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# FATTY ACID AMIDE HYDROLASE EXPRESSION DURING RETINAL POSTNATAL DEVELOPMENT IN RATS

**N. ZABOURI,[a,b] M. PTITO,[c] C. CASANOVA[a] AND J.-F. BOUCHARD[b]***

[a]*Laboratoire des Neurosciences de la vision, École d'optométrie, Université de Montréal, Montréal, QC, Canada*

[b]*Laboratoire de Neuropharmacologie, École d'optométrie, Université de Montréal, Montréal, QC, Canada*

[c]*Laboratoire de la plasticité visuelle, École d'optométrie, Université de Montréal, Montréal, QC, Canada*

**Abstract—The endocannabinoid (eCB) system is thought to participate in developmental processes in the CNS. The rodent retina represents a valuable model to study CNS development because it contains well-identified cell types with established developmental timelines. The distribution of cannabinoid receptor type 1 (CB1R) was recently revealed in the developing retina; however, the expression patterns of other elements of this system remain unknown. In this study, we investigated the expression pattern of the degradative enzyme fatty acid amide hydrolase (FAAH), a key regulator of the eCB system, in the rat retina during postnatal development. To identify the cells expressing the enzyme, co-stainings were carried out for FAAH and retinal cell type markers. FAAH was expressed at postnatal day (P) 1 in ganglion and cholinergic amacrine cells. In the course of development, it appeared in cones, horizontal, and bipolar cells. For most cell types (horizontal, cholinergic amacrine cells, and cone bipolar cells), FAAH was transiently expressed, suggesting an important redistribution of the enzyme during postnatal development and thus a potential role of the eCB system in developmental processes. Our results also indicated that, in the adult retina, FAAH is expressed in cones, rod bipolar cells, and some retinal ganglion cells. The presence of FAAH in adult animals supports the hypothesis that the eCB system is involved in retinal functions. Overall these results indicate that, as shown in other structures of the brain, the eCB system could play an instrumental role in the development and function of the retina.** 

**Key words: endocannabinoids, anandamide, CB1R, immunohistochemistry, bipolar cells, retinogenesis.**

In the last decade, interest in endogenous cannabinoids (eCBs), lipid-signaling molecules such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), has increased given their role in development as well as in adult neurotransmission. The best characterized eCB receptors are cannabinoid receptor types 1 and 2 (CB1R and CB2R). Due to their lipophilic nature, eCBs cannot be stored in vesicles. Consequently, the enzymes responsible for their synthesis and degradation become key regulators of the physiological actions of eCBs. One enzyme responsible for their degradation is fatty acid amide hydrolase (FAAH; Deutsch and Chin, 1993; Ueda et al., 1995, 1998; Cravatt et al., 2001). This enzyme is an integral membrane protein of the plasma membrane and the endoplasmic reticulum (Tsou et al., 1998; Gulyas et al., 2004) and is part of a large class of enzymes known as the amidase signature family (Chebrou et al., 1996). It degrades AEA and 2-AG as well as other eCBs, with a marked preference for the former (Deutsch and Chin, 1993; Cravatt et al., 1996; Goparaju et al., 1998; Ueda et al., 1998). FAAH converts AEA into arachidonic acid and ethanolamide through a hydrolytic reaction (Deutsch and Chin, 1993; Cravatt et al., 1996).

Some constituents of the eCB system—that are, ligands, synthesizing and degradative enzymes, and receptors—were localized in the retina or in specific retinal cell types in adults of several species, from fish to primates (Buckley et al., 1998; Straiker et al., 1999; Yazulla et al., 1999; Leonelli et al., 2005; Lalonde et al., 2006; Nucci et al., 2007; Warrier and Wilson, 2007; Zimov and Yazulla, 2007; Hu et al., 2010; Lopez et al., 2011; and see Yazulla, 2008 for review). For instance, in adult rodents, both CB1R and FAAH have been shown to be present in adult retinae. Namely, they are expressed in cone (Straiker et al., 1999; Yazulla et al., 1999; Zabouri et al., 2011), mutually exclusive populations of bipolar cells (Yazulla et al., 1999; Hu et al., 2010), some amacrine (Yazulla et al., 1999; Warrier and Wilson, 2007; Hu et al., 2010; Zabouri et al., 2011), and retinal ganglion cells (RGCs; Straiker et al., 1999; Yazulla et al., 1999; Yazulla, 2008; Hu et al., 2010; Zabouri et al., 2011). Both the protein and the mRNA of CB2R were shown to be expressed in adult rat retina (Lu et al., 2000; López et al., 2011).

The expression of the eCB system starts early during development of the CNS (embryonic day 13; Buckley et al., 1998) and is age dependent (Thomas et al., 1997; Fernández-Ruiz et al., 2004; Morishita et al., 2005; Goncalves et al., 2008; Zabouri et al., 2011). Its involvement in developmental processes such as cell fate determination and neurogenesis (Aguado et al., 2005; Galve-Roperh et al., 2006; Hill et al., 2006; Keimpema et al., 2010), axonal elongation and pathfinding (Williams et al., 2003; Berghuis et al., 2005, 2007; Keimpema et al., 2010; Argaw et al.,

*Corresponding author. Tel: +1-514-343-6111 ext 4083; fax: +1-514-343-2382.
E-mail address: jean-francois.bouchard@umontreal.ca (J.-F. Bouchard).
*Abbreviations:* AEA, anandamide; CB1R, cannabinoid receptor type 1; ChAT, choline acetyltransferase; eCB, endogenous cannabinoid; FAAH, fatty acid amide hydrolase; GCL, ganglion cell layer; GS, glutamine synthetase; INL, inner nuclear layer; IPL, inner plexiform layer; KO, knockout; NBL, neuroblast layer; ONL, outer nuclear layer; OPL, outer plexiform layer; P, postnatal day; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PFA, paraformaldehyde; PKC$\alpha$, protein kinase C alpha; RGC, retinal ganglion cell; 2-AG, 2-arachidonoylglycerol.

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2011), and synaptogenesis (Kim and Thayer, 2001; Berghuis et al., 2007; Gómez et al., 2007) has been demonstrated in recent years. For instance, a number of studies showed that 2-AG, synthesis enzymes, and degradative enzymes participate in axonal elongation and pathfinding (e.g. Berghuis et al., 2005, 2007; Keimpema et al., 2010). AEA and FAAH were also linked to developmental processes such as cell fate determination and neurogenesis (Aguado et al., 2005; Soltys et al., 2010).

The rodent retina presents a valuable model to study development, as it includes seven cell classes, comprising six neuronal types and one major glial cell type. These cell categories have distinct and well-documented developmental timelines (Rapaport et al., 2004). To our knowledge, very few studies examined the impact of the eCB system in the developing retina. For instance, the consequences of CB1R modulation in retinal cells of embryonic and young animals were studied by Lalonde et al. (2006) and Warrier and Wilson (2007). Very recently, our laboratory investigated the spatiotemporal distribution of CB1R expression during postnatal development (Zabouri et al., 2011). Our results demonstrated a differential distribution of CB1R during development, thereby supporting the hypothesis that eCB signaling is important for retinal development. We also revealed an age-dependent concentration change of FAAH and other elements of the eCB system during retinal development.

Apart from CB1R, the distribution pattern of retinal eCB system proteins during development remains unknown. Given that metabolic enzymes exert a key regulator function in the eCB system, knowing their expression pattern during development could prove to be instrumental in our efforts to decipher the role of eCBs in development processes. In this context, we investigated the spatiotemporal expression of FAAH in the retina during postnatal development until adulthood. The results reported here show that FAAH is already present at postnatal day (P) 1, but is greatly redistributed in the retina along postnatal development and maturation. In most cell types, its expression is transient and overlaps spatially with CB1R early in postnatal development. However, this overlap is present only in two cell types in adult retina. These results strengthen the hypothesis that the eCB system is involved in the development of the retina during the postnatal period.

## EXPERIMENTAL PROCEDURES

### Animals

Gestating or non-gestating adult Long–Evans rats were obtained from Charles River (St-Constant, QC, Canada) and maintained on a 12-h light/dark cycle. All procedures were in accordance with the guidelines set out by the Canadian Council on Animal Care and the American Association of Anatomists' Guiding Principles in the Care and Use of Animals, and were approved by the ethics committee on animal research of the Université de Montréal. For all ages considered, three to seven pups from at least three different litters were used for each co-labeling.

### Tissue preparation

The animals were sacrificed at various ages, namely P1, 3, 5, 7, 9, 11, 13, 15, 21, 30, 45, and adults (≥P60). Rats were deeply anesthetized either by hypothermia (pups younger than P5) or through excess of isoflurane inhalation. Simultaneously, a transcardiac perfusion was conducted with phosphate-buffered 0.9% saline (PBS, 0.1 M, pH 7.4), followed by phosphate-buffered 2% paraformaldehyde (PFA), until the head was lightly fixed. The nasal part of one eye was marked with a suture before it was removed. Two small holes were made in the cornea, prior to a postfixation in 2% PFA for a period varying between 1 h 30 min and 2 h, depending on the size of the eye. The cornea and lens were then removed and the eyecups postfixed for 10–30 min. Eyecups were washed in PBS, cryoprotected in 30% sucrose overnight, embedded in HistoPrep tissue embedding media (Fisher Scientific, Ottawa, ON, Canada), flash frozen, and kept at −80 °C until processing. Sections (14 $\mu$m thick) were cut with a cryostat (Leica Microsystems, Exton, PA, USA) starting at the nasal pole and placed on gelatin/chromium (Double frosted microscope slides, Fisher Scientific, Ottawa, ON, Canada)-coated slides.

### Immunohistochemistry

*Double- and triple-label immunohistochemistry.* Sections were washed in PBS, postfixed for 5 min in a 70% solution of ethanol, rinsed in 0.03% Triton X-100 in PBS, and blocked in 10% normal donkey serum (NDS, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and 0.5% Triton X-100 in PBS for 1 h. The sections were then co-incubated overnight in a rabbit anti-FAAH (Cayman Chemical, Ann Arbor, MI, USA) solution with one or two of the following antibodies tagged as mouse or goat and directed against protein kinase C alpha (PKC$\alpha$), syntaxin, glutamine synthetase (GS), choline acetyltransferase (ChaT), Chx10, Brn3a, and proliferating cell nuclear antigen (PCNA). It is to be noted that for the later antibody, a Tris 0.05 M, 0.9% based saline was used as a buffer. After incubation with the primary antibodies, the sections were washed in buffered saline, blocked for 30 min, and incubated for 1 h with the following secondary antibodies: Alexa donkey anti-rabbit 555 for FAAH and Alexa donkey anti-goat 647 and mouse 488 for cell type markers (Molecular Probes, Eugene, OR, USA). After washes, the sections were mounted with a homemade Dabco-PVD mounting media (Montreal, QC, Canada) (Ono et al., 2001).

*Sequential co-labeling immunohistochemistry.* In some cases, the antibodies that we selected came from the same host, making the use of simultaneous double-labeling protocol not suitable. Thus we used a sequential protocol previously described by our research group (Zabouri et al., 2011) and others (Sherry et al., 2003). Briefly, the sections were labeled in a serial manner: the exposition to the first primary antibody was conducted as described above, followed by incubation in a goat anti-Fab fragment solution (Jackson ImmunoResearch Laboratories; Brandon, 1985). This allowed us to tag the first primary antibody as a goat rather than a rabbit. The sections were revealed with a secondary Alexa donkey anti-goat 488. Following that, they were exposed to a second primary antibody overnight, the latter revealed with an Alexa donkey anti-rabbit 555 the following day. The markers that required this type of protocol were rabbit anti-recoverin (McGinnis et al., 1992, 1997) and anti-cone transducin (Johnson et al., 2001), and CB1R (Zabouri et al., 2011) and rabbit anti-CB1R (Zabouri et al., 2011), co-labeled with rabbit anti-FAAH.

*Antibody characterization.* Rabbit anti-CB1R (Sigma-Aldrich, Oakville, ON, Canada) was shown to be specific using retinal tissue from CB1R knockout (KO) and wild-type mice. Moreover, in rat retinal lysates, it was shown to react with one major band at 53 kDa (Zabouri et al., 2011). This is in agreement with

others using CB1R antibody on rat retinal tissue (Yazulla et al., 1999).

Rabbit anti-FAAH (Cayman Chemical, Ann Arbor, MI, USA) was shown to react with a dense band at about 66 kDa and a very light one below 37 kDa in rats (Fig. 1A). In mice retinal tissue, the antibody against FAAH (Cayman Chemical, Ann Arbor, MI, USA) displays a band around 60 kDa (Fig. 1A). Moreover, we tested the specificity of this antibody by immunolabeling adult retinal tissue where the *faah* gene has been deleted (*faah*$^{-/-}$; Cravatt et al., 2001). These samples were generously provided by Dr. Gabriella Gobbi from McGill University (Montreal, QC, Canada). To ensure the closest possible match between wild type and *faah*$^{-/-}$ C57BL/6 mice, Dr. Gobbi provided tissue from both wild-type and KO animals. Sections from both samples were processed together during the same experiment; pictures were taken under the same conditions and off-line processing was identical. The results are shown in Fig. 1B, C. The FAAH staining in wild-type animals yields a clear signal throughout the retina (Fig. 1B), while no staining was visible in the KOs (Fig. 1C). There is a small divergence (9%) between FAAH protein sequences in mice and rats. In the antigen sequence of the antibody used here, only three non-consecutive amino acids (out of a total of 18) differ between rat and mouse proteins. Using Western blot analysis, we found that our antibody recognizes one major band at about 66 kDa in retinal homogenates of mice (Fig. 1A). This observation is comparable to the band seen in rat homogenates. This strongly suggests that, despite the small discrepancy in protein sequence, recognition is not affected and *faah*$^{-/-}$ mice remain a valid control.

The protein PKC$\alpha$ is specifically expressed in rod bipolar cells and dopaminergic amacrine cells (Negishi et al., 1988). Mouse anti-PKC$\alpha$ (H7; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was shown to exclusively recognize these cells (Gaillard et al., 2008). These authors as well as others also reported that this antibody recognizes PKC$\alpha$, an 80-kDa protein, by Western blot (Nagar et al., 2009). The staining pattern we observed was similar to that reported in previous studies (Negishi et al., 1988; Morrow et al., 2008).

The goat polyclonal anti-Chx10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) labels a transcription factor that is expressed in bipolar cells (Rowan and Cepko, 2004). This antibody gives a single, 46-kDa band on Western blots of mouse eye extracts according to the manufacturer. The pattern of immunofluorescence that we obtained with this antibody was similar to that previously reported (Rowan and Cepko, 2004).

Goat anti-choline acetyltransferase (ChAT; Chemicon, Temecula, CA, USA) recognizes a single band at 70 kDa in mouse brain lysate (Chemicon datasheet) and in COS cells transfected with ChAT cDNA lysate (Ohno et al., 2001). It has been used by others to stain the cholinergic amacrine cells in rodent retina (Gunhan et al., 2002). The immunoreactivity we observed is in agreement with the reported ChAT distribution using the same antibody as well as others (Voigt, 1986; Jeon et al., 1998; Haverkamp and Wässle, 2000; Majumdar et al., 2008).

The protein syntaxin-1 was recognized as a specific marker of retinal amacrine and horizontal cells by several research teams (Barnstable et al., 1985; Hirano et al., 2005). Mouse anti-syntaxin (HPC-1; Sigma-Aldrich, Oakville, ON, Canada) recognizes syntaxin-1, a 35-kDa protein, from hippocampal, retinal, and cortical neurons (Inoue et al., 1992). The staining pattern observed in the present study was similar to that formerly reported (Li et al., 2004; Voinescu et al., 2009).

The expression of GS in Müller cells was demonstrated by Riepe and Norenburg (1977). Chang et al. (2007) established that the mouse anti-GS (Clone GS-6; Chemicon, Temecula, CA, USA) reacted with a single 45-kDa protein in adult retinal tissue. The staining obtained with this antibody was akin to that published elsewhere (Gargini et al., 2007).

PCNA was chosen as a neuroblast and mitotic cell marker because it provides the least false-positive and -negative immunolabeling of neuroblasts and mitotic cells in the retina (Barton and Levine, 2008). The specificity of this mouse anti-PCNA (Clone PC10, Dako, Burlington, ON, Canada) was fully characterized by Waseem and Lane (1990) and others (Ino and Chiba, 2000); it recognized a single band at 36 kDa.

Anti-Brn3a was used to identify retinal ganglion cells. The Brn3a antibody (clone 5A3.2; Chemicon, Temecula, CA, USA) labels only RGCs in retinae of several vertebrate including rats and mice (Xiang et al., 1995; Nadal-Nicolás et al., 2009). In addition, it was also shown that Brn3a—rather than the other members of the Brn3 family—was present in most RGCs subtypes in rats and mice (Nadal-Nicolás et al., 2009; Badea and Nathans, 2010). The staining pattern obtained in the present study is similar to that found by others (Quina et al., 2005; Liu et al., 2009). According to the manufacturer, this antibody does not stain tissue from Brn3a KO mice, and a recent study established that it reacted with expected band at 46 kDa (Hudson et al., 2008).

A number of laboratories showed that recoverin is expressed in a subset of bipolar cells (Milam et al., 1993; McGinnis et al., 1997; Haverkamp and Wässle, 2000; Haverkamp et al., 2003). The immunoreactivity obtained in our study with the rabbit antibody directed against recoverin revealed a pattern of immunoreactivity identical to that described in the mouse retina (Haverkamp et al., 2003; Gargini et al., 2007; Acosta et al., 2008). This antibody (Chemicon, Temecula, CA, USA) recognized a single 26-kDa band (manufacturer's technical information), in accordance with the reported size of recoverin (Yan and Wiechmann, 1997).

Rabbit anti-cone transducin-$\alpha$ subunit (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to identify cone photoreceptors. The specificity of this antibody was confirmed by preadsorption and Western blot studies (Coleman et al., 2004); it recognizes a single band at 46 kDa.

All working dilutions and provenance are provided in Table 1.

*Validity of the sequential labelings.* Validity of the sequential staining was verified for FAAH/recoverin, FAAH/cone-transducin, and FAAH/CB1R co-labelings with the following two controls: (1) the second primary antibody was omitted yielding a strong staining with the goat secondary 488 but no staining with rabbit secondary 555; (2) the first secondary and second primary antibodies were omitted, yielding no signal for the goat secondary 488 and some low residual signal for the rabbit secondary 555. An example is presented in Fig. 1D–L for FAAH and recoverin immunolabeling. The first column presents the goat signal (green), the second column the rabbit signal (magenta), and the last column the merged signals. The first line (Fig. 1D–F) shows the immunostaining with complete protocol: clear signals for both recoverin (D) and FAAH (E) can be seen and this co-labeling shows that FAAH and recoverin signals are overlapping (F). The second line (Fig. 1G–I) illustrates the result of the first control for the same co-immunolabeling. As expected, there was a clear signal for recoverin (G, I) with very faint staining for FAAH (H, I). The last line (Fig. 1J–L) presents the results for the second control where there was very little staining for both recoverin (Fig. 1J, L) and FAAH (Fig. 1K, L). Thus, these control data demonstrate that the sequential Fab fragment protocol remains specific even when there is a lot of non-overlapping between two proteins.

One can note that nuclear staining seems to be present in some cells such as the bipolar cells shown in Fig. 1E, F. Nuclear staining is not expected given that FAAH is located in the endoplasmic reticulum and at the membrane. This expression was observed in young animals only (up to 1 month of life); consequently, one could question the specificity of our antibody in younger animals. In a previous study (Zabouri et al., 2011), we used Western blot to investigate the age-dependent concentration change of FAAH with the same antibody; we observed that the same bands were visible at all ages. We are thus confident that

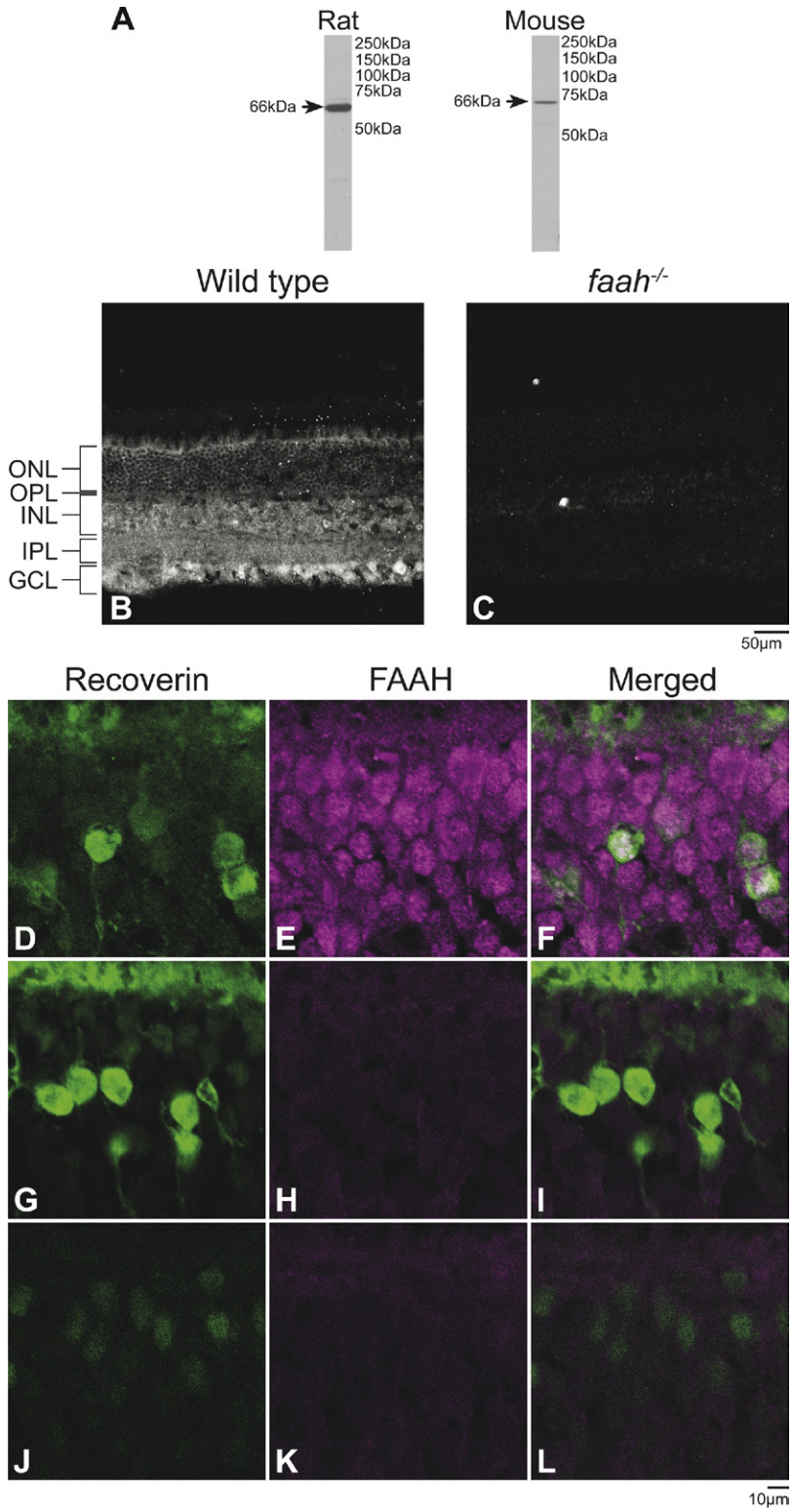


**Fig. 1.** (A) Immunoblots of FAAH immunoreactivity in rat and mice retinal homogenates following the Western blot protocol described by Zabouri et al. (2011). (B, C) FAAH immunoreactivity in the retina of a wild-type and a *faah*$^{-/-}$ mouse. (D–L) Recoverin (D, G, J), FAAH (E, H, K) signals, and their overlay (F, I, L) for the complete sequential protocol (D–F), for the control where the second primary antibody was omitted (G–I) and for the control where the first secondary and second primary antibodies were omitted (J–L) in rats. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

**Table 1.** Antibodies

| Antibody | Immunogen | Dilution | Host | Company |
|---|---|---|---|---|
| CB1R | A fusion protein with the sequence MKSILDGLADTT-FRTITTDLLYVGSNDIQYEDIKGDMASKLGYFPQK-FPLTSFRGSPFQEKMTAGDNSPLVPAGDT of rat CB1R | 1/150 | Rabbit | C1233, Sigma-Aldrich, Oakville, ON, Canada |
| FAAH | Synthetic peptide from rat FAAH with the sequence ELCLRFMREVEQLMTPQKQPS | 1/200 | Rabbit | 101600, Cayman Chemical, Ann Arbor, MI, USA |
| Chx10 | The antibody was raised against the aa sequence PPSSHPRAALDGLAPGHL from human Chx10 Martinez-Navarrete et al., 2008 | 1/100 | Goat | Sc-21690, Santa Cruz Biotechnology, Santa Cruz, CA, USA |
| ChAT | Human placental choline acetyltransferase | 1/50 | Goat | AB144P, Chemicon, Temecula, CA, USA |
| Recoverin | The full-length recombinant human recoverin | 1/200 | Rabbit | AB5585, Chemicon, Temecula, CA, USA |
| Cone transducin ($G_{\alpha t2}$) | A synthetic peptide with the sequence IDYAEVSCVD from bovine retina | 1/200 | Rabbit | Sc-390, Santa Cruz Biotechnology, Santa Cruz, CA, USA |
| Syntaxin, clone HPC1 | Synaptosomal plasma fraction of rat hippocampus | 1/500 | Mouse | S0664, Sigma-Aldrich, Oakville, ON, Canada |
| PKC clone H7 | A synthetic peptide with the sequence DFEGFSYVNPQFVHPILQSSV from the human protein (Zabouri et al., 2011) | 1/500 | Mouse | Sc-8393, Santa Cruz Biotechnology, Santa Cruz, CA, USA |
| GS (clone GS-6) | The full protein purified from sheep brain | 1/3000 | Mouse | MAB302, Chemicon, Temecula, CA, USA |
| Brn3a (clone 5A3.2) | Sequence: GGSAHPHPHMHGLGHLSHPAAAAAMN-MPSGLPHPGLVAA fused to the T7 gene 10 protein | 1/500 | Mouse | MAB1585, Chemicon, Temecula, CA, USA |
| PCNA, clone PC10 | Synthetic peptide with the sequence LVFEAPNQEK (Ino and Chiba, 2000) | 1/500 | Mouse | M0879, Dako, Burlington, ON, Canada |

the specificity of the antibody does not change with age. Furthermore, we also demonstrated that our protocol could cause intracellular protein redistribution (Zabouri et al., 2011) probably due to a mixture of light fixation and permeabilization (Goldenthal et al., 1985; Melan and Sluder, 1992; Mayor and Maxfield, 1995; Cinar et al., 2006). This leads us to believe that any nuclear staining observed in the present study is most probably due to the same artifacts as observed by Zabouri et al. (Zabouri et al., 2011), as the experiments were conducted together. The presence of this artifact has no impact on the study, as we investigate FAAH expression within cell types rather than its subcellular distribution.

### Confocal microscopy

The fluorescent specimens were visualized using a Leica TCS SP2 confocal laser-scanning microscope. Images were acquired using a 40× oil-immersion objective and were scanned at a 2048×2048 pixel resolution. Pictures of the central retina (within 200 $\mu$m of the optic nerve head) and the periphery were taken. Matching images were captured in the Alexa fluo 555/546, Alexa fluo 488/FITC, and Alexa fluo 647 channels, pseudo-colored, merged, and exported using Leica LCS software (version 2.61). Throughout this study, retinal cell markers are always presented in green and blue, while FAAH is shown in magenta for the double labeling or red for the triple staining. The pictures were taken sequentially to ensure no "bleed-through" between channels. When the co-expression of FAAH and retinal cell markers was ambiguous, the presence or absence of co-labeling was demonstrated by taking *z*-stacks with optimized steps. This allowed the visualization of the cells in the X–Y, X–Z, and Y–Z axes, thereby confirming the expression or the lack of FAAH in specific cell types. Pictures were selected to illustrate representative findings of the immunostaining at all ages studied. Contrast and brightness were adjusted when necessary using Adobe Photoshop (CS4).

As no differences were observed in the expression of FAAH between the center and the periphery—with the exception of the expected delay in development (Rapaport et al., 2004)—only pictures of the central retina are presented in the results section.

## RESULTS

### Spatial and temporal patterns of expression of FAAH in the retina

The expression of FAAH was studied at different postnatal ages ranging between P1 and adult rats. Briefly, at P1, the retina is characterized by neuroblast, plexiform, and ganglion cell layers (NBL, IPL, and GCL, respectively). The NBL contains mostly neuroblasts, dividing cells, differentiated but immature cones, horizontal and amacrine cells, as well as a few rods (Fletcher and Kalloniatis, 1997; Sharma et al., 2003; Rapaport et al., 2004). FAAH was already present at P1 in the rat retina, although its expression was rather scarce. It could be observed in a few cells in the deepest part of the NBL, as well as in the GCL (Fig. 2A). The rest of the NBL showed some fluorescence, although it was quite low in intensity.

Some amacrine, bipolar, and Müller cells as well as rods are generated during the first week of life (Sharma et al., 2003; Rapaport et al., 2004). Between P3 and P5, FAAH immunoreactivity was largely upregulated, as the immunopositive cells in the deepest part of the NBL and in the GCL presented an increased fluorescence. Some regularly spaced cells in the deepest part of the forming inner nuclear layer (INL) and in the GCL presented immunofluorescence in their cytosol and probably at the membrane with clear unmarked nuclei. Other somas in the GCL appeared completely stained (Fig. 2B, C). Moreover, a set of

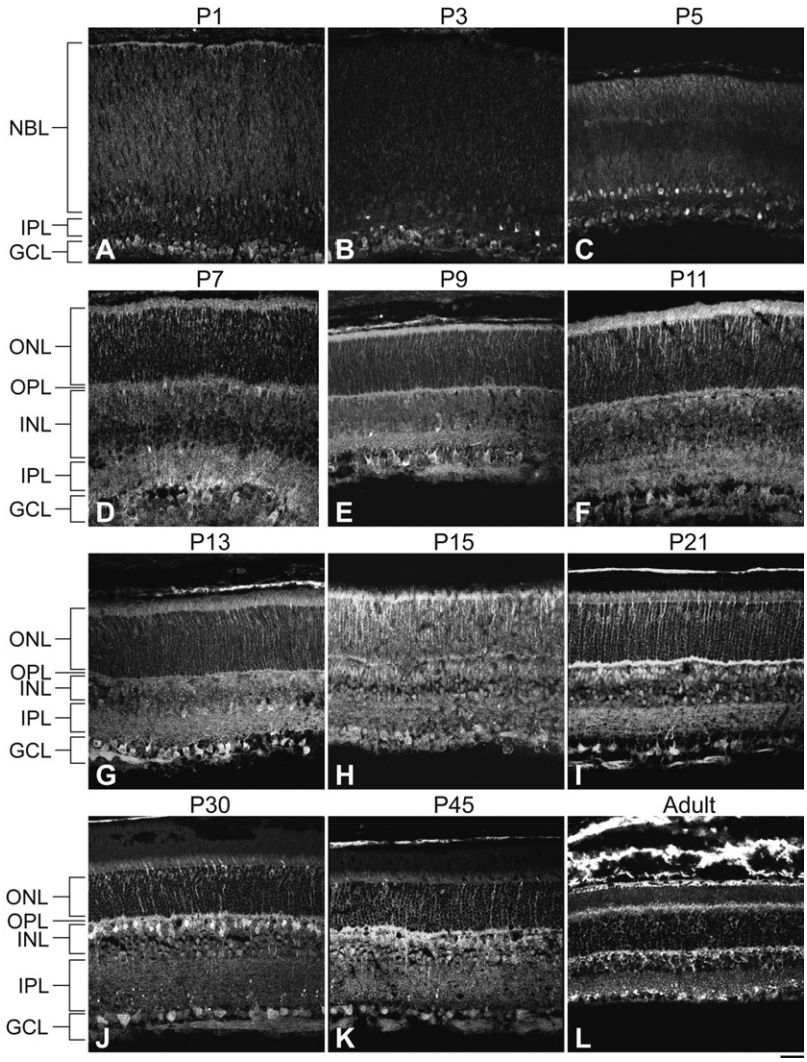


**Fig. 2.** FAAH immunoreactivity in the developing rat retina. (A–L) Vertical sections from P1 (A), P3 (B), P5 (C), P7 (D), P9 (E), P11 (F), P13 (G), P15 (H), P21 (I), P30 (J), P45 (K), and adult (L) rat retinae. For all ages, confocal images were obtained from a single focal plan. NBL, neuroblast layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar=40 $\mu$m.

regularly spaced cell bodies, strongly reminiscent of horizontal cells, close to the upper part of the NBL (P3, Fig. 2B) or just below the forming IPL (P5, Fig. 2C), became immunopositive. At P5, some photoreceptors became immunopositive for FAAH in the forming outer nuclear layer (ONL). Their distribution and morphology were indicative of cones (Fig. 2C). Between P5 and P7, FAAH immunofluorescence increased in the ONL, appearing in most photoreceptors, although suspected cones were more strongly stained than other surrounding cells bodies (Fig. 2D). FAAH was also present in the forming outer plexiform layer (OPL) and just below, in the upper half of the INL. In the deepest part of this layer and in the GCL, FAAH expression remained essentially the same as that observed at P5.

During the second week of life, one important process occurring is synaptogenesis and synaptic refinement (Weidman and Kuwabara, 1968; Kapfhammer et al., 1994), processes in which endocannabinoids are implicated (Kim and Thayer, 2001). The staining at P9 was very similar to that at P7, that is, cells in the GCL strongly expressed FAAH. The enzyme was also present in a set of equally distributed cells in the deepest part of the INL. Cells in the upper half of this layer display a light immunoreactivity of FAAH. Photoreceptors also expressed this protein, with one subpopulation more intensely stained than the rest of the cell bodies (Fig. 2E). At P11, FAAH expression in the GCL and INL remained stable, while in the ONL, expression in the suspected cones increased and became more defined (Fig. 2F).

By eye opening (P12–14), the retinal structure is basically complete, albeit still immature. During the weeks following eye opening, several maturation processes take place, including synapse consolidation or elimination and cell death (see Reese, 2011 for review). FAAH expression remained stable in the ONL and OPL throughout this period. In particular, it was strongly expressed in the inner segments, cell bodies, axons, and synaptic pedicles of suspected cones (Fig. 2G, H). The enzyme expression in

rods also remained stable. In the INL, however, several changes occurred. At P13–15, cells in the innermost part of the layer expressed FAAH strongly, while those in the upper half showed a light expression. In addition, suspected horizontal cells were strongly immunoreactive for the enzyme. Around P15, the immunofluorescence in some cells closer to IPL was upregulated with respect to those closer to the OPL (Fig. 2H). By P21, the expression in the upper half of the INL was increased even more and became more defined, while it remained stable in its inner part (Fig. 2I). Expression of FAAH in regularly spaced cell bodies in the inner part of the INL and in the GCL disappeared by P21. One week later, at P30, expression in the cells close to outer part of the INL appeared stronger and was present in the axon and synapses. From the morphology of these cells, we assume that they are rod bipolar cells (Fig. 2J). The timeline for this process was somewhat variable, as it was observed as early as P13 in some cases. In others, it was still absent at P21. However, for most animals, it started at P15 and was completed by P45. The expression in the rest of the INL was strongly downregulated between P21 and P30. This distribution of FAAH expression profile was maintained over the following weeks (Fig. 2K) until adulthood, where only suspected rod bipolar cells expressed FAAH in the INL and OPL (Fig. 2L). In the GCL, the expression of FAAH remained stable until P21 (Fig. 2I), at which point it was downregulated but remained present (Fig. 2J–L).

### Identification of cell types expressing FAAH

*Expression of FAAH in early-born neurons.* At P0, the bulk of early-born neurons—horizontal, amacrine, ganglion cells, and cone photoreceptors—are differentiated, with very few of these neurons being generated after birth (Morest, 1970; Rapaport et al., 2004).

We investigated the presence of FAAH in these cell types. Examples of FAAH expression in horizontal and amacrine cells at various ages are presented in Figs. 3 and 4, respectively. At P1, no expression of the enzyme could be observed in horizontal cells (Fig. 3A–C). By P3 and until P7, a light expression, often concentrated in punctae, could be detected in this cell type. Between P7 and P21, a clear somatic expression could be seen in horizontal cells (Fig. 3D–L). Over the following 2 weeks, however, this expression decreased (Fig. 3M–O), and from P45, no expression could be seen in horizontal cells (Fig. 3P–U).

At P1, FAAH was expressed in most amacrine cells (Fig. 4A–C). Starting around P3, a population of regularly dispersed cells stood out. They displayed immunofluorescence distributed over the cytosol and membrane. Because of the position and distribution of these cells, they were considered cholinergic amacrine cells (Fig. 4D–F, stars). The remaining amacrine cells displayed a light immunofluorescence. This pattern of expression was maintained until P15 (Fig. 4G–L). At P21 (Fig. 4M–O), FAAH expression in amacrine cells was decreased but still present. Over the second month of life, no expression of FAAH was seen in amacrine cells (Fig. 4P–X). It is worth noting that two patterns of staining were observed for amacrine cells: in some cases, the whole cells were immunopositive (see e.g. Fig. 4A, C), while in others, FAAH staining was in punctate structures as presented in Fig. 4D, F. These patterns were mutually exclusive and present in about half of our sample regardless of the age. We could not determine if these two patterns were caused by an intrinsic change within the retina or an artifact produced by our tissue preparation procedure, which was designed to maintain light fixation in order to preserve FAAH antigenicity. This sort of protein relocalization artifacts have been previously reported by several authors (Goldenthal et al., 1985; Melan and Sluder, 1992; Mayor and Maxfield, 1995; Cinar et al., 2006) and correlated with a combination of light fixation and tissue permeabilization. However, this does not influence our findings, as we investigated FAAH expression within cell types rather than its subcellular distribution.

The identity of the suspected amacrine cells was confirmed with co-staining with the cholinergic cell marker ChAT (Fig. 5). This marker identifies the following two distinct populations of cholinergic amacrine cells: OFF- and ON-starburst cells. The first group is characterized by somas situated in the INL and dendrites that form a narrow line defining the outer stratum of processes in the IPL. ON-starburst cells have their cell bodies in the GCL, and their dendrites mark the inner stratum of processes in the IPL (Voigt, 1986). Both populations were analyzed, and both expressed FAAH at P1 (Fig. 5A–C). Between P3 and P15, both populations of cholinergic cells remained immunopositive for FAAH (Fig. 5D–L). Starting at P21, OFF-starburst cells became immunonegative (arrows in Fig. 5M–O), while most ON-starburst cells remained immunofluorescence (arrowsheads, Fig. 5M–O). At P30 both populations of cholinergic cells were immunonegative (Fig. 5P–R). Over the following month of life and into adulthood, all amacrine cells were immunonegative or showed a very light FAAH immunoreactivity (Fig. 5S–X).

Examples of FAAH expression pattern in GCL are displayed in Fig. 6. The spatial organization of FAAH expression in ganglion cells did not vary with time. The enzyme was expressed in large ganglion cells (stars, Fig. 6A–U), while in others it was not (arrows, Fig. 6A–U). It is to be noted, however, that our data do not allow us to determine if FAAH was expressed in all large ganglion cells or in a subpopulation. FAAH expression in large ganglion cells was present until adulthood; however, a decrease in the intensity of FAAH immunoreactivity could be noted after P7, suggesting a decrease in FAAH expression after that age (compare Fig. 6A–F). As reported in numerous studies, a normal decrease in the number of ganglion cells with age was seen (e.g. Farah and Easter, 2005). FAAH was also present in Brn3a-negative somas situated in the GCL; these neurons were considered displaced amacrine cells (Xiang et al., 1995; arrowheads, Fig. 6A–I). These cells could only be seen during the first 2 weeks of life (arrowheads, Fig. 6A I), suggesting that they were not FAAH positive anymore (Fig. 6D–E).

Cones are the last class of early-born neurons to be generated (Rapaport et al., 2004). They did not express

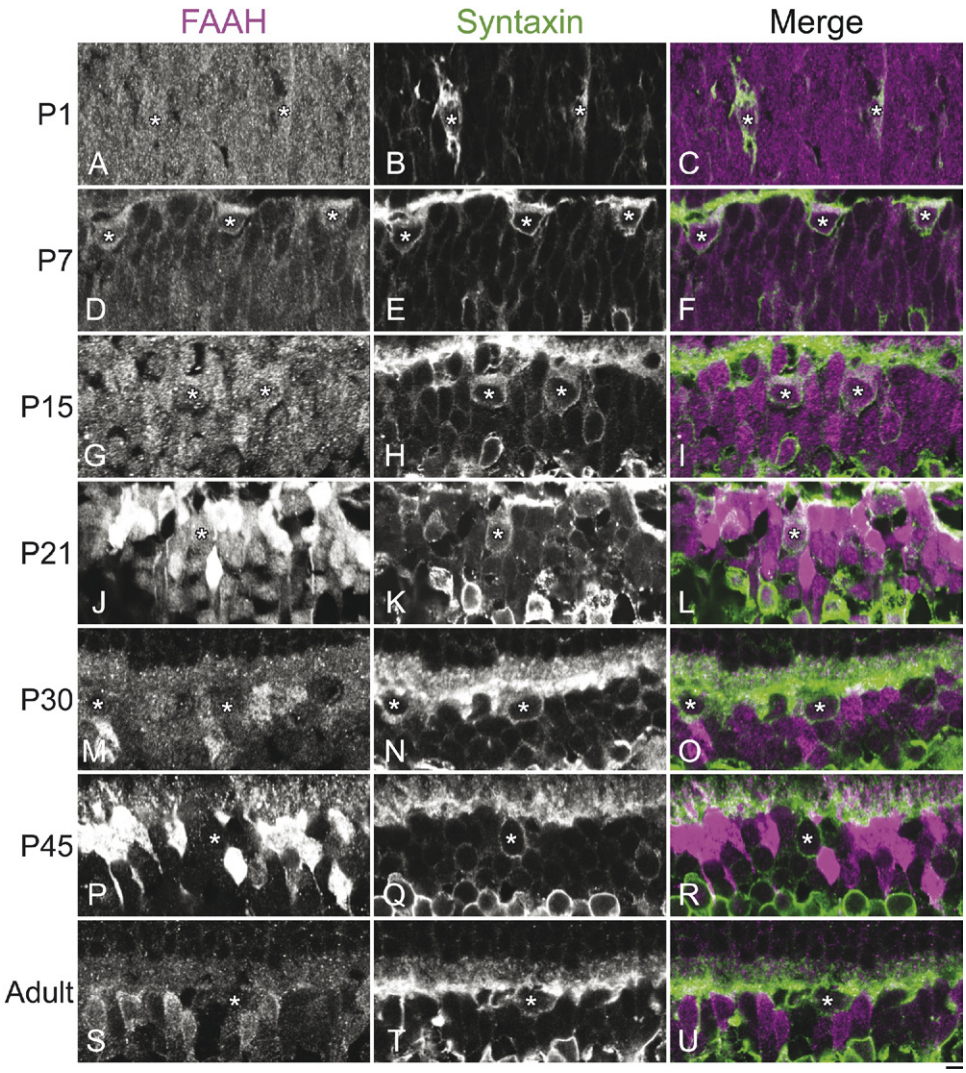


**Fig. 3.** FAAH immunoreactivity in horizontal cells. (A–U) Vertical sections from P1 (A–C), P7 (D–F), P15 (G–I), P21 (J–L), P30 (M–O), P45 (P–R), and adult (S–U) rat retinae. Confocal micrographs of retinae co-immunolabeled for FAAH and cell-type–specific marker for the retinal interneurons (horizontal and amacrine cells), syntaxin. Each protein is presented alone in gray scale: FAAH in the first column and syntaxin in the second, then the two are presented merged (third column—FAAH in magenta and syntaxin in green). Horizontal somas (stars) are immunonegative for FAAH at the youngest age (P1). They become positive for FAAH between P7 and P21, after which they lose their immunoreactivity for FAAH. Scale bar=10 $\mu$m. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

FAAH until P5 as shown in Fig. 2A–C. More detailed pictures of FAAH expression in cones are presented in Fig. 7A–U. Around P5, FAAH was visible in the cell bodies of cone photoreceptors (stars; Fig. 7A–C). FAAH was also expressed in cone pedicles at P5 (data not shown), and its levels remained high throughout the entire time interval investigated. Over the following days, FAAH expression increased in the inner segments as they developed (arrows; Fig. 7D–I). Although cone morphology and distribution varied over the following weeks, FAAH expression reached adult-like pattern at P15 and remained the same until adulthood (Fig. 7J–U).

*Expression of FAAH in late-born neurons.* During the first week, several developmental processes coincide. Late-born neurons are generated from neuroblasts mostly after birth and give rise to different classes of retinal cells: bipolar and Müller cells, as well as rod photoreceptors (Morest, 1970; Rapaport et al., 2004).

PCNA was expressed in the retina between P1 and P7 (Fig. 8). At P1, it was present in neuroblasts and dividing cells, which were visible at the outer edge of the NBL (Fig. 8A, C, E). Neither mitotic cells (stars; Fig. 8A) nor neuroblasts (arrows; Fig. 8B) expressed FAAH at P1. The same pattern was visible at P3 and P5, although the number of dividing cells decreased rapidly, as expected (Fig. 8C–E). At these two time points, however, the somas of mitotic cells appeared surrounded with FAAH-positive fibers (Fig. 8C, E). From the morphology and the position of those fibers as well as the developmental timeline, these structures could either be the neuroepi-

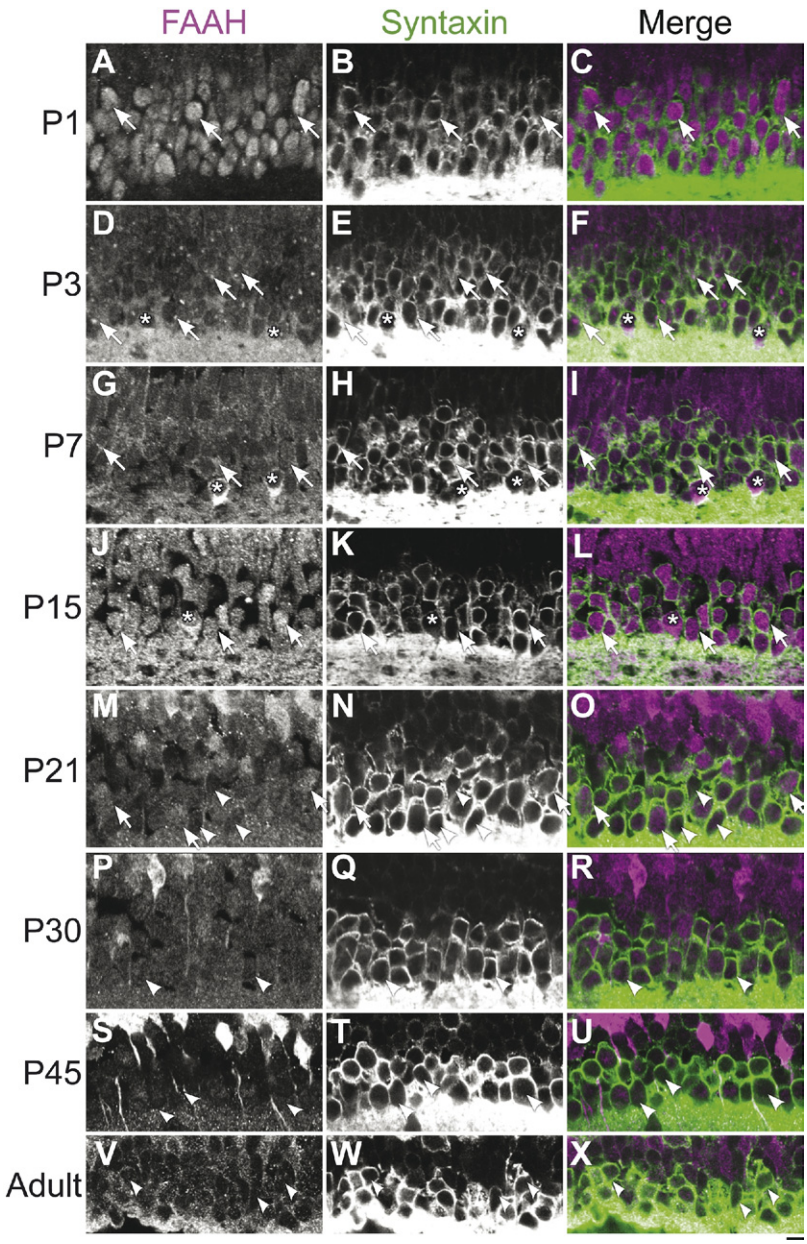


**Fig. 4.** FAAH immunoreactivity in amacrine cells. (A–X) Vertical sections from P1 (A–C), P3 (D–F), P7 (G–I), P15 (J–L), P21 (M–O), P30 (P–R), P45 (S–U), and adult (V–X) rat retinae. Confocal micrographs of retinae co-immunolabeled for FAAH and cell-type–specific marker for the retinal interneurons (horizontal and amacrine cells), syntaxin. Each protein is presented alone in gray scale: FAAH in the first column and syntaxin in the second, then the two are presented merged (third column—FAAH in magenta and syntaxin in green). FAAH immunopositive and immunonegative amacrine cells are indicated by arrows and arrowheads, respectively. Stars indicate a distinct population of amacrine cells. Scale bar=10 $\mu$m. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

thelial-like processes described by Morgan et al. (2006), which represent the future dendrites of bipolar cells or processes from young Müller cell. At P7, the few neuroblasts left showed a light immunofluorescence for FAAH (arrowheads, Fig. 8G).

FAAH expression in bipolar cells is presented in Figs. 9 and 10. We used PKC as a marker for rod bipolar cells, along with Chx10, a nuclear marker of all bipolar cells. Consequently, as seen in Fig. 9, Chx10-only–positive cells, in blue, would be cone bipolar cells, while Chx10- and PKC-positive cells, in green/blue, would be rod bipolar cells, and PKC-only–positive cells, in green, would be PKC-positive amacrine cells. At P3, most cells did not express FAAH (arrows, Fig. 9A), but some showed immunopositivity for the enzyme (arrowheads). At P7, an increase in FAAH immunoreactivity in the upper part of the INL, comprising both cone and rod bipolar cells, was observed (Fig. 9B, see also Fig. 2D). Between P13 and P30, FAAH expression in cone bipolar cells was progressively downregulated, whereas that in rod bipolar cells was up-

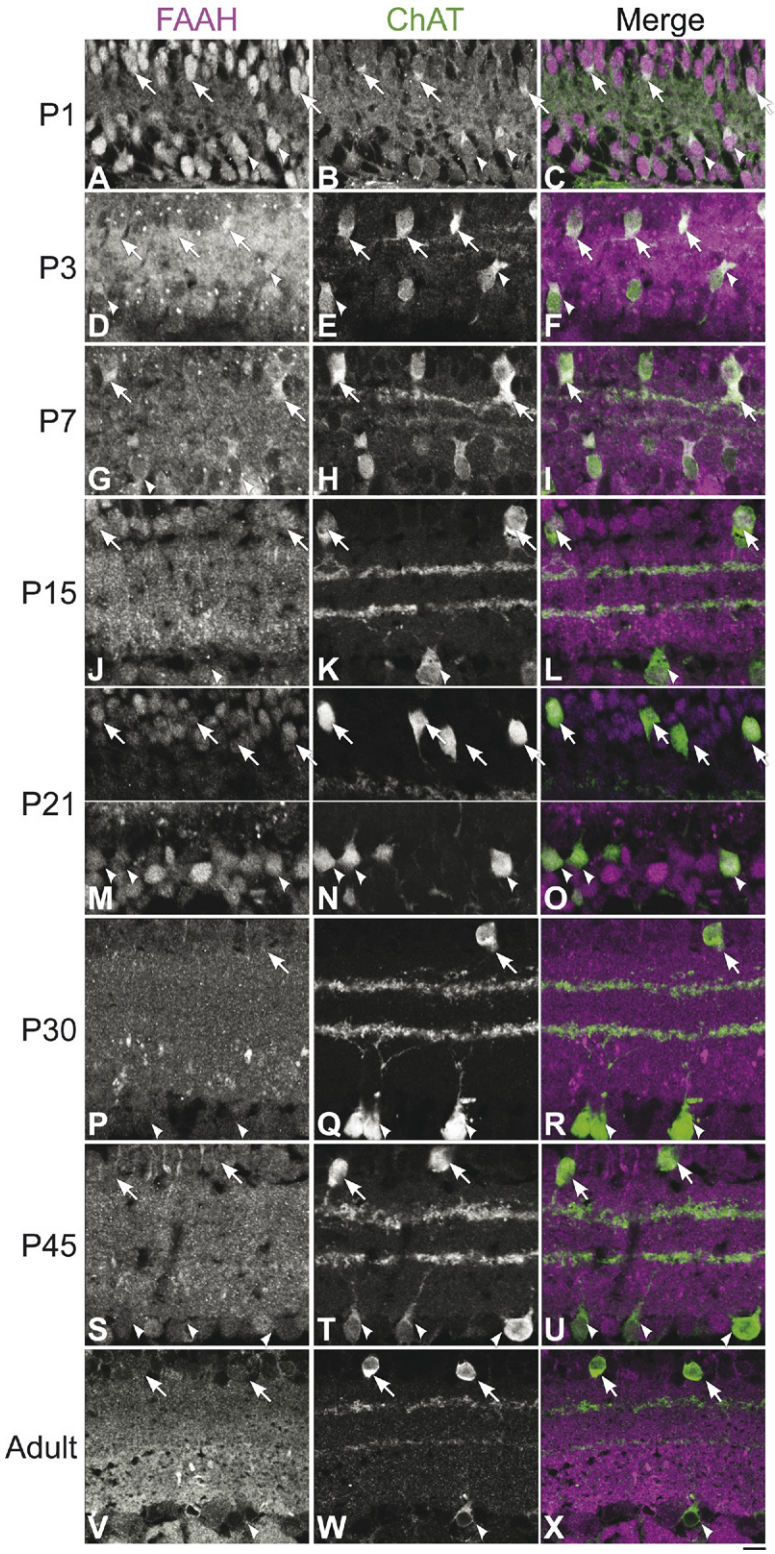


**Fig. 5.** FAAH immunoreactivity in cholinergic amacrine cells. (A–X) Vertical sections from P1 (A–C), P3 (D–F), P7 (G–I), P15 (J–L), P21 (M–O), P30 (P–R), P45 (S–U), and adult (V–X) rat retinae. Confocal micrographs of retinae co-immunolabeled for FAAH and cell-type–specific marker for the retinal cholinergic interneurons, ChAT. Each protein is presented alone in gray scale: FAAH in the first column and ChAT in the second, then the two are presented merged (third column—FAAH in magenta and ChAT in green). OFF and ON cholinergic amacrine cells are indicated by arrows and arrowheads, respectively. Scale bar = 10 $\mu$m. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

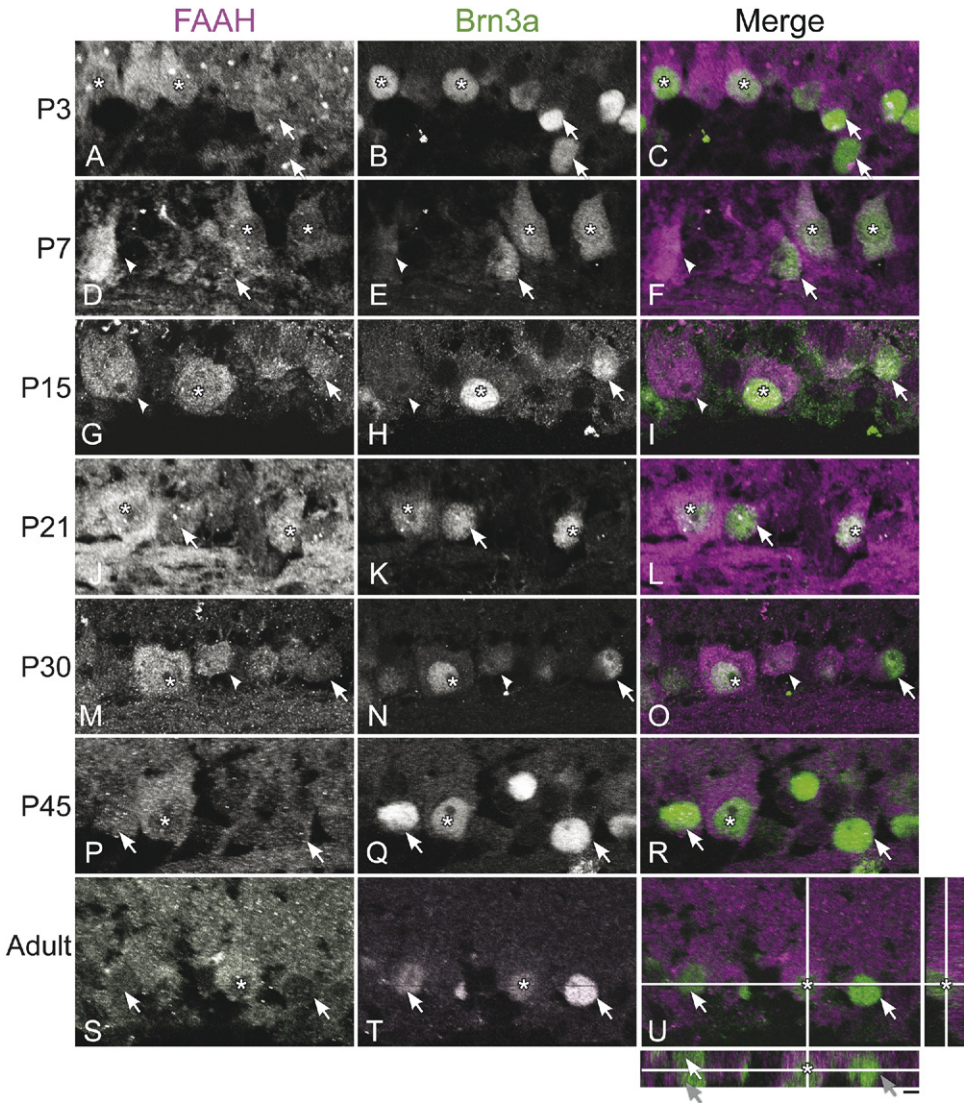


**Fig. 6.** FAAH immunoreactivity in the ganglion cell layer. (A–U) Vertical sections from P1 (A–C), P7 (D–F), P15 (G–I), P21 (J–L), P30 (M–O), P45 (P–R), and adult (S–U) rat retinae. Confocal micrographs of retinae co-immunolabeled for FAAH (magenta) and cell-type–specific marker for RGCs, Brn3a (green). In (U), the horizontal and vertical insets show the reconstruction of the section in the X–Z and Y–Z plains, respectively, along the white lines. Some RGCs are immunopositive for FAAH (stars) at all ages, while others are not (arrows). Other cells within the GCL (arrowheads), presumably displaced amacrine cells, are also immunopositive for FAAH across all ages. Scale bar = 10 $\mu$m. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

regulated (Fig. 9C–E, upper panels). Over this period, FAAH expression in rod bipolar cells was targeted to the membrane and distributed in the soma, axon, and synapses (Fig. 9C–E, lower panels) but was not present in the dendrites. It should also be noted that the time point at which this redistribution happened was relatively variable. In some animals, it was already present at P13, whereas in others it was not completed at P30. However, for most animals, this pattern was observed between P15 and P21. For the second month of life, the expression of FAAH in bipolar cells remained stable (Fig. 9F, G).

Recoverin was used as a marker to investigate FAAH expression in ON and OFF cone bipolar cells. This marker was chosen because it is present in type 2 and type 8 OFF and ON cone bipolar cells (Euler and Wässle, 1995; Chun et al., 1999), respectively, thereby allowing us to evaluate whether FAAH expression was expressed according to the type of cone bipolar cell. Examples of recoverin-positive cells are shown in Fig. 10 (Fig. 10A–L) during development. With this marker, bipolar cells are not clearly discernable until the OPL is formed (Sharma et al., 2003). At P7, most recoverin neurons were expressing FAAH (stars, Fig. 10A). By P15, however, one can see that all recoverin-positive cells were expressing FAAH (Fig. 10B). This demonstrates that at this age, both ON and OFF bipolar cells expressed FAAH. The staining of the somas remained the same at P21 and P30. By P45 and into adulthood, expression of FAAH was strongly downregulated if not completely

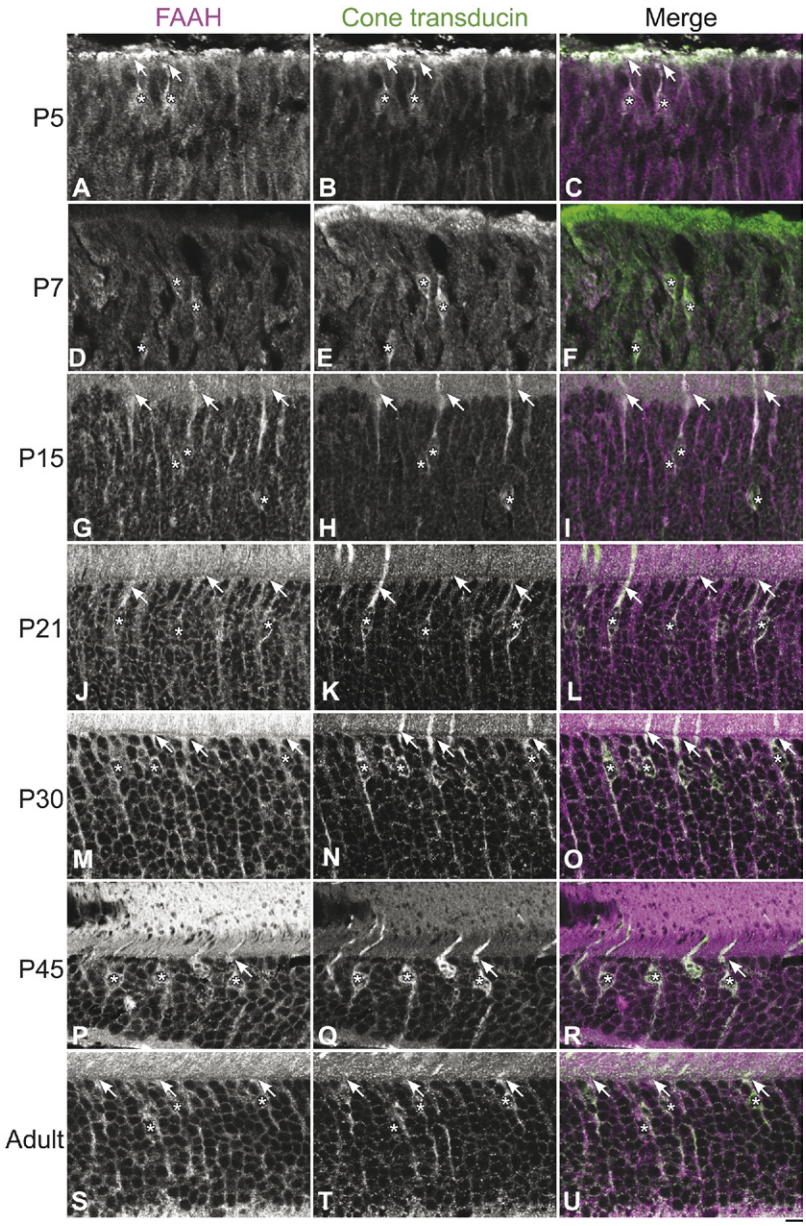


**Fig. 7.** FAAH immunoreactivity in cone photoreceptors. (A–U) Vertical sections from P5 (A–C), P7 (D–F), P15 (G–I), P21 (J–L), P30 (M–O), P45 (P–R), and adult (S–U) rat retinae. Confocal micrographs of retinae co-immunolabeled for FAAH and cell-type–specific marker for the cones, cone transducin. Each protein is presented alone in gray scale: FAAH in the first column and cone transducin in the second, then the two are presented merged (third column—FAAH in magenta and cone transducin in green). Cones become immunopositive for CB1R at P5 and remain so throughout development and into adulthood. CB1R is expressed in the inner segments of the cone (arrows), as well as the cell body (stars). Scale bar=10 $\mu$m. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

absent (Fig. 10H, K). FAAH was never observed at recoverin-positive axon terminals regardless of their type (Fig. 10B, D, F, H).

Rods constitute the vast majority of photoreceptors in the rat's retina (99% of the photoreceptors are rods; La Vail, 1976). We showed in Fig. 1 that some photoreceptors were more intensely immunopositive for FAAH and suggested that these cells were cones. This was verified using a cone-specific marker that also allowed us to analyze the surrounding cells, which were thus considered as rods. These cone transducin immunonegative cells also expressed FAAH (Fig. 7) albeit to a smaller extent than cones. Around P5, a light immunofluorescence could be discerned in rods, but was very close to the background signal (Fig. 7A–C). By P7, the immunofluorescence in rods increased above background level and remained stable over the following weeks of development and maturation (Fig. 7D–U).

Müller cells are glial cells and the most important support cells in the retina. They are generated late in devel-

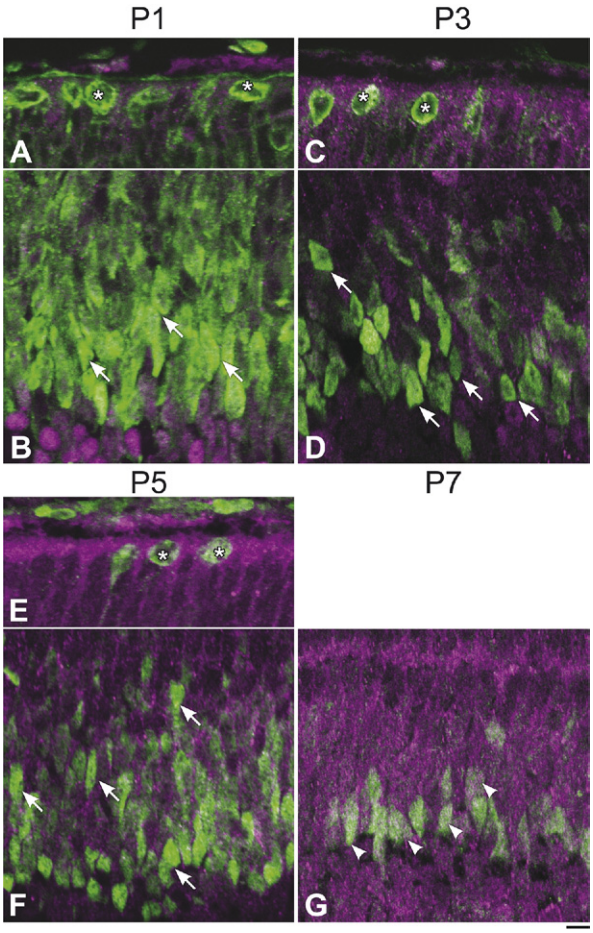


**Fig. 8.** FAAH immunoreactivity in retinal progenitor (B, D, F, and G) and mitotic cells (A, C, E). (A–G) Vertical sections from P1 (A, B), P3 (C, D), P5 (E, F), and P7 (G) rat retinae. Confocal micrographs of retinae co-immunolabeled for FAAH (magenta) and cell-type–specific marker for the retinal progenitor and mitotic cells, PCNA (green). Mitotic cells (stars) are not FAAH positive as long as they exist in the postnatal period, namely until P5–6. Neuroblasts are not FAAH positive (arrows) until P5, then they become immunoreactive to the enzyme FAAH at P7. Scale bar=10 $\mu$m. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

opment and start to express GS around P5 (Riepe and Norenberg, 1978). FAAH was never expressed in Müller cells (Fig. 11A–F).

*Comparison of the temporal profiles of FAAH and CB1R expression.* Fig. 12 shows the timelines of FAAH and CB1R expressions in all retinal cells studied. This timeline was constructed from co-immunolabeling of CB1R and FAAH (presented in Fig. 12A–E; CB1R and FAAH in green and magenta channels, respectively) and from detailed analysis of the expression of both proteins in each cell type. One can see that over the first 2 weeks of life, there is a large overlap in the expression of these two proteins. In details, over the first week of life, CB1R and FAAH were both expressed in horizontal, amacrine, and ganglion cells as well as in cones (Fig. 12A, B, F). During the second week, the two proteins were still co-expressed, although FAAH upregulation and CB1R downregulation in rod bipolar cells were perceptible at P15 (Fig. 12C, F). From P15, the amount of overlap in the expression of FAAH and CB1R progressively decreased. During the third and fourth weeks of life, CB1R and FAAH co-expression was maintained in cones (Fig. 12D, E, F), but decreased in the INL. In the latter, FAAH presence was progressively eliminated from all somas but rod bipolar cells, while CB1R was expressed in horizontal cells, recoverin-positive cone bipolar cells, and some amacrine cells. These data are summarized in Fig. 12F for all ages and cell types. One can observe that for the most part, CB1R and FAAH expression overlapped spatially during early postnatal development in all neuronal cell types except rods and Müller cells. In the adult retina, however, this overlap was greatly decreased, as it concurred only in two cell types (cones and ganglion cells).

## DISCUSSION

This study is the first one investigating the expression of FAAH, a key regulator of the eCB system, during postnatal development of the rodent retina. Using immunohistological staining, we showed that the pattern of expression of FAAH varies during postnatal development and maturation of the retina. Our data present evidence of an early and widespread expression of FAAH, that is, most cell types expressed the enzyme, though most demonstrated a transient expression. Around P3, FAAH was expressed by early-born neurons such as cones, horizontal and ganglion cells, as well as some amacrine cells. This expression was absent from interneurons by the end of third week of life. Late-born neurons such as rod photoreceptors also expressed FAAH but to a smaller extent than cones. Both ON and OFF recoverin-positive cone bipolar cells displayed a transient expression of FAAH between P7 and P30, whereas rod bipolar cells started expressing the enzyme around P15 and remained immunopositive into adulthood. Overall, these data, and particularly the pronounced redistribution of FAAH and transient nature of its expression in most cell types, suggest that the endocannabinoid system could be specifically involved in developmental and/or maturational processes, as well as information processing in adult retina. In brain regions such as hippocampus, the enzyme FAAH is predominantly linked to the metabolism of AEA, which is the main eCB ligand found in this structure in adult animals (Berrendero et al., 1999). A different picture is seen at the retinal level, as 2-AG is found in much higher concentration than AEA (by a factor of 25) in several adult animal models (Bisogno et al., 1999; Chen et al., 2005). In the rat retina, reports indicate that AEA is either present at low concentration (Nucci et al., 2007) or simply absent (Straiker et al., 1999). Given that FAAH is capable of degrading 2-AG (Goparaju et al., 1998; Ueda et al., 1998), it may well be that the close relation between AEA and FAAH found in brain regions does not stand in the retina. Consequently, FAAH could be as well involved in regulating retinal 2-AG concentration or other, less char-

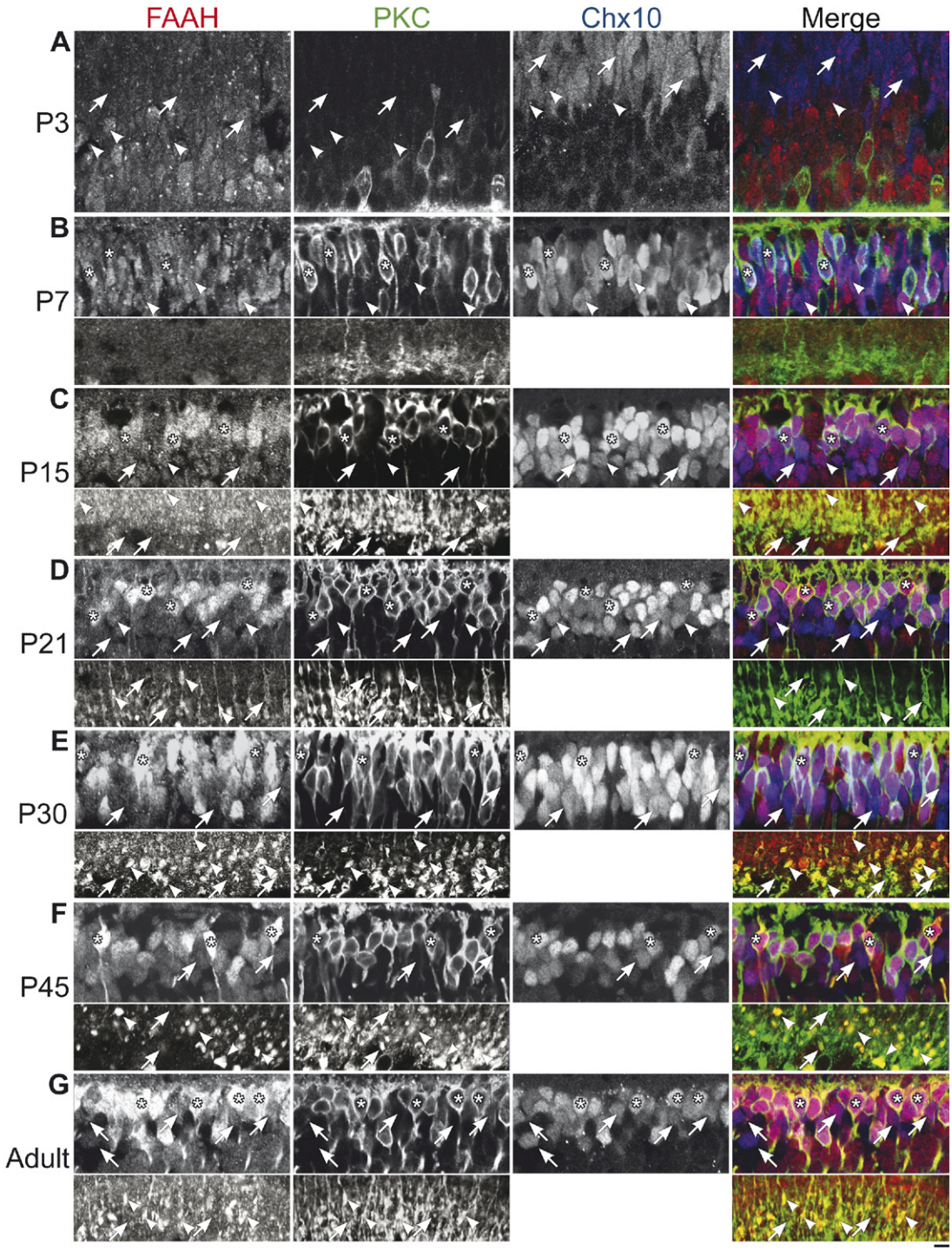


**Fig. 9.** FAAH immunoreactivity in bipolar cells. (A–G) Vertical sections from P3 (A), P7 (B), P15 (C), P21 (D), P30 (E), P45 (F), and adult (G) rat retinae. Confocal micrographs of retinae co-immunolabeled for FAAH (red) and cell-type–specific marker for the bipolar Chx10 (blue) and a third marker specific for rod bipolar and a subtype of amacrine cells, PKC$\alpha$ (green). Each protein is presented alone in gray scale: FAAH in the first column, PKC in the second, and Chx10 in the third, then all the proteins are presented merged (fourth column—FAAH in red, PKC in green, and Chx10 in blue). Each line—corresponding to a given age—presents two panels. The upper panels depict the cell bodies of bipolar cells, the cells immunopositive for both Chx10 and PKC$\alpha$ are rod bipolar cells (stars), while those cells immunoreactive for Chx10 (arrows and arrowheads) are cone bipolar cells. The lower panels display the synaptic terminals of rod bipolar cells in IPL. Please note that since Chx10 is a nuclear marker, there is no blue channel in these pictures. Scale bar=10 $\mu$m. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

acterized, eCBs as has been proposed by Di Marzo and Maccarrone (2008) in a recent review.

In contrast to classical neurotransmitters, eCBs are rather produced locally at the moment of their intended

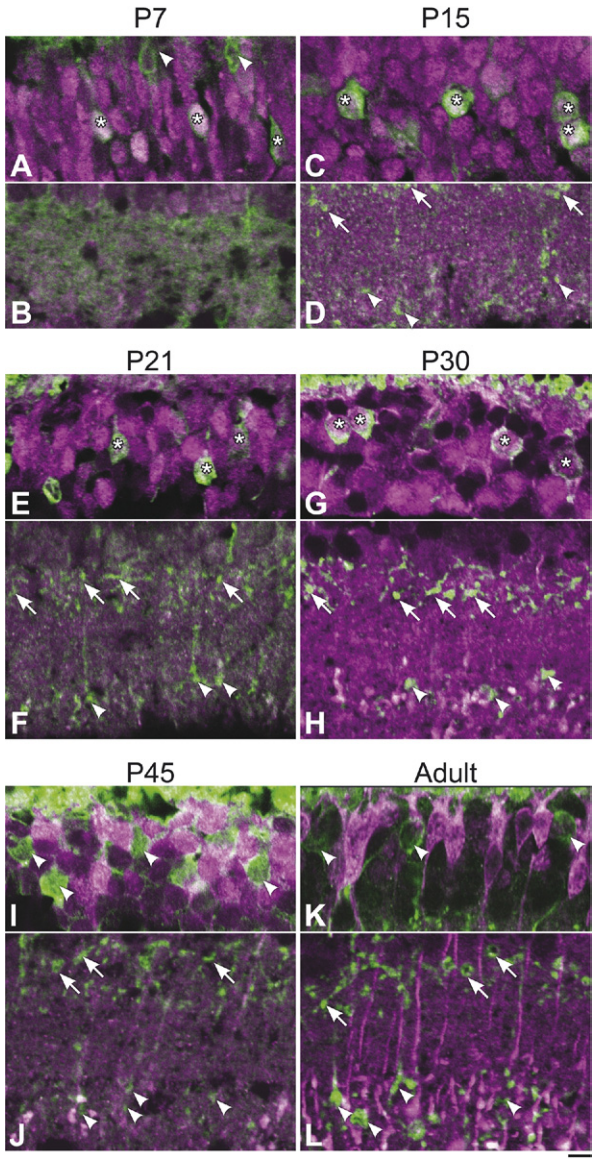


**Fig. 10.** FAAH immunoreactivity in types 2 and 8 cone bipolar cells. (A–L) Vertical sections from P7 (A, B), P15 (C, D), P21 (E, F), P30 (G, H), P45 (I, J), and adult (K, L) rat retinae. Confocal micrographs of retinae co-immunolabeled for FAAH (magenta) and cell-type–specific marker for the types 2 and 8 of cone bipolar cells, recoverin (green). The upper panels A, C, E, G, I, and K depict the cell bodies of FAAH positive (stars) and negative (arrowheads) bipolar cells of. The lower panels B, D, F, H, J, and L display the synaptic terminals of types 2 OFF (arrows) and 8 ON (arrowheads) cone bipolar cells in IPL. Scale bar=10 $\mu$m. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

action. This model places the enzymes responsible for biosynthesis and degradation of eCBs as critical regulators of their signaling. Using Western blot analysis, we established the temporal variation of the total amount of FAAH in our previous study (Zabouri et al., 2011). This variation followed a bimodal profile, as the total amount of the enzyme decreased during the first week of life, before being upregulated during the second one. In conjunction with our immunohistological data, this timeline suggests that during the first week of life, there was a redistribution of FAAH, as the pool of cells containing FAAH increased (addition of horizontal cells at P3 for example). The production of the enzyme increased during the second week of life as more cell types became FAAH positive. After the second week, FAAH was again redistributed, as its expression progressively disappeared from most cell types and became extremely strong in rod bipolar cells.

### FAAH expression during retinal development

Postnatal retinal development has been well studied and described by several groups, using different techniques (Fletcher and Kalloniatis, 1997; Koulen, 1997; Rich et al., 1997; Sharma and Johnson, 2000; Hack et al., 2002; Sharma et al., 2003; Rapaport et al., 2004). The order of the events described here is in line with those reports and has been described in details in our previous paper (Zabouri et al., 2011).

*RGCs and amacrine cells.* FAAH is expressed in most early-born neurons by P3. Our data demonstrate that the enzyme was expressed in some ganglion cells and that this pattern did not vary over time. Its presence in large

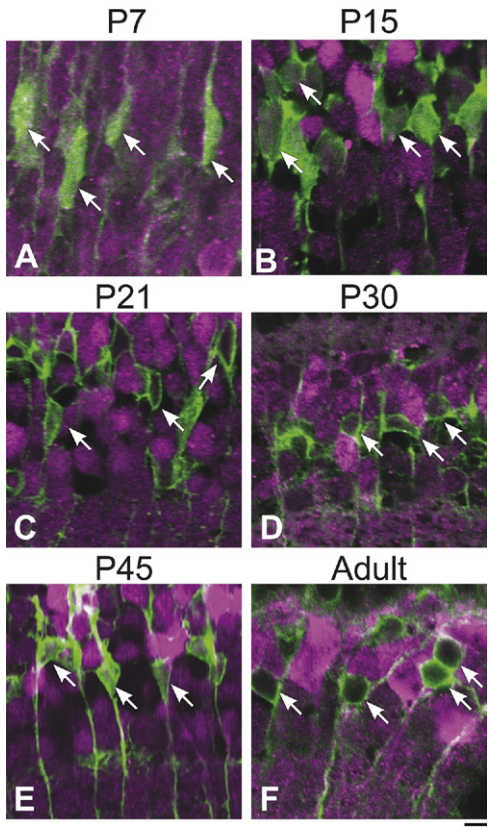


**Fig. 11.** FAAH immunoreactivity in Müller cells. (A–F) Vertical sections from P7 (A), P15 (B), P21 (C), P30 (D), P45 (E), and adult (F) rat retinae. Confocal micrographs of retinae co-immunolabeled for FAAH (magenta) and cell-type–specific marker for glial Müller cells, glutamine synthetase (GS—green). FAAH never expressed in Müller cells (arrows), regardless of the age. Scale bar=10 $\mu$m. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

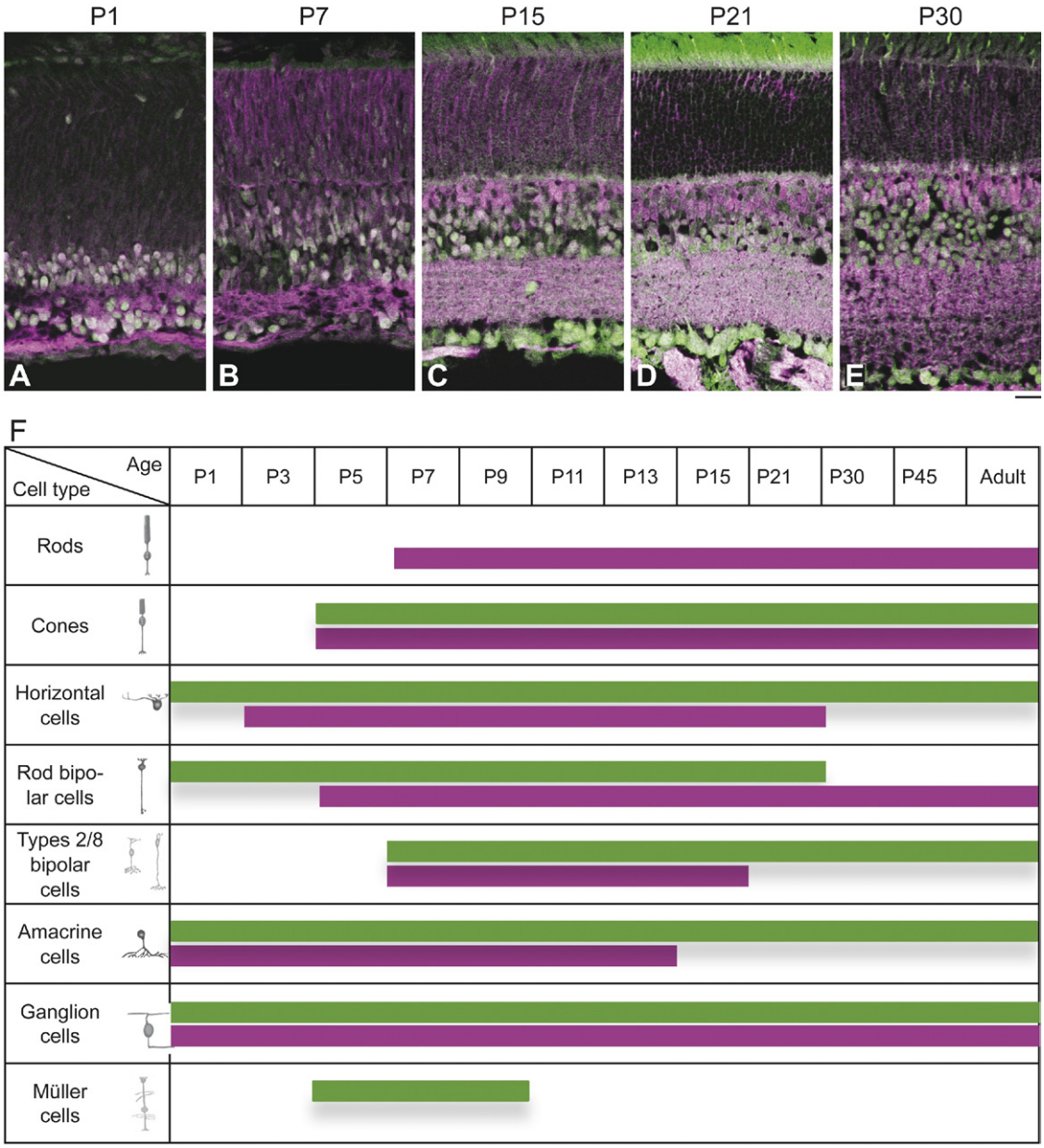


**Fig. 12.** Comparison of CB1R and FAAH expression. (A–E) Vertical sections from P1 (A), P7 (B), P15 (C), P21 (D), and P30 (E) rat retinae. Confocal micrographs of retinae co-immunolabeled for CB1R (green) and FAAH (magenta). Scale bar=10 $\mu$m. (F) Summary of CB1R (green bars) and FAAH (magenta bars) overlap or lack thereof over the period investigated (P1-adulthood). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

ganglion cells has been previously reported in adult mice and Sprague–Dawley rats (Yazulla et al., 1999; Hu et al., 2010). The expression of FAAH in the nerve fiber layer increased over the second month of life (data not shown). This expression is consistent with the demonstrated contribution of eCBs in the establishment of proper axon targeting in the visual system (Argaw et al., 2011) and other sensory systems (Li et al., 2009).

The transient expression of FAAH during development suggests that eCBs need to be controlled in the close environment of temporarily expressing FAAH cells. Such transient expression was observed in amacrine cells. It was present early on in most amacrine cells, and in particular, in ON and OFF cholinergic amacrine cells during the first 2 weeks of life. One can thus propose that eCB may be involved in developmental processes taking place within the inner retina, such as synapse establishment (Schubert et al., 2008), refinement of ganglion cells dendrites (Kim et al., 2010), and retinal spontaneous waves (see the section on Functional considerations). Moreover, a recent study demonstrated that cholinergic amacrine cells showed evidence of plasticity during early postnatal development (Zhang et al., 2005), a process in which eCBs could be involved.

In non-cholinergic amacrine cells, two patterns of expression were observed, namely a punctate pattern and a whole cell staining. The association of FAAH with cytoplasmic vesicle has been reported during postnatal hippocampal development (Morozov et al., 2004). In accordance with that study, we do not report any age relation between FAAH cytosolic punctate distribution and postnatal development.

*Cones and horizontal cells.* FAAH emerged in horizontal cells early during postnatal development, between P1 and P3. It remained stable through the first 2 weeks of life, after which it was greatly downregulated. As stated before, this transient expression suggests that eCBs need to be controlled in the close environment of these cells during development. FAAH absence from horizontal cells in adult retina is in agreement with a recent study on the expression of the eCB system in adult mouse retina (Hu et al., 2010). However, it is in disagreement with Yazulla et al. (1999), who reported a light expression of FAAH in rat horizontal cells. Since all three studies used antibodies tested against KO animals, these differences cannot be accounted for by a lack of antibody specificity. A more plausible explanation would be that this discrepancy comes from a species/strain difference. Yazulla et al. (1999) used albino rats, which have been shown to have anatomically and functionally different retinae from pigmented animals (Crespo et al., 1985; Rachel et al., 2002; Heiduschka and Schraermeyer, 2008).

FAAH appeared later in cones (around P5), in their cell bodies and pedicles, and remained strongly present into adulthood. In this cell type, FAAH and CB1R were co-expressed. These data are in agreement with previous findings reported by Yazulla et al. (1999) and Hu et al. (2010). Given the role of CB1R in retrograde signaling (see for review Kreitzer and Regehr, 2002), the presence of the degradative enzyme in the same cell as the receptor is logical. Since the timing of CB1R and FAAH expressions coincides with the appearance of the first cone horizontal cell synapses in the forming OPL (Dhingra et al., 1997; Rich et al., 1997; Hack et al., 2002), one could hypothesize that the eCB system may modulate early glutamatergic transmission between cone photoreceptors and horizontal cells (starting around P2; Raven et al. (2008)). This type of transmission is considered to participate in the organization of horizontal cell dendritic trees. This hypothesis is further supported by the fact that eCBs have been shown to modulate synaptogenesis in the hippocampus (Kim and Thayer, 2001). Another putative hypothesis is that horizontal cells could also affect cone development, as they are likely to modulate cone synaptogenesis and mosaic organization through GABAegic transmission (Schnitzer and Rusoff, 1984; Fletcher and Kalloniatis, 1997; Mitchell et al., 1999; Fei, 2003). However, a recent study reported only very subtle changes in the retina of animals in which GABA production was selectively knocked out of horizontal cells during their development (Schubert et al., 2010), making the above-mentioned hypothesis less attractive.

*FAAH expression in late-born cells.* All groups of late-born cells, except glial cells, expressed FAAH around P5–9, a period which corresponds to developmental processes such as cell migration, subtype identity acquisition, and morphological changes (Sharma et al., 2003; Morgan et al., 2006). Neuroblasts soma expressed FAAH lately (around P7).

Using a marker for all bipolar cell types in conjunction with one for rod bipolar cells only, we were able to differentiate cone and rod bipolar cells. Some cone bipolar cells transiently expressed FAAH during the first month of life. Conversely, the enzyme expression in rod bipolar cells appeared toward the end of the second week of life, and reached adult-like expression 1 month later. The presence of FAAH in adult rod bipolar cells only is at odds with the findings of Yazulla et al. (1999), who reported FAAH expression in at least one type of cone bipolar cells and its absence from rod bipolar cells in Sprague–Dawley rats. This study and ours were conducted using antibodies raised against similar antigens that were both tested on FAAH KO mice generated in Dr. Cravatt laboratory (Cravatt et al., 2001; see Experimental procedures). Thus, differences in FAAH distributions are not likely to be caused by the lack of specificity of the antibodies. It could arise from strain or protocol differences. To investigate this, we replicated our experiments in Sprague–Dawley rats. FAAH expression was clearly found in rod bipolar cells (as in pigmented rats) and in some cone bipolar cells, as in Yazulla et al. (1999; data not shown). This partial agreement suggests that FAAH is not equally revealed by all antibodies. In support of this last statement, we found a comparable distribution in pigmented rats using another FAAH antibody raised against the N-terminal portion of the protein (Alpha Diagnostics International, San Antonio, TX, USA). Overall, these data suggest that the enzyme is probably present in both cell types.

To investigate whether the eCB system could affect the development and signaling of both ON and OFF bipolar cells, we studied FAAH expression in ON and OFF recoverin-positive cells. During the second and third weeks of life, all recoverin-positive cells were immunoreactive for FAAH, after which they lost their immunoresponsiveness to this enzyme. These results suggest that the eCB system could be involved in the maturational processes of these cells. Indeed, during the second week of life, bipolar cells and photoreceptors develop the complete machinery to communicate (Sherry et al., 2003). The transient presence of elements of the eCB system in bipolar cells during this period supports their involvement in developmental processes, whether directly in synaptogenesis, as reported elsewhere (Kim and Thayer, 2001) or simply as an activity regulator.

Rod photoreceptors started to express FAAH around P7 and remained immunoreactive into adulthood. These data, cumulated with FAAH expression in rod bipolar cells, raise the possibility that the rod pathway could control the eCB degradation and modulate, for example, CB1R activity in cones (Yazulla et al., 1999; Fan and Yazulla, 2003;

Struik et al., 2006), thereby modulating the signal transfer from cones to bipolar cells.

### Functional considerations

During the first week of life, spontaneous activity could be an important mechanism by which visual map refinement occurs in thalamic nuclei (see for review Torborg and Feller, 2005).

Spontaneous discharges have been shown to originate from cholinergic amacrine and ganglion cells (Feller et al., 1996; Stacy et al., 2005), two cell types expressing CB1R (Zabouri et al., 2011) and FAAH during the first week of life. ACh is the major excitatory input onto ganglion cells in the developing retina, acting via nicotinic receptors and affecting the spontaneous bursting activity of these cells (Feller et al., 1996; Sernagor and Grzywacz, 1996). Although no studies were conducted during development, this neurotransmitter was shown to be modulated by eCB signaling in the adult cortex (Degroot et al., 2006). We previously proposed the hypothesis that a propagating wave of spontaneous activity could cause local changes in eCB concentration, which would in turn limit the spread of spontaneous activity through CB1R activation (Zabouri et al., 2011). The data presented here demonstrate that cholinergic amacrine cells are well suited to modulate CB1R activity during the days when spontaneous activity occurs, thereby further supporting this hypothesis.

Given the fact that during developmental and maturational stages, CB1R and FAAH are often found in the same cell types, a pattern that is seldom found in the adult retina, we suggest that this system is self-regulated during development. This idea has been raised by Harkany et al. (2008), who demonstrated that synthetic enzymes and CB1R were expressed in the same cells in developing neurons.

The adult pattern we reported, that is, the fact that FAAH and CB1R are not expressed in the same cells (with the exception of cones and some ganglion cells), is in agreement with that described in the retina (Yazulla et al., 1999; Hu et al., 2010) and elsewhere in the nervous system (Egertová et al., 1998). Since for most cells, FAAH and CB1R appear mutually exclusive, it may be that eCBs play a role in cell to cell communication.

## CONCLUSION

This study revealed the presence of FAAH in retinal cells of developing and mature rats. We showed that there is a redistribution of FAAH expression from birth to adulthood, which supports the notion that this enzyme is involved in developmental processes. In addition, the overlap of FAAH and CB1R expression patterns in young animals suggests that retinal cells autoregulate CB1R activation during the course of development. Such autoregulation is not likely to occur in adult animals given the absence of overlap expression. Overall these findings indicate that, as shown in other structures of the brain, the eCB system is likely to play an instrumental role in the development and function of the retina.

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