Chapter 4
Fatty Acid Amide Hydrolase: A Gate-Keeper of the Endocannabinoid System

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Abstract The family of endocannabinoids contains several polyunsaturated fatty acid amides such as anandamide (AEA), but also esters such as 2-arachidonoylglycerol (2-AG). These compounds are the main endogenous agonists of cannabinoid receptors, able to mimic several pharmacological effects of Δ^9-tetrahydrocannabinol (Δ^9-THC), the active principle of *Cannabis sativa* preparations like hashish and marijuana. The activity of AEA at its receptors is limited by cellular uptake, through a putative membrane transporter, followed by intracellular degradation by fatty acid amide hydrolase (FAAH). Growing evidence demonstrates that FAAH is the critical regulator of the endogenous levels of AEA, suggesting that it may serve as an attractive therapeutic target for the treatment of human disorders. In particular, FAAH inhibitors may be next generation therapeutics of potential value for the treatment of pathologies of the central nervous system, and of peripheral tissues. Investigations into the structure and function of FAAH, its biological and therapeutic implications, as well as a description of different families of FAAH inhibitors, are the topic of this chapter.

Keywords Cannabinoids · endocannabinoid system · FAAH · gene expression · inhibitor

Abbreviations AEA: anandamide; 2-AG: 2-arachidonoylglycerol; AMT: anandamide membrane transporter; AS: amidase signature; AD: Alzheimer's disease; CB1R: type 1 cannabinoid receptor; CB2R: type 2 cannabinoid receptor; DAG: 1-acyl-2-arachidonoylglycerol; DAGLs: diacylglycerol lipases; EAE: autoimmune encephalomyelitis; ECS: endocannabinoid system; ETTH: episodic tension-type headache; FAAH: fatty acid amide hydrolase; FAAs: fatty acid amides; HAEAs: hydroxyanandamides; HD: Huntington's

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disease; MAE2: malonamidase; MAFP: methoxy arachidonoyl fluorophosphonate; MAGL: monoacylglycerol lipase; MoA: migraine without aura; MS: multiple sclerosis; NAGly: *N*-arachidonoyl-glycine; NAPE-PLD: *N*-acylphosphatidylethanolamide-phospholipase D; NArPE: *N*-arachidonoylphosphatidylethanolamine; NAT: *N*-acyltransferase; PAM: C-terminal peptide amidase; PMSF: phenylmethylsulfonyl fluoride; SNPs: single nucleotide polymorphisms; Δ^9-THC: Δ^9-tetrahydrocannabinol; TM: N-terminal transmembrane; TRPV1, transient receptor potential vanilloid 1

4.1 Introduction

4.1.1 (Endo)cannabinoids

The recreational value of *Cannabis sativa* preparations is known to most people, largely as a result of the explosion in its use in the late 1960s; indeed, marijuana is still one of the most widespread illicit drugs of abuse in the world (Adams and Martin, 1996). The plant contains about 60 cannobinoid compounds (Ross and Elsohly, 1996), among which Δ^9-tetrahydrocannabinol (Δ^9-THC) (Fig. 4.1) is the primary psychoactive component and is thought to mediate most of the physiological effects associated with marijuana smoking (Dewey, 1986). Δ^9-THC was used in folklore medicine long before the discovery of its mechanism of action.

The stringent structural characteristics that cannabinoid compounds must possess in order to exert their psychotropic effects, and the key observation that cannabinoids inhibit adenylate cyclase, supported the presence of a specific, high-affinity binding site for these lipidic substances (Howlett and Fleming, 1984). Shortly afterwards, the first membrane receptor for Δ^9-THC was identified in rat brain (Devane et al., 1988). Its distribution was consistent with the pharmacological properties of psycotropic cannabinoids, and therefore it was

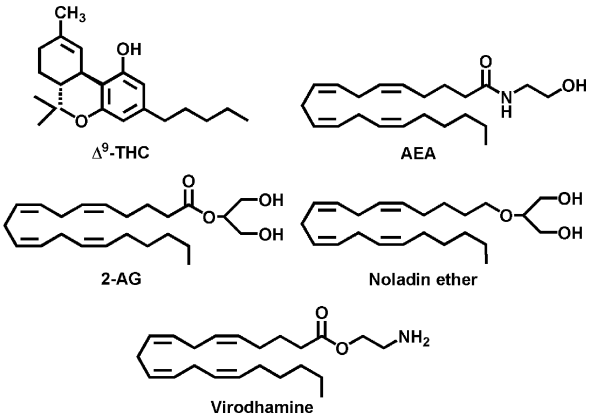


Fig. 4.1 Chemical structures of various (endo)cannabinoids

designated type 1 cannabinoid receptor (CB1R) (Devane et al., 1988). A peripheral cannabinoid-binding receptor was identified a few years later in spleen and immune cells, and was called type 2 cannabinoid receptor (CB2R) (Munro et al., 1993). Since then, a number of endogenous agonists of CB receptors were characterized, i.e. amides, esters and ethers of long chain polyunsaturated fatty acids collectively termed 'endocannabinoids'. Remarkably, these compounds are structurally different from Δ^9-THC or other plant cannabinoids (Mechoulam et al., 2002; Piomelli, 2003; De Petrocellis et al., 2004). In fact two arachidonate derivatives, the amide *N*-arachidonoylethanolamine (anandamide, AEA) (Devane et al., 1992) and the ester 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995), both shown in Fig. 4.1, are the most biologically active endocannabinoids described to date (Piomelli, 2003; De Petrocellis et al., 2004). Also the ether 2-arachidonoylglyceryl-ether (noladin ether) (Fig. 4.1) has been shown to act as an endocannabinoid (Hanus et al., 2001), but its actual physiological relevance remains a matter of debate (Oka et al., 2003). Furthermore an 'inverted anandamide', *O*-arachidonoylethanolamine (virodhamine) (Fig. 4.1), has been shown to behave as a partial agonist or as a full agonist at CB1 or CB2 receptors, respectively (Porter et al., 2002).

Instead, the amides *N*-oleoylethanolamine (OEA), *N*-palmitoylethanolamine (PEA) and oleamide are better considered 'endocannabinoid-like' compounds, because they do not activate directly CB receptors, but rather prolong the activity of true endocannabinoids within the cell by an 'entourage effect' (De Petrocellis et al., 2004). AEA and 2-AG are present in the central nervous system (CNS) and also in peripheral tissues (Sugiura et al., 2002), but exhibit important differences in their quantitative distribution; 2-AG is more abundant than AEA in the brain and behaves as a full agonist for CB1R and CB2R, while AEA acts as partial agonist for CB1R and as a weak partial agonist for CB2R (Sugiura et al., 2000a). AEA levels may vary by 4–6-fold in different regions of the rat brain, with the highest levels in the striatum and brainstem and the lowest levels in the cerebellum and cortex (Bisogno et al., 1999a; Yang et al., 1999). AEA was found in regions of both rat and human brains that contain high densities of CB1R (e.g., hippocampus, cerebellum, and striatum) and also in a region that is sparse in CB1R like the thalamus (Felder et al., 1996). It is clear from these data that for AEA the relative regional abundance in the brain does not correlate with the distribution of CB1R. AEA levels in the brain are equivalent to those of other neurotransmitters such as dopamine and serotonin, but at least 10-fold lower than the levels reported for GABA and glutamate. AEA has also been found in peripheral tissues such as human and rat spleen, which expresses high levels of CB2R. Small amounts of AEA were also detected in human serum, plasma, and cerebrospinal fluid (Felder et al., 1996).

The concentration of 2-AG can be up to ~200-fold higher than that of AEA in the brain (Bisogno et al., 1999a). Yet, there are reports showing much lower 2-AG:AEA ratios in rat striatum (~10), substantia nigra (~3), and globus pallidus (~4) (Di Marzo et al., 2000; Gubellini et al., 2002). These differences

may arise from different methodologies, for instance killing the animals by decapitation without immediate freezing instead of soaking in liquid nitrogen can increase 2-AG levels by ~15-fold (Sugiura et al., 2002). Discrepancies may also be a consequence of the high sensitivity of endocannabinoids to environmental factors like animal diets, caging and bedding systems, viral load, water quality, and pathogen infections. A recent example of the dramatic effect of these factors on endocannabinoid levels has been recently reported (Guo et al., 2005). In this context, it seems noteworthy that a recent study has shown that the extracellular concentrations of AEA and 2-AG are both in the nanomolar range (Caillè et al., 2007), suggesting that these two compounds have a similar availability for their CBR-mediated biological actions. On the other hand, the spatial distribution of the two endocannabinoids is similar in different regions of the brain. In fact, the highest concentrations of 2-AG were found in the brainstem, medulla, limbic forebrain, striatum, and hippocampus, and the lowest in the cortex, diencephalons, mesencephalon, hypothalamus, and cerebellum (Sugiura et al., 2002). Therefore, much alike AEA, no correlation was found between 2-AG concentrations and CB1R distribution. 2-AG was also detected in the peripheral nervous system, i.e. in the sciatic nerve, lumbar spinal cord, and lumbar dorsal root ganglion cells (Sugiura et al., 2002).

In just one decade, endocannabinoids have been shown to play manifold roles, both in the CNS and in the periphery. In particular, it is now widely accepted that the biological activity of AEA and 2-AG is largely dependent on a 'metabolic control', that modulates the effects of these substances by modulating their in vivo concentration (or endogenous tone) (Cravatt and Lichtman, 2002).

4.1.2 Overview of the Endocannabinoid System

Investigations of the pathways involved in the metabolism of endocannabinoids have grown exponentially in recent years following the discovery of cannabinoid receptors. As other lipid mediators, AEA and 2-AG are released from cells 'on demand' by stimulus-dependent cleavage of membrane phospolipid precursors (Di Marzo et al., 1994).

AEA biosynthesis has been shown to occur through several pathways mediated by *N*-acylphosphatidylethanolamide-phospholipase D (NAPE-PLD), a secretory PLA_2 and PLC. 2-AG is generated through the action of selective enzymes such as phosphatidic acid phsophohydrolase, diacylglycerol lipase (DAGL), phosphoinositide-specific PLC (PI-PLC) and lyso-PLC. A putative membrane transporter, catalyzing a facilitated diffusion process, is involved in the cellular uptake or release of endocannabinoids. AEA is metabolized by fatty acid amidohydrolase (FAAH) and 2-AG is metabolized by monoacylglycerol lipase (MAGL), and to a lesser extent by FAAH.

Taken together AEA and 2-AG, their congeners and metabolic enzymes, their purported transporters and molecular targets form the 'endocannabinoid system (ECS)'.

4.1.2.1 Molecular Targets

Endocannabinoids act primarily at cannabinoid receptors. These are seven trans-membrane spanning receptors that include type-1 cannabinoid receptors (CB1R), which are present mainly in the CNS but are also expressed in peripheral tissues and cells like lymphocytes (Börner et al., 2007), and type-2 cannabinoid receptors (CB2R), expressed predominantly by astrocytes, spleen and immune cells (Lunn et al., 2006), but also present in the brainstem (Van Sickle et al., 2005; Aguado et al., 2007). CB1R and CB2R belong to the rhodopsin family of G protein-coupled receptors (GPCRs), particularly those of the Gi/o family (Howlett et al., 2002). The binding of endocannabinoids to these receptors induces several biological actions, such as the inhibition of adenylate cyclase (AC), the regulation of ionic currents (inhibition of voltage-gated L, N and P/Q-type Ca^{2+} channels, activation of K^+ channels), the activation of focal adhesion kinase, of mitogen-activated protein kinase (MAPK), and of cytosolic phospholipase A_2, and the activation (CB1R) or the inhibition (CB2R) of nitric oxide synthetase (NOS). Additionally, a recent report has shown an unprecedented coupling of CB1R to Gq/11 proteins, suggesting further diversity of CB1R signaling pathways (Lauckner et al., 2005). Furthermore, there is some evidence that endocannabinoids induce a biological activity via other CB receptors, like a purported CB3 (GPR55) receptor (Sawzdargo et al., 1999; Baker et al., 2006; McPartland et al., 2006; Ryberg et al., 2007), via non-CB1/non-CB2 receptors, or via non-cannabinoid receptors. In the latter group, transient receptor potential vanilloid 1 (TRPV1) has emerged as an important target of AEA, but remarkably not of 2-AG. TRPV1 is a six transmembrane spanning protein with intracellular N- and C-terminals, and a pore-loop between the fifth and sixth transmembrane helices (Jung et al., 1999). This ligand-gated and non-selective cationic channel is activated by molecules derived from plants, such as the pungent component of 'hot' red peppers capsaicin, by noxious stimuli like heat and protons (Jordt and Julius, 2002), and by peptides contained in spider toxins (Siemens et al., 2006). Also AEA is considered a true 'endovanilloid' (van der Stelt et al., 2004; Starowicz et al., 2007), that behaves as an authentic (though weak) ligand of TRPV1.

4.1.2.2 Biosynthesis of Endocannabinoids

The main route for AEA biosynthesis occurs by two enzymatic steps involving the sequential action of a calcium dependent *N*-acyltransferase (NAT) and of a NAPE-specific phospholipase D (NAPE-PLD) (Okamoto et al., 2004). In the first step, NAT catalyzes direct transfer of arachidonic acid from the *sn*-1 position of phosphatidylcholine (PC), generating

N-arachidonoylphosphatidylethanolamine (NArPE), the AEA precursor (Fig. 4.2). This biosynthetic pathway is in agreement with the observation that AEA levels are generally lower than those of the other NAEs in most of the tissue analyzed so far, because the arachidonic acid levels in position 1 of phospholipids are very low.

In the last step, NArPE is hydrolyzed by NAPE-PLD which releases AEA and phosphatidic acid (PA). This enzyme has been cloned and purified from rat heart and classified as a member of the zinc metallo-hydrolase family of the β-lactamase fold (Okamoto et al., 2004). NAPE-PLD does not recognize phosphatidylcholine and phoshatidylethanolamine as substrates, and it is widely distributed in mouse organs, with highest concentrations in brain, kidney and testis (Okamoto et al., 2004). The same group who characterized NAPE-PLD also suggested that several PLA_1/A_2 isozymes can generate *N*-arachidonoyl-lysoPE (NAr-lysoPE) from NArPE, and that a lysoPLD may release AEA from NAr-lysoPE. Therefore, the sequential action of PLA_1/A_2 and lysoPLD may represent an alternative biosynthetic pathway for NAEs, including AEA (Sun et al., 2004) (Fig. 4.2).

Recently, it has been shown that in RAW264-7 macrophages, the lipopolysaccharide-induced anadamide production appears to depend mainly on a pathway whereby NAPE is hydrolysed to yield a phosphor-AEA, which is

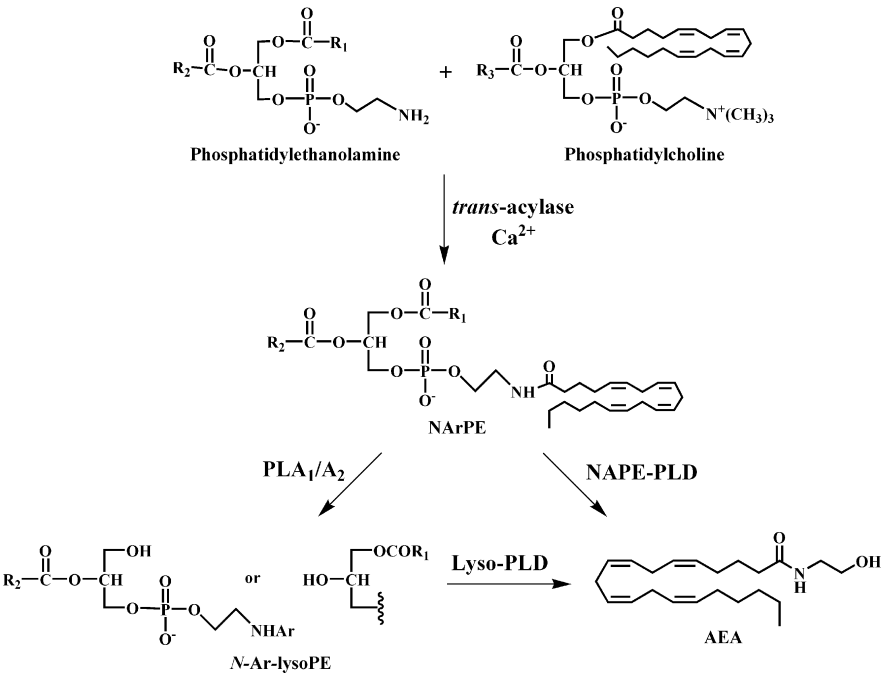


Fig. 4.2 Major biosynthetic pathways of AEA. See text for details

then dephosphorylated (Liu et al., 2006). Furthermore, a non exclusive role of NAPE–PLD in the conversion of NAPE to AEA is clearly indicated by the unchanged brain levels of AEA in NAPE–PLD knockout mice (Leung et al., 2006). In fact, an independent pathway may occur through a double-deacylation of NAPE to generate *lyso*-NAPE and then glycerophospho-NAE, that is rapidly cleaved to release the corresponding NAE. This novel route is driven by the sequential action of a fluorophosphonate-sensitive serine hydrolase and a metal-dependent phosphodiesterase (Simon and Cravatt, 2006).

The levels of 2-AG in tissues and cells are usually much higher than those of AEA, and in principle they are sufficient to activate both cannabinoid receptor subtypes (Sugiura et al., 1995). At any rate, the 2-AG found in cells and tissues is probably not uniquely used to stimulate cannabinoid receptors, as 2-AG is at the crossroads of several metabolic pathway. It is likely that a particular pool of 2-AG is produced via a special pathway only for the purpose of functioning as endocannabinoid. In line with this hypothesis, the extracellular levels of 2-AG are close to those of AEA, and are in the nanomolar range (Caillè et al., 2007).

A biosynthetic pathway for 2-AG provides for quick hydrolysis of inositol phospholipids by a specific PLC, generating 1-acyl-2-arachidonoylglycerol (DAG) (Di Marzo et al., 1999; Piomelli et al., 1998). DAG is then converted to 2-AG by a *sn*-1-DAG lipase (Stella et al., 1997; Bisogno et al., 2003). Another pathway for 2-AG formation involves the hydrolysis of phosphatidylinositol (PI) by PLA_1 into lysoPI, followed by hydrolysis by phospholipase C (PLC) to produce 2-AG (Sugiura and Waku, 2000b). Furthermore, 2-AG has been shown to be produced also by PLC-independent pathways (Bisogno et al., 1999b). Very recently, two *sn*-1-specific DAG lipases (α and β) responsible for the synthesis of 2-AG have been cloned by comparing human genome with Penicillium DAGL sequence. Both DAGL α and β are associated with the cell membrane and are stimulated by high concentrations of Ca^{2+} and, remarkably, by physiological concentrations of glutathione (Bisogno et al., 2003).

4.1.2.3 Degradation of Endocannabinoids

The endocannabinoid actions are relatively short-lasteing, due to the presence of effective mechanisms for their cellular removal and subsequent degradation. Because they are lipophilic compounds, endocannabinoids can diffuse through the cell membrane. However, in order to be rapid, selective and subject to regulation, the diffusion process needs to be facilitated by a carrier, or to be driven by a mechanism capable of rapidly reducing the intracellular concentration of endocannabinoids, or both.

Indeed, AEA appears to be taken up by several cells via a facilitated transport mechanism, possibly mediated by a purported anandamide membrane transporter (AMT) (Fig. 4.3). In fact, cellular uptake of AEA is saturable, temperature-dependent and sensitive to synthetic inhibitors, as expected for a protein-mediated process (Maccarrone et al., 1998, Bisogno et al., 2001a). However, some authors have reported evidence against the existence of AMT,

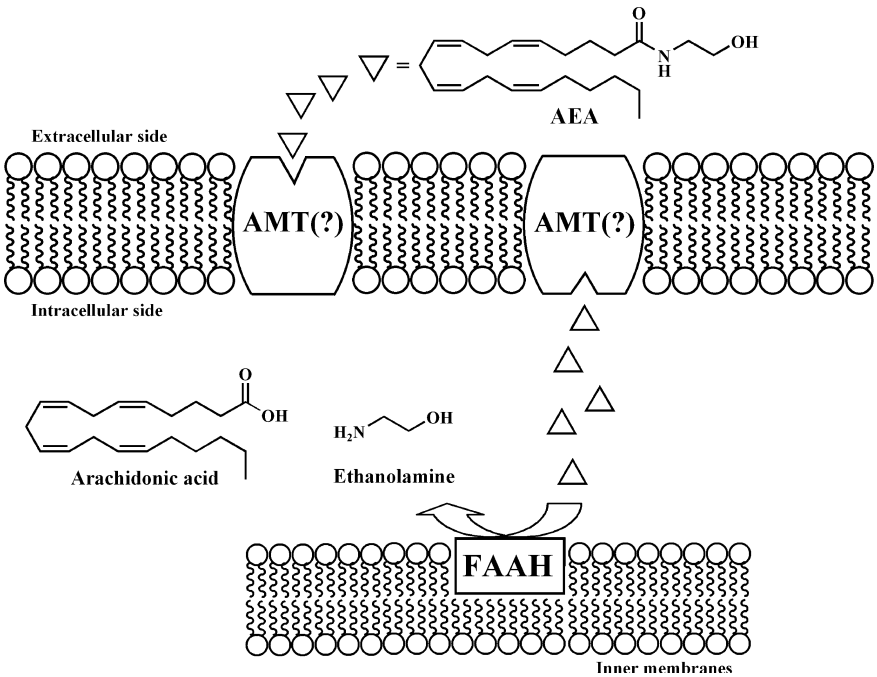


Fig. 4.3 Pathways of AEA inactivation. Once taken up by a purported transporter on the plasma membrane (AMT), AEA is rapidly cleaved by endomembrane-bound FAAH, realesing arachidonic acid (AA) and ethanolamine (EA)

suggesting that the enzyme mostly responsible for AEA hydrolysis, fatty acid amide hydrolase (FAAH) (Fig. 4.3), may be the sole responsible of AEA cellular uptake, by reducing its intracellular concentration (Bracey et al., 2002; Glaser et al., 2003).

On the other hand, several data are in agreement with a facilitated transport of AEA independent of FAAH. In fact, different cells that do not express FAAH are still able to rapidly take up AEA (Day et al., 2001); compounds that inhibit AEA cellular uptake without affecting FAAH activity have been synthesized (López-Rodríguez et al., 2001; Ortar et al., 2003); saturable AEA accumulation can be still observed in synaptosomes and cells prepared from FAAH-null mice (Ligresti et al., 2004; Fegley et al., 2004). Overall, from the available data it is possible to conclude that FAAH activity can contribute to facilitated AEA transport, yet it is not necessary; other mechanisms different from intracellular hydrolysis may also enhance the rate of endocannabinoid uptake. In line with this, a new model for AEA transport has been proposed, that might engage a caveolae/lipid rafts-related endocytic process (McFarland et al., 2004; Bari et al., 2005). On the other hand, it has been suggested that the 2-AG membrane transporter may be the same used by anandamide, i.e. AMT (Beltramo and Piomelli, 2000; Bisogno et al., 2001b).

Once inside the cell, endocannabinoids are degraded through mechanisms depending on their chemical nature. FAAH has been identified as the main responsible for AEA hydrolysis to arachidonic acid and ethanolamine (Cravatt et al., 1996; Cravatt and Lichtman, 2002) (Fig. 4.3). Although FAAH can catalyze also the hydrolysis of 2-AG (Di Marzo and Deutsch, 1998), the levels of the latter substance, unlike those of AEA, are not increased in FAAH 'knockout' mice (Lichtman et al., 2002). This observation is in agreement with the existence of other enzymes catalyzing 2-AG degradation (Di Marzo et al., 1999; Goparaju et al., 1999a). In fact, monoacylglycerol lipase (MAGL) is a cytosolic enzyme that cleaves efficiently 2-AG (Ben-Shabat et al., 1998; Di Marzo and Deutsch, 1998). In rat brain, MAGL is more abundant in regions where also CB1 receptors are highly expressed (hippocampus, cortex, anterior thalamus and cerebellum). Furthermore, immunohistochemical studies in the hippocampus suggested a presynaptic localization of MAGL, supporting its role in the degradation of 2-AG as retrograde messenger. Interestingly, recent studies have confirmed a sort of 'complementary localization' of MAGL and FAAH in the brain, pre-synaptic and post-synaptic respectively, suggesting different roles for AEA and 2-AG in endocannabinoid signaling within the CNS (Gulyas et al., 2004). Incidentally, the data on MAGL localization supplement previous observations showing that the diacylglycerol lipases (DAGLs) responsible for 2-AG production are instead post-synaptic in the adult brain (Dinh et al., 2002; Bisogno et al., 2003).

4.2 Properties of Fatty Acid Amide Hydrolase

The actual enzymes involved in fatty acid amides (FAAs) metabolism remained unknown until the late 1990s, when a rat liver oleamide hydrolase activity was affinity-purified and its cDNA was cloned (Cravatt et al., 1996). Oleamide amidase was connected to AEA hydrolysis, because AEA and oleamide were catalyzed by the same enzyme, called fatty acid amide hydrolase (FAAH; *N*-arachidonoylethanolamine amidohydrolase, EC 3.5.1.4) (Maurelli et al., 1995).

Later on, FAAH was cloned from human and mouse liver (Giang and Cravatt, 1997), and from porcine brain (Goparaju et al., 1999b). All these enzymes are composed of 579 amino acids, and their molecular weights are ~63 kDa. The porcine enzyme shows 80, 81, and 85% identity of the deduced amino acid sequence to mouse, rat and human FAAH respectively (Goparaju et al., 1999b).

In rats, FAAH is mainly distributed in liver, small intestine, brain, testis, uterus, kidney, ocular tissues and spleen, but not in skeletal muscle or heart (Deutsch and Chin, 1993; Desarnaud et al., 1995; Ueda et al., 1995). In humans, the distribution is different: FAAH is mainly detected in pancreas, brain, kidney, skeletal muscle (Giang and Cravatt, 1997), placenta (Park et al., 2003) and is less abundant in liver (Giang and Cravatt, 1997). FAAH activity can also

be detected in mouse uterus (Paria et al., 1996), and its expression is regulated during pregnancy (Paria et al., 1999; Maccarrone et al., 2000a).

FAAH is a membrane-bound serine hydrolase, that shows its maximal activity at pH 9 (Cravatt et al., 1996). This enzyme belongs to a protein family called 'amidase signature (AS)' (Chebrou et al., 1996), whose members share a common, conserved amino acid sequence comprising ~130 residues, the so-called 'amidase-signature sequence'. The AS family of enzymes is mainly represented among bacteria and fungi, and FAAH was, until recently, the only known representative of this class of proteins in mammals.

Further studies were conducted with the aim of identifying the primary-sequence of FAAH, to unravel the properties of the region that allows anchoring to the membrane. Although the amino acids in positions 9–29 were predicted with the aid of a sequence-analysis software to constitute the FAAH transmembrane domain, deletion of this segment did not release FAAH from lipid bilayers (Arreaza and Deutsch, 1999; Patricelli et al., 1998). Noteworthy, this so-called transmembrane domain, while not necessary for hydrolase activity, seems to be involved in the self-association of FAAH, because a mutant lacking the first 30 amino acids showed a reduced tendency to form oligomers (Arreaza and Deutsch, 1999).

4.2.1 Structural Features

FAAH has been crystallized in complex with an irreversible active site-directed inhibitor, the methoxy arachidonyl fluoro-phosphonate (MAFP), and its three-dimensional structure has been analyzed at a 2.8 Å resolution (Bracey et al., 2002). To obtain the crystalline structure, a catalytically active mutant was generated (ΔTM-FAAH), where the first 29 amino acids were deleted (Patricelli et al., 1998). ΔTM-FAAH is soluble and homogeneous in detergent-containing buffers, opening the avenue to the in vitro mechanistic and structural studies, and is still able to bind membranes (Fig. 4.3). The X-ray structure of this mutant confirmed that FAAH is an integral membrane enzyme with a globular shape: the enzyme crystallized as a homodimer, indicating that it is at least a dimer in solution (McKinney and Cravatt, 2005).

More than 100 members of the AS family of enzymes have been reported in the literature, but only for malonamidase (MAE2) (Shin et al., 2002) and C-terminal peptide amidase (PAM) (Labahn et al., 2002), two soluble bacterial enzymes, structural data are available. All three resolved structures of AS enzymes (FAAH, MAE2, and PAM) revealed a common core, consisting of a twisted β-sheet of 11 mixed strands, surrounded by a large number of α-helices (those of FAAH are shown in Fig. 4.3). Compared to other AS enzymes, which are mostly soluble proteins, FAAH displays two distinguished features: i) integration into membranes, and ii) strong preference for hydrophobic substrates.

Furthermore, three well-defined domains have been identified in FAAH: i) a transmembrane domain at the N-terminus which directs protein oligomerization, ii) a serine- and glycine-rich domain, and iii) a proline-rich domain.

FAAH has several elements of secondary structure: the twisted β-sheet consisting of 11 mixed strands (accounting for ~17% of the whole protein structure) is surrounded by 28 α-helices of various lengths (accounting for ~53% of the whole protein structure). Recently, the stability of ΔTM-FAAH has been studied as a function of chemical (guanidinium hydrochloride) or physical (high hydrostatic pressure) denaturation (Mei et al., 2007). The unfolding transition of the enzyme was observed to be complex and required a fitting procedure based on a three-state process with a monomeric intermediate. The first transition was characterized by dimer dissociation, with a free energy change of ~11 kcal/mol that accounted for ~80% of the total stabilization energy. This process was also paralleled by a large change in the solvent-accessible surface area, because of the hydration occurring both at the dimeric interface and within the monomers. As a consequence, the isolated subunits were found to be much less stable (ΔG ~3 kcal/mol). The addition of MAFP enhanced the stability of the dimer by ~2 kcal/mol, toward denaturant- and pressure-induced unfolding. FAAH inhibition by MAFP also reduced the ability of the protein to bind to the membranes. Taken together, these findings suggest that local conformational changes at the level of the active site might induce a tighter interaction between the subunits of FAAH, thus affecting the enzymatic activity and the interaction with membranes (Mei et al., 2007).

ΔTM-FAAH appears to bind membrane lipids via helices α-18 and α-19 (amino acid 410–438), which form a helix-turn-helix motif. This motif interrupts the AS fold and is comprised mainly of hydrophobic residues (with few basic amino acid) that are likely to constitute a membrane binding surface of FAAH. In addition, a predicted N-terminal transmembrane (TM) domain (amino acids 9–29) forms a membrane binding helix that strengthens the interactions of the α-18 and α-19 helices with membranes (McKinney and Cravatt, 2005). Remarkably, sequence comparisons revealed that this domain is not present in other AS enzymes (Cravatt et al., 1996).

The two monomers of FAAH have a parallel alignment, that allows both subunits to function concomitantly by recruiting substrates from the same membrane. The parallel orientation is required to have the α-18 and α-19 membrane cap on the same face of the dimer, thus enhancing membrane binding (McKinney and Cravatt, 2005). Noteworthy, the intimate relationship between the membrane binding surface and the active site of FAAH resembles the membrane-binding domains of two other integral membrane enzymes, like squalene cyclase (Wendt et al., 1997) and prostaglandin H_2 synthase (Picot et al., 1994). Also these enzymes act on lipid-soluble substrates and have hydrophobic caps surrounding the entrance of the corresponding active sites. These three enzymes share no sequence or fold homology, indicating that they have evolved independently similar strategies for membrane integration (Bracey et al., 2004). However, all three enzymes are dimeric proteins, with

the active site capped by a hydrophobic domain, that is surrounded by basic amino acids in order to interact with negatively charged phospholipids (McKinney and Cravatt, 2005).

It has been suggested that FAAH may have different structural alterations, allowing direct access from the cytosolic and the membrane side to its active site. In fact, X-ray analysis revealed several unusual features of the enzyme: the resolved crystal structure confirms that FAAH has different key regions, including a remarkable collection of channels that form a 'cytosolic port' and a 'membrane port' to facilitate substrate recognition, binding, hydrolysis and product release (thus improving the catalytic turnover). These ports might grant the simultaneous access to both membrane and cytosolic compartments of the cell, useful for substrate entry and/or product exit during the catalytic reaction (Cravatt and Lichtman, 2003).

A potential substrate entryway (which presents anphipathic residues possibly to accommodate polar substrate head groups towards the FAAH active site) has been identified next to α-18 and α-19 helices, and it may indicate direct connection between the FAAH active site and the hydrophobic membrane bilayer. The mode for membrane binding of FAAH may facilitate movement of the FAA substrates directly from the bilayer to the active site, with no need for transport of these lipids through the aqueous cytosol. In this model, the substrate would first enter via the membrane to the active site; following hydrolysis, the released fatty acid (hydrophobic) and amine (hydrophilic) products would then exit through the membrane-access and cytosolic-access channels, respectively. Moreover, the cytoplasmic port may serve the additional function of providing a way for a water molecule required for deacylation of the FAA-FAAH acyl-enzyme intermediate, which has been already characterized by LC-MS (Patricelli and Cravatt., 1999).

4.2.2 Catalytic Mechanism

FAAH presents unique biochemical properties due to an unusual serine-serine-lysine (Ser241-Ser217-Lys142) catalytic triad. In fact, differently from the substrate selectivity displayed by most serine hydrolases, which react with esters at rates several orders of magnitude faster than amides, FAAH reacts with esters and amides at equivalent rates. It has been demonstrated that this unusual property depends on a single lysine residue (Lys142), since its mutation to alanine greatly reduces the amidase activity of FAAH, without affecting the esterase activity (Patricelli and Cravatt., 1999). Many investigations have been focused to clarify the catalytic mechanism of FAAH. A number of mutagenesis, kinetic and chemical labeling studies have revealed that the FAAH nucleophile is Ser241 (Patricelli et al., 1999). Mutagenesis studies also invoked the participation of additional residues in the catalytic mechanism of FAAH: in particular, a serine residue (Ser217) mutated to alanine produced a mutant FAAH with

a significant reduction of hydrolytic activity (~2000 fold), reduction that was much less severe than that observed with mutants lacking either the serine nucleophile (Ser241) or the lysine base/acid (Lys142). Remarkably, the unusual catalytic core of FAAH is highly conserved among the AS family members. Lys142 appears to play a critical role as both base and acid in the hydrolytic cycle. In fact, several lines of experiments show that Lys142 plays a role as a base that activates the Ser241 nucleophile in FAAH, whereas other kinetic data seem to support a role for Lys142 as an acid that participates in the protonation of the substrate leaving group (Patricelli and Cravatt., 1999). The relative importance of acid-catalyzed leaving group protonation for amide hydrolysis compared to ester hydrolysis has been emphasized previously in semi-empirical studies (Fersht, 1971): consistent with these predictions, a tight coupling of base-catalyzed nucleophile activation and acid-catalyzed leaving group protonation might explain the ability of FAAH to normalize the acylation/hydrolysis rates of an amide or an ester substrate (McKinney and Cravatt, 2005). This hypothetical mechanism assumes that Lys142 would be deprotonated in the absence of bound substrate, leading to a constitutively activated nucleophile (Ser241). The structural arrangement analysis of catalytic residues indicates that in FAAH the impact of Lys142 on Ser241 nucleophile strands and the leaving group protonation likely occurs indirectly, via the bridging Ser217 of the triad; the latter may act as a 'proton shuttle'. In this model, FAAH would force protonation of the substrate leaving group early in the transition state of acylation, concomitantly with the nucleophile attack on the substrate carbonyl group (McKinney and Cravatt, 2005). The overall catalytic cycle of FAAH is shown in Fig. 4.4.

It should be pointed out that the comparable hydrolysis rate for amide and ester bonds has a biological meaning: FAAH must bind and hydrolyze its FAA substrates against a background of a large excess of structurally related esters such as monoacylglycerols (Mechoulam et al., 1995). To reach this goal, the

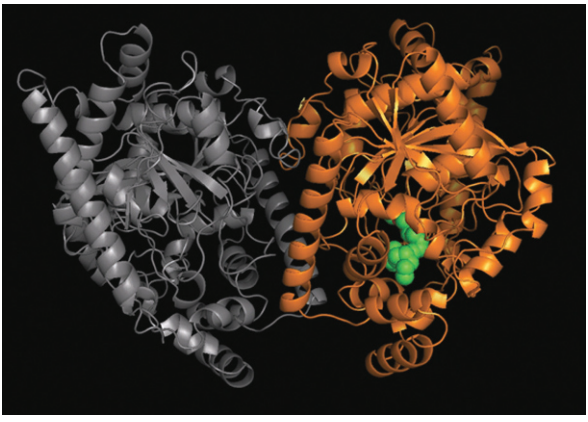


Fig. 4.4 Schematic representation of rat ΔTM-FAAH, complexed with the irreversible inhibitor MAFP (*in green*) (Protein Data Bank file 1MT5)

active site of FAAH has specifically evolved and adapted to hydrolysis of FAA substrates in a cellular environment with high concentration of fatty acid esters. Therefore, the unique biochemical proprieties of FAAH permit to this enzyme to act as a lipid amidase in vivo.

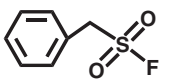
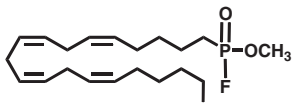
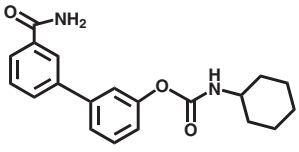
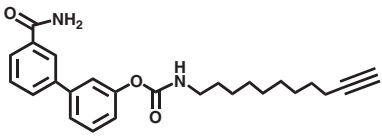
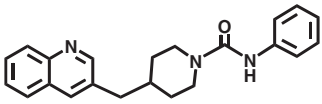
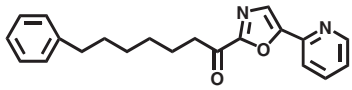
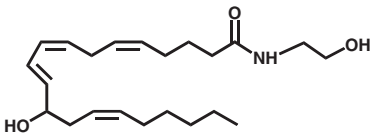
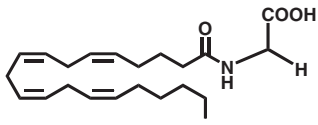
4.2.3 Synthetic and Natural Inhibitors

Growing evidence demonstrates that FAAH is the critical regulator of the endogenous levels of AEA, suggesting that it may serve as an attractive therapeutic target for the treatment of human disorders (Maccarrone, 2006). Unfortunately, AEA is rapidly inactivated by FAAH, which prevents its therapeutic exploitation. Yet, inhibitors of FAAH that block degradation of AEA and related endocannabinoids might be useful to tackle pathologies in which endocannabinoid levels are reduced.

The first non-specific inhibitor reported for FAAH was the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Table 4.1) (Deutsch and Chin, 1993). Since this compound was not selective for FAAH, there was a growing interest to design more potent and selective inhibitors. New compounds were obtained from the derivatization of various fatty acids with functional groups, previously reported to react and form covalent adducts with catalitycally active serine and cysteine residues. This method allowed the discovery of novel FAAH inhibitors, like diazomethylarachidonoyl ketone (Edgemond et al., 1998), stearylsulfonyl fluoride (Deutsch et al., 1997a), methyldodecyl fluorophosphonate (Martin et al., 2000), arachidonylsulfonyl fluoride (Segall et al., 2003), and the most potent methoxy arachidonoyl fluorophosphonate (MAFP) (Table 4.1) (Deutsch et al.,1997b; De Petrocellis et al., 1997). All these compounds are potent irreversible inhibitors of FAAH, however, they also have remarkable affinity for the CB1 receptor.

More recently, a series of irreversible aryl-carbamates inhibitors were described (Mor et al., 2004). URB597 (cyclohexyl carbamic acid 3'-carbamoyl-biphenyl-3-yl ester) (Table 4.1), the most potent member of this family, inhibited FAAH activity with an IC_{50} value of 4.6 nM in rat brain extracts (Mor et al., 2004), and of 0.5 nM in intact neurons (Piomelli et al., 2006), without affecting other serine hydrolases. In addition, introduction of small polar groups in meta-position of the distal phenyl ring, and in para-position of the proximal phenyl ring, were found to improve inhibition (Mor et al., 2004; Tarzia et al., 2006). These carbamates inhibit FAAH activity through irreversible interaction based on nucleophilic attack of Ser241 in the active site. Biochemical evidence (Alexander and Cravatt, 2005) showed that these inhibitors covalently modify the active site by adopting an orientation opposite of that originally predicted from modeling (Mor et al., 2004). Indeed, the *O*-biaryl substituents would reside in the cytoplasmic-access channel (rather than in the acyl-chain-binding channel), where they would be susceptible to enzyme-catalyzed protonation to

Table 4.1 Chemical structures of relevant-FAAH inhibitors

 **PMSF**	 **MAFP**
 **URB597**	 **JP-104**
 **PF-750**	 **OL-135**
 **12-OH-AEA**	 **NAGLy**

enhance their function as leaving groups. Based on these results, a series of carbamates were designed, in which the *N*-cyclohexyl unit was replaced with various *N*-alkyl groups mimicking the acyl chains of anandamide. These compounds, of which JP-104 (Undec-10-ynyl-carbamic acid 3'-carbamoyl-biphenyl-3-yl ester) is a prototype member (Table 4.1), generally exhibited enhanced potency (Alexander and Cravatt, 2005). More recently, Ahn and coworkers have described a new series of 'PF' urea-based inhibitors with piperidine/piperazine groups [see PF-750 (N-phenyl-4-(quinolin-3-ylmethyl)piperidine-1-carboxamide) in Table 4.1]. These compounds have been shown to covalently inactivate FAAH via carbamylation of the serine nucleophile in the active site, and did not show any detectable activity against other serine hydrolases in mammalian proteomes (Ahn et al., 2007).

In general, an irreversible mechanism of inhibition might reduce the versatility of a drug for in vivo applications. Thus, a major challenge for the ongoing pharmaceutical research is the development of potent and selective, but reversible, inhibitors of FAAH. Based on α-ketoheterocycle protease inhibitors (Edwards et al., 1995), potent reversible competitive inhibitors were developed, combining an unsaturated acyl chain and an α-keto-*N*4-oxazolopyridine, with incorporation of a second weakly basic N-atom. This class of compounds

showed potency in the subnanomolar range, with Ki values falling below 200 pM (Boger et al., 2000). The inhibition potency was strongly dependent on the hydrophobicity of the flexible acyl chain, and on the degree of α-substitution (Boger et al., 2001). For example, the compound OL-135 (1-oxo-1[5-(2-pyridyl)-2-yl]-7-phenylheptane) (Table 4.1) displayed an exceptional combination of high potency (Ki = 4.7 nM towards rat-recombinant FAAH) and high selectivity in vivo (Lichtman et al., 2004; Boger et al., 2005).

Several inhibitors have been tested in vivo and their ability to inactivate FAAH was shown to elicit pain and anxiety (Kathuria et al., 2003), without the side effects (hypomotility, hypothermia and catalepsy) that usually accompany activation of CB1 receptors by exogenous cannabinoids like Δ^9-THC (Piomelli et al., 2000; Fowler, 2003; Cravatt and Lichtman, 2003). To maintain this lack of 'cannabinoid side effects', FAAH inhibitors must be devoid of affinity for the cannabinoid receptors. The most studied of the FAAH inhibitors, URB597, does not exhibit affinity for cannabinoid receptors. Thus, at doses that inhibit FAAH and substantially raise brain levels of AEA, but not of 2-AG, this compound did not induce common side effects of typical CB1 agonists, suggesting that they it might be exploited as an innovative anti-anxiety therapeutic (Gaetani et al., 2003).

Also OL-135 (see above) allowed a profound increase of anandamide levels in the brain and spinal cord, and displayed CB1-dependent antinociceptive effects in the hot-plate, tail-immersion, and formalin tests (Lichtman et al., 2004).

Besides the design of synthetic molecules, several papers reported the presence of specific enzymatic reactions able to produce also in the cells compounds able to act as reversible FAAH inhibitors (Maccarrone and Finazzi-Agrò, 2004a). In particular, it has been found that oxidative metabolites of AEA generated by various lipoxygenases, i.e. the hydroxyanandamides (HAEAs; see 12-OH-AEA in Table 4.1), are powerful inhibitors of FAAH (van der Stelt et al., 2002). Instead, derivatives of AEA generated by cyclooxygenase-2, and termed prostamides, have been recently shown to be ineffective on FAAH activity (Matias et al., 2004).

Of interest is the fact that all HAEAs are reversible competitive inhibitors of FAAH (van der Stelt et al., 2002). In addition, the fact that various lipoxygenases (i.e., 5-, 12-, and 15-LOXs) generate different HAEAs with different inhibition profiles towards the proteins of the ECS, suggests that cells with different LOXs might contribute different selectivity to networks regulating endocannabinoids' actions. These compounds may be the 'physiological' inhibitors of FAAH, of potential utility in the control of emotional states and of those disorders whose onset or symptoms are associated with defective production or excessive degradation of AEA and congeners. More in general, it should be pointed out that HAEAs represent one of the newest paradigms of the ability of cells to make their own tools to regulate key targets like FAAH, and they seem to do it more simply than is done in the laboratory.

Moreover, the natural occurrence in mammalian tissues of several arachidonoylated amino acids prompted different research groups to evaluate the effects of these compounds as FAAH inhibitors. It has been shown that these derivatives may act as additional regulatory factors for FAAH. A typical example of these mediators is the *N*-arachidonoyl-glycine (NAGly) shown in Table 4.1 (Huang et al., 2001). NAGly has been shown to exert both analgesic and anti-inflammatory effects, despite its lack of activity at both CB1 and CB2 receptors, through inhibition of FAAH (Huang et al., 2001; Burstein et al., 2002).

4.2.4 Subcellular Localization

FAAH has been found mainly in microsomal and mitochondrial fractions of rat brain and liver (Deutsch and Chin, 1993; Desarnaud et al., 1995), and of porcine brain (Ueda et al., 1995). Recent studies performed with confocal microscopy, showed that FAAH is localized intracellularly as a vesicular-like staining, that has no association with the plasma membranes and is partially co-localized with the endoplasmic reticulum (Fig. 4.5). These morphological data were corroborated by biochemical assays of FAAH activity in subcellular fractions, showing that AEA hydrolysis was primarily confined to the endomembrane compartment (Oddi et al., 2005). Moreover, by means of reconstituted vesicles derived from purified membrane fractions, it was demonstrated that transport activity is retained by plasma membrane vesicles devoid of FAAH, thereby indicating that AEA hydrolase activity is not necessary for AEA membrane transport. Overall, by means of confocal microscopy, subcellular fractionation, and

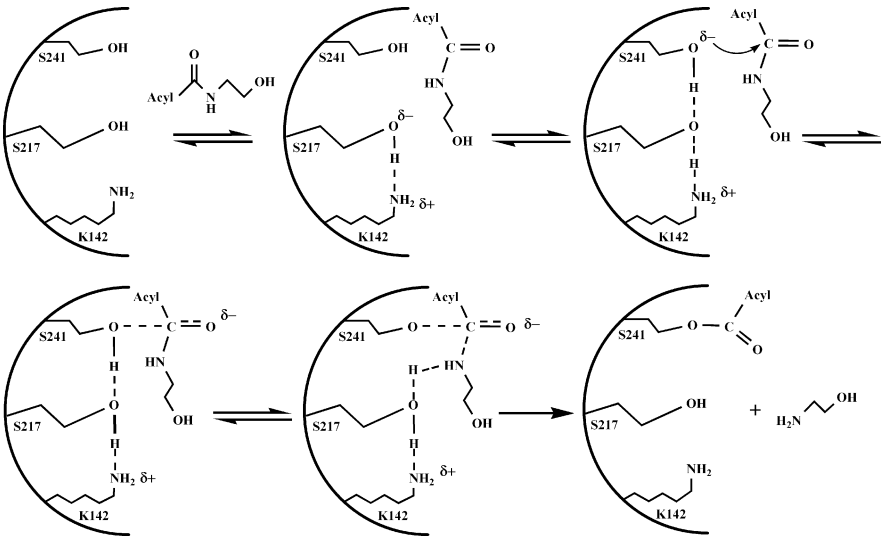


Fig. 4.5 Proposed mechanism for amide hydrolysis by FAAH. See text for details

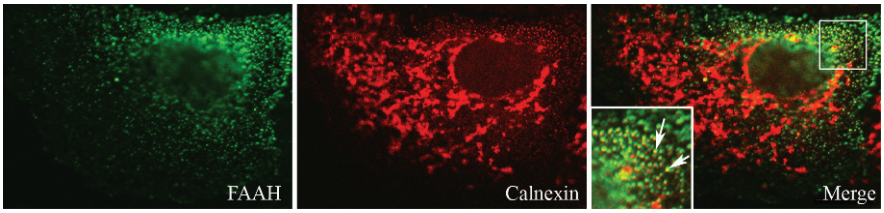


Fig. 4.6 Cellular localization of FAAH in human keratinocytes. Co-localization of FAAH with calnexin (marker to endoplasmic reticulum). Human keratinocytes (HaCaT cells) were co-stained with anti-FAAH (*in green*) and anti-calnexin (*in red*) antibodies. Superimposition of the two stainings (*merge*) revealed a vesicular region of the endoplasmic reticulum where FAAH and calnexin largely overlapped (*yellow*). Dot structures, where FAAH and calnexin co-localized, are indicated by the white arrows in the inset at the bottom of the merge panel. The remaining part of the reticulum, with lamellar appearence, did not display any co-localization of the two proteins. Courtesy of Dr. Sergio Oddi (University of Teramo, Italy)

biochemical analysis it can be demonstrated, at least in some cell types, that transport and hydrolysis of AEA are uncoupled also in cells with a normal genetic background for FAAH. Therefore, it can be concluded that the transport and the hydrolysis steps are two spatially and functionally independent events of the AEA inactivation pathway.

4.2.5 Regulation of Gene Expression

The genomic organization of mouse and human *FAAH* genes was reported in 1998 (Wan et al., 1998). In humans, the *FAAH* gene is localized to chromosome 1p, while in mice it is on chromosome 4 (Wan et al., 1998). The genomic configuration of the human and mouse *FAAH* exons is highly conserved, with 15 exons ranging in size from 40–207 bp. Each splice donor and acceptor sites are conserved and, with the exception of two introns (2 and 7), even the intron size of human and mouse *FAAH* gene is conserved (Wan et al., 1998). With the mouse genomic organization completed, another DNA region amenable to study was the *FAAH* promoter, and in fact a number of investigators turned their attention to understanding how *FAAH* gene expression is regulated.

A number of studies showed that in the periphery, estrogen and progesterone would in part regulate *FAAH* gene expression (Paria et al., 1996; Maccarrone et al., 2000a; Maccarrone et al., 2001). In 2001, a mouse *FAAH* promoter analysis using neuronal and muscle cell lines suggested that a tissue-specific expression of *FAAH* is accomplished via elements within 700 bp of the *FAAH* initiation codon ATG (Puffenbarger et al., 2001). In 2002, further mouse *FAAH* promoter analysis was published and while these first two studies did not mark identical sites for the start site of mouse *FAAH* mRNA (+1 of transcription), neither group identified a TATA box which might explain the variation of transcription initiation sites in brain and liver (Puffenbarger et al., 2001; Waleh et al., 2002). Human *FAAH* promoter studies have been performed in

human T lymphocytes, where leptin and progesterone activation of *FAAH* transcription was shown to occur via STAT3 (signal transduction and activator of transcription-3) and Ikaros transcription factors, respectively (Maccarrone et al., 2003a; Maccarrone et al., 2003b). Neither the human nor mouse *FAAH* promoters seem to have an active TATA box, while both contain several SP1 binding sites (Maccarrone et al., 2003b). The essential elements of human *versus* mouse *FAAH* promoter are schematically represented in Fig. 4.7. Interestingly, later on the human *FAAH* promoter was examined in lymphoma U937 *versus* neuroblastoma CHP100 cells (Maccarrone et al., 2004b). It was found that, while leptin and progesterone strongly enhanced *FAAH* promoter activity in lymphoma cells, neither leptin nor progesterone (alone or in combination) significantly changed *FAAH* expression in neuroblastoma cells, suggesting significant differences in the response of *FAAH* promoter sequences along the neuroimmune axis (Maccarrone et al., 2004b). Further work will be needed to understand the tissue-specific regulation of *FAAH* gene, and to determine which transcription factors drive FAAH expression within the CNS. Nowadays, with *FAAH* promoter sequences outlined and its genomic organization completed, another avenue of research could be to 'knockout' or silence *FAAH* gene expression, by using homologous recombination to target the *FAAH* gene. It seems noteworthy that tissue extracts from *FAAH*(−/−) mice displayed 50–100-fold lower hydrolysis rates towards AEA and other FAAs, indicating that FAAH is indeed the primary enzyme responsible for the hydrolytic degradation of these lipids in vivo. Consistent with this premise, the pharmacological administration of AEA produced greatly exaggerated behavioral effects in *FAAH*(−/−) mice, compared to wild-type littermates, including hypomotility, analgesia, hypothermia, and catalepsy. All of the effects of AEA in *FAAH* (−/−) mice were blocked by a CB1R antagonist, indicating that this substance acts as a selective CB1R ligand in these animal models (Lichtman et al., 2002).

4.2.6 *FAAH-1* versus *FAAH-2*

Recent proteomic data suggest the existence of a second mammalian AS enzyme with FAAH activity, called FAAH-2 (Wei et al., 2006). The FAAH-2

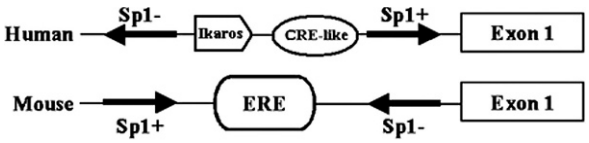


Fig. 4.7 The *FAAH* promoter. Comparison of the upstream transcription factor binding sites of human and mouse FAAH promoters (not drawn to scale). Arrows indicate SP-1 binding sites on the – (←) or + (→) strand. An Ikaros (Ik) binding site, a cyclicAMP responsive-like element (CRE), and an estrogen-responsive element (ERE) are schematically represented in boxes

gene was found in primates and in distantly related vertebrates but not in rodents like mice and rats. This enzyme exhibits an overlapping but distinct tissue distribution, substrate selectivity, and inhibitor sensitivity compared to the original FAAH enzyme (FAAH-1, discussed above). Both FAAH-1 and FAAH-2 share 20% amino acid sequence identity. Similarly to FAAH-1, FAAH-2 possesses an N-terminal transmembrane domain and an AS sequence containing the serine-serine-lysine catalytic triad, along with other amino acid residues required for enzyme activity (Wei et al., 2006). Interestingly, the C-terminal catalytic domains of FAAH-1 and FAAH-2 would be located in the cytoplasmic and luminal compartments of the cell, respectively, suggesting that the opposite relative orientation of these enzymes within the membrane could influence their respective access to specific FAA substrates in the cell, especially if these lipids show preferential localization to the inner or outer leaflet of the membrane bilayer (Wei et al., 2006).

Comparison of the enzymatic properties of FAAH-1 and FAAH-2 revealed that FAAH-1 has much higher hydrolytic activity than FAAH-2, with AEA (C20:4) as substrate. This differential activity contrasted with the similar rates of hydrolysis displayed by the two enzymes with oleamide (C18:10) and linoleamide (C18:1) FAAs. FAAH-2 thus appears to prefer monounsaturated over polyunsaturated acyl chains, while FAAH-1 exhibits the opposite selectivity.

These observations indicate that FAAH-2 may be important for the regulation of monounsaturated lipid amides in the CNS and peripheral tissues; however, further investigation is needed (Wei et al., 2006). To date, in addition to FAAH-1 and FAAH-2, other enzymes have been shown to be involved in the termination of endocannabinoid signaling, like the above-discussed MAGL and the *N*-acylethanolamine acid amidase (NAAA) (Tsuboi et al., 2005). The involvement of these additional hydrolases in regulating endocannabinoid tone needs to be clarified, and raises a question about the biological meaning of such a diversity of metabolic pathways (Vandevoorde et al., 2005; Dinh et al., 2004; Muccioli et al., 2007). Indeed, these proteins are able to hydrolyze, at least in vitro, a wide and overlapping panel of endocannabinoids, but the precise role played by each enzyme in vivo still remains unclear. Unfortunately, the lack of selective inhibitors to be used as pharmacological tools, as well as of knockout mice models, impairs a thorough characterization of the patho-physiological roles of these enzymes.

4.3 Involvement of Faah in Health and Disease

Experimental observations suggest that altered levels of endocannabinoids are associated with several physiopathological conditions, therefore the role of the endocannabinoid system is currently the subject of intense investigation. In particular several studies have provided strong evidence that FAAH, due to its

broad distribution, represents an attractive therapeutic target for the treatment of many diseases in both the CNS and the periphery (Maccarrone, 2006).

In the periphery, endocannabinoid signaling was soon identified as crucial for uterine receptivity for embryo implantation in mouse, with high AEA levels associated to an impairment of the implantation process. This effect was inhibited by the antagonist SR141716, indicating that anandamide is acting through CB1 receptors (Paria et al., 1996). The relevance of the effects of AEA on early pregnancy and on neuroendocrine function underlined the possible leading role played by FAAH in reproduction. In humans, decreased FAAH expression and activity, as well as elevated AEA concentrations in peripheral lymphocytes, are correlated with spontaneous abortion (Maccarrone et al., 2000b). Accordingly, the recent article on *FAAH(−/−)* mice outlined the capital importance of FAAH in the (pre-)implantation process and fertility (Wang et al., 2006). Indeed, an increase in AEA levels in knockout mice resulted in altered oviductal embryo transport and expression of genes required for differentiation and blastocyst implantation, ultimately leading to impairment of fertility. Based on the available data, it can be proposed that drugs that are able to enhance FAAH activity (e.g., by mimicking Ikaros or STAT3) might become useful therapeutic tools to correct defects in human fertility (Maccarrone and Finazzi-Agrò, 2004a).

Besides lymphocytes, other blood cells express an ECS that plays an important role in human pathologies. For instance, in platelets isolated from humans with headache, Cupini et al. (2006) found an increase in the activity of FAAH in two groups of headache subjects: migraine without aura (MoA) or episodic tension-type headache (ETTH) patients. In particular, this FAAH dysfunction was found in female but not male migraineurs. Furthermore, in a recent paper it has been reported a decrease of AEA and 2-AG levels in other two types of headache disorders (Rossi et al., 2007).

The alteration of the endocannabinoid system is implicated also in a number of human behaviors that seems to be, at least in part, determined by genetics. In fact, several articles have shown an association of genetic polymorphisms of FAAH with disease conditions (Norrod and Puffenbarger, 2007). Genetic polymorphisms are variations in DNA sequences from person to person. Polymorphic sequences may be single nucleotide polymorphisms (SNPs) or larger changes, including differences in the number of trinucleotide repeats.

In particular, the first mutations in the human FAAH sequence were discovered in 2002 (Sipe et al., 2002). In this study, it was described a natural SNP in the human gene that encodes for FAAH, that in homozygous form is strongly associated with risk factors for problem alcohol and drug use. This single nucleotide polymorphism results in a missense mutation (385C→385A), that converts a conserved proline residue to threonine (Pro129→Thr), producing a FAAH variant that displays normal catalytic properties but an enhanced sensitivity to proteolytic degradation. In the same line, studies on human T lymphocytes from genotyped blood donors, have revealed that 385A/385A donors who would have only Pro129→Thr type FAAH had less than half the

FAAH activity found in 385C/385C wild-type donors. Very interestingly, in a study of Japanese methamphetamine users, no significant linkage was found between the 385C→A and methamphetamine use (Chiang et al., 2004). Thus, it remains to be seen how the FAAH 385C→A polymorphism might affect the risk of drug and alcohol abuse in other ethnic groups.

The FAAH polymorphism has been associated also with eating disorders in subjects of multiple ethnic backgrounds. In fact, the homozygous FAAH 385A/A genotype was significantly associated with overweight and obesity in white subjects and in black subjects, but not in a small group of Asians. The median Body Mass Index (BMI) for all subjects was significantly greater in the FAAH 385A/A genotype group compared to heterozygote and wild-type groups. In white subjects, there was an increasing frequency of the FAAH 385A/A genotype with increasing BMI categories of overweight and obese, with the same trend in black subjects (Sipe et al., 2005).

In another study a group of 451 obese and dyslipidaemic participants were genotyped, and their biometric and metabolic outcome after a 6 week low fat diet was observed. Carriers of the P129T mutation in FAAH had a significantly great decrease in triglycerides and total cholesterol, as compared to wild-type. These findings remain to be elucidated, however, a hepatic down-regulation of endocannabinoid tone may contribute to the observed outcome in studied subjects (Aberle et al., 2007).

Several lines of evidence suggest that the endocannabinoid system is profoundly involved in neurodegenerative diseases, like Alzheimer's disease (AD), Huntington's disease (HD) and multiple sclerosis (MS). In fact, Benito and co-workers reported that FAAH expression and activity, and CB2 receptor expression are selectively enhanced in glial cells that are linked to the inflammatory process that accompanies AD. This is the first observation in a human tissue that suggests a role for the ECS in the progression of this neurodegenerative disease. FAAH exhibits upregulation in glial cells associated with senile plaques and its expression appears to be restricted to reactive astrocytes. While CB2 receptors are expressed only in activated microglial cells (Benito et al., 2003).

Alterations in FAAH activity seem to be implicated also in HD. Peripheral lymphocytes of HD patients are known to express mutant huntingtin (htt) (Ide et al., 1995), and to replicate some of the transcriptional abnormalities found in HD brain (Borovecki et al., 2005). According to that in a recent paper lymphocytes were used and it has been observed that FAAH activity (but not its expression) was dramatically decreased in HD compared to healthy subjects. In addition, FAAH activity was also decreased to approximately 50% of controls in specimens from HD brains, arguably suggesting that diminished FAAH activity in lymphocytes and brains are correlated (Battista et al., 2007).

A recent study has demonstrated that AEA, but not 2-AG, is increased in the cerebrospinal fluid of MS patients (Centonze et al., 2007a). Remarkably, increased synthesis, reduced degradation and increased levels of AEA were also observed in lymphocytes of MS patients, indicating that an important

source of AEA in the CNS of these subjects may be represented by activated inflammatory cells invading the brain from the periphery. Furthermore, by means of neurophysiological recordings from single neurons, it was confirmed that excitatory transmission is inhibited by CB1 receptor activation in mice with experimental autoimmune encephalomyelitis (EAE), a preclinical model of MS, and that GABA transmission becomes conversely insensitive to CB1 receptor stimulation (Centonze et al., 2007a). Overall, together with previous experimental findings, these results suggest that during immuno-mediated attack of the CNS, the activation of ECS represents a protective mechanism aimed at reducing both neurodegenerative and inflammatory damage through various and partially converging mechanisms that involve neuronal and immune cells (Centonze et al., 2007b).

4.4 Conclusions and Future Perspectives

In this chapter we have outlined the structural properties of FAAH, its catalytic mechanism, its gene expression and its pathophysiological roles, against the wider background of the endocannabinoid system to which FAAH belongs. Since there is a general consensus that endocannabinoid tone and activity are under a 'metabolic control', and that FAAH is the key-player in this process (McKinney and Cravatt, 2005; Maccarrone, 2006), it can be proposed that this enzyme could be viewed as a possible important target for the development of new drugs.

It seems that modulating endocannabinoid metabolism, rather than agonizing or antagonizing cannabinoid and noncannabinoid receptors, might be the way to better understand the pathophysiological implications of these bioactive lipids, and to exploit them for therapeutic purposes. In this context, it can be suggested that not only inhibitors of FAAH, but also drugs able to enhance its activity might become useful therapeutic tools for the treatment of human diseases. If not as therapeutic agents *per se*, FAAH inhibitors or activators could be used together with AEA analogues to lower the doses or to shorten the treatment necessary in vivo to observe an effect, and hence to minimize the possible psychotropic side effects of endocannabinoids when they are used as pharmaceuticals.

On a final note, it seems necessary to remind that: i) several endogenous endocannabinoid(-like) compounds, whose functions are not yet understood, are present in our body, and their biological activity might be affected in unexpected ways by drugs that modulate FAAH or other known proteins of the endocannabinoid system; ii) new metabolic enzymes have been recently identified, that catalyze the hydrolysis and synthesis of AEA or 2-AG, and it remains to be elucidated how these multiple pathways may contribute to the overall tone and biological activity of endocannabinoids.

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