candidate eCBs’ or simply ‘eCBs’.

Proteins proposed to produce, transport, and break down AEA and related acyl amides such as PEA include *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), FAAH and *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) (Cravatt et al., 2001; Guo et al., 2005) but several alternative pathways, particularly for synthesis, have been proposed including one involving α/β -hydrolase domain 4 (ABHD4) (Leung et al., 2006; Simon and Cravatt, 2006). 2-AG is thought to be synthesized through the activity of diacylglycerol

lipase α (DGL α) (Bisogno et al., 2003) on phosphatidylinositol bisphosphate (PIP₂), and broken down by the action of monoacylglycerol lipase (MGL) (Dinh et al., 2002), but also perhaps α/β -hydrolase domain 6, 12 (ABHD6, ABHD12) (Blankman et al., 2007) and/or cyclooxygenase-2 (COX-2) (Kozak et al., 2000).

Toward an understanding of the role of endocannabinoids in retinal function

The next step in elucidating the retinal eCB signaling system is to understand its functional role. A key obstacle to achieving this step is that we don't know where in the retina eCBs are produced and broken down. Cannabinoids often act in a retrograde manner, so if CB₁ resides at presynaptic terminals of cone and rod photoreceptors (as they do in monkey (Straiker et al., 1999a)), what post-synaptic cells produce eCBs? In principle, the source could be horizontal, bipolar (of which there are ten sub-types in the mouse (Wassle et al., 2009)), or even amacrine cells. We have shown, using salamander retina, that cannabinoids modulate presynaptic Ca and/or K ion channels in bipolar cells and two classes of photoreceptor (Straiker et al., 1999a; Straiker and Sullivan, 2003). Other groups using goldfish or chick have also described modulation of Ca and/or K ion channels as well as possible effects on the time course of cone offset regulation, albeit with a concentration of the synthetic agonist WIN-55212-2 known to produce CB₁-receptor-independent effects (Fan and Yazulla, 2003; 2004; 2005; Lalonde et al., 2006; Shen and Thayer, 1998; Straiker et al., 1999a; Straiker and Sullivan, 2003; Struik et al., 2006; Warrier and Wilson, 2007; Yazulla et al., 2000). But in mammalian systems, to date only one study using mammalian retinal neurons has been reported (Lalonde et al., 2006). This study describes cannabinoid receptor-mediated inhibition of calcium signaling in ganglion cells, in neurons cultured from rat retinæ. These studies represent important contributions and a promising start on the way to a circuit-based exploration of cannabinoid signaling in the mammalian retina. The advent of mouse models with specific populations of fluorescent neurons opens the possibility of targeted electrophysiological dissection of specific retinal circuits. However, for these studies to proceed in a rational fashion a comprehensive understanding of where eCBs are produced and broken down in the retina is required.

The pantheon of proteins involved in eCB signaling has been substantially expanded and refined over the past decade, and reliable antibodies against these proteins have slowly come to be available. Precise localization of proteins such as the DGL α and DGL β (expected to play roles in 2-AG production), MGL, ABHD6, FAAH, and NAAA (each able to hydrolyze eCBs and PEA) and other associated proteins will be essential to defining retinal cannabinoid circuitry (Bisogno et al., 2003; Blankman et al., 2007; Dinh et al., 2002; Tsuboi et al., 2005). To this end, we have conducted an extensive immunocytochemical study of cannabinoid-related proteins in retina of the mouse.

MATERIALS AND METHODS

All procedures used in this study were approved by the Animal Care Committee of the Indiana University and conform to the Guidelines of the National Institutes of Health on the Care and Use of Animals. Adult mice (CD1 strain (4), C57/B16 strain (4)), >5 weeks, of either sex, from breeding colony) were housed under a 12/12 hour day/night cycle then killed (during the light cycle) by rapid cervical dislocation. Eyes were removed, and the anterior eye section cut away, forming an eyecup, from which the retina was scooped. For immunocytochemistry, the eyecup was fixed in 4% paraformaldehyde followed by a 30% sucrose immersion for 24-72 hours at 4 °C. Tissue was then frozen in OCT compound and sectioned (10-15 μ m) using a Leica CM1850 cryostat. Tissue sections were mounted onto Superfrost-plus slides, washed, treated with a detergent (Triton-X100, 0.3% or saponin, 0.1%) and normal donkey serum (0.5%) or milk (5%), followed by primary antibodies overnight at 4 °C. Secondary antibodies (usually Texas Red or fluorescein isothiocyanate

(FITC), 1:200, Jackson ImmunoResearch Laboratories, Inc., West Grove PA) were subsequently applied at room temperature for 1.5 hours.

GAD67-GFP mice (2 animals, C57/Bl6 strain, >5 weeks, either sex) generated by Dr. Yuchio Yanagawa (Gunma University, Gunma, Japan (Tamamaki et al., 2003)) were provided by Dr. Albert Berger (University of Washington, Seattle WA), with Dr. Yanagawa's permission. The Gy13 antibody was the generous gift of Dr. Z. Kokrashvili (Mount Sinai School of Medicine, NY).

Cell Culture

Human Embryonic Kidney (HEK) cells were grown as previously described (Straiker et al., 2009).

Antibody Characterization

All primary antibodies, the immunogen used in their generation, their sources, and their working dilutions are listed in Table 1. The anti- Δ TM-FAAH antibody was raised against truncated and his-tagged recombinant FAAH protein purified (aa 38-579) from *E. Coli*. (Bracey et al., 2002; Patricelli et al., 1998).

For the current study, we generated rabbit polyclonal antibodies for rat NAAA (rNAAA) and human CRIP1a (hCRIP1a) and characterized their specificity as described below. For NAAA, four GST fusion protein expression constructs were produced by inserting the DNA coding for four portions of rat NAAA (rNAAA) (designated as A, B, C, and D and corresponding to the following peptides: QDSQGRIYHGRNLD, SPHKFTISGDERDK, EGVVITRDRGGPAD, TNYDHWEPVPRDD) into the pGEX-3X vector at the BamH I and EcoR I restriction sites. Each fusion protein was purified from BL21 *E. coli* lysates on a glutathione sepharose column and the cocktail mixture was injected into two rabbits to generate antisera (Cocalico Biologicals, Inc., Reamstown, PA) using standard approaches (Bodor et al., 2005). The antiserum was purified in two steps, first by exclusion on a GST column and then by binding to and elution from an affinity column made with the rNAAA fusion protein C. For CRIP1a, a GST fusion protein expression construct was produced by inserting the DNA coding for full-length

(MGDLPGLVRLSIALRIQPNDGPVFYKVDGQRFQNRITIKLLTGSSYKVEVKIKPSTLQVENISIGGVLPVLELKSKEPDGDRVVTGTGTYDTEGVPTKSGERQPIQITMPFTDIGTFETVWQVKFYNYHKRDHCQWGSPFSVIEYECKPNETRSLMWVNKESFL) hCRIP1a into a pGEX-3X vector at the BamH I and EcoR I restriction sites. The fusion protein was purified from BL21 *E. coli* lysates on a glutathione sepharose column and was injected into two rabbits to generate antisera (R & R Rabbitry, Sedro-Wooley, WA) using standard approaches (Bodor et al., 2005). The antiserum was purified in two steps, first by exclusion on a GST column and then by binding to and elution from an affinity column made with the hCRIP1a GST fusion protein.

The specificity of CB₁-L15, CB₁-CT, and FAAH have been previously characterized by using knockout mouse models and the immunostainings for CB₁ and FAAH were completely absent in their corresponding knockout (Bodor et al., 2005; Bracey et al., 2002; Hajos et al., 2000). In addition, the specificity of MGL, ABHD6, DGL α , DGL β , NAAA, and CRIP1a antibodies have either been previously characterized or were characterized in the current study by using HEK cells transiently transfected with the corresponding epitope-tagged plasmids (ensuring that antibody staining for the epitope tagged protein exclusively co-localized with staining for the antibody being tested), and then using the corresponding immunizing protein (5 μ g/mL, or 10 μ g/mL for NAAA) to block the primary antibody. Images were acquired with a Leica TCS SP5 confocal microscope (Leica Microsystems,

Wetzlar, Germany) using Leica LAS AF software and a 63X oil objective. Images were processed using ImageJ (available at <http://rsbweb.nih.gov/ij/>) and/or Photoshop (Adobe Inc., San Jose, CA). Images were modified only in terms of brightness and contrast.

The calbindin-D28K (CB) antibody recognized a single band of 28 kDa on Western blots of rat brain (manufacturer's datasheet), and stained a pattern of cellular morphology and distribution in the mouse retina that is identical with previous reports (Haverkamp and Wassle, 2000). Specificity of the parvalbumin antibody was demonstrated by the absence of immunostaining by this antibody in the parvalbumin knockout mouse (Burette et al., 2009). The specificity of the GAD65 antibody was established by the recognition of a single band of 64 kDa (representing glutamic acid decarboxylase (GAD)) by Western blotting (Chang and Gottlieb, 1988; Jevince et al., 2006).

The SV2 antibody recognizes a 78 kDa transmembrane proteoglycan that is present at synapses in the central and peripheral nervous systems by Western blotting (manufacturer's datasheet). In the embryonic chicken retina, antibodies to PSD95 and SV2 label the axon terminals of photoreceptors in the OPL (Bergmann et al., 2002; Wahlin et al., 2008). The MAP2 antibody stained a single band of 280 kDa on Western blots of adult rat brain extract (manufacturer's datasheet). PKC is a well-characterized marker for rod bipolar cells in retina of many species (Haverkamp and Wassle, 2000). The PKC antibody recognized the purified PKC protein as well as an 80 kDa band from whole-cell extracts of rat glioma and murine NIH3T3 cell lines on Western blots and specifically immunoprecipitated PKC from cell lysates of 328 glioma and SVK 14 cell lines. This antibody reacts with PKC- α/β -1/ β -2 isoforms (manufacturer's datasheet). The PSD95 antibody recognized a single band of 95 kDa on Western blots (manufacturer's datasheet).

The recoverin antibody recognized a 25 kDa band on Western blots of mouse retina (Hendrickson et al., 2009). This calcium-binding protein has been detected in both rod and cone photoreceptors and in three populations of cone bipolar cells in the mouse as well as in midget bipolar cells of the primate (Haverkamp et al., 2003; Haverkamp and Wassle, 2000; Milam et al., 1993). The G γ 13 antibody was reported to label the ON bipolar cells in retina. Its specificity in retina was established by correlating transcript (RT-PCR) and immunostaining (Huang et al., 2003). The NK3-R antibody stained a population of cone bipolar cells consistent with that described in previous reports (Casini et al., 2000; Gargini et al., 2007).

RESULTS

DGL α is present in postsynaptic terminals of Type 1 OFF cone bipolar cells

DGL α is a favored candidate to produce the eCB 2-AG (Bisogno et al., 2003). In most brain regions, DGL α has been localized to postsynaptic terminals at glutamatergic synapses (Katona et al., 2006; Matyas et al., 2008). Specifically, the DGL α staining is limited to a wide perisynaptic annulus in the membrane of dendritic spines. This is consistent with a role in postsynaptic production of eCBs followed by retrograde movement of 2-AG across the synapse to act at presynaptic CB $_1$ receptors. Consistent with this, pharmacological inhibition of DGL α prevents cannabinoid inhibition of synaptic transmission (Chevalleyre et al., 2006). In the retina, DGL α appears in the two synaptic layers: the outer plexiform layer (OPL) and inner plexiform layer (IPL) (Fig 1A, C). This staining is absent when the DGL α antibody is coincubated with the immunizing protein (5 μ g/mL, Fig 1B). The OPL labeling is restricted to large intermittent clusters in the proximal OPL, suggestive of the postsynaptic terminal of a cone bipolar cell. Using a guinea pig DGL α antibody we stained for DGL α and an assortment of rod and cone bipolar cell markers. The rod bipolar cell marker PKC (Negishi et al., 1988) did not costain with DGL α in the OPL (Fig 1D-E). Nor did an antibody against

G γ 13, which labels ON-cone bipolar cells (Fig 1F, (Huang et al., 2003)). Recoverin, which is specific to Type 2 OFF cone bipolar cells (Fig 1G, (Shiells and Falk, 1992)), also did not colocalize with DGL α . However, an antibody against the neurokinin 3 receptor (NK3R) clearly costained with DGL α (Fig 1H). NK3R stains OFF cone bipolar cells 1 and 2 (Ghosh et al., 2004; Haverkamp et al., 2003; Pignatelli and Strettoi, 2004), but since recoverin did not costain with DGL α , we may conclude that OPL labeling of DGL α is restricted to postsynaptic terminals of Type 1 OFF cone bipolar cells.

Cannabinoid CB₁ receptors have been detected in retinas of mouse and other species (Straiker et al., 1999a), most prominently in rod photoreceptor terminals. Although CB₁ clearly labeled cone photoreceptor terminals in primate, it was important to establish that cone terminals in mouse similarly express CB₁. We examined this in part by costaining CB₁ with calbindin and DGL α . Calbindin in the OPL of the mouse labels the processes and somata of horizontal cells (Rabie et al., 1985; Rohrenbeck et al., 1987; Schreiner et al., 1985) - it clearly labels the terminals of horizontal cell processes where they invaginate into photoreceptor terminals. CB₁ staining was detected in the same region as calbindin, but did not colocalize with calbindin (Fig 1I). In a compressed Z-series, CB₁ costaining with DGL α clearly shows a dense CB₁ OPL staining in the OPL distal to DGL α . Notably, the CB₁ staining also closely juxtaposes to DGL α , suggesting that CB₁ may be present in the same cone terminals that lie presynaptic to Type I OFF cone bipolar cells (Fig 1J). To test this further we costained with CB₁ and PSD95, a retinal marker that outlines rod spherules and cone pedicles (Fig 1K, (Koulen et al., 1998)). CB₁ is clearly present within both rod spherules (arrowheads) and cone terminals (angled arrows). Thus, we can conclude that pre- and post-synaptic components of an eCB signaling system are closely juxtaposed in the OPL.

In addition to the intense Type I OFF cone bipolar cell staining, we also observed a more uniformly distributed and punctate DGL α staining throughout the OPL. Colabeling with PSD95 showed that this additional DGL α staining lay partly within rod spherules but chiefly outside, just proximal to the spherules (Fig 2A). This DGL α likely sits in the processes of bipolar cells, though we have not determined which subtype because the fine processes are not delineated by the bipolar cell markers that we tested.

DGL α labeling is widely distributed throughout the IPL

In the IPL, DGL α is widely and diffusely distributed. To investigate distribution of DGL α relative to inhibitory neurons, we made use of retinæ from GAD67-GFP mice, in which neurons that express GAD67 also express GFP (Tamamaki et al., 2003). These mice have been used as model system for the study of inhibitory neurons in the brain (Fukushima et al., 2009; Kaneda et al., 2008; Kaneko et al., 2008) and retina (May et al., 2008). DGL α (Fig 2B) did not overlap with GAD67-GFP positive neurons nor with CB₁ immunostaining (Fig 2C).

MGL is present in rod spherules and cone pedicles in the OPL and is expressed prominently in two laminae of the IPL

As revealed by functional and biochemical studies, several enzymes are involved in 2-AG degradation, chief among them including MGL, blockade of which has been shown to sharply prolong depolarization-induced suppression of inhibition (DSI (Hashimoto et al., 2007; Straiker and Mackie, 2009)). As a consequence, we sought to determine where MGL is expressed in the retina, particularly relative to CB₁ and DGL α . Since DGL α is localized in OPL and IPL one would predict MGL is also in both layers. This proved to be the case. MGL staining was particularly marked in the IPL and the ganglion cell layer (GCL) but was also seen in the OPL (Fig 3A, C). This staining is absent when the MGL

antibody is coincubated with the immunizing protein (5 $\mu\text{g/mL}$, arrows, Fig 3B). In the IPL, MGL was particularly prominent in two laminae, one in the central IPL and another in the distal IPL. MGL did not colocalize with DGL α in the IPL, (Fig 3D). Nor did MGL colocalize with GAD65, a marker for inhibitory neurons in the retina (Brecha et al., 1991) (Fig 3E). In the OPL, MGL stained distal to DGL α , suggesting it is present in photoreceptor terminals (Fig 3D). This was confirmed for rod spherules by costaining with SV2 (Fig 3G) and for cone pedicles by costaining with PSD95 (Fig 3F).

ABHD6 staining is widely distributed in IPL, GCL and INL, and present in calbindin and GAD67-positive amacrine cells as well as dendritic MAP2-positive processes

As mentioned above, ABHD6 also participates in 2-AG breakdown. We have found ABHD6 to strongly label targets in the IPL, inner nuclear layer (INL) and GCL (Fig 4A, C). This staining is absent when the ABHD6 antibody is coincubated with the immunizing protein (5 $\mu\text{g/mL}$, arrows, Fig 4B). If ABHD6 were to play a role in the breakdown of DGL α -produced 2-AG after its action on presynaptic CB $_1$, one might expect a juxtaposition of these two enzymes. However, this was not observed (Fig. 4D). In addition, ABHD6 did not costain with parvalbumin (data not shown) nor with PKC (Fig 5A). Instead, ABHD6 was present in calbindin positive amacrine cells in the inner margin of the INL (Fig 5B). ABHD6 also labeled GAD67-positive amacrine cells in the INL (Fig 5D). In hippocampal cultures, ABHD6 is primarily found in postsynaptic processes (Straiker et al., 2009). We therefore tested ABHD6 against a dendritic marker, MAP2. MAP2 stains puncta and processes in the IPL (Okabe et al., 1989). Interestingly, MAP2 and ABHD6 costained processes in the proximal IPL (Fig 5C), suggesting that the ABHD6 process staining in the proximal IPL is located in dendrites of ganglion cells or displaced amacrine cells. The GCL was prominently stained by the ABHD6 antibody (Fig 4A, C).

Cannabinoid receptor interacting protein 1a (CRIP1a) expression is limited to a population of calbindin-positive amacrine cells and to presynaptic terminals of cone photoreceptors juxtaposed to Type 1 OFF cone bipolar cells

The cannabinoid receptor interacting protein 1a (CRIP1a) remains an enigma. Identified in a yeast two hybrid screen (Niehaus et al., 2007), compelling evidence remains to be presented for a specific functional interaction with CB $_1$ receptors *in vivo*. However, the protein remains the focus of considerable interest and our group developed an antibody against it to investigate whether and to what extent the protein colocalized with CB $_1$. If the protein does not colocalize with CB $_1$, this would argue against functional interaction. The present study represents the first effort to examine this putative colocalization.

To determine where CRIP1a is expressed in mouse retina, we developed an antibody against this protein. Transient expression of hCRIP1a with a FLAG epitope (hCRIP1a-FLAG) allowed us to confirm the specificity of our newly developed antibody against CRIP1a. As shown in figure 6C-F, the CRIP1a antibody selectively labeled HEK293 cells in which CRIP1a is over-expressed and the overlap was almost complete (Fig 6C-F).

We found that the antibody against CRIP1a targets a population of calbindin positive amacrine cells in the central INL (Fig 6G) and a flat disc-like substrate in the OPL (Fig 6A, G, H & Fig 7A-D). This staining is absent when the CRIP1a antibody is coincubated with the immunizing protein (5 $\mu\text{g/mL}$, arrows, Fig 6B). Therefore, CRIP1a clearly does not colocalize with the bulk of CB $_1$ staining in rod photoreceptors and in the IPL. Costaining with the marker calbindin which labels horizontal cells including their dendritic invaginations into rod photoreceptor terminals shows the relative location of the CRIP1a staining is proximal to the mass of rod photoreceptor terminals (Fig 5G, H, arrows). Costaining with DGL α , a favored candidate for the postsynaptic production of 2-AG,

yielded an intriguing juxtaposition: DGL α exhibits a one-for-one juxtaposition with CRIP1a (Fig 7A), with DGL α occupying a region at the OPL/INL border strongly suggestive of a postsynaptic localization relative to a large presynaptic terminal. We therefore proceeded to test CRIP1a against PSD95, which generally labels postsynaptic densities in the brain, but instead reliably outlines rod spherules and cone pedicles in retina (Koulen et al., 1998). CRIP1a fully colocalized with PSD95 exclusively in cone terminals (Fig 7B-D). CRIP1a appears to display a one-for-one presynaptic localization relative to postsynaptic DGL α at cone-photoreceptor/Type 1 OFF cone bipolar cell synapses. Since CRIP1a is hypothesized to interact with CB₁, we also tested whether CRIP1a colocalized with CB₁ receptors, finding that in many cases CB₁ staining lay close to CRIP1a (Fig 7E).

DGL β is expressed exclusively in blood vessels

The role of diacylglycerol lipase β (DGL β) is not well understood (Bisogno et al., 2003). Our staining for DGL β is strongly suggestive of blood vessel localization (Fig 8A, C-D). This staining is absent when the DGL β antibody is coincubated with the immunizing protein (5 μ g/mL, Fig 8B). DGL β is therefore not in a position to play a classical role in regulation of synaptic transmission, though it may play in the vascular effects of endocannabinoids (Begg et al., 2003; Randall et al., 1996; Randall and Kendall, 1997; 1998b).

FAAH staining is widely distributed in IS, ONL, and GCL, and is present in a subpopulations of amacrine and cone bipolar cells in the INL, and is co-localized with CB₁ in the axon terminals of rod photoreceptors in OPL

FAAH is the major enzyme responsible for degrading AEA, oleamide, and related acyl amides (McKinney and Cravatt, 2005). Its localization has been described in the retina of the rat and goldfish (Yazulla et al., 1999). We found that as expected FAAH staining largely resembles that found in rat, with strong labeling in the IS, ONL, OPL and GCL (Fig 9A, H). This staining is absent in FAAH KO mouse retina (Fig 9B). FAAH is colocalized with the rod spherule marker PSD95 and also CB₁ in rod terminals in OPL (Fig 9C, I). FAAH is also present in a subpopulation of amacrine cells in the INL (Fig 9D, E). FAAH is not present in PKC-positive rod bipolar cells but rather in at least one population of cone-bipolar cells. In contrast to its distribution in the rat, FAAH does not appear to colocalize with horizontal cell bodies or processes as labeled by calbindin (Fig 9F, G). Finally, FAAH colocalizes with CB₁ not only in rod terminals as noted above, but also in amacrine and ganglion cells. (Fig 9H).

NAAA staining is limited to the retinal pigment epithelium

Although FAAH has long been considered the chief enzyme for the breakdown of AEA (Cravatt et al., 1996), another enzyme has recently been implicated. NAAA degrades AEA, but is active only at a very low pH (4-5), and has been proposed to serve as a lysosomal enzyme for the breakdown of AEA and related acyl amides (Tsuboi et al., 2005). However, a recent study showed that NAAA inhibition did not change AEA levels, but instead elevated those of palmitoyl ethanolamine (PEA) (Solorzano et al., 2009).

To determine where NAAA is expressed in mouse retina, we developed an antibody against this protein. Transient expression of rNAAA with a FLAG epitope (rNAAA-FLAG) allowed us to confirm the specificity of our newly developed antibody. As shown in figure 10A-D, the NAAA antibody selectively labeled HEK293 cells in which NAAA was over-expressed and the overlap was almost complete.

In mouse retina, NAAA labeling is highly restricted to the retinal pigment epithelial layer (RPE) (Fig. 10E) and the staining is absent when the NAAA antibody is coincubated with the immunizing protein (10 μ g/mL, Fig 10F).

DISCUSSION

Our most striking finding is evidence for a retrograde cannabinoid signaling module at Type 1 OFF cone bipolar cell-cone photoreceptor synapses, consisting of presynaptic CB₁ receptors and post-synaptic DGL α . We have also localized several other cannabinoid-related proteins, including widely distributed FAAH and ABHD6, a strong RPE presence for NAAA, cone terminal localization for CRIP1a and a presence in blood vessels of DGL β .

These findings are important because although the presence of cannabinoid CB₁ receptors as well as several candidate eCBs in the vertebrate retina is now well-established (Bisogno et al., 1999; Straiker et al., 1999a; Straiker and Sullivan, 2003; Straiker et al., 1999b; Yazulla, 2008; Yazulla et al., 1999), we are far from ascertaining the functional role of eCB signaling in the retina. Little is known regarding the likely synthetic sites of the chief candidate eCBs —2-AG and AEA. Nor has a systematic study of enzymes involved in eCB metabolism been done in murine retina. Thus, although CB₁ is believed to be present in rod and cone photoreceptor terminals and in rod bipolar cells in the IPL, the remaining components of these circuits remain unknown. A series of functional studies have demonstrated that cannabinoids affect retinal physiology, however these studies were performed in salamander and goldfish, or in cultured neurons (Fan and Yazulla, 2003; 2004; 2005; Lalonde et al., 2006; Straiker et al., 1999a; Straiker and Sullivan, 2003; Struik et al., 2006; Warrier and Wilson, 2007; Yazulla et al., 2000). The current study fills important gaps in our current understanding of cannabinoid retinal circuitry in an important, genetically pliable model — the mouse.

Enzymes involved in eCB production

In our most notable finding, prominent DGL α expression is found in postsynaptic terminals of one population of OFF cone bipolar cell — Type I —situated across from presynaptic CB₁ receptors in cone photoreceptor terminals. Given the suspected role of DGL α in production of the eCB 2-AG, this strongly implies that a chief source of 2-AG in the OPL is a single bipolar cell population. Recent efforts to systematically categorize mouse bipolar cells suggest that the mouse retina is home to one population of rod bipolar cells and nine cone bipolar cells (Ghosh et al., 2004; Haverkamp et al., 2003). Among these, Type I OFF bipolar cells remain poorly characterized. They are known to terminate in the most proximal portion of the IPL and exhibit a kainate response (Hartveit, 1997). As OFF bipolar cells, they are expected to activate when a cone-stimulating light signal is turned off. Because glutamate release is decreased with light, and increased in darkness, OFF bipolar cells have a sign-conserving configuration in terms of glutamate release and post-synaptic activation. As such, the standard model of cannabinoid retrograde feedback inhibition would predict an inhibition of presynaptic glutamate release via activation of presynaptic CB₁ receptors. This could occur via inhibition of Ca channels as it does in salamander cone photoreceptors (Straiker and Sullivan, 2003). Thus the DGL α juxtaposition at Type I OFF bipolar cells and cone terminals likely represents a feedback circuit. In addition a separate circuit is seen at rod photoreceptors, with DGL α likely producing 2-AG at rod bipolar cell terminals since it is juxtaposed to rod spherules but not present in horizontal cell terminals. Because rod bipolar cells represent more than 95% of the photoreceptors in the rod-dominant mouse retina, cannabinoid signaling via rods will form the bulk of OPL cannabinoid signaling, and perhaps the bulk of signaling in the retina. Given this density of apparent 2-AG production, it is notable that eCB spillover of as much as 20 μ m has been observed in brain slices (Kreitzer et al., 2002; Wilson and Nicoll, 2001). This means that cone bipolar cell-derived 2-AG may spill over to rods in a 40 μ m sphere of influence, resulting in a biphasic response. The reverse may also be true.

The mechanisms for production of AEA are less clear. Initial evidence suggested a central role for NAPE-PLD (Di Marzo et al., 1994; Okamoto et al., 2004). However, the decreased levels of long-chain saturated *N*-acyl ethanolamides with unchanged levels of AEA in NAPE-PLD knockout mice suggested that the former acyl amides may be preferred substrates for NAPE-PLD (Leung et al., 2006). Subsequent studies have yielded several candidate pathways for AEA biosynthesis including one involving ABHD4 (Simon and Cravatt, 2006). Interestingly, NAPE-PLD has been found to co-express with the transient receptor potential vanilloid type-1 (TRPV1) in retinal ganglion cell axons of the developing superior colliculus (Maione et al., 2009). These findings suggest a developmental role of NAPE-PLD in retinal function.

Sites of 2-AG hydrolysis in the retina

The two chief eCBs – 2-AG and AEA – are thought to activate CB₁ receptors followed by their rapid breakdown. While the chief candidate for 2-AG breakdown remains MGL several other proteins can metabolize 2-AG: ABHD6 and ABHD12 (Blankman et al., 2007), COX-2 (Kim and Alger, 2004; Kozak et al., 2000), and, in principle FAAH (Cravatt et al., 1996). In brain homogenates, MGL still accounts for the bulk of 2-AG breakdown, but ABHD6 and 12 have been found to account for a small but significant portion of eCB breakdown. Because brain homogenates do not distinguish between neuronal subpopulations of specific circuits, this leaves open the possibility that ABHD6 and/or ABHD12 play significant roles breaking down eCBs in subpopulations of neurons or glia. As noted below, the widespread distribution of ABHD6 in retinal IPL is consistent with this possibility, while study of ABHD12 will require development of an antibody against this protein.

MGL immunoreactivity is present in the OPL and IPL, with a distribution that was more diffuse and punctate than DGL α and CB₁, but clearly present in the photoreceptor terminals. MGL is therefore well-positioned to break down 2-AG after retrograde release onto both rod and cone terminals. In the IPL, the MGL localization is more difficult to interpret. MGL positive processes were widely distributed, suggesting roles in numerous circuits, but we were able to rule out expression in GAD65 containing inhibitory neurons, indicating that MGL is limited to excitatory neurons in the IPL.

ABHD6 proved to be highly abundant, particularly in neurons, with abundant INL staining in both calbindin- and putative GAD67-positive amacrine cells. At least some of ABHD6 staining is post-synaptic, consistent with dendrites of ganglion or displaced amacrine cells. The postsynaptic staining is less consistent with a role in breaking down eCBs after retrograde transmission. However, there is evidence for post-synaptic actions of CB₁ (Bacci et al., 2004; Kreitzer et al., 2002) and ABHD6 could also play a role in breakdown of extrasynaptic 2-AG that has diffused beyond its intended target synapses. It is also possible ABHD6 has other functions independent of 2-AG hydrolysis.

In the absence of information regarding likely AEA synthesis, it is impossible to identify cannabinoid circuits for AEA. AEA breakdown likely occurs by the action of FAAH (Cravatt et al., 1996), and/or NAAA (Tsuboi et al., 2005). Yazulla and colleagues detected FAAH immunoreactivity in large ganglion cells with processes proximal to the ganglion cell layer, in the somata of horizontal cells, a population of cone bipolar cells, large dopaminergic amacrine cells and in the dendrites of starburst amacrine cells in rat retina (Yazulla et al., 1999). As expected, our FAAH staining in mouse resembles that for the rat, with FAAH widely distributed in IS, ONL, a subpopulation of amacrine cells in INL, and in the GCL. The mouse labeling is consistent with rat in terms of the presence of a FAAH positive cone bipolar cell population, at least one amacrine cell population and substantial neuronal labeling in the ganglion cell layer. The most notable difference between mouse and rat staining is the absence of staining in horizontal cells of the mouse, possibly a function of

species difference. Our use of mouse FAAH knockout controls add confidence both to our results and to those previously obtained for the rat.

FAAH expression in photoreceptors is consistent with the observed AEA uptake in photoreceptors (Glaser et al., 2005), where FAAH (but not NAAA) is well-placed to play a role in AEA breakdown. Interestingly, FAAH co-localizes with CB₁ in rod photoreceptor terminals and also in amacrine cells and in GCL neurons. The OPL FAAH staining stands in contrast to previous findings in the hippocampus and cerebellum where FAAH is mainly expressed *post*-synaptically, across the synapse from CB₁ (Gulyas et al., 2004). Outside the OPL, CB₁ colocalizes more with FAAH than with MGL, suggesting that in the retina FAAH (and perhaps therefore AEA and other *N*-acyl amides) may play a central role in cannabinoid signaling.

NAAA is capable of breaking down AEA but a recent study suggests that NAAA may play more of a role in the breakdown of PEA, a structurally similar acylethanolamide that does not act reliably at CB₁ or CB₂: a selective inhibitor of NAAA elevated PEA levels but not those of AEA (Solorzano et al., 2009). Indeed, *in vitro* NAAA shows an at least 10-fold higher preference for PEA over AEA as a substrate (Solorzano et al., 2009). As such, the presence of NAAA in the RPE suggests that the RPE may be a site for PEA breakdown. The study of acyl amides in ocular pathology remains in its infancy, but there is evidence that these lipids are involved in ocular pathology. Decreased levels of PEA and 2-AG have been observed in ocular tissues of glaucoma patients, while increased levels of acyl amides including modest increases in PEA levels have been seen including modest increases in PEA levels have been seen in retinæ and assorted portions of anterior eyes of patients with diabetic retinopathy (Chen et al., 2005; Matias et al., 2006). As noted above, the enzymatic pathways for production of AEA remain the least well-characterized of the eCB production/breakdown machinery. Consequently, NAAA localization will remain difficult to interpret until we have more information about the enzymes that produce PEA and AEA.

CRIP1a was named 'cannabinoid interacting protein' after its isolation from the Y-79 retinoblastoma cell line with a yeast two hybrid approach (Niehaus et al., 2007). However it remains uncertain whether such an interaction actually occurs naturally in cells, particularly in neurons. If CB₁ is present in the same subcellular compartment as CRIP1a, then such an interaction is possible. We detected a close juxtaposition of CRIP1a and CB₁ in cone terminals, leaving open the possibility that they associate in these cells. More work remains to be done to address potential functional interactions between CRIP1a and CB₁ in cone cells. However the clear lack of colocalization of CRIP1a with the majority of CB₁ receptors in the OPL (i.e. rod photoreceptors) argues that CRIP1a only interacts with a subset of CB₁ receptors, at least in retina.

The DGL β staining is non-neuronal, but may still have a role in cannabinoid signaling. There is considerable evidence for a non- CB₁/non-CB₂ cannabinoid-responsive endothelial receptor with a role in regulating vascular function (Begg et al., 2003; Randall et al., 1996; Randall and Kendall, 1997; 1998a). DGL β may participate in the production of 2-AG acting at this unknown receptor. Once this receptor is identified, it will be highly interesting to explore the localization of this receptor relative to DGL β .

Functional implications

OFF cone bipolar cells hyperpolarize with light activation of cones. Endocannabinoids can be produced either following depolarization and calcium influx or through activation of metabotropic glutamate 1/5 or muscarinic M1/3/5 receptors in a calcium-enhanced process (at times other G_q-coupled receptors may play a similar role)(Kano et al., 2009). One plausible mechanism of 2-AG production in OFF 1 cone bipolar cells would be a darkness-

induced release of glutamate followed by calcium-permeable kainate receptor activation in bipolar cells. Retrograde 2-AG may then act on photoreceptor calcium currents. For instance, in the salamander, cone calcium currents are inhibited by the cannabinoid receptor agonist WIN55212-2 (Straiker and Sullivan, 2003). This would represent a feedback inhibition of the OFF cone bipolar cells. Depending on the timing and degree of spread of 2-AG, neighboring rod cells might be modulated by the 2-AG produced by cone bipolar cells. In salamander, rod calcium current responses are potentiated by cannabinoids. However, how cannabinoids influence calcium dynamics in murine rod photoreceptor terminals remains to be determined.

In conclusion, the CB₁ cannabinoid signaling system is composed of CB₁ receptors and an assortment of proteins that produce and break down eCBs as well as proteins that may interact with CB₁. We have made use of antibodies against many of the proteins suspected or known to fill these roles. This gives us an intriguing view of the synaptic architecture of cannabinoid signaling in the murine retina. Our results point to separate retrograde signaling circuitry at Type 1 OFF bipolar cell-cone photoreceptor synapses and at bipolar cell-rod photoreceptor synapses. Our results also identify additional possibilities for other cannabinoid-related proteins, including widely distributed ABHD6 and FAAH, a strong RPE presence for NAAA, cone terminal localization for CRIP1a and presence in blood vessels for DGL β .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations

Δ^9 -THC	Δ^9 -tetrahydrocannabinol
CB ₁ and CB ₂	cannabinoid receptor 1 and 2
eCBs	endocannabinoids
DGL α/β	diacylglycerol lipase α and β
2-AG	2-arachidonoyl glycerol
MGL	monoacylglycerol lipase
ABHD6 and 12	α/β -hydrolase domain 6 and 12
CRIP1a	cannabinoid receptor interacting protein 1a
FAAH	fatty acid amide hydrolase
NAAA	<i>N</i> -acylethanolamine-hydrolyzing acid amidase
anandamide or AEA	<i>N</i> -arachidonoyl ethanolamine
RPE	retinal pigment epithelium

IS	inner segments
IPL	inner plexiform layer
OPL	outer plexiform layer
INL	inner nuclear layer
GCL	ganglion cell layer
PEA	palmitoyl ethanolamine
NAPE-PLD	<i>N</i> -acyl phosphatidylethanolamine phospholipase D
TRPV1	transient receptor potential vanilloid type-1
COX-2	cyclooxygenase-2

LITERATURE CITED

- Bacci A, Huguenard JR, Prince DA. Long-lasting self-inhibition of neocortical interneurons mediated by endocannabinoids. *Nature*. 2004; 431(7006):312–316. [PubMed: 15372034]
- Begg M, Mo FM, Offertaler L, Batkai S, Pacher P, Razdan RK, Lovinger DM, Kunos G. G protein-coupled endothelial receptor for atypical cannabinoid ligands modulates a Ca²⁺-dependent K⁺ current. *J Biol Chem*. 2003; 278(46):46188–46194. [PubMed: 12952947]
- Bergmann M, Grabs D, Roder J, Rager G, Jeromin A. Differential expression of neuronal calcium sensor-1 in the developing chick retina. *J Comp Neurol*. 2002; 449(3):231–240. [PubMed: 12115677]
- Bisogno T, Delton-Vandenbroucke I, Milone A, Lagarde M, Di Marzo V. Biosynthesis and inactivation of N-arachidonylethanolamine (anandamide) and N-docosahexaenylethanolamine in bovine retina. *Arch Biochem Biophys*. 1999; 370(2):300–307. [PubMed: 10577359]
- Bisogno T, Howell F, Williams G, Minassi A, Cascio MG, Ligresti A, Matias I, Schiano-Moriello A, Paul P, Williams EJ, Gangadharan U, Hobbs C, Di Marzo V, Doherty P. Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. *J Cell Biol*. 2003; 163(3):463–468. [PubMed: 14610053]
- Blankman JL, Simon GM, Cravatt BF. A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. *Chemistry & biology*. 2007; 14(12):1347–1356. [PubMed: 18096503]
- Bodor AL, Katona I, Nyiri G, Mackie K, Ledent C, Hajos N, Freund TF. Endocannabinoid signaling in rat somatosensory cortex: laminar differences and involvement of specific interneuron types. *J Neurosci*. 2005; 25(29):6845–6856. [PubMed: 16033894]
- Bracey MH, Hanson MA, Masuda KR, Stevens RC, Cravatt BF. Structural adaptations in a membrane enzyme that terminates endocannabinoid signaling. *Science*. 2002; 298(5599):1793–1796. [PubMed: 12459591]
- Brecha NC, Sternini C, Humphrey MF. Cellular distribution of L-glutamate decarboxylase (GAD) and gamma-aminobutyric acidA (GABAA) receptor mRNAs in the retina. *Cell Mol Neurobiol*. 1991; 11(5):497–509. [PubMed: 1660350]
- Buckley NE, Hansson S, Harta G, Mezey E. Expression of the CB1 and CB2 receptor messenger RNAs during embryonic development in the rat. *Neuroscience*. 1998; 82(4):1131–1149. [PubMed: 9466436]
- Burette AC, Strehler EE, Weinberg RJ. “Fast” plasma membrane calcium pump PMCA2a concentrates in GABAergic terminals in the adult rat brain. *J Comp Neurol*. 2009; 512(4):500–513. [PubMed: 19025983]
- Casini G, Brecha NC, Bosco L, Rickman DW. Developmental expression of neurokinin-1 and neurokinin-3 receptors in the rat retina. *J Comp Neurol*. 2000; 421(2):275–287. [PubMed: 10813787]

- Chang YC, Gottlieb DI. Characterization of the proteins purified with monoclonal antibodies to glutamic acid decarboxylase. *J Neurosci*. 1988; 8(6):2123–2130. [PubMed: 3385490]
- Chen J, Matias I, Dinh T, Lu T, Venezia S, Nieves A, Woodward DF, Di Marzo V. Finding of endocannabinoids in human eye tissues: implications for glaucoma. *Biochem Biophys Res Commun*. 2005; 330(4):1062–1067. [PubMed: 15823551]
- Chevalere V, Takahashi KA, Castillo PE. Endocannabinoid-mediated synaptic plasticity in the CNS. *Annu Rev Neurosci*. 2006; 29:37–76. [PubMed: 16776579]
- Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, Lichtman AH. Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc Natl Acad Sci U S A*. 2001; 98(16):9371–9376. [PubMed: 11470906]
- Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB. Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature*. 1996; 384(6604):83–87. [PubMed: 8900284]
- Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science*. 1992; 258(5090):1946–1949. [PubMed: 1470919]
- Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC, Piomelli D. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature*. 1994; 372(6507):686–691. [PubMed: 7990962]
- Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, Kathuria S, Piomelli D. Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc Natl Acad Sci U S A*. 2002; 99(16):10819–10824. [PubMed: 12136125]
- Fan SF, Yazulla S. Biphasic modulation of voltage-dependent currents of retinal cones by cannabinoid CB1 receptor agonist WIN 55212-2. *Vis Neurosci*. 2003; 20(2):177–188. [PubMed: 12916739]
- Fan SF, Yazulla S. Inhibitory interaction of cannabinoid CB1 receptor and dopamine D2 receptor agonists on voltage-gated currents of goldfish cones. *Vis Neurosci*. 2004; 21(1):69–77. [PubMed: 15137583]
- Fan SF, Yazulla S. Reciprocal inhibition of voltage-gated potassium currents ($I_K(V)$) by activation of cannabinoid CB1 and dopamine D1 receptors in ON bipolar cells of goldfish retina. *Vis Neurosci*. 2005; 22(1):55–63. [PubMed: 15842741]
- Fukushima T, Ohtsubo T, Tsuda M, Yanagawa Y, Hori Y. Facilitatory actions of serotonin type 3 receptors on GABAergic inhibitory synaptic transmission in the spinal superficial dorsal horn. *J Neurophysiol*. 2009; 102(3):1459–1471. [PubMed: 19369358]
- Gargini C, Terzibasi E, Mazzoni F, Strettoi E. Retinal organization in the retinal degeneration 10 (rd10) mutant mouse: a morphological and ERG study. *J Comp Neurol*. 2007; 500(2):222–238. [PubMed: 17111372]
- Ghosh KK, Bujan S, Haverkamp S, Feigenspan A, Wassle H. Types of bipolar cells in the mouse retina. *J Comp Neurol*. 2004; 469(1):70–82. [PubMed: 14689473]
- Glaser ST, Deutsch DG, Studholme KM, Zimov S, Yazulla S. Endocannabinoids in the intact retina: 3 H-anandamide uptake, fatty acid amide hydrolase immunoreactivity and hydrolysis of anandamide. *Vis Neurosci*. 2005; 22(6):693–705. [PubMed: 16469181]
- Gulyas AI, Cravatt BF, Bracey MH, Dinh TP, Piomelli D, Boscia F, Freund TF. Segregation of two endocannabinoid-hydrolyzing enzymes into pre- and postsynaptic compartments in the rat hippocampus, cerebellum and amygdala. *Eur J Neurosci*. 2004; 20(2):441–458. [PubMed: 15233753]
- Guo Y, Wang H, Okamoto Y, Ueda N, Kingsley PJ, Marnett LJ, Schmid HH, Das SK, Dey SK. N-acylphosphatidylethanolamine-hydrolyzing phospholipase D is an important determinant of uterine anandamide levels during implantation. *J Biol Chem*. 2005; 280(25):23429–23432. [PubMed: 15890658]
- Hajos N, Katona I, Naiem SS, MacKie K, Ledent C, Mody I, Freund TF. Cannabinoids inhibit hippocampal GABAergic transmission and network oscillations. *Eur J Neurosci*. 2000; 12(9):3239–3249. [PubMed: 10998107]

- Hartveit E. Functional organization of cone bipolar cells in the rat retina. *J Neurophysiol.* 1997; 77(4): 1716–1730. [PubMed: 9114231]
- Hashimoto-dani Y, Ohno-Shosaku T, Kano M. Presynaptic monoacylglycerol lipase activity determines basal endocannabinoid tone and terminates retrograde endocannabinoid signaling in the hippocampus. *J Neurosci.* 2007; 27(5):1211–1219. [PubMed: 17267577]
- Haverkamp S, Ghosh KK, Hirano AA, Wassle H. Immunocytochemical description of five bipolar cell types of the mouse retina. *J Comp Neurol.* 2003; 455(4):463–476. [PubMed: 12508320]
- Haverkamp S, Wassle H. Immunocytochemical analysis of the mouse retina. *J Comp Neurol.* 2000; 424(1):1–23. [PubMed: 10888735]
- Hendrickson A, Troilo D, Djajadi H, Possin D, Springer A. Expression of synaptic and phototransduction markers during photoreceptor development in the marmoset monkey *Callithrix jacchus*. *J Comp Neurol.* 2009; 512(2):218–231. [PubMed: 19003975]
- Herkenham M, Lynn AB, Johnson MR, Melvin LS, de Costa BR, Rice KC. Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *J Neurosci.* 1991; 11(2):563–583. [PubMed: 1992016]
- Howlett AC, Breivogel CS, Childers SR, Deadwyler SA, Hampson RE, Porrino LJ. Cannabinoid physiology and pharmacology: 30 years of progress. *Neuropharmacology.* 2004; 47(Suppl 1):345–358. [PubMed: 15464149]
- Huang L, Max M, Margolskee RF, Su H, Masland RH, Euler T. G protein subunit G gamma 13 is coexpressed with G alpha o, G beta 3, and G beta 4 in retinal ON bipolar cells. *J Comp Neurol.* 2003; 455(1):1–10. [PubMed: 12454992]
- Jevince AR, Kadison SR, Pittman AJ, Chien CB, Kaprielian Z. Distribution of EphB receptors and ephrin-B1 in the developing vertebrate spinal cord. *J Comp Neurol.* 2006; 497(5):734–750. [PubMed: 16786562]
- Kaneda K, Isa K, Yanagawa Y, Isa T. Nigral inhibition of GABAergic neurons in mouse superior colliculus. *J Neurosci.* 2008; 28(43):11071–11078. [PubMed: 18945914]
- Kaneko K, Tamamaki N, Owada H, Kakizaki T, Kume N, Totsuka M, Yamamoto T, Yawo H, Yagi T, Obata K, Yanagawa Y. Noradrenergic excitation of a subpopulation of GABAergic cells in the basolateral amygdala via both activation of nonselective cationic conductance and suppression of resting K⁺ conductance: a study using glutamate decarboxylase 67-green fluorescent protein knock-in mice. *Neuroscience.* 2008; 157(4):781–797. [PubMed: 18950687]
- Kano M, Ohno-Shosaku T, Hashimoto-dani Y, Uchigashima M, Watanabe M. Endocannabinoid-mediated control of synaptic transmission. *Physiol Rev.* 2009; 89(1):309–380. [PubMed: 19126760]
- Katona I, Urban GM, Wallace M, Ledent C, Jung KM, Piomelli D, Mackie K, Freund TF. Molecular composition of the endocannabinoid system at glutamatergic synapses. *J Neurosci.* 2006; 26(21): 5628–5637. [PubMed: 16723519]
- Kim J, Alger BE. Inhibition of cyclooxygenase-2 potentiates retrograde endocannabinoid effects in hippocampus. *Nat Neurosci.* 2004; 7(7):697–698. [PubMed: 15184902]
- Koulen P, Fletcher EL, Craven SE, Brecht DS, Wassle H. Immunocytochemical localization of the postsynaptic density protein PSD-95 in the mammalian retina. *J Neurosci.* 1998; 18(23):10136–10149. [PubMed: 9822767]
- Kozak KR, Rowlinson SW, Marnett LJ. Oxygenation of the endocannabinoid, 2-arachidonylglycerol, to glyceryl prostaglandins by cyclooxygenase-2. *J Biol Chem.* 2000; 275(43):33744–33749. [PubMed: 10931854]
- Kreitzer AC, Carter AG, Regehr WG. Inhibition of interneuron firing extends the spread of endocannabinoid signaling in the cerebellum. *Neuron.* 2002; 34(5):787–796. [PubMed: 12062024]
- Kreitzer AC, Regehr WG. Cerebellar depolarization-induced suppression of inhibition is mediated by endogenous cannabinoids. *J Neurosci.* 2001; 21(20):RC174. [PubMed: 11588204]
- Lalonde MR, Jollimore CA, Stevens K, Barnes S, Kelly ME. Cannabinoid receptor-mediated inhibition of calcium signaling in rat retinal ganglion cells. *Mol Vis.* 2006; 12:1160–1166. [PubMed: 17093402]

- Leung D, Saghatelian A, Simon GM, Cravatt BF. Inactivation of N-acyl phosphatidylethanolamine phospholipase D reveals multiple mechanisms for the biosynthesis of endocannabinoids. *Biochemistry*. 2006; 45(15):4720–4726. [PubMed: 16605240]
- Maione S, Cristino L, Migliozi AL, Georgiou AL, Starowicz K, Salt TE, Di Marzo V. TRPV1 channels control synaptic plasticity in the developing superior colliculus. *J Physiol*. 2009; 587(Pt 11):2521–2535. [PubMed: 19406878]
- Matias I, Wang JW, Moriello AS, Nieves A, Woodward DF, Di Marzo V. Changes in endocannabinoid and palmitoylethanolamide levels in eye tissues of patients with diabetic retinopathy and age-related macular degeneration. *Prostaglandins Leukot Essent Fatty Acids*. 2006; 75(6):413–418. [PubMed: 17011761]
- Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*. 1990; 346(6284):561–564. [PubMed: 2165569]
- Matsuda S, Kanemitsu N, Nakamura A, Mimura Y, Ueda N, Kurahashi Y, Yamamoto S. Metabolism of anandamide, an endogenous cannabinoid receptor ligand, in porcine ocular tissues. *Exp Eye Res*. 1997; 64(5):707–711. [PubMed: 9245900]
- Matyas F, Urban GM, Watanabe M, Mackie K, Zimmer A, Freund TF, Katona I. Identification of the sites of 2-arachidonoylglycerol synthesis and action imply retrograde endocannabinoid signaling at both GABAergic and glutamatergic synapses in the ventral tegmental area. *Neuropharmacology*. 2008; 54(1):95–107. [PubMed: 17655884]
- May CA, Nakamura K, Fujiyama F, Yanagawa Y. Quantification and characterization of GABA-ergic amacrine cells in the retina of GAD67-GFP knock-in mice. *Acta Ophthalmol*. 2008; 86(4):395–400. [PubMed: 17995983]
- McKinney MK, Cravatt BF. Structure and function of fatty acid amide hydrolase. *Annu Rev Biochem*. 2005; 74:411–432. [PubMed: 15952893]
- Milam AH, Dacey DM, Dizhoor AM. Recoverin immunoreactivity in mammalian cone bipolar cells. *Vis Neurosci*. 1993; 10(1):1–12. [PubMed: 8424920]
- Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature*. 1993; 365(6441):61–65. [PubMed: 7689702]
- Negishi K, Kato S, Teranishi T. Dopamine cells and rod bipolar cells contain protein kinase C-like immunoreactivity in some vertebrate retinas. *Neurosci Lett*. 1988; 94(3):247–252. [PubMed: 3205402]
- Niehaus JL, Liu Y, Wallis KT, Egertova M, Bhartur SG, Mukhopadhyay S, Shi S, He H, Selley DE, Howlett AC, Elphick MR, Lewis DL. CB1 cannabinoid receptor activity is modulated by the cannabinoid receptor interacting protein CRIP 1a. *Mol Pharmacol*. 2007; 72(6):1557–1566. [PubMed: 17895407]
- Okabe S, Shiomura Y, Hirokawa N. Immunocytochemical localization of microtubule-associated proteins 1A and 2 in the rat retina. *Brain Res*. 1989; 483(2):335–346. [PubMed: 2706525]
- Okamoto Y, Morishita J, Tsuboi K, Tonai T, Ueda N. Molecular characterization of a phospholipase D generating anandamide and its congeners. *J Biol Chem*. 2004; 279(7):5298–5305. [PubMed: 14634025]
- Patricelli MP, Lashuel HA, Giang DK, Kelly JW, Cravatt BF. Comparative characterization of a wild type and transmembrane domain-deleted fatty acid amide hydrolase: identification of the transmembrane domain as a site for oligomerization. *Biochem*. 1998; 37(43):15177–15187. [PubMed: 9790682]
- Pignatelli V, Strettoi E. Bipolar cells of the mouse retina: a gene gun, morphological study. *J Comp Neurol*. 2004; 476(3):254–266. [PubMed: 15269969]
- Porcella A, Casellas P, Gessa GL, Pani L. Cannabinoid receptor CB1 mRNA is highly expressed in the rat ciliary body: implications for the antiglaucoma properties of marihuana. *Brain Res Mol Brain Res*. 1998; 58(1-2):240–245. [PubMed: 9685662]
- Porcella A, Maxia C, Gessa GL, Pani L. The human eye expresses high levels of CB1 cannabinoid receptor mRNA and protein. *Eur J Neurosci*. 2000; 12(3):1123–1127. [PubMed: 10762343]

- Qin N, Neep MP, Liu Y, Hutchinson TL, Lubin ML, Flores CM. TRPV2 is activated by cannabidiol and mediates CGRP release in cultured rat dorsal root ganglion neurons. *J Neurosci*. 2008; 28(24): 6231–6238. [PubMed: 18550765]
- Rabie A, Thomasset M, Parkes CO, Clavel MC. Immunocytochemical detection of 28 000-MW calcium-binding protein in horizontal cells of the rat retina. *Cell Tissue Res*. 1985; 240(2):493–496. [PubMed: 3995565]
- Randall MD, Alexander SP, Bennett T, Boyd EA, Fry JR, Gardiner SM, Kemp PA, McCulloch AI, Kendall DA. An endogenous cannabinoid as an endothelium-derived vasorelaxant. *Biochem Biophys Res Commun*. 1996; 229(1):114–120. [PubMed: 8954092]
- Randall MD, Kendall DA. Involvement of a cannabinoid in endothelium-derived hyperpolarizing factor-mediated coronary vasorelaxation. *Eur J Pharmacol*. 1997; 335(2-3):205–209. [PubMed: 9369375]
- Randall MD, Kendall DA. Anandamide and endothelium-derived hyperpolarizing factor act via a common vasorelaxant mechanism in rat mesentery. *Eur J Pharmacol*. 1998a; 346(1):51–53. [PubMed: 9617751]
- Randall MD, Kendall DA. Endocannabinoids: a new class of vasoactive substances. *Trends Pharmacol Sci*. 1998b; 19(2):55–58. [PubMed: 9550942]
- Rohrenbeck J, Wassle H, Heizmann CW. Immunocytochemical labelling of horizontal cells in mammalian retina using antibodies against calcium-binding proteins. *Neurosci Lett*. 1987; 77(3): 255–260. [PubMed: 3302765]
- Schlicker E, Timm J, Gothert M. Cannabinoid receptor-mediated inhibition of dopamine release in the retina. *Naunyn Schmiedebergs Arch Pharmacol*. 1996; 354(6):791–795. [PubMed: 8971741]
- Schreiner DS, Jande SS, Lawson DE. Target cells of vitamin D in the vertebrate retina. *Acta Anat (Basel)*. 1985; 121(3):153–162. [PubMed: 3885666]
- Shen M, Thayer SA. The cannabinoid agonist Win55,212-2 inhibits calcium channels by receptor-mediated and direct pathways in cultured rat hippocampal neurons. *Brain Res*. 1998; 783(1):77–84. [PubMed: 9479052]
- Shiells R, Falk G. Retinal on-bipolar cells contain a nitric oxide-sensitive guanylate cyclase. *Neuroreport*. 1992; 3(10):845–848. [PubMed: 1358250]
- Simon GM, Cravatt BF. Endocannabinoid biosynthesis proceeding through glycerophospho-N-acyl ethanolamine and a role for alpha/beta-hydrolase 4 in this pathway. *J Biol Chem*. 2006; 281(36): 26465–26472. [PubMed: 16818490]
- Sjostrom PJ, Turrigiano GG, Nelson SB. Neocortical LTD via coincident activation of presynaptic NMDA and cannabinoid receptors. *Neuron*. 2003; 39(4):641–654. [PubMed: 12925278]
- Solorzano C, Zhu C, Battista N, Astarita G, Lodola A, Rivara S, Mor M, Russo R, Maccarrone M, Antonietti F, Duranti A, Tontini A, Cuzzocrea S, Tarzia G, Piomelli D. Selective N-acylethanolamine-hydrolyzing acid amidase inhibition reveals a key role for endogenous palmitoylethanolamide in inflammation. *Proc Natl Acad Sci U S A*. 2009; 106(49):20966–20971. [PubMed: 19926854]
- Stella N, Schweitzer P, Piomelli D. A second endogenous cannabinoid that modulates long-term potentiation. *Nature*. 1997; 388(6644):773–778. [PubMed: 9285589]
- Straiker A, Hu SS, Long J, Arnold A, Wager-Miller J, Cravatt BF, Mackie K. Monoacyl glycerol lipase (MGL) limits the duration of endocannabinoid-mediated depolarization-induced suppression of excitation (DSE) in autaptic hippocampal neurons. *Mol Pharmacol*. 2009
- Straiker A, Mackie K. Cannabinoid signaling in inhibitory autaptic hippocampal neurons. *Neuroscience*. 2009
- Straiker A, Stella N, Piomelli D, Mackie K, Karten HJ, Maguire G. Cannabinoid CB1 receptors and ligands in vertebrate retina: localization and function of an endogenous signaling system. *Proc Natl Acad Sci U S A*. 1999a; 96(25):14565–14570. [PubMed: 10588745]
- Straiker A, Sullivan JM. Cannabinoid receptor activation differentially modulates ion channels in photoreceptors of the tiger salamander. *J Neurophysiol*. 2003; 89(5):2647–2654. [PubMed: 12740409]

- Straiker AJ, Maguire G, Mackie K, Lindsey J. Localization of cannabinoid CB1 receptors in the human anterior eye and retina. *Invest Ophthalmol Vis Sci*. 1999b; 40(10):2442–2448. [PubMed: 10476817]
- Struik ML, Yazulla S, Kamermans M. Cannabinoid agonist WIN 55212-2 speeds up the cone response to light offset in goldfish retina. *Vis Neurosci*. 2006; 23(2):285–293. [PubMed: 16638179]
- Tamamaki N, Yanagawa Y, Tomioka R, Miyazaki J, Obata K, Kaneko T. Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. *J Comp Neurol*. 2003; 467(1):60–79. [PubMed: 14574680]
- Tsuboi K, Sun YX, Okamoto Y, Araki N, Tonai T, Ueda N. Molecular characterization of N-acyl ethanolamine-hydrolyzing acid amidase, a novel member of the cholesteryl glycerophosphorylcholine hydrolase family with structural and functional similarity to acid ceramidase. *J Biol Chem*. 2005; 280(12):11082–11092. [PubMed: 15655246]
- Wahlin KJ, Moreira EF, Huang H, Yu N, Adler R. Molecular dynamics of photoreceptor synapse formation in the developing chick retina. *J Comp Neurol*. 2008; 506(5):822–837. [PubMed: 18076030]
- Warrier A, Wilson M. Endocannabinoid signaling regulates spontaneous transmitter release from embryonic retinal amacrine cells. *Vis Neurosci*. 2007; 24(1):25–35. [PubMed: 17430607]
- Wassle H, Puller C, Müller F, Haverkamp S. Cone contacts, mosaics, and territories of bipolar cells in the mouse retina. *Journal of Neuroscience*. 2009; 29(1):106–117. [PubMed: 19129389]
- Wilson RI, Nicoll RA. Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. *Nature*. 2001; 410(6828):588–592. [PubMed: 11279497]
- Yazulla S. Endocannabinoids in the retina: from marijuana to neuroprotection. *Prog Retin Eye Res*. 2008; 27(5):501–526. [PubMed: 18725316]
- Yazulla S, Studholme KM, McIntosh HH, Deutsch DG. Immunocytochemical localization of cannabinoid CB1 receptor and fatty acid amide hydrolase in rat retina. *J Comp Neurol*. 1999; 415(1):80–90. [PubMed: 10540359]
- Yazulla S, Studholme KM, McIntosh HH, Fan SF. Cannabinoid receptors on goldfish retinal bipolar cells: electron-microscope immunocytochemistry and whole-cell recordings. *Vis Neurosci*. 2000; 17(3):391–401. [PubMed: 10910107]

Figure 1. Diacylglycerol lipase α (DGL α) is present in the postsynaptic terminals of Type 1 OFF cone bipolar cells

A, B) Paired images taken under same settings in mouse retina show DGL α staining alone (A) and DGL α coincubated with immunizing protein (B, IP 5 μ g/mL) which blocks the DGL α staining. Scale bar 50 μ m. C) Combined fluorescence and Nomarski image shows relative localization of DGL α staining, prominent in clusters in the outer plexiform layer (OPL) and more diffusely distributed in the inner plexiform layer (IPL). Scale bar: 50 μ m. D) In a compressed Z-series DGL α (red) does not costain with PKC (green), a marker for rod bipolar cells, in the OPL. Scale bar: 25 μ m. E) In higher magnification image of single plane, DGL α (red) clearly does not overlap with PKC (green) in OPL. Scale bar: 15 μ m. F) DGL α (red) does not colocalize with G γ 13 (green, a marker for ON-bipolar cells). Scale bar: 25 μ m. G) DGL α (red) does not overlap with Type 2 OFF bipolar cell marker recoverin (green). Scale bar: 25 μ m. H) DGL α (red) colocalizes with post-synaptic terminals of Type 1 OFF bipolar cells, as marked by NK3R (green, arrows). Scale bar: 25 μ m. I) CB $_1$ (red) does not colocalize with horizontal cell marker calbindin (green) in OPL. Scale bar: 15 μ m. J) Flattened Z-series indicates CB $_1$ (red) closely juxtaposes distal to DGL α (green), consistent with rod spherule and cone pedicle localization. K) CB $_1$ (red) costaining with PSD95, which outlines rod spherules (arrows) and cone pedicles (angled arrows) shows CB $_1$ present within terminals of both rod and cone photoreceptor terminals. Scale bar: 25 μ m. A magenta-green version of this figure is available as Supplementary Figure 1.

Figure 2. DGL α labeling is widely distributed throughout the IPL

Aside from the pronounced cone bipolar cell staining, dimmer punctate DGL α is also present in the OPL. A) punctate DGL α is present proximal to rod spherules. In the IPL, DGL α is chiefly present in the distal two-thirds of the IPL, but without prominent lamination. Scale bar: 10 μ m. B) DGL α (red) does not costain with putative GAD67-GFP neurons and processes (green). Scale bar: 25 μ m. C) DGL α (red) does not costain with CB₁ (green). Scale bar: 20 μ m. A magenta-green version of this figure is available as Supplementary Figure 2.

Figure 3. MGL is present in rod spherules and cone pedicles in the OPL and is expressed prominently in two laminae of the IPL

A, B) Paired images taken under same conditions in mouse retina show MGL staining alone (A) and MGL coincubated with immunizing protein (B, IP 5 μg/mL) which blocks the staining. Scale bars: 60 μm. C) Combined fluorescence and Nomarski image shows relative localization of MGL staining, most prominent in the two laminae of the IPL (arrows), and in the ganglion cell layer (GCL), but also in the OPL. Scale bar: 50 μm. D) MGL (red) staining with DGLα (green) shows their apparent non-overlap and relative localization. Scale bar: 25 μm. E) MGL costaining with GAD65, a marker for inhibitory neurons, shows little if any colocalization. Scale bar: 10 μm. F) MGL (red) costaining with PSD95 (green), a marker that outlines rod and cone terminals shows MGL within a cone terminal (arrow) as well as punctate staining within multiple rod spherules. Scale bar: 15 μm. G) MGL (red) costaining with SV2 (green), a marker for rod spherules, shows substantial overlap in the OPL (arrows). Scale bar: 25 μm. A magenta-green version of this figure is available as Supplementary Figure 3.

Figure 4. ABHD6 staining is widely distributed in IPL, GCL and INL

A) Paired images taken under same settings in mouse retina show ABHD6 staining alone (A) and ABHD6 coincubated with immunizing protein (B, IP 5 $\mu\text{g/mL}$) which blocks the ABHD6 staining. Scale bars: 50 μm . C) Flattened Z-series shows relative localization of ABHD6 staining, with clearly labeled neuronal populations in the proximal INL (downward arrows) and in the GCL (upward arrowheads). Scale bar: 50 μm . D) Costaining of ABHD6 (green) with DGL α (red) reveals neither colocalization nor apparent juxtaposition. Scale bar: 15 μm . A magenta-green version of this figure is available as Supplementary Figure 4.

Figure 5. ABHD6 is present in calbindin and GAD67-positive amacrine cells as well as dendritic MAP2-positive processes

A) ABHD6 costaining with rod bipolar cell marker PKC shows no overlap with rod bipolar cell terminals or processes. Scale bar: 25 μ m. B) Costaining with calbindin reveals clear overlap with calbindin-positive amacrine cells (arrows). Scale bar: 15 μ m. C) ABHD6 colocalizes with MAP2 in some processes of the proximal IPL. Scale bar: 10 μ m. D) ABHD6 colocalizes with GAD67-GFP presumptive inhibitory amacrine cells in the INL. Scale bar: 40 μ m. A magenta-green version of this figure is available as Supplementary Figure 5.

Figure 6. Cannabinoid receptor interacting protein 1a (CRIP1a) expression is limited to a population of calbindin-positive amacrine cells

A, B) Coincubation with immunizing protein (IP 5 $\mu\text{g/mL}$) blocks the staining (control image taken at same settings). Scale bars: 50 μm . C-F) CRIP1a antibody recognizes HA-hCRIP1a transiently expressed in HEK cells. HEK cells were transiently transfected with HA-tagged hCRIP1a and stained using HA- and hCRIP1a- antibodies. C) DAPI staining identifies cell nuclei (arrowheads). D) HA antibody (red) staining shows two HA-hCRIP1a transfected cells (nuclei indicated by arrows). E) hCRIP1a staining (green) labels the same cells identified with the HA antibody. F) Overlay of HA- and hCRIP1a-images shows both antibodies overlap completely (yellow), DAPI fluorescence identifies all cells in the field. The non-transfected cells are not labeled with the hCRIP1a antibody. Scale bar: 15 μm . G) CRIP1a (red) and calbindin (green) show occasional CRIP1a/calbindin-positive amacrine cells (arrows). Scale bar: 25 μm . H) The same combination in the OPL shows the relative position of CRIP1a in OPL. Scale bar: 25 μm . A magenta-green version of this figure is available as Supplementary Figure 6.

Figure 7. Cannabinoid receptor interacting protein 1a (CRIP1a) expression is limited to presynaptic terminals of cone photoreceptors juxtaposed to Type 1 OFF cone bipolar cells
 A) In a compressed Z-series, CRIP1a (red) juxtaposes distal to DGL α (green) in OPL. Scale bar: 25 μ m. B-D) CRIP1a costaining with PSD95, shows that CRIP1a is limited to presynaptic terminal of cone photoreceptors. Scale bar: 5 μ m. E) CRIP1a (green) staining is close to that of CB₁ (red) in OPL suggesting that both are present in the same cone photoreceptor presynaptic terminals. Scale bar: 5 μ m. A magenta-green version of this figure is available as Supplementary Figure 7.

Figure 8. DGL β is expressed exclusively in blood vessels

A) Image shows DGL β staining in mouse retina. B) Coincubation with immunizing protein (IP 5 μ g/mL) blocks the DGL β staining (control image taken at same settings). C) Combined fluorescence and Nomarski image shows relative localization of DGL β staining (arrows). D) Flattened Z-series shows that the staining for DGL β is restricted to blood vessels. Scale bars: 60 μ m. A magenta-green version of this figure is available as Supplementary Figure 8.

Figure 9. Fatty acid amide hydrolase (FAAH) is widely expressed in the inner segments (IS), outer nuclear layer (ONL), and ganglion cell layer (GCL), and in the axon terminals of photoreceptors in the outer plexiform layer (OPL)

FAAH is widely expressed in wild-type (WT) mouse retina (A), but not in FAAH knockout (KO) mouse retina (B). Scale bar: 25 μm . C) FAAH (green) costaining with PSD95 (red), a marker that outlines rod and cone terminals, shows FAAH within rod terminals (arrows). Scale bar: 10 μm . D, E) In a compressed Z-series FAAH staining (green, arrows) does not overlap with PKC (red), a marker for rod bipolar cells, in the OPL. Scale bar: 5 μm . F) FAAH (green) is not expressed in calbindin (red)-positive horizontal cell body. G) FAAH (green) also does not costain with calbindin (red)-positive horizontal axonal terminals (arrows). Scale bar: 15 μm . H) Costaining of FAAH (green) and CB₁ (red) shows overlap (in yellow) in subpopulation of amacrine cells in INL and in cells in the GCL. Scale bar: 25 μm . I) In a higher magnification image of a compressed Z-series FAAH (green) costains with CB₁ (red) (arrows) within rod photoreceptor terminals in OPL (arrows). Scale bar: 10 μm . A magenta-green version of this figure is available as Supplementary Figure 9.

Figure 10. *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) expression is limited to retinal pigment epithelium (RPE)

A-D) NAAA antibody recognizes rNAAA transiently expressed in HEK cells. HEK cells were transiently transfected with FLAG-tagged rNAAA and stained using FLAG and NAAA antibodies. A) DAPI staining identifies cell nuclei (arrowheads). B) FLAG antibody (red) staining shows four FLAG-rNAAA transfected cells (nuclei indicated by arrows). C) rNAAA staining (green) labels the same cells identified with the FLAG antibody. D) Overlay of FLAG- and rNAAA-images shows both antibodies overlap completely, DAPI fluorescence identifies all cells in the field. The non-transfected cells are not labeled with the rNAAA antibody. Scale bar: 15 µm. E) Fluorescence image shows NAAA expression limited to RPE. F) Image taken at same settings of NAAA coincubated with immunizing protein (IP 10 µg/mL). Scale bar: 60 µm. A magenta-green version of this figure is available as Supplementary Figure 10.

Table 1

Antibodies used in this study.

Antibody	Immunogen	Manufacturer, catalog number, species, mono- vs. polyclonal	Dilution used
mMGL	MGL-GST fusion protein, aa 172-206 of mouse MGL	Mackie lab (Straiker et al., 2009), rabbit polyclonal	1:400
mABHD6	ABHD6-GST fusion protein, aa 104-141 of mouse ABHD6	Mackie lab (Straiker et al., 2009), rabbit polyclonal	1:1000
rNAAA	NAAA-GST fusion protein, aa 261-275 of rat NAAA	Mackie lab (characterized in this study), rabbit polyclonal	1:400
rDGL α	DGL α -GST fusion protein, aa 790-908 of rat DGL α	Mackie lab (Berghuis et al., 2007), guinea pig polyclonal	1:600
rDGL α	DGL α -GST fusion protein, aa 790-908 of rat DGL α	Mackie lab (Berghuis et al., 2007), rabbit polyclonal	1:600
rDGL β	DGL β -GST fusion protein, aa 203-283 of rat DGL β	Mackie lab (Berghuis et al., 2007), rabbit polyclonal	1:600
rCB1-L15	CB1-GST fusion protein, aa 460-473 of rat CB1	Mackie lab (Bodor et al., 2005), rabbit polyclonal	1:1000
rCB1-CT	CB1-GST fusion protein, aa 401-473 of rat CB1	Mackie lab (Hájos et al., 2000), rabbit polyclonal	1:1000
hCRIP1a	CRIP1a-GST fusion protein, full-length protein of human CRIP1a	Mackie lab (characterized in this study), rabbit polyclonal	1:400
FAAH	Δ TM-FAAH which was raised against the purified transmembrane-deleted FAAH-GST fusion protein, aa 38-579 of rat FAAH	Cravatt lab (Bracey et al., 2002; Patricelli et al., 1998), rabbit polyclonal	1:100
Calbindin	Purified bovine kidney calbindin-D28K	Sigma-Aldrich, St. Louis, MO, #C9848, mouse monoclonal	1:2000
Parvalbumin	Purified frog muscle parvalbumin	Sigma-Aldrich, St. Louis, MO, #P3088, mouse monoclonal	1:1000
GAD65	Affinity-purified glutamic acid decarboxylase (GAD65) from rat brain	Developmental Studies Hybridoma Bank, Iowa City, IA, mouse monoclonal	1:600
SV2	Synaptic vesicles purified from the <i>Ommata</i> electric organ	Developmental Studies Hybridoma Bank, Iowa City, IA, mouse monoclonal	1:2000
MAP2	Microtubule-associated protein 2 (MAP2) purified from rat brain	Millipore, Temecula, CA, #MAB3418, mouse polyclonal	1:1000
PKC (α , β 1, β 2)	PKC purified from bovine brain, and reacts with PKC α , β 1, and β 2 isoforms	BioDesign, Saco, ME, #K01107M, mouse monoclonal	1:200
PSD95	Purified recombinant rat PSD-95 (Post Synaptic Density 95 kDa)	Genetex, Irvine, CA, #GTX80682, mouse monoclonal	1:1000
Recoverin	Recombinant human recoverin protein	Chemicon, Temecula, CA, #AB5585, rabbit polyclonal	1:2000
G γ 13	Synthetic peptide aa 47–59 of mouse G γ 13	Dr. Zaza Kokrashvili, Mount Sinai School of Medicine, New York City, NY, rabbit polyclonal	1:200
NK3R	Synthetic peptide of the C-terminus of rat NK-3 (aa 438–452) conjugated to bovine thyroglobulin	Abcam, Cambridge, MA, #ab7, rabbit polyclonal	1:2000