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Endocannabinoids in the Retina: From Marijuana to Neuroprotection

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Abstract

The active component of the marijuana plant *Cannabis sativa*, Δ^9-tetrahydrocannabinol (THC), produces numerous beneficial effects, including analgesia, appetite stimulation and nausea reduction, in addition to its psychotropic effects. THC mimics the action of endogenous fatty acid derivatives, referred to as endocannabinoids. The effects of THC and the endocannabinoids are mediated largely by metabotropic receptors that are distributed throughout the nervous and peripheral organ systems. There is great interest in endocannabinoids for their role in neuroplasticity as well as for therapeutic use in numerous conditions, including pain, stroke, cancer, obesity, osteoporosis, fertility, neurodegenerative diseases, multiple sclerosis, glaucoma and inflammatory diseases, among others. However, there has been relatively far less research on this topic in the eye and retina compared with the brain and other organ systems. The purpose of this review is to introduce the “cannabinergic” field to the retinal community. All of the fundamental work on cannabinoids has been performed in non-retinal preparations, necessitating extensive dependence on this literature for background. Happily, the retinal cannabinoid system has much in common with other regions of the central nervous system. For example, there is general agreement that cannabinoids suppress dopamine release and presynaptically reduce transmitter release from cones and bipolar cells. How these effects relate to light and dark adaptation, receptive field formation, temporal properties of ganglion cells or visual perception are unknown. The presence of multiple endocannabinoids, degradative enzymes with their bioactive metabolites, and receptors provides a broad spectrum of opportunities for basic research and to identify targets for therapeutic application to retinal diseases.

Keywords

retina; eye; endocannabinoid; cannabinoid; marijuana

1. Introduction

Marijuana, pot, hashish, reefer, clip, roach, bong and munchies are words that need no introduction or definition for most people. Depending on your age, they may conjure up images of Vietnam-era hippies, coffee houses, the New Orleans jazz scene, student dorm life, “Harold and Kumar”, and so on. For many others, these words evoke images of a gateway drug and serious drug addition. Marijuana has been cultivated for thousands of years for manufacturing and recreational use. Its negative image as a dangerous drug of abuse has hampered research on the mechanisms by which marijuana exerts its physiological and psychotropic effects. A

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certain “giggle” factor accompanies the Introduction to a general audience of any speaker who studies the biology or pharmacology of marijuana. Chuckles devolve into laughter when I mention that, on two occasions, my studies were carried out in Maarten Kamerman's laboratory - in Amsterdam. Such a reaction has not and does not occur with a researcher who studies the opiates or dopamine transporters. No one glibly questions as to whether samples of morphine or heroin were injected or cocaine snorted during experiments on pain or drug addition. The fact is that the effects of marijuana can be detected in virtually every organ system in the body. We now know that the active component of marijuana, Δ^9-tetrahydrocannabinol (THC), acts on specific receptors that are distributed throughout the body. These receptors, of course, respond to endogenous ligands. Given the global distribution of this system, there has been extensive research effort into its fundamental properties, and interest from pharmaceutical companies for potential therapeutic use of drugs that modulate these receptors. Progress in this field has exploded in the last ten years. Understanding how marijuana exerts its numerous physiological and psychological effects has advanced greatly since the identification of an endogenous system that is activated by, and mimics many of the effects of marijuana. There is a huge literature, growing by the day, investigating this endogenous system in neural and non-neural tissues. This review will focus on the retina and include, to some extent, other ocular tissues. All of the fundamental work on the characterization and function of the components of this endogenous system has been done in non-retinal preparations. As a result, the background for interpretation of the retinal data must necessarily come from these other studies. Numerous excellent reviews have appeared in the last several years that treat the history, biochemistry, pharmacology and therapeutic potential of this system (for example, Piomelli, 2003; Lambert and Fowler, 2005; Hohmann and Suplita, 2006; Pertwee, 2006; Kogan and Mechoulam, 2007). A comprehensive treatment of all these topics was published by CRC press (Onaivi et al., 2006). In addition, for an entertaining and informative introduction to this topic, I recommend the highly readable book “The Science of Marijuana” by L. Iversen (2000). Also, two older reviews that appeared in Science (Weil et al., 1968; Hollister, 1971) are of particular interest because they were written during the height of the Vietnam War and long before the discovery of the endocannabinoid system. They point out the difficulty of obtaining and interpreting psychophysical data on the effects of smoking marijuana, due largely to problems in adequate controls for concealing the placebo and effects of the drug on attention.

The marijuana plant *Cannabis sativa* has been cultivated as hemp for thousands of years in the Middle East, India and Europe for food and to make rope and fabric. Hemp seeds contain an excellent balance of amino acids, omega 3 and omega 6 fatty acids. With the increased awareness of the importance of dietary omega 3 fatty acids for the control of cholesterol and overall cardiac health benefits, food products containing hemp seed are becoming more available; these seeds do not contain any appreciable THC. “Canvas” is derived from “cannabis”, appearing in English usage in the 13th century. Jamestown, the first settlement in the United States, had a law in 1619 requiring farmers to grow hemp. By 1850 there were over 8000 *Cannabis* plantations in the United States. In antiquity, *Cannabis* also was used therapeutically to relieve pain, reduce inflammation and as a sedative. Its psychotropic effects, though known, were less important in ancient China, Rome and Greece but not so in 15th century Iraq and Egypt. Napoleon returned to France from Egypt with *Cannabis* seeds in 1799; and therein began its widespread use in Europe as an intoxicant. In the latter half of the 19th century, *Cannabis* was freely available and used extensively to treat migraine headaches and ulcers. The word “marijuana” or “marihuana” came from ballads sung by Pancho Villa and his men in the 1890s, giving *Cannabis* its current popular name and English translation “MaryJane”. By the 1930s, the therapeutic use of marijuana was being replaced by more effective drugs to relieve pain and nausea. Also, its long reputation as a drug that corrupted morals and stilted personal initiative grew. For historical context, recall that the U.S. was still in the Prohibition era (1919−1933) and the effects of the Stock Market crash of 1929 were yet to be fully felt. Pulp fiction of the 1930s and the hilariously inept propaganda film “Reefer

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Madness" of 1936 portrayed users of marijuana as hopelessly depraved and driven to homicidal madness. The U.S. Congress (1937) passed the Marijuana Tax Act that made it so expensive and difficult to get marijuana that it was effectively banned. The coup de grace came in 1970 when marijuana was classified as a Class 1 drug, on a par with heroin, LSD and methamphetamine, as lacking any medicinal value with highest addictive properties. The controversy over the biomedical use of smoking marijuana to relieve pain, nausea and appetite stimulation continues today with court battles between State and Federal Laws.

2. Marijuana and the Endocannabinoids

The active component of the marijuana plant *Cannabis sativa* was first identified as Δ^9-tetrahydrocannabinol (THC) (Gaoni and Mechoulam, 1964; Mechoulam and Gaoni, 1967). THC, also known as dronabinol, produces numerous beneficial effects, including analgesia, appetite stimulation, nausea reduction and reduction of intraocular pressure (IOP). THC also affects bone remodeling, fertility, short term memory, tumor growth and motor coordination (Mechoulam, 2002; Iversen, 2003). The therapeutic use of THC has been hampered by psychotropic effects that have prevented general acceptance by the Federal Drug Administration (FDA). Marinol™ is the only FDA-approved cannabinoid agonist for use in the U.S. It is synthetic THC in sesame oil that is prescribed as an appetite stimulant in AIDS, gastric bypass and chemotherapy patients and also as an anti-emetic for chemotherapy. Sativex™ was approved by Health Canada in 2005 to relieve pain and spasticity in multiple sclerosis. Sativex™ is a mouth spray that is made by blending two of the main active ingredients of cannabis, THC and cannabidiol (CBD), in a nearly 1:1 ratio. Sativex™ also is used for cancer pain and should enter Stage III clinical trials for treatment of pain in the U.S. by 2008. An antagonist of a type of cannabinoid receptor (CB1), marketed as Acomplia™ by Sanofi Aventis, has been approved for use as an anti-obesity drug in the European Union. It also blocks the weight gain associated with nicotine withdrawal, reduces visceral fat content and lowers LDL levels. However, Stage III clinical trials of Acomplia™ were halted in the US in June 2007 due to unacceptable dose-dependent side effects. Data, combined from the one year RIO-Lipids, RIO Europe, RIO North America Trials, showed that there was a 2-fold increase in psychiatric (depression, anxiety, irritability) and gastrointestinal (nausea) side effects with 20 mg Acomplia™ compared with placebo. Sanofi subsequently withdrew its application for approval of this drug, renamed Zimulti™, in July 2007. Additional warnings have been issued with its use in the EU.

THC and synthetic cannabinoid agonists mimic endogenous chemicals, referred to as "endocannabinoids" (eCBs) that activate specific membrane receptors. Endocannabinoids are the most recently identified neuromodulation systems. Endocannabinoids are ubiquitous among deuterostomes and are found in neural and non-neural tissues throughout the body. It seems ironic that cannabinoids have not been identified in protostomes, so that the classic genetic preparations of drosophila and C-elegans are not appropriate. The study of eCBs started in earnest with the identification and cloning of specific receptors (Devane et al., 1988; Matsuda et al., 1990; Munro et al., 1993) and the identification of endogenous ligands for these receptors (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995). Endocannabinoids belong to a very large group of bioactive lipids that are derivatives of arachidonic acid, a 20-carbon omega-6 polyunsaturated fatty acid with four double bonds. Arachidonic acid, in addition to being a second messenger, is a precursor of eicosanoids (after the Greek *eikos*- meaning 20, after the 20 carbons). The eicosanoids that are derived from arachidonic acid include the series 2 prostaglandins, series 2 prostacyclines, series 2 thromboxanes, series 3 and 4 leukotrines, all of which are involved in immunity, blood clotting and inflammatory responses to injury, among other actions. Arachidonic acid is a precursor in the synthesis of eicosanoids and a metabolite of eCB degradation.

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Endocannabinoids mediate a wide array of physiological functions, ranging from tumor growth to synaptic plasticity (Chaperon and Thiebot, 1999; Howlett et al., 2004; Bradshaw and Walker, 2005; Fowler et al., 2005; Lambert and Fowler, 2005). Entire journal issues have been devoted to reviews of these topics, including their therapeutic potential (Neuropharmacology 2005:48 (8); Curr Drug Targets, CNS Neurol Disord 2005:4(6); J Neuroendocrinology 2008:20 (Suppl 1); Addiction Biology 2008:13(2)). Russo (2004) reviewed a concept he called "Clinical Endocannabinoid Deficiency" that is based on the idea of an endocannabinoid tone that is important in homeostasis. Analogous to serotonin or catecholamines, disruptions in endocannabinoid tone may underlie some chronic disease states, including migraine, fibromyalgia and irritable bowel syndrome. Endocannabinoids include a large variety of amide-, ester- and ether-derivatives of arachidonic acid. The most widely studied of these are arachidonoyl ethanolamide (anandamide, AEA), *sn*-2 arachidonoyl glycerol (2-AG) and N-arachidonyl dopamine (NADA) (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995; Bisogno et al., 2000) (Fig. 1). The term "anandamide" refers to the Sanskrit "ananda" meaning bliss and to "amide" for the chemical nature of the compound (Devane et al., 1992). Other putative eCBs have been identified that have varying degrees, or no affinity for cannabinoid receptors, but also compete with AEA and 2-AG for the metabolizing enzymes. In this way, they may modulate eCB activity by competition at the receptors or by affecting substrate availability for metabolism. These other eCBs include: O-arachidonyl ethanolamine (virodhamine), 2-arachidonoyl glyceryl ether (noladin ether), N-palmitoyl ethanolamine (PEA) oleoylethanolamide (oleamide, OEA) and others (Lambert and Fowler, 2005). These cannabimimetic fatty acid derivatives, as well as their bioactive metabolites, have been referred to as an "endocannabinoid soup" to describe the diversity of interacting lipid modulators, enzymes and receptors (Fowler, 2007).

2.1. Synthesis and Release

Unlike water soluble transmitters (e.g., amino acids and biogenic amines), AEA and 2-AG are highly lipophilic and not stored in synaptic vesicles. Rather, membrane phospholipids are metabolized 'on demand' to liberate AEA and 2-AG by calcium-dependent phospholipases. There are numerous reviews that illustrate the details of these pathways (i.e., Piomelli, 2003; Bisogno et al 2005; Hohmann and Suplita, 2006). Briefly, the precursor of AEA is the membrane phospholipid, N-arachidonyolphosphatidyl ethanolamine (NAPE) that is formed by the transfer of arachidonic acid from the sn-1 position of 1,2-sn-diarachidonoylphosphatidylcholine (diAPC) to phosphatidylethanolamine (PE), a process that is catalyzed by a calcium-dependent N-acyltransferase (NAT). In a one-step pathway, NAPE is hydrolyzed by a phospholipase D to release AEA and phosphatidic acid (Di Marzo et al 1994). In a two-step pathway, NAPE is hydrolyzed to N-Acyl-lyso-PE by phospholipase A_1/A_2, then, AEA is released from N-Acyl-lyso-PE by lysophospholipase D (Sun et al., 2004). A third pathway has been described in which phospholipase C (PLC) cleaves NAPE to generate phosphoanandamide, which is dephosphorylated by phosphotases to liberate AEA (Liu et al., 2006). It has been suggested that this latter "PLC" pathway is involved in the 'on demand' synthesis of AEA rather than in maintaining basal tissue levels of AEA (Liu et al 2008). The primary pathway for 2-AG synthesis appears to involve hydrolysis of diacylglycerols (DAG) by two DAG lipase isozymes, DAGLα and DAGLβ (Stella et al., 1997; Bisogno et al., 2003; 2005). DAGs may be produced either by the phospholipase C β catalyzed hydrolysis of phophotidylinositol or the hydrolysis of phosphatidic acid by a phosphohydrolase (Bisogno et al., 2005, for review). In addition to arachidonic acid, these same enzymes incorporate other fatty acids into membrane phospholipids which then serve as precursors for numerous other endocannabinoids, (i.e., palmitic acid and oleic acid into N-palmitoyl ethanolamine (PEA) and oleoylethanolamide (OEA), respectively).

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As indicated in the previous paragraph, eCBs are stored as fatty acid components of membrane phospholipids. The liberation of eCBs from the phospholipids is due to an increase in intracellular calcium that leads to phosphorylation by calcium-dependent phospholipases. The increase in intracellular calcium may be achieved by depolarization and resultant Ca^{2+} influx through N-type calcium channels (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001), activation of postsynaptic $G_{q/11}$-coupled receptors such as mAChR or group I mGluRs (Maejima et al., 2001), or a combination of the two (Ohno-Shosaku et al., 2002; Maejima et al., 2005; Ohno-Shosaku et al., 2005).

As AEA and 2-AG are components of plasma membrane phospholipids, direct histological localization of endocannabinoids has not been possible. Their presence and localization have been inferred from the distribution of synthesizing and inactivating enzymes as well as physiological effects on identified cells. Even though AEA and 2-AG are released from membrane phospholipids by calcium-dependent phospholipases, the problem is how and if the eCBs leave the plasma membrane to affect target receptors. Unlike water-soluble transmitters (i.e., amino acids, acetylcholine, peptides), eCBs are highly lipophilic with a tendency to remain in the membrane. The eCBs freely diffuse within the membrane where they can interact with the active sites of degradative enzymes and receptors (see below). However, the phenomenon of retrograde transmission clearly demonstrates that eCBs can leave the membrane, traverse the aqueous medium of the intercellular space to act on presynaptic cannabinoid receptors as far as 20 μm from a release site (Wilson and Nicoll, 2001). It is not clear how eCBs leave the lipid phase of the membrane once they are released from the phospholipids or how they are able to travel in the aqueous phase of the tissues. AEA binds reversibly to serum albumin and it is likely that such binding is critical for the movement of AEA, 2-AG, etc in blood, the extracellular matrix and the cytoplasm (Bojensen et al., 2003).

2.2. Inactivation

AEA and 2-AG are inactivated following intracellular accumulation by several enzymes including: fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MGL), cyclooxygenase-2 (COX-2) and lipoxygenase (LOX). FAAH is an integral membrane protein with its hydrolytic site facing the lipid bilayer (McKinney and Cravatt, 2005). COX-2 is structurally similar to FAAH in that it is a monotopic membrane protein that is present in the lumen of the endoplasmic reticulum. MGL, in contrast, is a cytosolic enzyme. An issue of continuing contention is the means by which the highly lipophilic AEA and 2-AG cross the plasma membrane prior to their enzymatic inactivation. Although eCB movement across the plasma membrane is a sodium- and ATP-independent process that is driven by a concentration gradient, there are three competing ideas regarding the mechanism of uptake: a high affinity membrane transporter, inward transport by lipid rafts, and passive diffusion through the plasma membrane. A high-affinity membrane transporter for AEA has not yet been identified or cloned. The argument seems to center on the observations that most drugs that are used to block uptake of AEA, also block AEA hydrolysis by FAAH. Since FAAH is an integral membrane protein of the endoplasmic reticulum, one cannot differentiate the action of these drugs (i.e., AM404, MAFP) on a transporter or the enzyme. Detailed arguments for the relative merits of these mechanisms are beyond the scope of this review and may be found in older and more recent reviews (Hillard and Jarrahian, 2003; Glaser et al., 2005b; Hermann et al., 2006; Dainese et al., 2007).

AEA and 2-AG are hydrolyzed by FAAH into arachidonic acid and ethanolamine or glycerol, respectively (Deutsch & Chin 1993; Yu et al., 1997; Goparaju et al., 1998). In a similar manner 2-AG, but not AEA, is hydrolyzed by MGL (Goparaju et al., 1999; Dinh et al., 2002). Following hydrolysis of AEA or 2-AG, arachidonic acid is promptly incorporated into membrane phospholipids. In vivo, AEA is hydrolyzed principally by FAAH (Deutsch and Chin, 1993;

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Cravatt et al., 2001), while MGL appears to be the predominant 2-AG hydrolyzing enzyme (Goparaju et al., 1999; Saario et al., 2004). COX-2 oxidizes arachidonic acid, AEA and 2-AG to prostamides or prostaglandin glyceryl esters, leading to prostaglandins that also are biologically active (Yu et al., 1997; Kozak et al., 2000). In addition, oxidation of arachidonic acid by lipoxygenase produces 12-(S)-hydroperoxyeicosatetraenoyl acid (15-(S)-HPETE) 5-(S)-HETE and leukotriene B_4, all of which are agonists of TRPV1 receptors (Hwang et al., 2000). It is important to note that all these enzymes have other fatty acid derivatives as substrates. The above pathways are not all present in any given tissue. Rather, the effects of AEA and 2-AG may be modulated by the balance of metabolic enzymes that is specific to each cell type. A summary schematic of these pathways is shown in Figure 2.

2.3. Receptors

Effects of cannabinoids in the brain are mediated by metabotropic and ionotropic receptors (Diaz-Laviada & Lorez-Llorente, 2005, for review) (Fig 2). Cannabinoid 1 receptors (CB1R) are the most numerous G protein coupled receptor in the brain, accounting for much of the physiological and psychotropic actions of marijuana. In general, activation of CB1R, via Gi/o, modulates voltage-gated K^+ and Ca^{2+} conductances, resulting in a reduction of neurotransmitter release, particularly GABA and glutamate (McAllister and Glass, 2002;Pertwee and Ross, 2002; Straiker and Mackie, 2005). CB2 receptors also signal through Gi/o (Munro et al., 1993). CB2 receptors, though mainly expressed in cells of the immune system, also are found in the CNS, particulalry in astrocytes (Pazos et al., 2005;Martínez-Orgado et al., 2007;Cabral et al., 2008). There also is mounting evidence based on the persistence of cannabinoid-mediated effects in CB1R knockout mice for additional unidentified targets, or ‘CB3R’ (Zimmer et al., 1999;Begg et al., 2005). There is evidence that G-protein-coupled receptor 55 (GPR55), is the novel cannabinoid receptor (Baker et al., 2006). Some endocannabinoids (i.e., AEA but not 2-AG) activate the ionotropic transient receptor potential type vanilloid 1 receptor (TRPV1), previously referred to as vanilloid receptor 1 (VR1) (Ross, 2003;Toth et al., 2005). CB1R and TRPV1 are widely distributed in the CNS (Tsou et al., 1998;Toth et al., 2005), and along with CB2R, are expressed in the periphery, and in non-neural tissues (Szallasi et al., 1993;Galiegue et al., 1995;Funakoshi et al., 2005;Watanabe et al., 2005). Prostamides and prostaglandin glycerol esters, produced by endocannabinoid oxidation by COX-2 bind to a variety of prostaglandin receptors. Recently, a presynaptic PGE2 EP2 receptor has been implicated in synaptic transmission in the hippocampus, indicating a role for COX-2 and perhaps AEA in this mechanism (Zhu et al., 2005;Sang et al., 2005). Endocannabinoids also have been identified as ligands for peroxisome proliferator-activated receptors (PPAR), members of the nuclear receptor superfamily that are involved in lipid metabolism, insulin sensitivity, regulation of inflammation and cell proliferation (Bernstein, 2005). Independent of receptors, endocannabinoids exert additional effects upon signal transduction pathways and lipid rafts (Sarker et al., 2003; Barnett-Norris et al., 2005)

2.4. Distribution

The cellular localization of CB1 receptors in the mammalian central nervous system (CNS) was first described by *in vitro* autoradiography (ARG) of receptor binding and *in situ* hybridization of CB1 mRNA. These studies show enrichment of CB1 receptors in the hippocampus, basal ganglia, cerebellum, pyriform and cerebral cortices (Herkenham et al., 1991; Mailleux and Vanderhaeghen, 1992; Westlake et al., 1994), consistent with regions associated with the psychomotor effects of cannabis. Higher resolution was obtained with the independent introduction of three antibodies against CB1 receptors at about the same time, 1997 and 1998. These were rabbit polyclonal antibodies against the N-terminus, aa 1−77 (Twitchell et al., 1997; Tsou et al., 1998a), aa 83−98 (Pettit et al., 1998) and C-terminus 13 amino acids (Egertová et al 2000). A fourth rabbit polyclonal antibody against the N-terminus,

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aa 1−14 (McIntosh et al 1998) was not used in the mammalian CNS but was used in the retinas of rat, goldfish and zebrafish (Yazulla et al., 1999, 2000; Yazulla and Studholme, 2001) and the developing chick retinotectal pathway (Leonelli et al., 2005). Fukodome et al. (2004) produced a guinea pig polyclonal antibody against the C-terminus aa 443−473; this antibody was used to study the comparative distributions of CB1 receptors and diacylglycerol lipase α (DGL-α) in mouse hippocampus (Katona et al., 2006). In general, and quite happily, the immunocytochemistry supported the ARG localization of CB1 receptors throughout the rat brain. However, one difference was noted. The N-terminus antibodies labeled the dendrites, axon and cell body (Twitchell et al., 1997; Tsou et al., 1998; Pettit et al., 1998) whereas the C-terminus antibody did not label the cell body (Egertová et al., 1998). All of the aforementioned antibodies appeared to be well characterized. This issue of differential labeling will come up again in the discussion on CB1R-IR in the retina.

The role of each endocannabinoid metabolizing enzyme is dictated, in part, by its expression pattern. Of the three endocannabinoid inactivating enzymes, FAAH expression was the first to be described (Thomas et al., 1997) and is the most widely studied. Comparative distributions of FAAH-IR and CB1R-IR show complimentary, overlapping and unrelated distributions. In the complimentary pattern, FAAH is very prominent in the cerebellum, hippocampus, neocortex, olfactory bulb and amygdala in which it is present in large caliber processes of major output neurons (i.e., pyramidal cells) that are postsynaptic to processes containing CB1R. This pattern suggested presynaptic retrograde regulation of transmitter release by endocannabinoids (Thomas et al ., 1997; Egertová et al., 1998; Tsou et al., 1998b; Egertová et al., 2003). In the overlapping pattern, CB1 mRNA has been detected in cell types known to contain FAAH-IR, that is, some pyramidal cells in the mouse hippocampus, amygdala and entorhinal cortex (Marsicano and Lutz, 1999) and in pyramidal neurons of rat cortex (Hill et al., 2007). Even in the absence of double-labeling, the inference is that FAAH and CB1 receptors would co-localize in these pyramidal cells. In the unrelated pattern, there are FAAH-immunoreactive neurons in the thalamus, mid-brain and hind brain that are not associated with CB1 receptors (Egertová et al., 2003). As mentioned, FAAH has substrates in addition to AEA and 2-AG that are not ligands for CB1R, for example oleamide that may interact with serotonin receptors in the thalamus (Thomas et al., 1997). GABAergic output terminals from the striatum to the globus pallidus, entopenduncular nucleus and substantia nigra are rich in CB1 receptors, but FAAH is markedly reduced or absent. One possibility it that FAAH has a different role in regulating retrograde transmission in GABAergic synapses compared with glutamatergic synapses.

MGL, though it has a somewhat similar distribution as FAAH, is localized presynaptically rather than postsynaptically, with a near complimentary distribution of MGL and FAAH immunoreactivities in rat hippocampus, cerebellum and amygdala; with MGL in presynaptic and FAAH in postsynaptic processes (Dinh et al., 2002; Gulyas et al., 2004). An ultrastructural study in the hippocampus showed that diacylglycerol lipase α (DGL-α) was present in postsynaptic dendritic spines (Katona et al., 2006). The presynaptic contacts contained CB1R-IR. These data were consistent with the presence of MGL presynaptically as well as the identity of 2-AG as a retrograde transmitter at excitatory synapses as described below. COX-2, as with FAAH is present in postsynaptic dendrites, particularly spines, of large output neurons in the rat cortex, hippocampus CA3, and amygdala (Kaufmann et al., 1996). Although FAAH, COX-2 and MGL overlap in CNS distribution, they are not identical, and together may complement the overall distribution of CB1, TRPV1 and EP2 receptors in the brain. The unique cellular and subcellular distribution of endocannabinoid metabolizing enzymes provides multiple opportunities of local regulation of endocannabinoid tone. In addition to the spatial regulation, endocannabinoid activity is also temporally regulated, with *in vivo* AEA and 2-AG levels varying with a circadian pattern in the nucleus accumbens, prefrontal cortex, striatum, and hippocampus. Specifically, AEA levels are at a maximal in the dark-phase, and 2-AG at a

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maximal in the light phase. This corresponded to observed reductions of FAAH activity of these regions in the dark phase. MGL activity, however, only significantly differed in the striatum, suggesting additional mechanism(s) regulating 2-AG levels (Egertová et al., 2000; Valenti et al., 2004).

2.5. Functions

AEA and 2-AG have short and long term effects. In general, short term mechanisms involve presynaptic modulation of ion channels that result in inhibiting glutamate or GABA release; while in the long term, endocannabinoids modulate protein kinases and gene transcription (van der Stelt and Di Marzo, 2005, for review). The effects on synaptic plasticity and neuroprotection appear to depend on retrograde transmission in which postsynaptic dendrites release an eCB that binds to presynaptic CB1 receptors to reduce transmitter release (Wilson and Nicoll, 2001). Retrograde transmission of eCBs can be evoked by two mechanisms: 1) voltage-dependent and 2) activation of $G_{q/11}$ coupled metabotropic receptors. In the voltage dependent mechanism, depolarization of postsynaptic dendrites opens calcium channels, increases intracellular Ca^{2+}, activates Ca-dependent PLD to release an eCB that diffuses to the presynaptic cell to reduce transmitter release. In the brain, strong depolarization of postsynaptic cells elicits a transient suppression of an inhibitory input. This was termed DSI (depolarization-induced suppression of inhibition) and was shown by Wilson and Nicoll (2001) to be due to activation of presynaptic cannabinoid receptors that reduced GABAergic input, thereby relieving the postsynaptic cell from inhibition. Analogous to this in excitatory synapses, retrograde inhibition of glutamate release was the basis of depolarization-induced suppression of excitation (DSE) (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001). A complication of DSI is that the GABAergic neurons in the hippocampus that contain CB1R also contain CCK, which is excitatory. Thus the retrograde suppression of these hippocampal neurons reduces the output of excitatory and inhibitory substances, the combined effect of which is still not clear (Beinfeld et al 2001). The second mechanism involves activation of $G_{q/11}$ coupled metabotropic receptors, usually Group I mGluR (mGluR1 and mGluR5) and muscarinic (M1 and M3) receptors. The events, metabotropic-induced suppression of excitation and inhibition, may be abbreviated as MSE and MSI, respectively. DSE, DSI, MSE and MSI are not potentiated by FAAH inhibitors but are blocked by inhibitors of DAG-lipase, implicating 2-AG rather than AEA as a retrograde transmitter (Kim and Alger, 2004; Slanina and Schweitzer, 2005; Fan and Yazulla, 2007).

In summary, the eCB-induced reduction of glutamate and GABA release contributes to short-term synaptic plasticity, while the reduction of glutamate release inhibits excitotoxicity following ischemia (Diana and Marty, 2004; Pertwee, 2005a, for reviews). Evidence implicates 2-AG rather than AEA in plasticity (Kim and Alger, 2004; Slanina and Schweitzer, 2005; Fan and Yazulla, 2007), while both AEA and 2-AG are involved in neuroprotection, in that they are released in response to brain trauma and confer protection against inflammation and excitotoxicity. Antioxidant properties of endocannabinoids also contribute to neuroprotection (Pertwee, 2005b). Whether both AEA and 2-AG serve as substrates for COX-2 to modulate synaptic transmission via EP2 receptors as demonstrated for AEA in the hippocampus (Sang et al., 2005) remains to be determined.

3. Cannabinoids and Ocular Tissues

It has been known for a long time that smoking marijuana induces corneal vasodilation (so called red eye) and a reduction of intraocular pressure (IOP) (Adams et al., 1978; Green, 1979, for historical review). The popular idea that marijuana dilates the pupil has not been supported by experimental data (Weil et al., 1968). Initially, it was thought that marijuana exerted its effects systemically through the CNS. It is now clear that the effects of THC and other cannabinoids reduce IOP by local action on CB1R in the eye (Straiker et al., 1999b; Song

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and Slowey, 2000; Porcella et al.,, 2001; Stamer et al., 2001; for reviews on application to glaucoma: Järvinen et al., 2002; Lograno and Romano, 2004; Chen et al., 2005; Tomida et al., 2007). Physiological and biochemical studies clearly show the presence of endocannabinoids and their effects on ocular tissues, including the ciliary body, iris, choroid and trabecular meshwork in porcine, bovine, monkey and human tissues (Gawienowski et al., 1982; Matsuda et al., 1997; Stamer et al., 2001; Chien et al., 2003; Lograno and Romano, 2004; Chen et al., 2005; Stumpff et al., 2005; Njie et al., 2008). CB1 mRNA has been localized in the ciliary body of rat, bovine and human (Porcella et al., 1998, 2000; Stamer et al., 2001) and in bovine and human trabecular meshwork (Stamer et al., 2001). Levels of CB1 mRNA were ten times higher in the ciliary body than in the retina in both rat and human. THC, as low as 10^{-12}M, increases monoamine oxidase (MAO) activity in the bovine trabecular meshwork, choroid and ciliary processes but not in the iris (Gawienowski et al., 1982). Hydrolysis of anandamide has been measured in the porcine iris, choroid, lacrimal gland, and optic nerve (Matsuda et al., 1997). CB1R-immunoreactivity (IR) was detected in the non-pigmented ciliary epithelium (NPE) in human and bovine tissues (Straiker et al., 1999b; Stamer et al., 2001) and conjunctival epithelium in mouse and human (Iribarne et al., 2008). In addition, AEA, 2-AG and PEA have been measured by gas chromatography in human ocular tissues, except for the lens (Chen et al., 2005; Matias et al., 2006). In addition, the content of eCBs varies in certain disease states, suggesting the importance of eCBs in maintaining ocular homeostasis. For example, 2-AG levels were found to decrease in the ciliary body of patients with glaucoma (Chen et al., 2005). However, the eyes of patients with diabetic retinopathy showed higher levels of 2-AG only in the iris. Increased levels of AEA were more widespread in the eye with significantly higher levels of AEA in the retina, ciliary body and cornea. Eyes of patients with AMD also showed widespread increases of AEA levels in the retina, choroid, ciliary body and cornea (Matias et al., 2006).

Topically applied AEA appears to reduce IOP by two mechanisms: via activation of CB1R and via activation of EP2R after conversion of AEA to prostamides (Järvinen et al., 2002, for review). Indeed, COX-2, prostaglandin E(2) and EP2 receptor activity have been identified in cells of the human ciliary body (Liang et al., 2003, 2004; Rosch et al., 2005) perhaps accounting for the ability of Butaprost™ (an EP2 agonist) to lower IOP. Of great interest is that cholinergic and adrenergic anti-glaucoma drugs induce the production of PGE2 *in vitro* from the iris-ciliary body of rabbits and irises of glaucoma patients (Kaplan-Messas et al., 2003), perhaps accounting, in part, for the hypotensive effects of pilocarpine and epinephrine on IOP. Furthermore, COX-2 expression is virtually absent in the NPE cells in patients with primary open-angle glaucoma (Maihöfner et al., 2001). Administration of either AEA or THC to human NPE cells induces COX-2 expression, indicating a relationship among prostaglandins, COX-2 and endocannabinoid in lowering IOP (Rosch et al., 2006). In addition, EP2 receptors have been localized in the NPE of mouse, porcine and human ciliary body by *in vitro* autoradiography and immunohistochemistry (Biswas et al., 2004; Sharif et al., 2004). However, Schlotzer-Schrehardt et al. (2002) reported that EP1 rather than EP2 receptors were most prominent in the human ciliary body, with EP2 receptors found in the cornea and choriocapillaries.

4. Cannabinoids and Vision

Considering the widespread use of marijuana there is little information on the psychophysical effects of marijuana use on visual function. Well controlled studies are difficult to obtain due to numerous factors (Weil et al., 1968; Carlin et al., 1972). First, standardizing the dose of the drug depends on the route of administration. Whether TCH is inhaled as smoke or ingested as a pill/food affects the bioavailability, time to onset and duration of any effects. Also, since THC is so lipid soluble, the pharmacokinetics can be affected by the fat content of the subjects. Second, placebos are difficult to administer because of the distinctive odor and taste of

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marijuana in experienced users. Third, social setting and expectations have a great effect on the perceived state of "intoxication" induced by marijuana. Fourth, the effects of marijuana on attention make it very difficult to identify effects on threshold detection. In early studies, comments on the effects of marijuana on vision appeared almost as an afterthought because the goal of the studies was either to investigate the combined effects of marijuana with alcohol on attention and a variety of motor behaviors or were concerned with effects of marijuana on pain perception. Adams et al. (1975) reported that THC plus alcohol did not affect static visual acuity even at low contrast levels, but there was a marked reduction in acuity of moving targets when coordinated eye movements were required. It is likely that the effect of THC on moving targets was due to alcohol because Flom et al. (1976) reported that ocular motor tracking was affected by alcohol but not by marijuana. Adams et al. (1978) reported that 15 mg THC increased the time to recovery from bright foveal glare by several seconds (5−10%) at low contrast but not high contrast. This effect of THC was similar to what was observed with alcohol in an earlier study (Adams and Brown, 1975) but the effects of THC and alcohol were not additive. Noyes et al. (1975) reported that 10 mg of THC was a mild analgesic for cancer pain but 20 mg of THC produced side effects that included blurred vision. In a survey of 112 people with multiple sclerosis who used marijuana to reduce spasticity and pain, side effects included double vision and vision dimness (Consroe et al., 1997). Also, there have been numerous anecdotal reports that smoking marijuana improves dim light vision, including the now famous report that Jamaican fishermen smoked marijuana to improve night vision when they went fishing (Reese, 1991; West,1991; See Russo et al., 2004b for a nice review of this issue). Acute effects of marijuana smoking on vision include a reduction in vernier and Snellen acuity, alterations in color discrimination and in increase in photosensitivity (Kiplinger et al., 1971; [Argurell et al., 1976; Shapiro, 1974, cited in Dawson et al., 1977)]; Russo et al., 2004a). Chronic effects of long term-marijuana use were reported for 10-year abstainers by Dawson et al. (1977). Relative to the non-user group, users showed strong trends toward an increase in photosensitivity and a decrease in dark adaptation, color-match limits and Snellen acuity, with probabilities ranging from $p< 0.07$ to $p <0.001$. Adams et al. (1978) argued that it was unlikely that all of these effects of marijuana on vision were due either to cortical or pre-retinal sites because light- and dark- adaptation take place in the retina and the increased glare recovery was accompanied by a reduction of pupil size, the reverse of what would be expected. Adams et al. (1978) stated that ..."it consequently seems likely that marijuana or a metabolic product of marijuana acts directly on the retina to produce the delay in glare recovery". This suggestion of 30 years ago is now receiving much support and likely applies to the effects of marijuana on acuity and color vision.

Evidence for central effects of marijuana use on visual perception has been presented for an optical illusion called "binocular depth inversion". This illusion occurs when a three-dimensional object is presented pseudoscopically (i.e., single stereoscopic images intended for the right eye are presented to the left eye and vice versa). Presented in this way, a face that appears convex should appear concave when the stereoscopic images are reversed. In normal subjects this inversion does not occur, presumably because the "top down" processing of perceptual networks corrects implausible data. Impairments of this illusion have been used as diagnostic tools in psychotic and intoxicated states. Acute use of cannabis does not affect normal depth perception, but it does reduce binocular depth inversion (Emrich et al., 1991; Leweke et al., 1999). Furthermore, Semple et al. (2003) reported that chronic cannabis users had a reduction of binocular depth inversion that was not related to the time since their last dose of cannabis. The conclusion was that there was a long term effect of cannabis use on this central component of visual processing.

Studies on the central effects of cannabis on visual perception have largely focused on the P300, a positive electrical event recorded around the midline of the central-parietal cortex in response to an auditory or visual stimulus. Consistent results have not been obtained in these

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studies (Skosnik, 2006, for review) partly because the P300 is not purely sensory. Also, some early studies drew subjects from psychiatric wards and may have had conflicts with drug history and other symptoms. This problem was shown by Patrick et al. (1995) who showed a decrease in P300 amplitude in cannabis users versus non-users when all subjects were psychiatric inpatients, but there was no difference in P300 amplitude when users and non-users were carefully screened normal subjects.

Skosnik et al. (2006) examined visual function in cannabis users via the steady state visual evoked potential (SSVEP), an EEG response of the occipital cortex, and N160, a response of later stages of the visual pathway, to flickering lights. They showed several effects of cannabis use on these potentials. First, the SSVEP for an 18 Hz stimulus was reduced in female users but not in males. Second, the reduction was greater for those who started to use cannabis at a younger age. Third, amplitude of the transient N160 component, unlike the SSVEP, was reduced in male and female cannabis users, suggesting a disruption of later-stage visual processing. Alternatively, Skosnik et al. (2006) suggested that because the N160 is generated in more central regions of cortex, the effects of cannabis could be due to affects on attention and have little to do with visual processing. This suggestion is consistent with the contradictory data obtained with P300 as well as the reliability of psychophysical data. As of yet I know of no studies on the effect of cannabis alone on the electroretinogram.

5. Retinal Endocannabinoids — Content and Distribution

There is growing evidence for a retinal endocannabinoid system that is likely to be responsible for some of the visual effects of ingesting THC. For example, CB1-receptor agonists stimulate monoamine oxidase in bovine retina (Gawienowski et al., 1982), inhibit dopamine release from guinea pig retina via Gi/o (Schlicker et al., 1996; Weber and Schlicker, 2001) and inhibit [^{3}H]-aspartate release from bovine retina (Opere et al., 2006). CB1-receptor mRNA was detected in rat retina by *in situ* hybridization and RT-PCR (Buckley et al., 1998; Porcella et al., 1998), while CB2-receptor mRNA was reported in the retina of adult rats (Lu et al 2000) but not in rat embryos (Buckley et al., 1998). AEA hydrolysis was measured in homogenates of porcine, bovine and goldfish retinas (Matsuda et al., 1997; Bisogno et al., 1999; Glaser et al., 2005a). 2-AG and AEA were detected in rat, bovine and human retinas by gas chromatography/mass spectrometry (Straiker et al., 1999a; Bisogno et al., 1999; Stamer et al., 2001; Chen et al., 2005; Matias et al., 2005). Cellular localization of [^{3}H]-AEA uptake was described in goldfish retina (Glaser et al., 2005a). FAAH-immunoreactivity (IR) is widely distributed in the rat, zebrafish and goldfish retinas (Yazulla et al., 1999; Yazulla and Studholme, 2001; Glaser et al., 2005a) and CB1R-IR was localized in non-mammalian and mammalian retinas (Straiker et al., 1999a,b; Yazulla et al., 1999, 2000; Begbie et al., 2004; Lionelli et al., 2005). Cannabinoid agonists modulate [^{35}S]GTPγS-binding in rat retina (Savinainen and Laitinen (2005) as well as voltage-dependent membrane currents in photoreceptors, bipolar cells and ganglion cells (Straiker et al., 1999a; Yazulla et al., 2000; Straiker and Sullivan, 2003; Fan and Yazulla, 2003, 2004, 2005, Lalonde et al., 2006). Struik et al. (2006) reported that a cannabinoid agonist, WIN 55212−2, accelerated the cone response to light offset. Finally, retrograde transmission was demonstrated in goldfish cones, with bipolar cell dendrites as the likely source of the endocannabinoid, 2-AG (Fan and Yazulla, 2007). The rest of this review explores these studies in more detail.

5.1. Biochemical assay

The earliest evidence for direct action of a cannabinoid agonist on the retina was provided by Gawienowski et al. (1982) who showed that THC increased MAO activity in a sampling of ocular tissues. The effect of THC was biphasic with the largest increase (~66%) in MAO activity occurring with 10^{-12}M THC in the retina, followed by the trabecular meshwork (~44%), ciliary process (~26%), iris (~17%) and choroid (~12%). There was no further work

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on the retina until the seminal studies on the identification and characterization of endocannabinoids, their receptors and biosynthetic pathways in other tissues that occurred between 1988 and 1992. The ability of the retina to hydrolyze ^{3}H-AEA was shown first in homogenates of porcine retina (Matsuda et al., 1997), followed by bovine retina (Bisogno et al 1999) and goldfish retina (Glaser et al., 2005a). Hydrolysis of AEA in these studies was inhibited by a variety of FAAH inhibitors including MAFP, an irreversible blocker of FAAH (Deutsch et al., 1997), strongly indicating that the hydrolysis of AEA was accomplished by FAAH. FAAH hydrolysis of AEA was comparable in the retina and brain of rat and goldfish. However, the measured FAAH activity was much higher in porcine retina compared to bovine and goldfish retinas probably because a saturating concentration of the substrate (AEA) was used in porcine retina. Matsuda et al. (1997) also showed that AEA could be synthesized in porcine retina by reversal of FAAH activity in the presence of a high concentration (250 mM) of ethanolamine. It is unlikely that this ‘condensation’ pathway operates in vivo to synthesize AEA in membrane phospholipids because of the very high concentration of ethanolamine required to drive FAAH activity in reverse.

The first report of endocannabinoids in the retina was the detection of AEA and 2-AG by gas chromatography in bovine retina (Bisogno et al., 1999). Straiker et al. (1999a) detected 2-AG but not AEA in rat retina, while AEA, 2-AG and PEA were detected later in human retina (Chen et al., 2005; Matias et al., 2005). An important observation was that AEA could be released from bovine retinal extracts by incubation in a physiological buffer a time-dependent fashion (Bisogno et al., 1999). This finding implied that the retinal extracts contained both the precursor N-acylphosphotidylethanolamine and phospholipase D that releases AEA from the membrane phospholipids. The presence of endocannabinoids along with their synthetic and hydrolytic enzymes was demonstrated in the retina; the localization of eCBs to specific cell types has been accomplished by in situ hybridization, autoradiography and immunohistochemistry.

5.2. Localization — cannabinoid receptors

The first cellular localization of CB1 receptors in the retina was by *in situ* hybridization in rat retina (Buckley et al., 1998). This study was part of a developmental study of CB1 mRNA in the entire rat embryo. CB1 mRNA appeared in retinal ganglion cells by E15 and “another cell layer within the retina” by E20. Given the low cellular resolution of the images, it is likely that the additional CB1 labeled cells at E20 were in the inner nuclear layer. Begbie et al. (2004) reported CB1 receptor expression by *in situ* hybridization in ganglion cells at E18 in chick embryo. CB2 mRNA was not detected in embryonic rat retina at E20 by *in situ* hybridization (Buckley et al., 1998). However, CB2 mRNA was detected by RT-PCR in adult mouse retina and in all cellular layers of adult rat retina by *in situ* hybridization (Lu et al., 2000). From the data presented by Lu et al. (2000), it was not possible to determine if the CB2 mRNA label was in neurons, Müller's cells (INL) or astrocytes associated with the vascular system or optic fiber layer.

Straiker et al. (1999a,b) described the localization of CB1R-immunoreactivity (CB1-IR) in the retinas of human, monkey, mouse, rat, chick, salamander, and goldfish. All species showed prominent label in the OPL and IPL. Labeling in the OPL appeared to be derived from the synaptic terminals of cones and perhaps rods. The intense labeling in the IPL could not be ascribed to a cell type as labeling of cell bodies in the INL appeared sporadic. It also was reported that ganglion cells of all the species except goldfish were labeled by CB1R-IR. It was mentioned that the GCL and optic fiber layer were labeled intensely in the human, chick and salamander retina. However, from the images shown in Straiker et al. (1999a) in chick and salamander, it is difficult to see labeled ganglion cell bodies. In any event, the cells in the GCL were labeled far less intensely than the IPL and even appear surrounded by label in the GCL.

Leonelli et al. (2005), in a developmental study of chick retina, showed a pattern at E18 that was similar to that shown by Straiker et al. (1999a) in chick, with intense label in the IPL and optic fiber layer. Although numerous amacrine cells were labeled, only few ganglion cells were labeled at E18. Yazulla et al. (1999) showed that CB1R-IR in rat was intense in the OPL and IPL, as well as in rod bipolar cells and PKC-immunoreactive GABAergic amacrine cells, but not in ganglion cells. The difference in the reported patterns of CB1R-IR in rat retina between the two studies (Straiker et al., 1999a; Yazulla et al., 1999) is disconcerting. Straiker et al. (1999a) used the Twichell et al. (1998) antibody against the N-terminus aa 1−77, while Yazulla et al. (1999) used the McIntosh et al. (1998) antibody against N-terminus aa 1−14. Both studies showed preadsorbed controls in rat retina and Yazulla et al. (1999) showed appropriate labeling in immunoblots of rat retina. Considering that CB1 receptor-mediated activity was demonstrated in rat retinal ganglion cells by [^{35}S]GTPγS autoradiography (Savinainen and Laitinen, 2004) and by RT-PCR and immunocytochemistry in a purified culture of rat ganglion cells (Lalonde et al., 2006), it is likely that the punctate label in the rat IPL described by both studies was derived from ganglion cell dendrites, while somatic labeling of ganglion cells in rat was not detected by the antibody used by Yazulla et al. (1999). However, we found that the McIntosh antibody labels ganglion cells in mouse (Fig. 3) as reported, but not illustrated by Straiker et al (1999). As in rat, we verified that the vertical streaks of CB1R-IR in the INL and IPL co-localized with PKC-IR and thus were rod bipolar cells. The labeled ganglion cells in mouse are not nearly as frequent as is observed with FAAH or MGL (to be described). As will be discussed in more detail below, the presence of CB1R-IR in bipolar cells is consistent with the presence of CB1 receptors on glutamatergic neurons in other regions of the mammalian CNS.

The distribution of CB1R-IR in goldfish retina reported by Yazulla et al. (2000) differs from Straiker et al. (1999a) who reported intense label in the OPL, associated with rod and cone terminals, and IPL. In addition to the CB1R-IR in the OPL and IPL, Yazulla et al. (2000) showed intense label over Müller's cells and lighter label over bipolar cell bodies and synaptic terminals (Fig. 4). At the ultrastructural level, CB1R-IR was preferentially located on the presynaptic membrane of cone pedicles and bipolar cell terminals. Rod spherules were not labeled. Yazulla et al. (2000) used the McIntosh CB1−14 antibody and showed a single band at ~70 kDa in immunoblots of goldfish retina as well as preadsorbed controls in immunoblots and immunocytochemistry. Of great interest in this regard is that Valenti et al. (2005) studied the goldfish brain with the same Twitchell antibody as Straiker et al. (1999a). In immunoblots of goldfish brain, that antibody labeled a single band at about 53 kDa and was blocked by preadsorption. Given that the appropriate controls appear to have been done it is difficult to determine which labeling pattern in the goldfish retina is more accurate. For that decision, corroborative data from other techniques are required. Electrophysiological studies to be described demonstrate the presence of CB1 receptors on goldfish cones and ON mixed rod/cone bipolar cells (Fan and Yazulla, 2003, 2004, 2005, 2007) supporting the localization data of Yazulla et al (2000).

CB1R-IR is present on cone terminals of all species. Ultrastructural analysis showed that the CB1R-IR in goldfish cone pedicles was located on plasma membrane at the perimeter of the pedicle as well as within the invagination. CB1R-IR was not immediately apposed to the synaptic ribbon, but was at some distance from it. In this regard, the absence of CB1R-IR near the arciform density within the invagination is similar to that reported for $GABA_A$ receptors (Yazulla et al., 1989; Yazulla and Studholme, 1997), perhaps consistent with this region being the site of active vesicular release and membrane fusion due to exocytosis. Studies also agree that CB1R-IR is found on a small population of amacrine cells. However, only in the rat has a population been identified, namely a relatively rare type that is immunoreactive for both PKC and GABA (Yazulla et al., 1999).

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In the goldfish, CB1R-IR was located presynaptically in amacrine cell synaptic boutons. These boutons were found throughout the full depth of the IPL and were presynaptic to either bipolar cell terminals or small processes that likely were derived from ganglion cells. Given the relative rarity of these CB1R-immunoreactive processes it seems likely that they were derived from a single type of amacrine cell that ramifies throughout the IPL. There is about a 75% chance that an amacrine cell type is GABAergic (Marc et al., 1995). Cannabinoids presynaptically inhibit GABA release from neurons in the rat substantia nigra, corpus striatum and hippocampus (Szabo et al., 1998, 2006; Katona et al., 1999), and it is likely that a similar function would be found in the retina.

A striking finding in rat and goldfish was the localization of CB1R-IR in bipolar cells. In rat, doubling labeling of antisera against CB1R and PKC showed that every PKC-immunoreactive cell was double labeled for CB1R-IR, including all rod bipolar cells and large PKC-immunoreactive amacrine cells. CB1R-immunoreactive cell bodies in the distal INL were identified as horizontal cells by double labeling with calbindin-IR. Curiously, we found that CB1R-IR in dendrites in the OPL was due exclusively to dendrites of the rod bipolar cells and not horizontal cells (Yazulla et al., 1999). In goldfish, CB1R-IR was present on the plasma membrane of bipolar cell synaptic terminals (Yazulla et al., 2000). Furthermore, there was a significant difference in the ON and OFF sublayers of the IPL with respect to frequency of membrane-bound CB1R-IR. All Mb bipolar cell terminals were CB1R-IR as were the vast majority (86%) of Cb bipolar cell terminals. This is in contrast to far lower frequency of labeling on the Ma (38%) and Ca (27%) terminals that occupy the OFF sublayer. Thus, there is a marked preference for ON bipolar cells in the membrane-associated labeling of CB1R-IR. This labeling of the membrane, as with the cone pedicles, was not adjacent to the synaptic ribbons; rather the CB1R-IR was always some distance removed from the ribbon. This is not surprising because presynaptic modulation of neural function by receptor-activated second messengers does not require spatial contiguity of the receptors with the presynaptic zone. The functional implications of these findings of CB1R-IR on cones and bipolar cells were investigated with whole cell recordings and will be discussed in the sections on physiology.

A relatively new and unexplored area of research is the presence of CB1 receptors on neuroglia. CB1R-IR was reported on Müller's cells in goldfish retina (Yazulla et al., 2000) but not in any other retinal preparation. There are also inconsistent reports of CB1R-IR in mammalian astrocytes, microglia and oligodendrocytes (Pazos et al 2005, for review). Recent studies show that activation of CB1 receptors can inhibit high affinity excitatory amino acid transport in rat cortical astrocytes (Shivachar, 2007) and induce release of glutamate from hippocampal astrocytes (Navarrete and Arague, 2008). This modulation of extracellular glutamate could be critical in controlling neuronal excitability in normal and pathological states. There is evidence that CB1 receptors and, particularly CB2 receptors are involved in gliotic responses to injury and are targets of investigation for pathological conditions, for example multiple sclerosis (Martínez-Orgado et al., 2007; Cabral et al., 2008). As far as I know the interaction of endocannabinoids and glia has not been investigated in the retina. It is possible that the CB2 mRNA described in all cellular layers of rat retina (Lu et al., 2000) could include glial labeling, particularly the Müller's cells in the proximal INL.

5.3. Localization — metabolizing enzymes

FAAH - The existence of FAAH in the retina was inferred by studies demonstrating that retinal tissue was able to hydrolyze AEA (Matsuda et al., 1997; Bisogno et al., 1999; Glaser et al., 2005a). There are only four studies that have localized FAAH-IR in the retina. In brain, FAAH-IR often is present in large caliber processes of major output neurons (i.e., Pyramidal cells, Purkinje cells) that are postsynaptic to processes containing CB1R (Thomas et al., 1997; Egertová et al.,1998; Tsou et al., 1998b; Egertová et al., 2003). This is the case in rat and mouse

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retina but not in goldfish or zebrafish. In rat retina, FAAH-IR was most prominent in medium and large ganglion cells, whose dendrites projected to narrow bands in the IPL. Weaker FAAH-IR was observed in the soma of horizontal cells (identified by calbindin-IR), the soma of large, but not small, dopaminergic amacrine cells (identified by tyrosine hydroxylase-IR), dendrites of orthotopic- and displaced-starburst amacrine cells (identified by choline acetyltransferase-IR), but in less than 50% of the starburst amacrine cell somata (Yazulla et al., 1999). Labeling at the inner limiting membrane, surrounding cell bodies in the ONL, the inner segments and occasionally vertical streaks in the IPL suggested that Müller's cells were FAAH-immunoreactive. This finding is consistent with the report of CB2 mRNA in rat retina (Lu et al., 2000) and the role of endocannabinoids in glia function (Martínez-Orgado et al., 2007; Cabral et al., 2008). The pattern of FAAH-IR in mouse retina (Fig. 5A) is very similar to what was found in rat, with the most prominent label in ganglion cells and around rods. The major difference in the mouse IPL was the presence of a single rather than the double lamination of starburst amacrine cell dendrites. As in rat (Yazulla et al., 1999), we found that FAAH-immunoreactive bipolar cells in mouse are not PKC-immunoreactive and thus, are cone bipolar cells rather than rod bipolar cells. This is in contrast to CB1R-IR, which is exclusively in rod bipolar cells. One possibility is that CB1 receptors in rod bipolar cells respond to retrogradely released 2-AG, which is not an *in vivo* substrate for FAAH, whereas FAAH in cone bipolar cells may be involved in hydrolysis of AEA with targets other than rod bipolar cell CB1 receptors. In contrast to the exclusive distribution of FAAH and CB1 receptors in bipolar cells, FAAH and CB1 receptors colocalize in horizontal cells and some ganglion cells. The possibility of autoregulation of amacrine cell and ganglion cell activity by endocannabinoids has been raised in studies on cultured rat ganglion cells and chick amacrine cells (Lalonde et al., 2006; Warrier and Wilson, 2007). These will be discussed in a following section.

The distribution of FAAH-IR and the activity of FAAH have been studied in goldfish retina (Glaser et al., 2005a; Zimov and Yazulla, 2007). Only one of the four commercially available antibodies against FAAH that we tried was appropriate for goldfish. This was a rabbit polyclonal against the C-terminus (aa 651−571) of rat FAAH that was obtained from ALEXIS Biochemical. This antibody labeled a single band in immunoblots at ~61 kDa in homogenates of goldfish retina and brain and rat brain (Glaser et al., 2005a). Labeling in immunoblots and tissue was blocked by preadsorption with the immunizing peptide antigen. FAAH-IR was most prominent over Müller's cells, cone inner segments and some large amacrine cells (Fig. 6). FAAH-activity assays showed that goldfish-retinal and brain homogenates hydrolyzed anandamide (AEA) at rates comparable to rat brain homogenate, and the hydrolysis was inhibited by methyl arachidonyl fluorophosphonate (MAFP), an irreversible inhibitor of FAAH (Deutsch et al., 1997) with an IC_{50} of 21 nM. The first demonstration of cellular accumulation of an endocannabinoid in an intact neural system was performed in goldfish retina by Glaser et al. (2005a). ^{3}H-AEA uptake was determined by *in vitro* autoradiography. As illustrated in Fig. 2, following uptake, ^{3}H-AEA is hydrolyzed by FAAH with ^{3}H-arachidonic acid rapidly incorporated into membrane phospholipids. Silver-grain deposition then represents the trapping of ^{3}H-arachidonic acid in plasma membrane following glutaraldehyde and osmium fixation. Silver-grain density was most prominent over cone photoreceptors and Müller's cells, with some accumulation over amacrine cell bodies (Fig. 7). Uptake was significantly reduced when retinas were preincubated with 100 nM MAFP. The co-distribution of FAAH-IR and ^{3}H-AEA uptake in cones, Müller's cells and amacrine cells indicated that the bulk clearance of AEA from the extracellular space in the retina occurs as a consequence of a concentration gradient across the plasma membrane created by FAAH activity. These experiments did not address the issue of a membrane transporter for AEA but demonstrated the importance of FAAH activity in selective AEA accumulation.

In goldfish retina, CB1R-IR is localized all over Müller's cells and presynaptically on the membrane of photoreceptor- and bipolar cell-synaptic terminals (Yazulla et al., 2000).

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FAAHIR as well as ^{3}H-AEA uptake are predominately found in Müller's cells, cone-inner segments and occasionally in amacrine cells. Thus, although CB1R-IR and FAAH-IR colocalize to cones and Müller's cells in goldfish retina, there is a difference. In Müller's cells, CB1R-IR and FAAH-IR are distributed over the entire cell with the same overall distribution within the cells. However, in cones, CB1R-IR is restricted to the synaptic terminal, whereas FAAH-IR is concentrated at the inner segment. The FAAH-IR in the OPL is rather diffuse and it is not clear what structures in the OPL are labeled. As will be discussed in a later section, the CB1 receptors on the cone terminals are involved in retrograde transmission (Fan and Yazulla, 2007). The retrograde endocannabinoid is 2-AG, not AEA; the hydrolyzing enzyme is MGL, not FAAH. Although there is no evidence yet for a function of AEA and FAAH in cones and Müller's cells, a role in protection against excitotoxicity as reported in rat hippocampus (Karanian et al., 2005) and most recently in rat retina (Nucci et al., 2007) could prove to be a fruitful line of research.

AEA also is an endogenous ligand for the ionotropic TRPV1 (formerly VR1) receptor (Zygmunt et al., 1999; van der Stelt and Di Marzo, 2004). Activation of TRPV1 increases intracellular calcium either by entry through the plasma membrane or by calcium release from intracellular stores (Olah et al., 2001; Liu et al., 2003; Karai et al., 2004). There is evidence that the binding site of TRPV1 receptors for AEA is on an intracellular domain (Jung et al., 1999, 2002; De Petrocellis et al., 2001). As FAAH and TRPV1 are integral membrane proteins of the endoplasmic reticulum and plasma membrane, respectively, it is likely that FAAH activity regulates the levels of AEA for TRPV1 activation (Ross, 2003; van der Stelt & Di Marzo, 2004). Such an effect has been demonstrated for increases in intracellular calcium (i.e., De Petrocellis et al., 2001; Millns et al., 2006). In addition, following hydrolysis of AEA by FAAH, lipoxygenase metabolites of arachidonic acid could activate TRPV1 (Hwang et al., 2000). Histological support for this idea in intact tissue was first reported in retina by Zimov and Yazulla (2007) who showed colocalization of TRPV1-IR with FAAH-IR in three anatomically distinct types of amacrine cell of goldfish. The cells are rare and include fusiform cells that ramify in sublaminae *a* and *b*, ovoid cells that ramify in sublamina *a* and pyriform cells that ramify in sublamina *b*. In addition, the ovoid and pyriform cell types labeled for GAD67-IR and are subsets of GABAergic amacrine cells, whereas the fusiform cells are not. Based on the lamination of dendrites in sublaminae *a*, *a/b* and *b*, TRPV1/FAAH amacrine cells function in the OFF, ON/OFF and ON pathways of the retina. The effect of TRPV1 activation would depend on the downstream cascade triggered by the increase in cytosolic calcium concentration, and presumably would differ for the GABAergic and non-GABAergic amacrine cells. A recent report (Cristino et al., 2008) showed co-localization of TRPV1 and FAAH in (CA3) hippocampal pyramidal neurons and in some Purkinje cells in mouse brain. These data in fish retina and mouse brain support the idea that AEA can act as an intracellular mediator by being produced from and/or degraded by the same neurons that express TRPV1 receptors. These data also are consistent with Glaser et al. (2005a) who showed ^{3}H-AEA uptake in a rare population of amacrine cells in goldfish retina, although it has not been demonstrated directly that these are the same cells that contain FAAH-IR and TRPV1-IR.

MGL and COX-2 - There are no published studies on the distribution of MGL-IR in the retina. Our preliminary data in mouse retina (Fig. 5B) show that MGL-IR is most prominent over most cells in the GCL (presumably including ganglion cells), proximal and distal INL. Double label with PKC-IR showed co-localization in all rod bipolar cells. MGL-IR was far less intense on rod bipolar cell axons and dendrites of amacrine and ganglion cells. There were numerous MGL-immunoreactive boutons throughout the IPL that could not be assigned to any particular cell type. Immunoreactivity was absent after preadsorption of the MGL antiserum with the immunizing peptide antigen.

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Ju et al. (2003) reported that in rat retina, COX-2-IR was constitutive in horizontal cells, amacrine cells and ganglion cells. Following transient ischemia, COX-2 was upregulated in these same cell types and was induced in Müller's cells. This pattern in rat differs from our preliminary data in mouse in which constitutive COX-2 is largely restricted to several layers of bipolar cell bodies and their axons in the IPL (Fig. 5C). Double labeling with PKC showed that 65% of COX-2 bipolar cells were rod bipolar cells while the remaining 35% presumably were cone bipolar cells. Unexpectedly, only 68% of rod bipolar cells were COX-2-immunoreactive, indicating that rod bipolar cells were of two types, those with and those without COX-2-IR. The same rabbit anti-COX-2 from Cayman Chemical was used in our study and by Ju et al. (2003). We have not as yet tried to replicate the COX-2 pattern in rat (Ju et al., 2003).

The cannabinergic system in vertebrate retinas, as indicated by CB1R-IR, FAAH-IR, COX-2-IR and MGL-IR, is concentrated in the ‘through pathway’, that is, in photoreceptors, bipolar cells and ganglion cells. These are the same cells that for the most part, use L-glutamate as their neurotransmitter. Cells of the inhibitory lateral pathways, horizontal cells and amacrine cells, do not feature as prominently. Exceptions seem to be the horizontal cells, dopaminergic amacrine cells and some cholinergic starburst amacrine cells that label weakly for FAAH-IR. Bipolar cells appear to be differentiated depending on whether they receive rod input. CB1RIR and MGL-IR are restricted to rod bipolar cells, FAAH-IR is restricted to cone bipolar cells and COX-2-IR is found in subtypes of rod and cone bipolar cells. As of now there are no electrophysiological data in mammalian retina to do more than speculate on the function of the differential distribution of CB1 receptors and related enzymes among the retinal cell types. Electrophysiological studies in salamander and goldfish and transmitter release studies in mammalian retina have provided tantalizing glimpses at the richness of cannabinoid function at all stages of retinal processing.

6. Effects on Transmitter Release

Interactions of endocannabinoids with other transmitter systems in the retina have been reported for catecholamines, acidic amino acids and GABA. The first reports were in regard to dopamine. Retinal discs of guinea pig were preloaded with [^{3}H]-noradrenaline or [^{3}H]-dopamine (Schlicker et al., 1996). Voltage- and Ca^{2+}-evoked release of these substances was reduced by CB1 agonists CP-55,940 and WIN 55,212−2 (IC_{50s} 7.0 and 6.7 μM, respectively for [^{3}H]-noradrenaline). The inhibitory effect of WIN 55,212−2 was blocked by CB1 antagonist SR141716A, which by itself, increased the evoked release of [^{3}H]-noradrenaline. This result indicated either the presence of a tonic cannabinoid tone acting on the dopamine neurons, or that SR141716A functioned as an inverse agonist, as reported in other regions of the CNS. However, Neal et al. (2001) reported that 5 μM SR141716A had no effect on the resting or evoked release of [^{3}H]-dopamine from isolated rabbit retina; they did not try any CB1 agonists. Weber and Schlicker (2001) later reported that the inhibition of [^{3}H]-noradrenaline release by WIN 55,212−2 in guinea pig retina was through action by G(i)-protein coupled receptors not Gs or Gq. A further demonstration that action of CB1 receptors was via Gi/o was provided by Savinainen and Laitinen (2004) who showed stimulation of [^{35}S] GTPγS binding in rat retinal membranes. By *in vitro* autoradiography they showed that this binding activity was most evident in the ganglion cell layer.

Effects of cannabinoids on acidic amino acids were studied utilizing uptake of [^{3}H]D-aspartate, which is used as a substitute to identify high affinity uptake sites for L-glutamate and L-aspartate. [^{3}H]D-aspartate is species dependent. In general it is selectively taken up by cones in rabbit, guinea pig, pigeon, goldfish and monkey (Ehinger, 1981; Marc and Lam, 1981; Ladanyi and Beaudet, 1986), by rods in human and rat (Brandon and Lam, 1981). In addition, [^{3}H]D-aspartate is taken up by a small percentage (5−10%) of ganglion cells in pigeon, guinea

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pig and rabbit (Ehinger, 1981) and by Müller's cells in goldfish and monkey retina (Marc and Lam, 1981; Ladanyi and Beaudet). Agonists of CB1receptors, but not CB2 receptors, inhibited K^+ - and ischemia evoked [^{3}H]D-aspartate release from isolated bovine retina (Opere et al., 2006). The rank order of potency differed for K^+-evoked and ischemia-evoked release: K^+-evoked - AEA, arachydonyl-2-chloroethylamide (ACEA), methanandamide (methAEA) and WIN55,212−2; ischemia-evoked: methAEA, ACEA and WIN 55,212−2 (Opere et al., 2006). The uptake pattern of [^{3}H]D-aspartate in bovine retina is not known. However as mentioned above, D-aspartate is accumulated photoreceptors, a subpopulation of ganglion cells and Müller's cells in mammals (Ehinger, 1981). As photoreceptors are more resistant to ischemia than ganglion cells, the relative release of [^{3}H]D-aspartate from cones and ganglion cells should differ for the K^+ and ischemia conditions. The difference then in the rank order of CB1 agonists could be due to the relative potencies of these agonists on CB1 receptors on cones versus ganglion cells. Also, the inhibition of [^{3}H]D-aspartate release from cones and ganglion cells in bovine retina is consistent with the recent data on CB1-mediated inhibition of voltage-gated currents of goldfish cones (Fan and Yazulla, 2003, 2004, 2007) and rat ganglion cells (Lalonde et al., 2006). These studies will be discussed in a following section.

Warrier and Wilson (2007) studied the CB1 regulation of GABA release from cultured chick embryonic amacrine cells as measured by spontaneous minis, $GABA_A$ receptor-mediated inward currents. They found 2 types of cell based on the initial rate of spontaneous minis. WIN 55,212−2 (10 μM) induced an increase in spontaneous transmission (WIST) only in cells with a low initial release rate of minis. WIST was CB1 mediated, calcium-independent and mediated by a Gi/o reduction in cAMP. Spontaneous minis in cells with a high initial rate of minis were unaffected by CB1 agonists, but mini frequency was decreased irreversibly with CB1 antagonist (1 μM SR141716A). It was suggested that the cells with the high initial rate were maintained by an endocannabinoid tone acting presynaptically on these cultured amacrine cells. In contrast to the spontaneous mini frequency, evoked mini frequency was reduced by WIN 55,212−2 in a manner that was independent of CB1 receptors. In fact, co-application of SR141716A suppressed the evoked minis even further. Results of this study were unusual in at least three regards. First, the concentration of WIN 55,212−2 at 10 μM was very high, particularly for cultured cells without any diffusion barrier. Despite the inhibition by 1μM SR141716A, it is possible that WIN 55,212−2 was acting at an “off target”. Second, activation of CB1 receptors ordinarily suppresses transmitter release, as in DSE and DSI, rather than increase it as with the spontaneous minis reported by Warrier and Wilson (2007).Third, in other systems CB1-mediated suppression of transmitter release is calcium-dependent, unlike that reported for these cultured amacrine cells. These differences could be due to the developmental stages of the cells involved in these studies, embryonic *versus* mature. This factor could be complicated by the observation of ‘agonist-specific trafficking’ in which the downstream effect of CB1 activation depends on the agonist involved. This is because of differences in intracellular G protein binding sites that are exposed in response to particular CB1 agonists that bind to the intra-membrane sites of the CB1 receptor. Thus, the response of a cell to WIN 55,212−2 may not predict the response of that cell to other agonists or to the endocannabinoids, AEA or 2-AG (Bonhaus et al., 1998; Lauckner et al., 2005; McIntosh et al., 2007). This unhappy situation makes it difficult to infer *in vivo* functions of endocannabinoids from data obtained with synthetic agonists. This point will be made again when discussing the retrograde suppression of cone currents (Fan and Yazulla, 2007) and the effect of WIN 55,212−2 on the cone light response (Struik et al., 2006) in following sections.

Electrophysiological evidence for cannabinoid function in the retina has been provided by whole-cell recordings from ganglion cells, bipolar cells and photoreceptors. As of yet, recordings have not been reported for amacrine cells or horizontal cells.

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7. Effects on Ganglion cells

High-voltage activated (HVA) Ca^{2+} currents were recorded by patch-clamp from cultured rat ganglion cells in response to WIN 55,212−2 (Lalonde et al., 2006). The HVA Ca^{2+} current was inhibited in a concentration dependent manner up to 50% by 0.5 − 5 μM WIN 55,212−2. This effect was blocked by either of the CB1 antagonists, SR141716A and AM281 at 0.1 μM each. No mention was made of whether SR141716A had an effect on its own, indicating potential action as an inverse agonist. The concentrations of WIN 55,212−2 used on the cultured cells was quite high; 0.5 μM elicited only a 20% suppression of the Ca^{2+} current. Suppression of Ca^{2+} by CB1 agonists is a general finding in neural systems. The presence of CB1 receptor function on rat retinal ganglion cells appeared unusual in that in other 'non-retinal' regions of the mammalian CNS, CB1 receptors were thought not to be on the major output neuron (Purkinje cell, pyramidal cell) but rather on presynaptic interneurons. However, there is evidence for CB1 mRNA in what were referred to as "projecting principal neurons", likely to be pyramidal cells, in the mouse hippocampus, amygdala and entorhinal cortex (Marsicano and Lutz, 1999). Most recently, CB1 receptors have been detected by RT-PCR in pyramidal neurons of rat cortex (Hill et al., 2007). The effect of CB1 agonists on these cells is to reduce glutamate release from the axon terminals of layers II/III and V pyramidal neurons; that is, the effect is still presynaptic. One possibility is that the CB1 receptors are present on associational ganglion cells, whose axons and axon collaterals do not leave the retina (Gallego and Cruz, 1965; Dacey, 1985). Associational ganglion cells in a variety of species are immunoreactive for somatostatin, purinergic P2X1 receptors and vanilloid receptor like 1 protein (Sagar and Marshall, 1988; Yazulla and Studholme, 2004). If ganglion cells are a source of eCBs for retrograde suppression of bipolar cell and amacrine cell activity, these eCBs also could serve an autoregulatory function to modulate the output of associational ganglion cells. There is no evidence for this as yet, but as will be described for bipolar cells and photoreceptors, there are multiple stages for eCB modulation of retinal processing.

8. Effects on Bipolar Cells

Straiker et al. (1999a) were the first to report on CB1-mediated effects on membrane currents of a retinal neuron. They showed that WIN 55,212−2 (0.6 μM and 1.5 μM) reversibly inhibited (70%) the L-type calcium current (I_{ca}) in salamander bipolar cells. The effects were blocked by SR141716A (1 μM). Yazulla et al (2000), with whole-cell recordings from large ON-type Mb bipolar cells in goldfish, showed that the delayed rectifier ($I_{K(V)}$) was rapidly and reversibly inhibited (40%) by either 1 μM CP 54490 or 1 μM WIN 55,212−2, the effects of which were blocked completely by 1 μM SR141716A. Straiker et al. (1999a) did not comment on independent effects of SR141716A, while Yazulla et al. (2000) reported that SR141716A had no effect of its own on some cells and enhanced $I_{K(V)}$ on other cells. Thus, evidence for SR141716A acting as an inverse agonist or for a cannabinoid tone present in the retinal slice is not strong. The CB1 agonists did not alter the voltage-activation range of the currents, but simply scaled down the amplitude of the evoked currents over the entire activation range. Suppression of calcium and potassium currents suggests that bipolar cells would be subject to retrograde inhibition from ganglion cells. The data from salamander and goldfish were obtained from ON bipolar cells. Yazulla et al. (2000) showed by electron microscopy that CB1 receptors were present on virtually all ON bipolar cells but only about one-third of the OFF bipolar cells. This bias toward the ON pathway indicates that retrograde suppression of bipolar cell activity would be more of a factor with increments in light intensity than in decrements about the ambient background.

Activation of dopamine D1 receptors enhances I_{Ca} and $I_{K(V)}$ in Mb bipolar cells of goldfish (Heidelberger and Matthews, 1994; Fan and Yazulla, 1999). As Mb bipolar cells have D1 receptors (Mora-Ferrer et al., 1999) that act via G protein G_s, and CB1 receptors (Yazulla et

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al., 2000) that act via G protein $G_{i/o}$, it was determined whether CB1 receptor agonists and dopamine interacted to modulate $I_{K(V)}$ of Mb bipolar cells (Fan and Yazulla, 2005). Dopamine (10 μM) consistently increased $I_{K(V)}$ by a factor of about 1.6, whereas WIN 55,212−2 (0.25 − 1 μM) had no effect on its own. However, when co-applied WIN 55,212−2 (0.1 − 0.25 μM) reversibly blocked the effect of 10 μM dopamine even though these low concentrations of WIN 55,212−2 had no effect when applied alone (Fig. 8). All effects of WIN 55,212−2 were blocked by co-application of SR141716A and pretreatment with pertussis toxin (a blocker of $G_{i/o}$) indicating action via CB1 receptor activation of G protein $G_{i/o}$. Co-activation of CB1 and D1 receptors on Mb bipolar cells produced reciprocal effects on $I_{K(V)}$. The CB1-evoked suppression of $I_{K(V)}$ was mediated by G protein $G_{i/o}$, whereas the D1-evoked enhancement was mediated by G protein G_s. The silent effect of WIN 55,212−2 suggests that the basal level of G_s-activation in Mb bipolar cells is normally low, and consequently, activation of $G_{i/o}$ by low concentrations of WIN 55212−2 would have no effect unless there was a concomitant increase in G_s; for example, by stimulation of D1 receptors. Effects of dopamine and cannabinoid agonists on $I_{K(V)}$ occur within the physiological range of Mb bipolar cell function (~ −25 to 0 mV). $I_{K(V)}$ would be activated during the ON portion of the response and, as a counter current, would modulate the peak:plateau ratio of the response. All things being equal, CB1 receptor activation should make the Mb bipolar cell ON response more tonic by suppressing the hyperpolarizing effect of $I_{K(V)}$, whereas D1 receptor activation should make the ON response more phasic by enhancing $I_{K(V)}$. If cannabinoids and dopamine constitute opposing modulators of retinal sensitivity, the effect on ganglion cells that are innervated by Mb bipolar cells should be relatively tonic responses in scotopic conditions and relatively phasic responses in photopic conditions. Suppression of the calcium currents should reduce transmitter release and reset sensitivity to further increments. As dopamine is a retinal "light" signal, these data supported the idea that endocannabinoids could function as a counter "dark" signal, interacting with dopamine to modulate the temporal aspects of signaling in the retina as well as in maintaining sensitivity.

9. Effects on Photoreceptors

9.1 Voltage gated currents

Effects of the CB1 agonist WIN 55,212−2 on membrane currents of photoreceptors were reported in goldfish cones and tiger salamander rods and cones (Fan and Yazulla, 2003; Straiker and Sullivan, 2003). There is considerable agreement in the data from these two studies: 1. all effects of WIN 55,212−2 were blocked by SR141716A that had no effect of its own, and 2. all effects were mediated by PKA. Salamander rods and cones responded differently to 1 μM WIN 55,212−2. I_K was suppressed in single cones and rods whereas I_{Ca} was suppressed in cones but enhanced in rods. It was suggested that the differential effect on I_{Ca} and I_K in rods would increase transmitter release, the result of which would be to reduce sensitivity. This positive feedback mechanism appears to be counter-adaptive and at odds with virtually all other data that show a CB1-mediated inhibition of calcium currents, including salamander cones. This issue will be addressed again in the discussion on the biphasic effect of WIN 55,212−2 and retrograde inhibition of goldfish cones.

Fan and Yazulla (2003) recorded from goldfish cones and reported a biphasic response to WIN 55,212−2. At concentrations <1 μM there was an enhancement of I_K, I_{Cl} and I_{Ca}, while at concentrations >1 μM, I_K, I_{Cl} and I_{Ca} were suppressed. The voltage-activation ranges of these currents were not affected (Fig. 9). All effects of WIN 55,212−2 were blocked by the SR 141716A as well as the PKA inhibitor, Wiptide. The enhancement was blocked by 0.5 nM cholera toxin and the suppressive effect was blocked by pertussis toxin. The conclusion drawn from these data was that activation of CB1 receptors enhanced I_K, I_{Cl} and I_{Ca} by G protein G_s and suppressed these currents by G protein $G_{i/o}$. This biphasic regulation of cone currents seemed consistent with reports of CB1 receptor coupling to G_s and $G_{i/o}$ G-proteins in other

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preparations (Glass and Felder, 1997; Maneuf and Brotchie, 1997; Sulcova et al., 1998; Calandra, 1999). As both D2 receptors and CB1 receptors are located on goldfish cones (Wagner and Behrens, 1993; Yazulla and Lin, 1995; Yazulla et al., 2000), the next study was to investigate potential interactions between the cannabinoid and dopamine systems.

The idea was that dopamine is a light-adaptive signal that desensitizes the retina, while endocannabinoids increase photosensitivity. At first, an opposing action of CB1 and D2 receptors on cone function seemed unlikely because both receptor types ordinarily act by decreasing cAMP via $G_{i/o}$. However, the enhancement of cone currents by low concentrations of Win 55,212−2 by G_s seemed to solve this potential problem. Indeed a D2 agonist, quinpirole (<10 μM), completely blocked the enhancement of I_K and I_{Ca} seen with 0.7μM WIN 55,212 −2. The effect of quinpirole was blocked by sulpiride and pertussis toxin, indicating action via a D2 receptor-$G_{i/o}$ coupled mechanism. The suppressive action of 50 μM quinpirole was not additive with the suppressive effect of high concentrations (3 μM) of WIN 55,212−2 suggesting that both agonists decreased membrane currents via the same transduction pathway, $G_{i/o}$ PKA. The opposite effects of CB1 and D2 receptor activation on goldfish cone membrane currents occurred only with low concentrations of WIN 55,212−2. Assuming that the effects of WIN 55,212−2 could be generalized to endocannabinoids, we suggested that the physiological condition in fish involves very low extracellular concentrations of endocannabinoids in the OPL. It was only in this condition that a push-pull effect of CB1 receptor with D2 receptor activation on membrane currents could be observed. However, subsequent experiments proved that the best laid plans of fish and men are often gone astray (with apologies to John Steinbeck).

9.2 Retrograde endocannabinoid transmission onto cones

Despite evidence for the presence and actions of endocannabinoids in the retina, a functional role for them had not been determined. In other regions of the CNS it was clear that endocannabinoids, particularly 2-AG, serve as a retrograde transmitter and are responsible for the properties of short term plasticity, including DSI and DSE (Diana and Marty, 2004; Ohno-Shosaku et al., 2005). We attempted to demonstrate and characterize endocannabinoid-mediated retrograde suppression of membrane currents of goldfish cones in a retinal slice (Fan and Yazulla, 2007). Stimulation of retrograde release was achieved by applying a single 50 msec puff of saline with 70 mM KCl or an mGluR1 agonist DHPG through a pipette within a few μm of an Mb bipolar cell body while recording $I_{K(V)}$ from cone inner segments under whole-cell voltage clamp (Fig. 10A). $I_{K(V)}$ rather than I_{Ca} was used to monitor effects on the cone because $I_{K(V)}$ was more robust and stable and showed the same effects and pharmacology to application of cannabinoid ligands as I_{Ca} (Fan and Yazulla, 2003). Retrograde inhibition of $I_{K(V)}$ was reversible and stable over several hours (Fig. 10B,C). It had a latency of about 200 msec after a K^+ puff, was reduced by an average of 25% and a half time to recovery of 3.4 minutes (Fig. 10D). Puff durations less than 30 msec had no effect on $I_{K(V)}$ and puffs longer than 50 msec had little additional suppressive effect on $I_{K(V)}$ (Fig. 10E). Retrograde suppression of $I_{K(V)}$ was unaffected by a combination of picrotoxin and CNQX but blocked completely by SR141716A. Experiments with the FAAH inhibitor URB597, Nimesulide (a COX-2 inhibitor), URB754 (a MGL inhibitor), and Orlistat (a blocker of 2-AG synthesis), indicated that 2-AG rather than AEA was the retrograde endocannabinoid. Data also showed that calcium influx or release from intracellular stores could mediate retrograde 2-AG release. The conclusions of this study were that retrograde suppression of $I_{K(V)}$ is mediated by Ca^{2+}-dependent release of 2-AG from Mb bipolar cell dendrites by separate mechanisms (Fig. 11): 1. a voltage-dependent mechanism, mimicked by the K^+ puff, that would be activated by the depolarizing ON response to light, and 2. a voltage-independent mechanism, occurring under ambient illumination, mediated by tonic activation of mGluR1 that are present on Mb bipolar cells (Klooster et al., 2001).

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Based on our observation that low concentrations (<1 μM) of WIN 55,212−2 enhanced $I_{K(V)}$ and I_{Ca} by a CB1/PKA/cholera toxin sensitive (G_s) mechanism (Fan and Yazulla, 2003), it was expected that the concentration of the retrograde endocannabinoid would be very low and therefore enhance cone currents when released. It was thus surprising that the retrograde effect always suppressed the currents. There was no initial enhancement followed by suppression as if there were a buildup of endocannabinoid concentration. The likely explanation appeared to be in ‘agonist-specific’ trafficking in which binding of different agonists to CB1 receptors favors coupling to different G proteins (Bonhaus et al., 1998). For example, WIN 55,212−2 increases intracellular calcium by $G_{q/11}$ coupling in hippocampal neurons and human trabecular meshwork cells, while other CB1 agonists including THC, 2-AG, CP55940 and methanandamide may couple to $G_{i/o}$ but not to $G_{q/11}$ (Lauckner et al., 2005; McIntosh et al., 2007). It seemed likely that the reciprocal interaction between CB1/G_s- and dopamine D2/$G_{i/o}$-mediated processes that was proposed, based on data obtained with WIN 55,212−2 (Fan and Yazulla, 2004), may have been peculiar to WIN 55,212−2 and did not apply to the endocannabinoid, 2-AG. In the absence of the tidy ‘push-pull’ idea, what could be the function of the retrograde release that is mediated by the same $G_{i/o}$ mechanism as activated by D2 receptors?

The unwarranted assumption is that activation of the D2 and CB1 receptors on the cones occurs under the same lighting conditions. Two conditions evoke 2-AG release from Mb bipolar cells, strong depolarization and activation of mGluR1, corresponding to voltage-dependent and voltage-independent mechanisms (Fan and Yazulla, 2007). Paradoxically, these two conditions occur in opposite lighting situations. It is presumed that rods and cones release L-glutamate at a steady rate under any ambient illumination; this rate is increased by decrements of light intensity and decreased by increases in light intensity. Also, it was presumed that the data on the retrograde suppression of cone $I_{K(V)}$ could be generalized to I_{Ca} with implications for transmitter release from the cones.

The voltage-dependent release of 2-AG from Mb bipolar cells would provide a positive feedback that would amplify the reduction in cone transmitter release caused by increasing light intensity (Fig. 11B). This situation would occur because large increments in light would decrease L-glutamate release from cones, depolarize the Mb bipolar cell, trigger retrograde release of 2-AG that would subsequently reduce L-glutamate release from cones. It is not known how physiologically relevant this sequence would be because the half-time to recovery from a 50 msec pulse of retrograde stimulation is several minutes. This prolonged effect would seem to mitigate any response to rapidly changing intensities of light.

It was suggested that the voltage-independent mechanism was more likely to operate under normal conditions in that it would provide a negative feedback control of the tonic release of glutamate from cones (Fig. 11A). This is because as L-glutamate is released during ambient illumination, mGluR1α on Mb bipolar cells would be activated tonically and 2-AG would be released via a calcium-dependent $G_{q/11}$ mechanism. The retrograde inhibition of transmitter release from photoreceptors would provide negative feedback that would dampen the steady transmitter release under ambient illumination; the dimmer the background, the stronger the negative feedback. As background was increased, the feedback would be reduced. In this manner ambient illumination would produce a resting endocannabinoid tone that maintained a steady release of transmitter within relatively narrow limits, regardless of background. In this case, retrograde transmission would occur even though Mb bipolar cells were hyperpolarized by L-glutamate acting on either the EAAT or mGluR6 (Grant and Dowling, 1996;Wong et al., 2004). Also, the long half life of the retrograde effect would make this system insensitive to rapid changes around the background.

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Endocannabinoids are only the latest of the neuromodulators that affect membrane currents in photoreceptors. Others include adenosine (Stella et al., 2002, 2003), dopamine (Fan and Yazulla, 2004), GABA (Tachibana and Kaneko, 1984; Picaud et al., 1998), glycine (Balse et al., 2006), nitric oxide (Kurreny et al., 1994; Kourennyi et al., 2004), polyunsaturated fatty acids (Vellani et al., 2000), protons (Barnes and Bui, 1991; Harsanyi and Mangel, 1993), somatostatin (Akopian et al., 2000), as well as modulation of hemichannels (Kamermans et al., 2001). It seems unlikely that all these operate on transmitter release at the same time. Even if paired off as opposing modulators, this makes for excessive control if occurring at the same time. Perhaps reciprocal modulation of photoreceptor activity occurs under multiple conditions of ambient lighting (scotopic, mesopic, photopic), circadian shifts in sensitivity, or even large changes in intensity for a given ambient background condition. The area of influence also may be a factor. Endocannabinoids, nitric oxide, protons and hemichannels could have a very local effect, down to a single synapse, whereas adenosine, dopamine and somatostatin could have global effects on the retina as they can function as paracrine modulators or volume transmitters.

9.3. WIN 55,212−2 affects the cone light response

Goldfish cone photoreceptors contain CB1 receptors at the synaptic terminal, selectively accumulate ^{3}H-anandamide, contain fatty acid amide hydrolase-immunoreactivity, and possess voltage-gated calcium and potassium currents that are modulated by CB1 ligands (Yazulla et al 2000, Fan and Yazulla, 2003, Glaser et al., 2005a). The question was whether effects were restricted to the synaptic output or was there any effect on cone light responses. The previous studies were performed on retinal slices whereas these experiments were done in the isolated retina. Cones were stimulated with a spot of light of variable wavelength and intensity in combination with voltage- and current-clamp protocols (Fig. 12). Bath application of WIN 55,212−2 (10 μM) had no effect on the absolute sensitivity of the cones or on the kinetics of the onset response. However, the light offset response became faster and the depolarizing overshoot was enhanced. This effect was seen at all but dim intensities (Fig. 12A) and was independent of holding potential (Fig. 12B). This was found under current-clamp as well as under voltage-clamp conditions, indicating that the effect of WIN 55,212−2 was mediated by modulation of the cGMP-gated channels in the outer segment of the cones rather than by voltage-dependent currents. The effects of WIN 55,212−2 were not blocked by SR141716A, indicating that the effect was not mediated by CB1 receptors. With a train of 'dark' flashes from a steady background, the photocurrent recovered toward baseline more quickly with WIN 55,212−2 than in Control, with the result that the peak-to-peak response to succeeding flashes was greater. It was concluded that WIN 55,212−2 sped up the dynamics of the phototransduction deactivation cascade in the cone outer segments. The functional consequence of this effect was to shorten the recovery time to the offset of bright flashes, perhaps resulting in an increase in contrast sensitivity. A prediction from these experiments is an increase in the flicker fusion frequency at photopic intensities. However, Adams et al. (1978) reported that 15 mg of THC introduced a modest 8% delay in the recovery from a bright flash under photopic conditions. This result is counter to what would be predicted from our finding that during the light offset response, the membrane potential/current returned to baseline more quickly with WIN 55,212−2. Our data indicate that cones would actually recover more quickly at the offset of a flash under a wide range of backgrounds. This effect, if preserved through the visual pathway, could result in increases in contrast detection or critical flicker frequency.

These experiments raised several issues. The first has to do with the application of drugs. Concentrations of WIN 55,212−2 (0.2 − 4 μM) that modulated $I_{K(V)}$ of cones in a retinal slice (Fan and Yazulla, 2003) did not affect the cone-light response when applied to the outer surface of the isolated retina; and 10 μM WIN 55,212−2, when applied to the retinal surface had inconsistent effects on membrane currents. CB1 receptors are concentrated at the cone synaptic

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terminal (Yazulla et al., 2000). In the slice preparation, access to these receptors is immediate on the exposed surface of the cone terminal. However, in the isolated retina, the cone synaptic terminal is about 100 μm from the outer segments on the surface of the retina. The voltage-independence and insensitivity of WIN 55,212−2 to inhibition by SR141716A in the isolated retina indicates that the ligand did not reach CB1 receptors on the cone terminal. A resolution to this issue could come from recording light responses from cones in a slice preparation. The second issue is the mechanism by which WIN 55,212−2 accelerated the light OFF response in a CB1-independent manner. It was suggested that WIN 55,212−2 stimulated a component involved in the deactivation of $G_{T\alpha}$/PDE, leading to a quicker response to light offset without affecting the kinetics of the onset of the light response (Struik et al., 2006). This idea remains to be tested. A third issue is whether the effect of WIN 55,212−2 on the photoresponse would be observed with other CB1 agonists and the endocannabinoids, AEA and 2-AG. Recall that the enhancing effect of low concentrations of WIN 55,212−2 on cone membrane currents was not observed with retrograde endocannabinoid release (Fan and Yazulla 2003, 2007). A combination of agonist-specific trafficking and unidentified CB receptors on cone inner or outer segments could be factors in these findings. There is no evidence that endocannabinoids directly modify the cone light response, and the effect of WIN 55,212−2 may turn out to be agonist specific. For example, a prediction from our experiments is that WIN 55,212−2 would cause an increase in the flicker-fusion frequency at photopic intensities. However, Adams et al, (1978) reported that THC prolonged the recovery time from a bright flash under photopic conditions. Direct comparison of these studies is difficult because of the many potential sites of action of inhaled THC *versus* the localized sites of action of WIN 55,212−2 in the goldfish isolated retina.

Electrophysiological studies show that endocannabinoids presynaptically suppress the output of the ‘through pathway’ of the retina, photoreceptors and bipolar cells. The effect is subtle and modest as might be expected since smoking marijuana does not render the smoker blind. Evidence for effects of eCBs on amacrine cells is strongest for their suppression of dopamine release. Dopamine, a signal for light adaptation in the retina, also antagonizes the action of cannabinoids on bipolar cells. Historically, psychophysical studies of the effects of cannabinoids on vision were often done in concert with alcohol in the 1970s to determine effects on visual motor behaviors as they may relate to driving (Dawson et al., 1977, for review). Endocannabinoids constitute a major transmitter system in the central and peripheral nervous systems where they are critically involved in neuronal plasticity. Light and dark adaptation also involve processes of neuronal plasticity that are confined to the retina. Given the action of cannabinoids on photoreceptors and bipolar cells and their interaction with dopamine, perhaps it is worth revisiting the issue of the visual consequences of cannabinoids in model animal systems as a prelude to further work in human.

10. Role in development

Considering the well established role of endocannabinoids in synaptic plasticity it is not surprising that there is great interest regarding the role of the endocannabinoids in neural development, particularly the effects of prenatal-marijuana use on offspring in humans. Earlier studies in humans were summarized in the Ottawa Prenatal Prospective Study (OPPS) by Fried (1995) and more recently by Ramos et al. (2005). Effects were observed on visual habituation, tremors and startle responses in neonates (4−30 days age), but no effects on children 1−6 years of age. However, for children 6−9 years old there were problems with behavior, decreased performance on visual perceptual tasks, language comprehension, sustained attention and memory. These deficits were attributed to effects on the prefrontal cortex, an area enriched in CB1 receptors, at least in rat (Herkenham et al., 1991). There is an extensive and growing literature on the role of endocannabinoids on pre- and postnatal brain development by affecting dopamine, endogenous opiods, gene expression for neuron-glia adhesion molecules, effects

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on neuron and glial differentiation, apoptosis and so on (Fernández-Ruiz et al., 1999, 2004; Galve-Roperh et al., 2006; Fride, 2008, for reviews). Although CB1 receptors have been localized in embryonic rat and chick retinas (Buckley et al., 1998; Begbie et al., 2004; Leonelli et al., 2005), I could find no studies that investigated or commented on the effects of manipulating endocannabinoids on retinal development. The effects of prenatal marijuana use on visual perceptual tasks were ascribed to cortical effects, although some participation of the retina cannot be excluded without more experimentation.

11. Neuroprotection of ganglion cells

In addition to its function in synaptic plasticity, there is exciting progress in the role of cannabinoids in affording protection against traumatic, ischemic, inflammatory and neurotoxic damage in the CNS (i.e., van der Stelt and Di Marzo, 2005b; de Lago and Fernández-Ruiz, 2007; Micale et al., 2007; Mechoulam and Shomai, 2007). The end point of glaucoma is the selective death of ganglion cells by apoptosis (Quigley, 1999) that may be caused by optic nerve injury following compression or ischemia. Activation of NMDA receptors by glutamate release during ischemia causes an increase in intracellular calcium that triggers a cascade leading to cell death (Osborne et al., 2004). Activation of CB1 receptors provides protection against neuron death caused by transient global ischemia, or excitotoxicity *in vitro* (Nagayama et al., 1999; Marsicano et al., 2002; Veldhuis et al., 2003; Sommer et al., 2006; Gilbert et al., 2007). Several studies have shown in the retina that CB1 agonists (THC and cannabidiol) protect ganglion cells from glutamate-excitotoxicity (El-Remessy et al., 2003; Opere et al., 2006) and ischemia caused by increased IOP (Crandall et al., 2007; Nucci et al., 2007). So far these studies in retina have been performed in rat and have involved mostly modulation of CB1 and TRPV1 receptors to alter the course of apoptosis. An alternative strategy is to modulate the activity of metabolizing enzymes: FAAH, MGL and COX-2. The rationale is that since eCB tone is low, modulation of enzyme activity would avoid the side effects of receptor modulation (Pertwee, 2005b). For example, inhibition of FAAH protects against excitotoxicity in rat hippocampus (Karanian et al., 2005) and increases AEA tone in the brain with beneficial effects in rat models of depression and analgesia without the side effects of CB1 receptor stimulation (Jayamanne et al., 2006; Bortolato et al., 2007). Most recently in rat retina, Nucci et al, (2007) showed that inhibition of FAAH significantly prevented the decrease in Thy-1 levels and the reduction in the number of cells in the ganglion cell layer in response to increased IOP. The conclusion was that AEA protects against ganglion cell death by activation of CB1 and TRPV1 receptors. Adenosine, via A1 receptors, also protects against NMDA-toxicity and glutamate induced Ca influx in cultured rat ganglion cells (Hartwick et al., 2004; Oku et al., 2004). This is of interest because effects of micromolar concentrations of CB1 antagonists, SR141716A and AM251, can be blocked by adenosine A1 antagonists; such effects are seen even in preparations from CB1-receptor knockout mice (Fowler et al., 2005, for review).

COX-2 contributes to neuronal cell death following ischemia or NMDA-toxicity in cortex, retinal glial cells, RPE and ganglion cells (for example, Nogawa et al., 1997; Hewett et al., 2000; Chin et al., 2001; Nakamichi et al., 2003; Zheng et al., 2007), while COX-2 blockers prevented NMDA-induced ganglion cell apoptosis in rat retina (Ju et al., 2003). A very recent study showed that 2-AG is neuroprotective in the hippocampus by suppressing COX-2 elevation in response to proinflammatory and excitotoxic stimuli (Zhang and Chen, 2008). Curiously, PGE_2, a downstream COX-2 metabolite of AEA, arachidonic acid and 2-AG, acting via EP2 receptors, was neuroprotective following cerebral ischemia and NMDA-induced toxicity in brain (e.g., McCullough et al., 2004; Liu et al., 2005; Ahmad et al., 2006). Conversely, some report that activation of EP2 receptors promotes apoptosis in neurons of rat hippocampus and cortex (Takadera et al., 2002, 2006; Sang et al., 2007). It appears that the neuroprotective effect of EP2 receptors is due to increased cAMP levels in the affected cells that occur downstream of COX-2. If so, then stimulation of COX-2 and EP2 receptors could

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have opposite effects on ganglion cell apoptosis. Progress made regarding the role of endocannabinoids in other regions of the CNS for a wide variety of diseases and traumatic events hopefully will stimulate similar research for retinal and ocular diseases.

12. Future Directions and Implications

An alternative to studying effects of chronic or acute drug use in rats is to use knockout (KO) mice in which FAAH or CB1 receptors have been inactivated. Such mice provide opportunities to study chronic suppression of enzyme and receptor function as they relate to synaptic plasticity, neuroprotection and compensatory changes that may occur with chronic drug therapy. The mouse is a marvelous preparation to study the genetic bases of numerous diseases, including ocular and retinal disorders (LaVail and Sidman, 1974). Issues of *Vision Research* 44 (28) 2004 and *Visual Neuroscience* **22** (5) 2005 were devoted to the topics of “Mouse Visual System” and “The Laboratory Mouse in Vision Research”, with emphasis on mouse models of ocular diseases. As eCBs are found in neural and non-neural tissues, knockouts of FAAH and CB1 receptor have systemic effects that involve memory, motor coordination, pain sensitivity, the immune system, weight control, response to addictive drugs and many others (for example, Ledent et al., 1999; Cravatt et al., 2001, 2004; Lichtman et al., 2004; Varvel et al., 2006; Wise et al., 2007). The few studies on neurotoxicity in CB1 KO mice show that CB1 receptors are critical for the survival of cerebellar neurons and hippocampal progenitor cells in response to excitotoxicity (Marsicano et al., 2002; Aguado et al., 2007). As of now, I know of no studies on the effects of the FAAH KO on neuron survival in any tissue following ischemia or excitotoxicity.

The study of cannabinoids has exploded in other parts of the CNS, PNS and peripheral organ systems but not in retina or other ocular tissues. For example, a PubMed search for the last 5 years on cannabinoid + brain yielded 1,279 papers; cannabinoid + hippocampus yielded 276 papers and cannabinoid + obesity yielded 316 papers. However, cannabinoid + glaucoma yielded 20 papers, and cannabinoid + retina yielded only 23 papers. Endocannabinoids constitute the newest of the neuromodulators that are found in neural and non-neural tissues throughout the body. There is great interest in cannabinoids not only for their role in neuroplasticity but also for therapeutic use in numerous conditions, including pain, stroke, cancer, obesity, osteoporosis, fertility, neurodegenerative diseases, multiple sclerosis, glaucoma, inflammatory diseases and diabetic retinopathy, among others. The presence of multiple endocannabinoids, degradative enzymes and receptors provides a broad spectrum of targets for therapeutic application (Mackie, 2006; Kogan and Mechoulam, 2007). In terms of retinal function, there is general agreement that cannabinoids suppress dopamine release and presynaptically reduce transmitter release from cones and bipolar cells. How this relates to light and dark adaptation, receptive field formation and temporal properties of ganglion cell responses are just three of the many questions that need to be answered. Modulation of Müller's cells and their downstream effects on retinal physiology, including development and neuroprotection, is a completely open area of research. It cannot be left to researchers who study the hippocampus, frontal cortex or cerebellum to explain endocannabinoid function in the retina. The ability to control the photic environment gives retinal scientists a powerful advantage in the study of any neurobiological event; we know and can control the input and we know the output. A big problem dealing with the physiology and pharmacology of endocannabinoids is how lipophilic they are and the difficulty of obtaining reversible effects. Still considerable progress has been made and the payoff in this rapidly expanding and exciting field is worth the effort. I close with quotes from my doctoral and postdoctoral mentors. If I commented on the difficulty of a project, my Ph.D. advisor, Allen Granda would simply say “If it were easy, everyone would do it”. When we would chat about a new project, my postdoctoral advisor, John Dowling would often say “That sounds like fun, let's do it.” Well, the last ten years my collaborators and I have spent studying retinal cannabinoids have been

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difficult and often solitary. However, it has been fun. Hopefully, others will follow, hop on and enjoy the ride.

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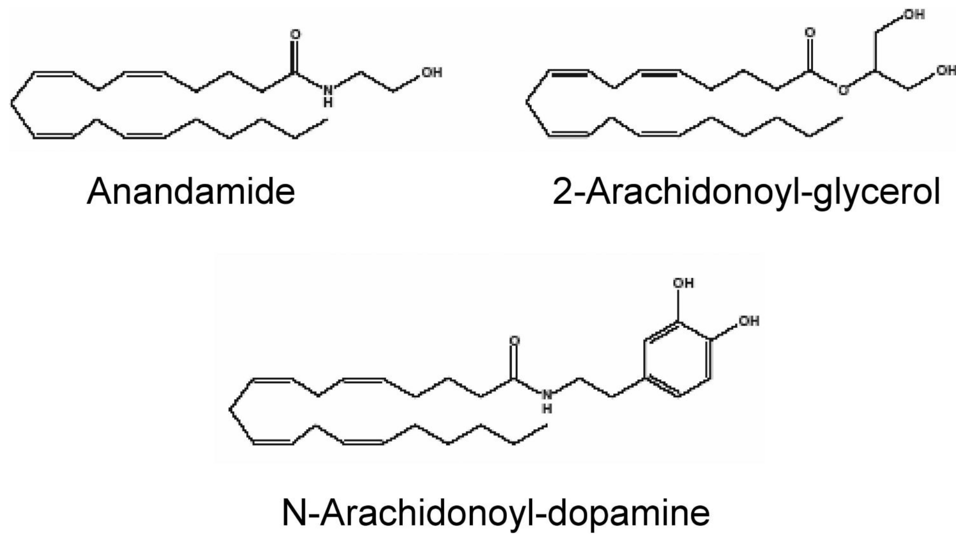

Fig. 1.
Chemical structures of three endocannabinoids: arachidonoyl-ethanolamide (Anandamide, AEA), 2-arachidonoyl-glycerol (2-AG) and N-arachidonoyl-dopamine (NADA).

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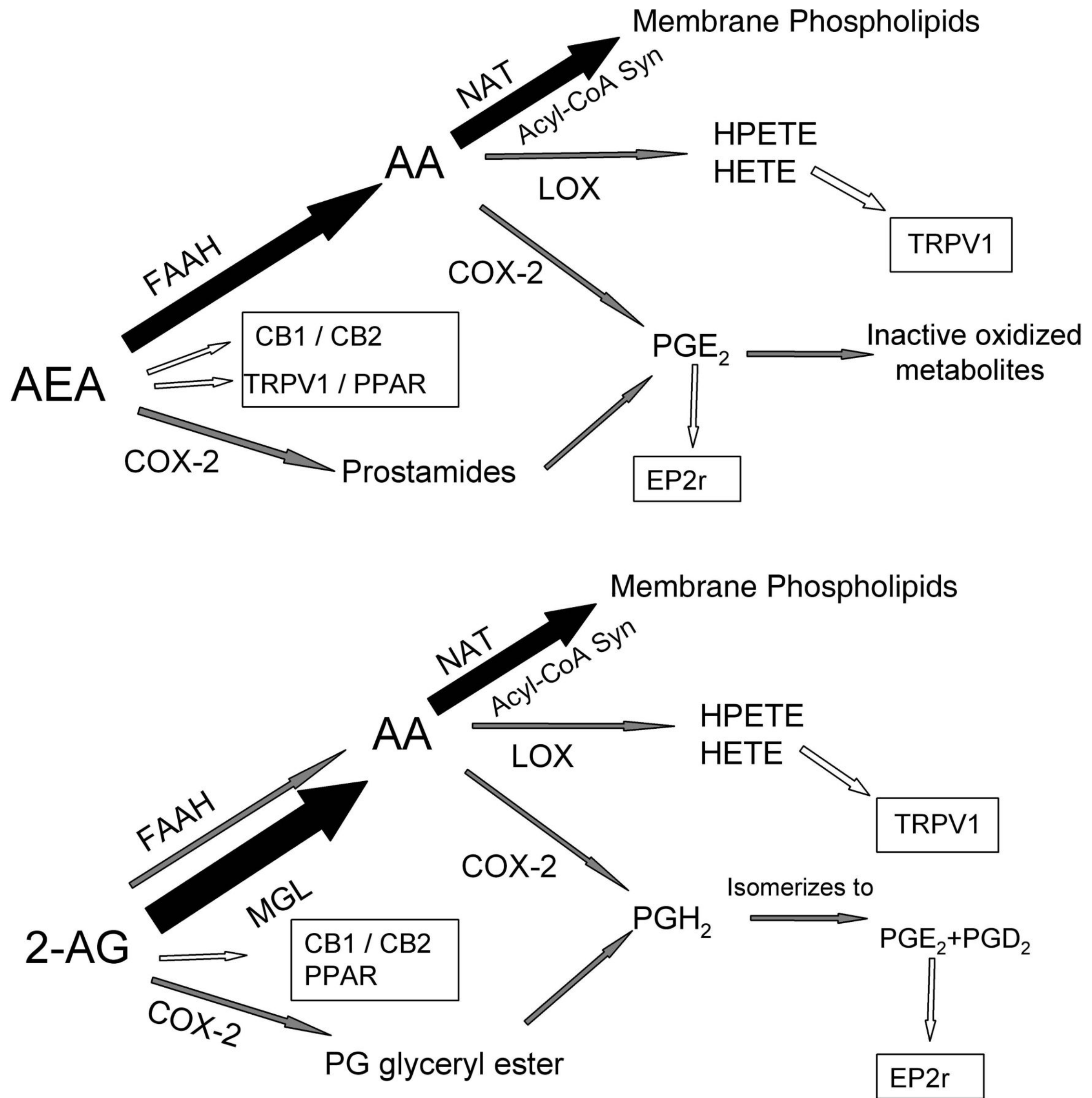

Fig. 2.

This schematic illustrates some of the metabolic pathways of the degradation of AEA and 2-AG. In the dominant pathways (**bold arrows**), AEA and 2-AG are hydrolyzed to arachidonic acid (AA) and then rapidly incorporated into membrane phospholipids via N-acyltransferase (NAT) and Acyl-Coenzyme A synthetase. Lesser pathways (shaded arrows) involve oxidation by cyclooxygenase-2 (COX-2) of AEA, 2-AG and AA to prostaglandins (PGE_2 and PGD_2). Additionally, AA may be oxidized by lipoxygenase (LOX) to 12-(S)- and 15-(S)-HPETE and 5-(S)-HETE. Hollow arrows show that AEA and 2-AG are endoligands for CB1, CB2 and PPAR receptors, while AEA also activates TRPV1 receptors. Metabolites of COX-2 oxidation activate EP2 receptors, and metabolites of LOX oxidation activate TRPV1 receptors.

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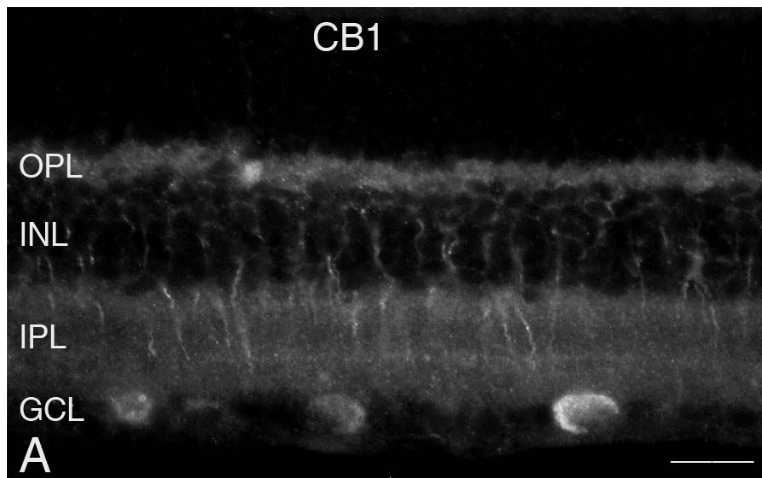

Fig. 3. Localization of CB1R-IR in mouse retina (unpublished observation)
CB1R-IR was observed over the photoreceptor inner segments, the outer plexiform layer (OPL), scattered cell bodies in the inner nuclear layer (INL), in two broad bands in the inner plexiform layer (IPL) and cells in the ganglion cell layer (GCL). We found that CB1R-IR in the vertical streaks in the INL and IPL, along with cell bodies in the distal INL was co-localized with PKC-IR, and thus are rod bipolar cells. With the exception of CB1RIR in mouse ganglion cells, this pattern is very similar to what was reported in rat retina with this same CB1 antiserum (Yazulla et al., 1999). Cal bar = 10 μm.

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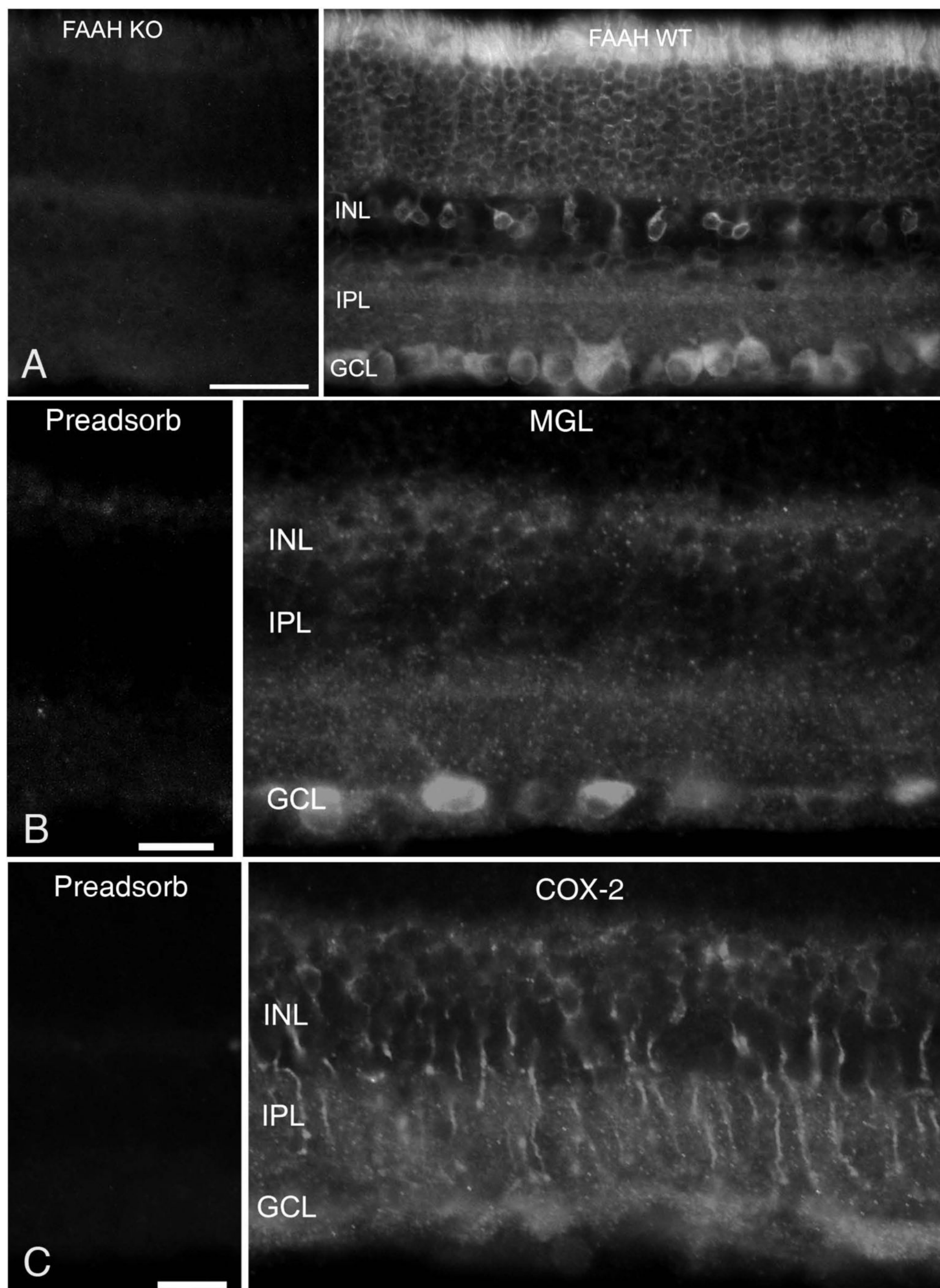

Fig. 4. Distribution of CB1R-IR in goldfish retina (from Yazulla et al., 2000)

A) Preadsorption of the CB1 antiserum with the immunizing peptide antigen abolished CB1R-IR over the entire retina except for the bright band over the basal lamina, indicating non-specific labeling in this region. ONL — outer nuclear layer, OPL — outer plexiform layer, INL — inner nuclear layer, IPL — inner plexiform layer, GCL — ganglion cell layer. Calibration bar for **A** and **B** = 20 µm.

B) CB1R-IR was prominent over cell bodies in the proximal margin of the INL, diffuse labeling over cone cell bodies, but not rod cell bodies in the ONL, as well as diffuse and punctate labeling in the OPL (white arrows) and IPL. The streaks of CB1R-IR that extended vertically through the retina were identified as Müller's cells (black arrow). Bright patches of CB1R-IR in the

proximal IPL (arrowheads) were identified as the synaptic terminals of Mb bipolar cells by double labeling with PKC-IR.

C. Electron micrograph of CB1R-IR in the outer plexiform layer of goldfish retina. Membrane associated CB1R-IR (arrowhead) was located away from a synaptic ribbon (small arrow). H — horizontal-cell dendrite. Calibration bar = 0.5 μM.

D. Electron micrograph of CB1R-IR in the inner plexiform layer of goldfish retina. CB1R-IR in the presynaptic terminal of an Mb bipolar cell was along the bipolar cell membrane (arrowheads) that was apposed to two amacrine cell processes (ac). Calibration bar = 0.25 μm.

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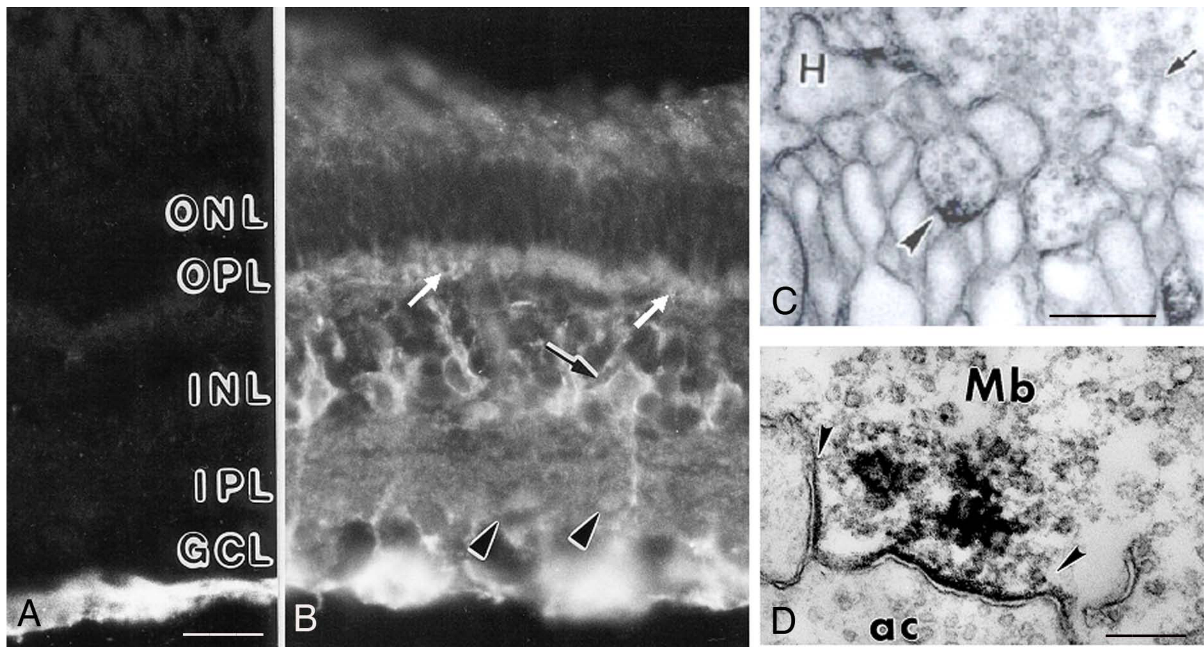

Fig. 5. Distribution of endocannabinoid metabolizing enzymes in mouse retina. (unpublished observations)

A. FAAH — FAAH-IR was absent in the FAAH knockout (KO) mouse, attesting to the specificity of the FAAH antisera. The distribution of FAAH-IR in wild type (WT) retina was essentially identical to that in rat retina with this antisera (Yazulla et al 1999). The most prominent label was in virtually all cells in the ganglion cell layer (GCL), bipolar cells, rod inner segments and nuclei, and lighter label over amacrine cell bodies in the proximal inner nuclear layer (INL, not seen in this section) and in a band in the middle of the inner plexiform layer (IPL). As in rat (Yazulla et al 1999), FAAH-immunoreactive bipolar cells in mouse are **not** PKC-immunoreactive and thus are cone BC rather than rod BC. Cal bar = 10 μm.

B. MGL — MGL-IR was absent after preadsorption of the MGL antiserum with the immunizing peptide antigen. MGL-IR was prominent over ganglion cells in the GCL and proximal INL and in rod bipolar cells (these colocalize with PKC-IR). MGL-IR was far less intense on bipolar cell axons and dendrites of amacrine and ganglion cells. There were numerous MGL-immunoreactive boutons throughout the IPL that could not be assigned to any particular cell type.

C. COX-2 — No COX-2-IR was observed in retinas after preadsorption of the COX-2 antiserum with the immunizing peptide antigen. The distribution of COX-2-IR was most prominent over several layers of bipolar cell bodies in the INL and their axons in the IPL. Double labeling with PKC-IR showed that COX-2-immmunoreactive bipolar cells were a mixed population of rod bipolar cells and cone bipolar cells.

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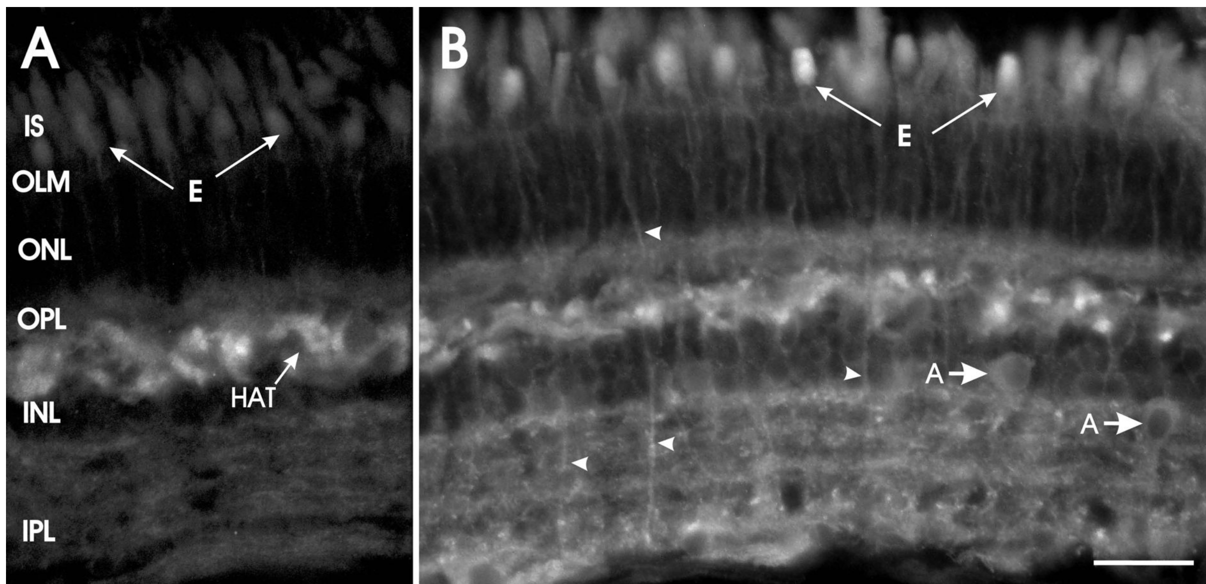

Fig. 6. FAAH-IR in the goldfish retina (from Glaser et al., 2005a)
A. Preadsorption of the FAAH antisera with peptide antigen revealed heavy labeling over horizontal cell axon terminals (HAT) in the middle of the inner nuclear layer (INL) and weak label over cone ellipsoids (E — arrows), indicating non-specific labeling of these structures. **B**. FAAH-IR was prominent in the perinuclear region of cone inner segments (IS), vertical fibers (arrowheads) that extended from the outer limiting membrane (OLM) through the inner plexiform layer (IPL) and cell bodies at the proximal INL that were identified as processes of Müller's cells by double labeling with GFAP. There were also labeled amacrine cells (arrows — A). Inner segment — IS, Outer limiting membrane — OLM, Outer nuclear layer — ONL, Outer plexiform layer — OPL, Horizontal cell axon terminals — HAT, inner plexiform layer — IPL. Scale bar = 20 μm.

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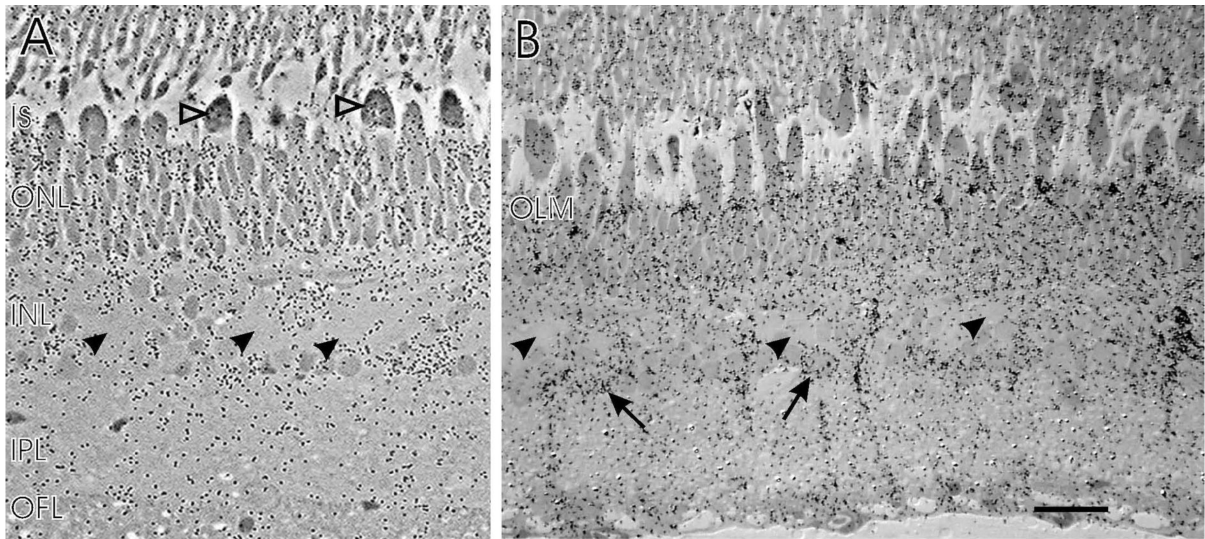

Fig. 7. ***In vitro*** **autoradiographs of ^{3}H-AEA uptake in intact goldfish retina, incubated at 20°C at two exposures. The silver grain pattern represents the deposition of ^{3}H-AEA and its metabolites (from Glaser et al., 2005a)**

A. At 60 days exposure, there was a relative increase in grain density over cone inner segments (IS, open arrowhead). Despite numerous grains in the outer nuclear layer (ONL), rod nuclei tended to be surrounded by grains rather than filled with them, suggestive of Müller's cell processes. There were also streaks of grains through the inner nuclear layer (INL), inner plexiform layer (IPL) to the optic fiber layer (OFL). Also note there was a complete lack of grain deposition over the axon terminals of horizontal cells (HAT, black arrowheads) in the mid INL.

B. At 151 days exposure, a grain pattern indicative of Müller's cells was far more apparent, with clusters of grains at the outer limiting membrane (OLM) and prominent vertical streaks through the retina, particularly in the INL and IPL and along the end feet at the inner limiting membrane. There appeared to be labeled cell bodies in the proximal INL (arrows), but their identity as amacrine or Müller's cells is not clear. A relative lack of label over rod nuclei and HAT was still apparent even at this long exposure. Scale bar = 20 µm.

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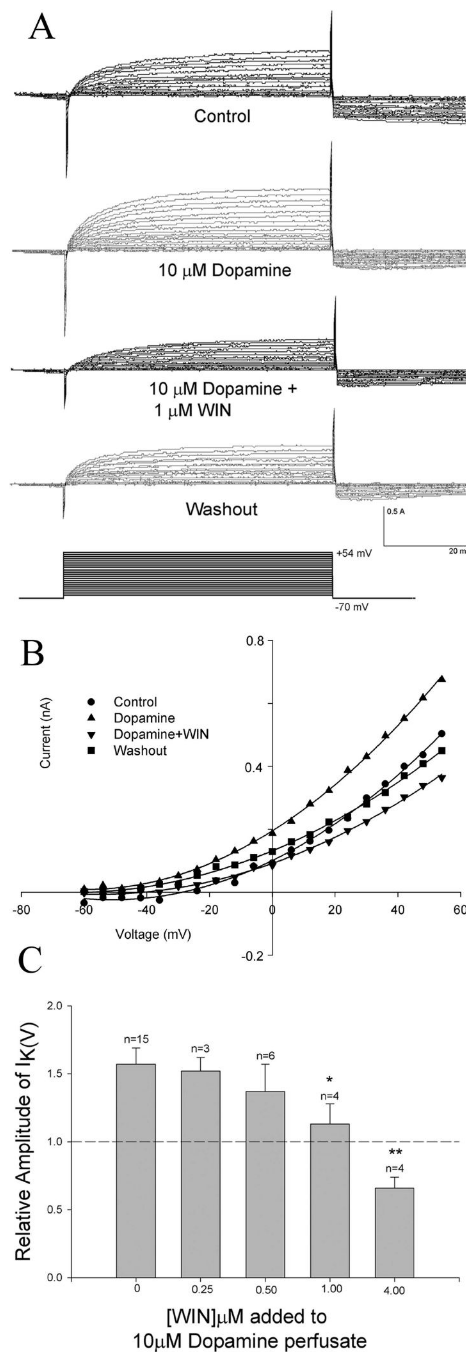

Fig. 8. Interaction of WIN 55,212−2 and dopamine on $I_{K(V)}$ in goldfish Mb bipolar cells (from Fan and Yazulla, 2005)

WIN 55,212−2 blocked the enhancing effect of 10 μM dopamine on $I_{K(V)}$ as illustrated in the current records (**A**) and I-V curves (**B**) for one Mb bipolar cell. Application of 10 μM dopamine enhanced $I_{K(V)}$. The addition of 1 μM WIN 55,212−2 suppressed $I_{K(V)}$, blocking the effect of dopamine. There was a return to control with washout of drugs. Depolarizing steps were applied from a holding potential of −70 mV. The sequence of conditions is indicated, in order, by the identified symbols in the upper left side of the I-V axis.

C. WIN 55,212−2 blocked the enhancing effect of 10 μM dopamine on $I_{K(V)}$ in a concentration dependent manner. In these experiments, the amplitude of $I_{K(V)}$ was measured in response to

application of 10 μM dopamine and again after the addition of the nominal concentration of WIN 55,212−2, as indicated on the abscissa. Each Mb bipolar cell was subjected to only one concentration of WIN 55,212−2. The effect of 10 μM dopamine was effectively negated at 1 μM WIN 55,212−2 ($p < 0.05$; n=5) and reversed to an net inhibition at 4 μM WIN 55,212−2 ($p < 0.01$; n=4). Depolarizing steps to +48 mV were applied from a holding potential of −70 mV. Current amplitudes are plotted relative to that elicited in the absence of dopamine. "0" on the abscissa is the response to 10 μM dopamine in the absence of any WIN 55,212−2.

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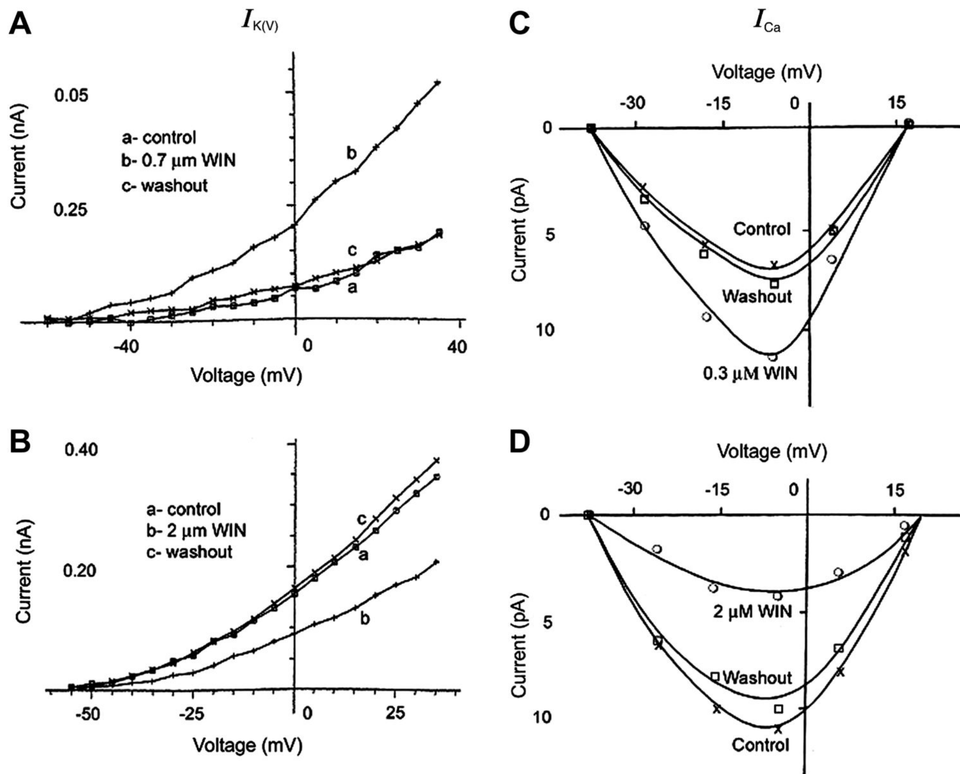

Fig. 9. Reversible biphasic effects of WIN 55,212−2 on voltage-dependent currents in goldfish cones (from Fan and Yazulla, 2003)

A, B. $I_{\mathbf{K(V)}}$ was increased by WIN 55,212−2 at concentrations < 1 µM (**A**), and was decreased at concentrations > 1 µM (**B**). The effect of WIN 55,212−2 was mostly on the amplitude of $I_{K(V)}$; the activation ranges appeared unaffected. Note, that control amplitudes were achieved with washout of WIN 55,212−2.

C, D. I_{Ca} was increased by WIN 55,212−2 at concentrations < 1µM (**C**) and decreased at concentrations > 1 µM (**D**). As with $I_{K(V)}$, only the amplitude of I_{Ca} was affected; there appeared to be no effect on the voltage-activation range. A concentration of 1 µM was the crossover from enhancement to suppression for $I_{K(V}$ and I_{Ca}.

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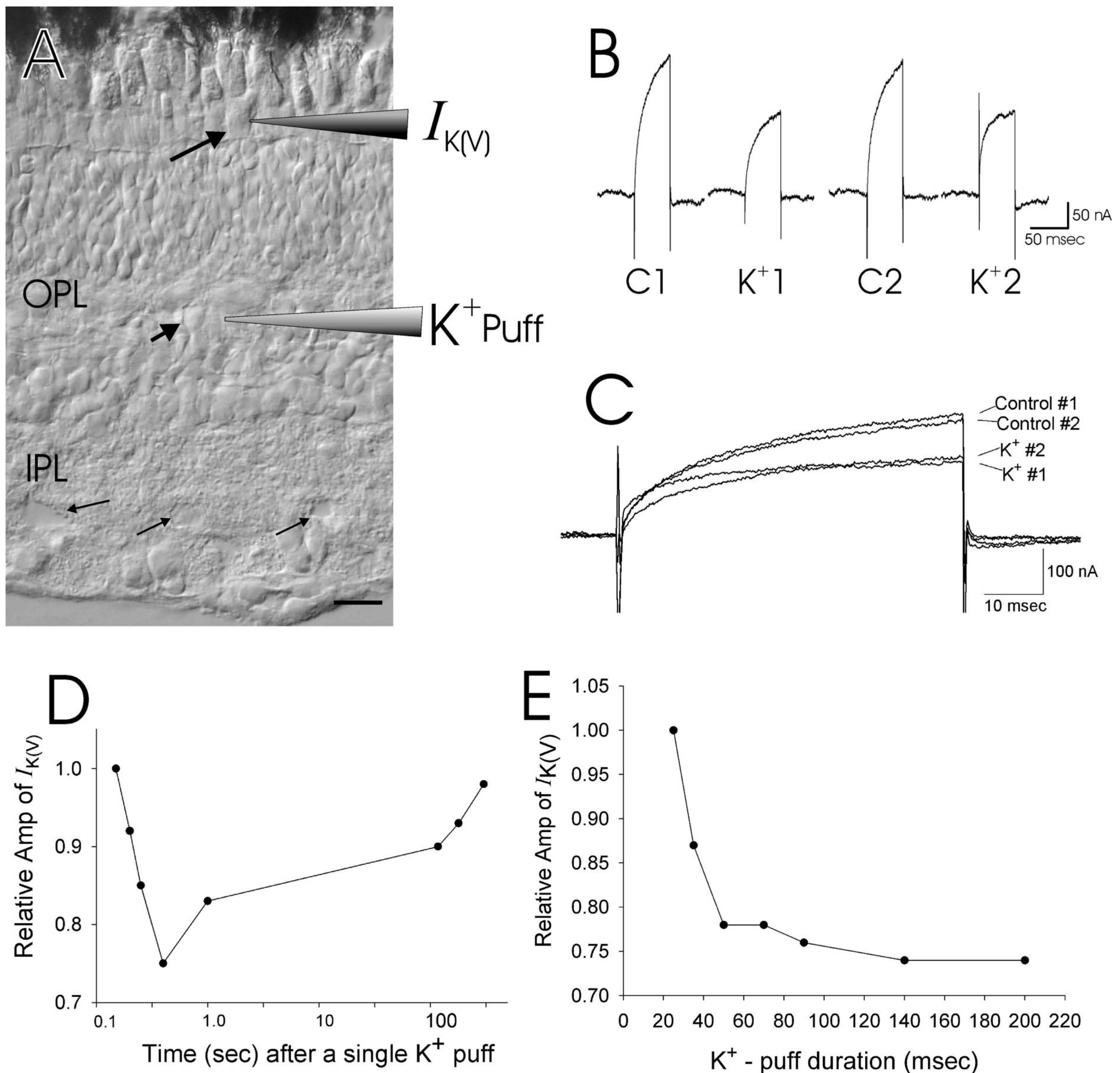

Fig. 10. Properties of the retrograde responses of cones (from Fan and Yazulla, 2007)

A. An illustration of the method used to detect retrograde responses in goldfish cones in a retinal slice. Whole cell recordings of $I_{K(V)}$ were obtained from long-single cones (long arrow). A puff pipette, containing 70 mM KCl, was positioned slightly upstream and at the cell body of a Mb bipolar cell (short arrow). Thin arrows indicate the synaptic terminals of Mb bipolar cells. OPL — outer plexiform layer, IPL — inner plexiform layer. Calibration bar = 20 μm.

B, C. Sequential and overlay of raw records of $I_{K(V)}$ from a single cone evoked by a 50 msec depolarizing pulse to +54 mV from a holding potential of −70 mV. The records have not been normalized. A 50 msec K^+ puff was delivered twice. $I_{K(V)}$ in response to K^+ puff #1 was reduced compared to that evoked for the pre-puff control #1. The cone was allowed to recover for 30 min after K^+ puff #1. $I_{K(V)}$ returned to control amplitude (C2, Control #2). The K^+ puff #2 produced an equivalent reduction in $I_{K(V)}$.

D. Time course (log scale) of the reduction of $I_{K(V)}$ in response to a single 50 msec puff of K^+ shows a latency of about 200 msec following the puff, a peak response at about 500 msec and a gradual return to control level by 5 min.

E. Effect of K^+ puff duration on $I_{K(V)}$. These data were obtained from a single cone over 4 hours. After a pre-puff control value of $I_{K(V)}$ was obtained, a 25 msec K^+ puff was administered and the effect on $I_{K(V)}$ was determined. The cell was allowed to recover for 30 min and another pre-puff control and a K^+ puff of a longer duration was administered. This sequence was followed for all puff durations. Thus, the value plotted for each puff-duration is relative to its own pre-puff control. There was no effect with a puff of ≤ 25 msec duration. Near maximal suppression of $I_{K(V)}$ at about 25% was achieved with a puff of 50 msec and there was little additional effect with puffs as long as 200 msec.

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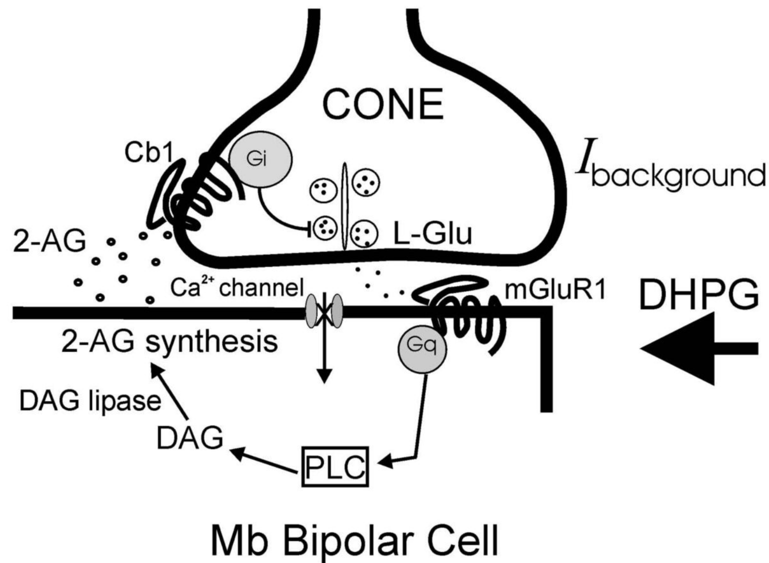

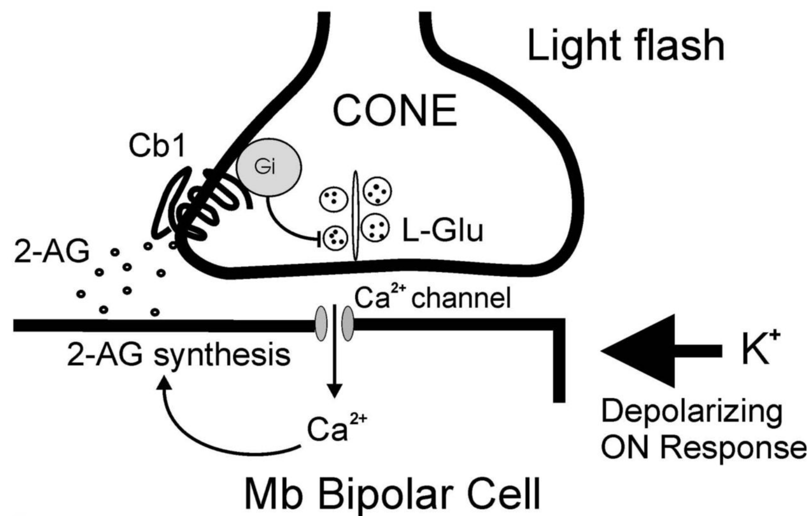

Fig. 11. Schematic illustrations of proposed modulation of retrograde release of 2-AG from Mb bipolar cell dendrites (from Fan and Yazulla, 2007)

A. Voltage-independent — Under any steady background illumination ($I_{background}$), there will be basal release of L-glutamate (L-Glu) from the cones. Independent of any other glutamate receptors on Mb bipolar cells, L-Glu will activate mGluR1 that, via a $G_{q/11}$ cascade involving phospholipase C (PLC), will result in a calcium dependent synthesis and release of 2-AG from the Mb bipolar cell plasma membrane. Upon release, 2-AG will activate pre-synaptic CB1 receptors on the cone pedicle with a resulting inhibition of membrane currents via $G_{i/o}$. The effect will be a negative feedback that maintains L-Glu release within some limit.

B. Voltage-dependent — In response to an increase in light intensity, there will be a reduction in the release of L-Glu from the cone, resulting in a depolarizing response in the Mb bipolar cell. The depolarization could open Ca^{2+} channels resulting in the synthesis and release of 2-AG from the Mb bipolar cell. Retrograde inhibition of cone transmitter release would be a positive feedback, enhancing the light response. As the retrograde effect has a slow onset and is long-lasting, the voltage-dependent mechanism may be of less relevance compared with the negative feedback maintenance of L-Glu release that would result from the Voltage-independent mechanism.

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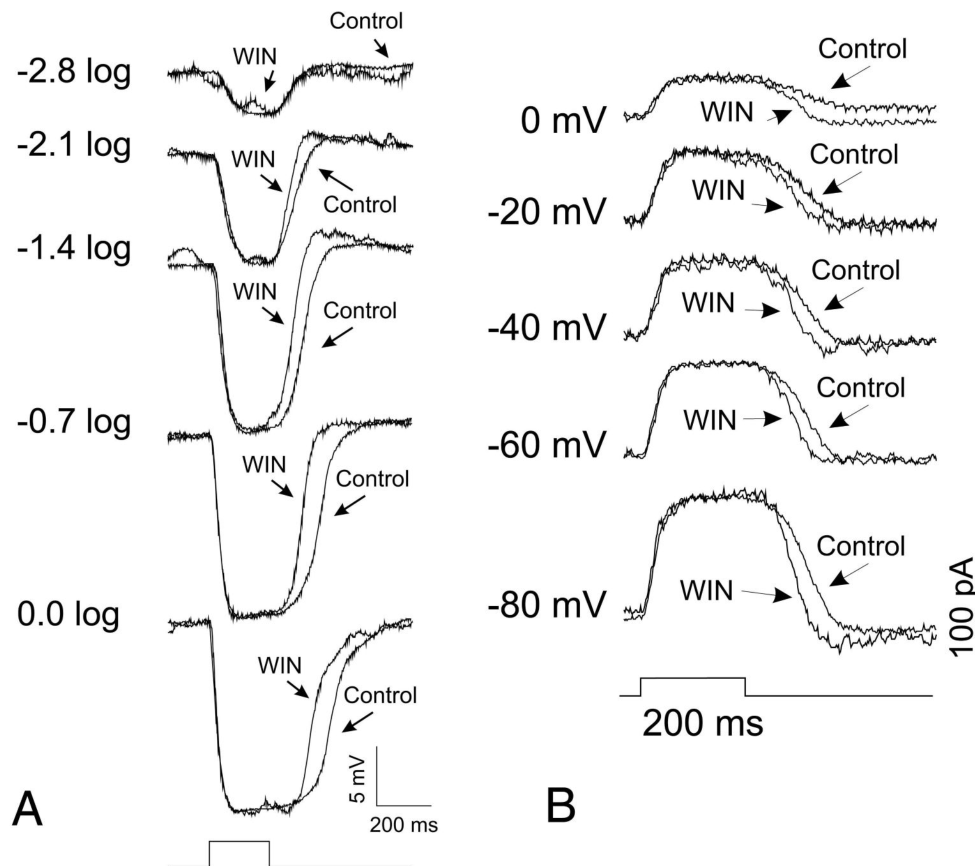

Fig. 12. Effect of WIN 55,212−2 on the responses of goldfish cones in an isolated retinal preparation to flashes of light (from Struik et al., 2006)

A. Voltage-light responses of an L-cone under current clamp to a 200 ms light stimulus of different intensities for Control conditions and after 8 min in 10 μM WIN 55,212−2. Indicated at the right are relative stimulus intensities. The response amplitudes in the Control and WIN conditions differed from each other by about 10%. To facilitate comparison, the traces were normalized and superimposed. Except for the dimmest intensity, there was a speeding up of the response to light offset and an enhancement of the overshoot at two intermediate intensities. There was no effect on the response to light onset or on the plateau phase of the response. The 5 mV calibration refers to the control response.

B. Current-light responses of an L-cone at different holding potentials to a 200 ms light stimulus of approximately half-maximal intensity in Control and 10 μM WIN 55,212−2. The timing of the light stimulus is indicated at the bottom of the figure. The amplitude of the light response decreased with decreasing holding potential because the holding potential approached the reversal potential of the photocurrent. The response amplitudes in the Control and WIN conditions differed from each other by 5−20%. To facilitate comparison, the traces were normalized and superimposed. Speeding up of the response to light offset in response to WIN 55,212−2 is apparent at all holding potentials. There was no effect of WIN 55,212−2 on the response to light onset or plateau phases of the light response. The holding potential did not change the kinetics of the light responses. The 100 pA calibration refers to the control response.