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DOI:

[10.1016/bs.apha.2017.03.006](https://doi.org/10.1016/bs.apha.2017.03.006)

Document Version

Peer reviewed version

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Citation for published version (APA):

Fowler, C. J., Doherty, P., & Alexander, S. P. H. (2017). Endocannabinoid Turnover. *Advances in Pharmacology*, 80. <https://doi.org/10.1016/bs.apha.2017.03.006>

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Endocannabinoid turnover

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Contents

1. Introduction
2. Biosynthesis of AEA and related NAEs
3. DAGL-dependent synthesis of 2-AG
4. 2-AG, AA and AEA levels in DAGL knockout mice
5. Pharmacological inhibition of the DAGLs mimics the knockout results
6. DAGL-dependent eCB signalling regulates synaptic function
7. Release and re-uptake of endocannabinoids
8. Enzymatic hydrolysis of AEA and related NAEs
9. Enzymatic hydrolysis of 2-AG and related monoacylglycerols
10. Pharmacological inhibitors of monoacylglycerol hydrolase activities
11. Oxidative metabolism of endocannabinoids
12. Integrating the catabolism of the endocannabinoids and related NAEs in health and disease
13. Conclusions

Acknowledgements

Conflict of interest

References

1. Introduction

In this report, we assess the principle routes by which the endocannabinoids anandamide and 2-arachidonoylglycerol are produced in biological systems, and their hydrolysis and biotransformation.

Anandamide (AEA, N-arachidonylethanolamine) and 2-arachidonoyl-sn-glycerol (2-AG) are arachidonic acid derivatives which are synthesised and hydrolysed by independent, parallel pathways (Figure 1). However, a feature they share with arachidonic acid is the possibility of biotransformation, via oxidative metabolism (Figure 1, see below). We review the enzymes responsible for endocannabinoid turnover, as well as the potential for therapeutic exploitation.

2. Biosynthesis of AEA and related NAEs

The synthesis of AEA and NAEs has been well reviewed elsewhere (Ueda *et al.*, 2013) and so will be dealt with briefly here. The pioneering work on NAE synthesis was undertaken by Harald and Patricia Schmidt and colleagues who delineated the key steps. The first step is the transacylation of membrane phosphatidylethanolamine-containing phospholipids to form *N*-acyl-phosphatidylethanolamines (NAPEs) by a calcium-dependent *N*-acyl transferase (Natarajan *et al.*, 1982; Natarajan *et al.*, 1983) that has recently been identified as the cytosolic enzyme PLA2G4E (Ogura *et al.*, 2016). Calcium-independent formation of NAPEs from phospholipids are catalysed by a family of phospholipase A/acyltransferase (PLA/AT) enzymes, where PLA/AT 1, 2 and 5 have greater *N*-acyltransferase activity than phospholipase 1/2 activity (Uyama *et al.*, 2012). NAPEs are thereafter metabolised to the corresponding NAEs by a variety of pathways, including hydrolysis catalysed by NAPE-hydrolysing phospholipase D (Schmid *et al.*, 1983); production and subsequent dephosphorylation of NAE phosphates (Liu *et al.*, 2006; Okamoto *et al.*, 2004a), and production and hydrolysis of lyso-NAPE (Sun *et al.*, 2004). Finally, NAEs can be produced from *N*-acylethanolamine plasmalogen (by both NAPE-PLD dependent and independent pathways (Rahman *et al.*, 2016; Tsuboi *et al.*, 2011). Consistent with this multiplicity of pathways, genetic deletion of NAPE-PLD reduces, but does not remove completely, NAE levels in the brain (Leishman *et al.*, 2016; Leung *et al.*, 2006;

Tsuboi *et al.*, 2011). The situation is further complicated by the finding that NAPE-PLD can be allosterically modulated by bile acids (Margheritis *et al.*, 2016; Schmid *et al.*, 1983).

Given the central position of NAPE as a precursor to NAE, it would be expected that the levels of the NAE species match those of NAPE and of the membrane phospholipids. In the brain, this is the case. As an example, data from Hansen and colleagues (Hansen *et al.*, 2001a; Hansen *et al.*, 2001b) investigating levels of these lipids in young rat cerebral cortex are shown in Figure 2. There is a very good correlation between the levels of the NAPE and corresponding NAE species, with a large increase in both following intrastriatal injection of the glutamatergic excitotoxin *N*-methyl-D-aspartate (NMDA). In all cases, the NAE levels are ~10% of the corresponding NAPE levels. However, there are no rules without exception (a rule in itself!) and the exception in this case is the uterus, where endocannabinoids play a key role in reproductive behaviour (review, see (Paria *et al.*, 2002)). In this case, the NAEs other than AEA are again roughly 10% of the corresponding NAPE levels, whereas AEA levels are much higher than expected (Figure 3).

A second observation relates to the calcium sensitivity of PLA2G4E (Ogura *et al.*, 2016), and of NAPE-PLD (Okamoto *et al.*, 2004b). Such calcium sensitivity presents an obvious regulation point, whereby extracellular events leading to an increased intracellular calcium will stimulate NAE synthesis. Indeed, NAPE synthesis in cultured cortical neurons is increased following treatment with the calcium ionophore ionomycin in a manner potentiated by treatments increasing cyclic AMP levels in the cells (forskolin, vasoactive intestinal peptide) (Cadas *et al.*, 1996). In vivo, toxic events leading to calcium influx, such as NMDA treatment (Hansen *et al.*, 2001a; Hansen *et al.*, 2001b) [see Figure 2], traumatic injury (Garcia-Ovejero *et al.*, 2009) and oxygen deprivation (Amantea *et al.*, 2007; Franklin *et al.*, 2003; Schmid *et al.*, 1995) lead to increased NAPE-PLD activity and NAE levels. However, in murine peritoneal macrophages, treatment with the calcium ionophore A23187 does not increase the levels of either NAEs or NAPEs (Kuwaie *et al.*, 1999). In macrophage cell lines, AEA (as well as *N*-acyltransferase and NAPE-PLD activities) but not 2-AG levels can be increased by lipopolysaccharide

treatment, whereas the reverse is true when platelet-activating factor is used as a stimulus (Berdyshev *et al.*, 2001; Liu *et al.*, 2003). This is compounded by different expression levels of the AEA and 2-AG synthetic enzymes in different cells. A nice example of this comes from the work of Freund, Katona and colleagues who demonstrated that NAPE-PLD is located pre-synaptically in hippocampal glutamatergic axon terminals (Nyilas *et al.*, 2008)), in contrast to the synthetic machinery for 2-AG. Clearly, AEA/NAE and 2-AG syntheses are regulated differentially in a cell- and stimulus-dependent manner.

3. DAGL-dependent synthesis of 2-AG

2-AG was the second putative endocannabinoid to be identified a few years after AEA, acting as a full agonist at CB₁ and CB₂ receptors (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995). Interestingly, prior to this 2-AG was largely studied as an intermediate in a two-step pathway that releases arachidonic acid (AA) from diacylglycerol (DAG) in platelets and other cells. For example, thrombin treatment of human platelets was postulated to stimulate the generation of diacylglycerol (DAG) by a phospholipase C (PLC), followed by the sequential hydrolysis of DAG by a diacylglycerol lipase (DAGL) acting at the sn-1 position to generate 2-AG, and a monoacylglycerol lipase (MAGL) acting at the sn-2 position to liberate AA (Bell *et al.*, 1979; Prescott and Majerus, 1983). Subsequently other groups went on to confirm the release of AA through a DAGL/MAGL pathway in platelet-derived growth factor-stimulated Swiss 3T3 cells (Hasegawa-Sasaki, 1985) and in dorsal root ganglion neurons (Allen *et al.*, 1992). Remarkably, the above pathway for AA synthesis is conspicuous by its absence from some sections of the mainstream literature based on the continuing dogma that AA levels are largely governed by phospholipase A₂ (PLA₂) activity (Bazinet and Laye, 2014). We will discuss recent experiments that have provided unequivocal evidence for DAGL regulating the level of AA in the nervous system and some other tissues, and confirm the importance of DAGL activity for 2-AG biosynthesis and eCB signalling throughout the nervous system.

The first reported biochemical purification of a DAGL was in 1984 (Farooqui *et al.*, 1984); the authors identified a ~27 kDa enzyme that was not directly activated by calcium, but subsequently reported to be phosphorylated and activated by cAMP-dependent protein kinase (Rosenberger *et al.*, 2007). However, it was a bioinformatics strategy that resulted in the cloning and characterisation of the first specific DAGLs (Bisogno *et al.*, 2003). A single enzyme is present in lower species such as *Drosophila* and nematodes, but a gene duplication event has resulted in the presence of two very closely related enzymes, namely DAGL α and DAGL β , in vertebrates and other species. These enzymes are not obviously related to the above 27 kDa DAGL, as both have an N-terminal four transmembrane domains followed an intracellular canonical α/β hydrolase domain resulting in a molecular mass of 70-125 kDa. The presence of a C-terminal tail (~300 amino acids) in DAGL α is the main distinguishing feature between itself and DAGL β . The transmembrane domain and the C-terminal tail are not required for enzyme activity (Singh *et al.*, 2016) and most likely regulate trafficking and compartmentalisation of the enzymes within polarised cells (Reisenberg *et al.*, 2012).

Functional evidence suggests differential expression of the two enzymes in the CNS, with DAGL α being present in isolated neurones and DAGL β expressed in microglia (Viader *et al.*, 2016). The catalytic activity of both enzymes has been characterised in detail; they are *sn*-1 specific DAGLs that can make and release 2-AG from cells in response to calcium stimulation (Bisogno *et al.*, 2003). Interestingly, although the enzymes selectively act at the *sn*-1 position, they show little preference for substrates with differing lipid groups at the *sn*-2 position which can include arachidonic, oleic, linoleic and stearic acid suggesting that they can synthesise a range of monoacylglycerols. This makes sense of their expression in species such as *Drosophila* that do not synthesise AA; here the enzymes are more likely to synthesise lipids such as 2-linoleoyl glycerol (Tortoriello *et al.*, 2013).

4. 2-AG, AA and AEA levels in DAGL knockout mice.

The contribution that the cloned DAGLs make to the biosynthesis of 2-AG, and the requirement of this for eCB signalling, has been directly addressed by the generation of independent lines of

knockout mice. Given the importance of eCB function for behaviour, we largely focus our attention on eCB levels in the adult brain and on the requirement of the cloned DAGLs for synaptic function. Other DAGL-dependent functions, including roles in axonal growth and guidance in the developing brain and the regulation of neurogenesis in the postnatal brain have recently been reviewed elsewhere (Maccarrone *et al.*, 2014; Oudin *et al.*, 2011).

Expression will dictate function and in the first cloning paper DAGL α transcripts were reported to be enriched in the nervous system with protein expression seen at relatively high levels in neuronal dendrites that appose CB₁ positive synaptic terminals (Bisogno *et al.*, 2003). However, DAGL α and DAGL β are present in neural stem cells in the adult brain (Goncalves *et al.*, 2008) and more recent RNA-sequencing experiments on isolated cell types from the mouse cerebral cortex clearly show DAGL α transcripts to be present in astrocytes, neurons, oligodendrocyte precursor cells and microglia (Zhang *et al.*, 2014). In contrast, the same database shows that DAGL β transcripts are found almost exclusively in microglia. Thus DAGL α is well placed to regulate the 2-AG levels in several cell types in the brain, with DAGL β perhaps contributing alongside DAGL α in a more restricted manner within neural stem cells and microglia, the "resident macrophages" within the brain.

At the level of the whole brain and spinal cord 75-80% reductions in 2-AG levels have been observed in DAGL α knockout mice, with ~50% reductions seen in adipose tissue and liver (Gao *et al.*, 2010). Similar reductions of ~80% (or more) have been reported in cerebellum, hippocampus, striatum, whole cortex, prefrontal cortex and amygdala in three additional DAGL α knockout lines (Jenniches *et al.*, 2016; Shonesy *et al.*, 2014; Tanimura *et al.*, 2010), with a complete loss of stimulus generated 2-AG (Tanimura *et al.*, 2010) and no difference between the sexes (Shonesy *et al.*, 2014). The one anomaly appears to be the forebrain where significant, but less dramatic decreases have been reported (Shonesy *et al.*, 2014). Overall, these data indicate that DAGL α is responsible for

maintaining nearly all of the basal 2-AG in the nervous system, as well as being required for stimulus induced increases.

Nonetheless, residual 2-AG is found in the nervous system of DAGL α knockout mice implicating DAGL β and/or other enzymes in the biosynthesis of this lipid. 2-AG levels were variable but not significantly different in the cerebellum, hippocampus and striatum of one line of DAGL β knockout mice (Tanimura *et al.*, 2010). However, a 50% decrease in 2-AG at the level of the whole brain has been reported in an independent DAGL β knockout line (Gao *et al.*, 2010). Thus, it would appear that DAGL α is primarily responsible for the biosynthesis of 2-AG in the brain, but DAGL β can make a contribution, possibly within a restricted set of cell types (see above for details).

As discussed above, DAGL activity was first studied in the context of a DAGL/MAGL pathway that generates AA in cells yet much of the mainstream literature remains focussed on the notion that the bulk and signalling pools of AA are governed by the activities of the PLA₂ family of enzymes (Bazinet and Laye, 2014). In this context, it was a major surprise to see parallel reductions in AA and 2-AG levels in the brains of DAGL α knockout mice. For example, in the brain and spinal cord there are 75-80% reductions in AA in DAGL α knockout mice (Gao *et al.*, 2010). Similar dramatic reductions in AA are seen in the forebrain, prefrontal cortex, amygdala and striatum in independent lines of DAGL α knockout animals (Jenniches *et al.*, 2016; Shonesy *et al.*, 2014; Tanimura *et al.*, 2010). It follows that the DAGL α /MAGL pathway is responsible for the biosynthesis of most of the 2-AG and AA throughout the brain. Importantly, this pathway also appears to provide the signalling pool of AA that is mobilised to generate eicosanoids in the brain, with up to 80% reductions in the inflammatory prostaglandins PGD₂ and PGE₂ seen in DAGL α knockout mice (Ogasawara *et al.*, 2016). Inhibition of the eicosanoid pathway at the level of MAGL dramatically reduces much of the neurodegeneration seen in animal models of Alzheimer's and Parkinson's disease (Nomura *et al.*, 2011; Piro *et al.*, 2012), with inhibition at the level of DAGL impairing lipopolysaccharide-induced anapyrexia (Ogasawara *et al.*, 2016). Thus it is clear that the DAGL/MAGL pathway can generate prostaglandins that promote

neuroinflammation in the brain. Interestingly, in the periphery evidence from knockout and pharmacological studies also show that DAGL β regulates proinflammatory responses by regulating 2-AG, AA and eicosanoid levels in a manner that is distinct but complementary with cytosolic PLA $_2$ (Hsu *et al.*, 2012).

A perhaps even more surprising observation made in the DAGL α knockout mice studies was a reduction in the level of AEA in the brain. In this context, there is a significant 40-50% reduction of AEA in the whole brain of DAGL α knockout mice (Gao *et al.*, 2010; Ogasawara *et al.*, 2016). The reason for this unexpected cross-talk between the biosynthesis of the two major eCBs does not appear to involve an obviously straightforward biosynthetic pathway (Di Marzo, 2011), but it is clearly a robust finding and on that basis it can be concluded that DAGL α is the key enzyme in a simple pathway that generates the bulk of the 2-AG and AA found in the brain, but it can also contribute in a more complex manner to the steady-state levels of AEA levels in some brain regions. We do not as yet know the consequence of knocking out DAGL α and DAGL β on 2-AG levels in the brain, and it remains possible that a small pool of 2-AG (perhaps 10-20% of the total) might be maintained by a different biosynthetic pathway. In this context, an enzyme previously characterised as a phospholipase A $_1$ (and termed DDHD2) has recently been purified from the brain and cloned based on exhibiting DAGL activity with evidence obtained that it can contribute to bulk 2-AG levels following overexpression in CHO cells (Araki *et al.*, 2016). The importance of this enzyme in regulating the bulk levels or stimulus induced signalling pools of 2-AG in cells and tissues remains to be determined.

5. Pharmacological inhibition of the DAGLs mimics the knockout results

Genetic approaches to study enzyme function need to be interpreted with some caution given that the relatively long-term loss of function in one pathway can lead to alterations in other pathways with these secondary changes influencing the measured endpoints. On the other hand, whereas pharmacological approaches have the advantage of temporal resolution, they can be limited by drug

availability and selectivity; [for review, see Janssen and van der Stelt \(2016\)](#). In the case of the DAGLs, the historical pharmacological studies have largely relied on the use of RHC80267 and/or tetrahydrolipstatin (THL), but results obtained with them have sometimes been contentious as although they can inhibit DAGL activity they also inhibit other serine lipases and can show poor tissue penetration (Hoover *et al.*, 2008; Janssen *et al.*, 2015). A set of centrally active DAGL inhibitors has recently been developed that, when used with appropriate controls, has allowed for the analysis of the effects of acute inhibition of DAGL activity on lipid networks in the brain (Ogasawara *et al.*, 2016). These [1,2,3-triazole urea-based](#) drugs are highly selective across the discernible family of [\$\alpha\$ / \$\beta\$ -domain](#) serine hydrolases, but show limited selectivity between DAGL α and DAGL β . Within 2 hr administration to animals, these novel inhibitors (DH376 and DO34) fully mimic the reductions in 2-AG (>80%), AA (>80%) and AEA (~50%) measured in parallel studies on DAGL α knockout mice. Concomitant increases in the level of the 2-AG precursor 1-stearoyl-2-arachidonyl-*sn*-glycerol were also seen in the drug treated and knockout animals. Importantly, the levels of the prostaglandins PGD₂ and PGE₂ were also rapidly reduced by ~80% following pharmacological block of DAGL activity, as were the LPS induced increases in these prostaglandins and inflammatory cytokines demonstrating the importance of the DAGL α /MAGL pathway for the rapid generation of inflammatory mediators in the brain. [LEI105 represents a distinct class of reversible DAGL-selective inhibitors, based on \$\alpha\$ -ketoheterocycles](#) (Baggelaar *et al.*, 2015). [The use of this compound in electrophysiological experiments reinforced the role of DAGL in short-term plasticity of synaptic activity in the hippocampus.](#)

6. DAGL-dependent eCB signalling regulates synaptic function.

The above genetic and pharmacological studies have established that DAGL α plays a central role in regulating 2-AG, AA and prostaglandin synthesis in the brain and other tissues. However, they do not directly address the role of DAGL α or DAGL β in eCB signalling pathways. 2-AG has a short half-life (Jarai *et al.*, 2000) and a relatively low affinity for the CB₁/CB₂ receptors (Sugiura and Waku, 2000),

thus it is reasonable to assume that “on-demand” physiological eCB signalling via the DAGLs will require their close proximity to the cannabinoid receptors.

It has long been recognised that AEA and/or 2-AG serve as retrograde synaptic messengers throughout the nervous system (Alger, 2012). DAGL α is well placed to be the key enzyme in this pathway as its expression in neurons is restricted to dendrites in the adult brain (Bisogno *et al.*, 2003) with high resolution imaging studies showing enrichment in all postsynaptic sites that juxtapose CB₁-positive synaptic terminals (Katona *et al.*, 2006; Uchigashima *et al.*, 2007; Yoshida *et al.*, 2006; Yoshida *et al.*, 2011). Live cell imaging has recently shown DAGL α to be localised to an endosomal compartment that can shuttle the enzyme to and from the surface of the dendritic spine in dynamic manner (Zhou *et al.*, 2016). It follows that 2-AG might be made within endosomes possibly providing a regulated vesicular release mechanism to be considered alongside the current model that posits passive diffusion from the cell membrane. 2-AG mobilisation at synapses is established to be triggered by excitatory neurotransmitters (glutamate, acetylcholine, dopamine) acting on their respective postsynaptic receptors and involves a G-protein and/or calcium stimulated synthesis of substrate by a PLC β , however there also appears to be an additional calcium-dependent step operating independently of substrate synthesis (Ohno-Shosaku and Kano, 2014). One possibility is that calcium (and other signals) might stimulate phosphorylation and opening of a regulatory lid on DAGL α to facilitate substrate access to the catalytic site (Reisenberg *et al.*, 2012).

Depolarisation induced suppression of inhibition or excitation (DSI/DSE) is perhaps the best characterised eCB function in the adult brain (Ohno-Shosaku and Kano, 2014). Numerous studies on DAGL α knockout mice have identified it as the pivotal enzyme controlling this phasic form of synaptic plasticity throughout the nervous system (Gao *et al.*, 2010; Tanimura *et al.*, 2010; Yoshino *et al.*, 2011). In contrast there is little if any effect of knocking out DAGL β on this almost ubiquitous form of synaptic plasticity (Gao *et al.*, 2010; Tanimura *et al.*, 2010). It is now clear that DAGL α generates the signalling pool of 2-AG that acts on presynaptic CB₁ receptors to dampen the release

of excitatory and inhibitory neurotransmitters to regulate phasic and tonic forms of synaptic plasticity (Lee *et al.*, 2015; Ramikie *et al.*, 2014; Younts and Castillo, 2014). This in turn impacts on a myriad of fundamental behaviours including appetite, pain and cognition (Alger and Kim, 2011). Indeed, it is noteworthy that DAGL α and CB₁ receptor KO mice share the same disrupted phenotypes (Powell *et al.*, 2015) implicating 2-AG as the "workhorse" eCB in the nervous system. The adverse psychiatric consequences of inhibiting CB₁ function in humans revealed in clinical trials for obesity with a CB₁ antagonist (Moreira and Crippa, 2009) are also likely to be consequential to the disruption of 2-AG signalling at synapses as DAGL α KO mice have been used to demonstrate a causative link between synaptic eCB signalling and anxiety and depressive behaviours (Jenniches *et al.*, 2016; Shonesy *et al.*, 2014). In addition, dysfunction of tonic 2-AG signalling appears to play a key and possible reversible role in autism (Anderson *et al.*, 2015; Busquets-Garcia *et al.*, 2013; Foldy *et al.*, 2013; Jung *et al.*, 2012; Kerr *et al.*, 2013). Importantly, pharmacological modulation of 2-AG signalling is being investigated as a treatment for major disorders including depression, pain, obesity and neurodegeneration (Kohnz and Nomura, 2014).

7. Release and reuptake of endocannabinoids

The mechanism(s) by which endocannabinoids are released from cells and subsequently cleared from the extracellular space have been a matter of controversy, the key question being whether or not there exists an as yet unidentified plasma membrane transporter for these lipids (for reviews of the data from proponents and opponents, respectively, of the concept of a plasma endocannabinoid membrane transporter, see (Fowler, 2013; Nicolussi and Gertsch, 2015). It is possible that certain cell types may utilise proteins like pannexin-1 for the release of endocannabinoids (Hill *et al.*, 2015), but more data are needed. What is clear is that endocannabinoids (and NAEs) can be cleared from the extracellular space, and that there are intracellular proteins with other primary functions that can shuttle these lipids, once they have crossed the plasma membrane, to their intracellular

locations, be it the TRPV1 receptor (for AEA), PPAR α (for palmitoylethanolamide, PEA), or their catabolic enzymes (Kaczocha *et al.*, 2009; Oddi *et al.*, 2009).

The best characterised of these shuttling proteins with respect to endocannabinoids are FABP 5 and 7. Crystallographic studies have indicated that AEA binds, albeit with a lower affinity than arachidonic acid, to FABP5 with its lipophilic chain forming a loop in the substrate-binding pocket of this protein and with hydrogen bonding between the hydroxyl group of the ethanolamine to a Tyr¹³¹ residue. The interaction of FABP5 with 2-AG is largely similar, albeit with looser hydrophobic contacts and more hydrogen bonding (Sanson *et al.*, 2014). Pharmacological inhibition of FABP5/7 reduces uptake of AEA into wild-type HeLa cells and by other FABP5-expressing cell types, but does not inhibit AEA uptake by FABP5 shRNA-expressing HeLa cells (Berger *et al.*, 2012; Bjorklund *et al.*, 2014). Mice lacking FABP5 and 7 show increased brain levels of AEA, PEA and oleoylethanolamide (OEA), but not 2-AG (Kaczocha *et al.*, 2015). A fatty acid amide hydrolase-like transporter protein has also been proposed as an intracellular carrier protein, but its existence has been disputed (Fu *et al.*, 2012; Leung *et al.*, 2013).

8. Enzymatic hydrolysis of AEA and related NAEs

The enzymes responsible for the hydrolysis of AEA and related NAEs to their respective long-chain fatty acids are fatty acid amide hydrolase (FAAH) and *N*-acylethanolamine-hydrolysing acid amidase (NAAA). FAAH is a membrane-bound homodimer belonging to the serine hydrolase family of enzymes and with a wide substrate-selectivity including *N*-acylethanolamines, *N*-acylamides and *N*-acyltaurines (Bachur and Udenfriend, 1966; Boger *et al.*, 2000; Deutsch and Chin, 1993; McKinney and Cravatt, 2006; Schmid *et al.*, 1995). Molecular dynamics and crystallographic studies have indicated that the substrate enters the active site via a membrane access channel, where a triad of residues (Ser²⁴¹, Ser²¹⁷ and Lys¹⁴²) are necessary for hydrolysis to occur, and where additional residues (Phe⁴³² and Trp⁵³¹) may act as a “dynamic paddle” to ensure correct substrate orientation and localisation (Bracey *et al.*, 2002; Mileni *et al.*, 2008; Palermo *et al.*, 2015). In the hippocampus,

FAAH has a post-synaptic location, consistent with an orthograde release of AEA synthesized presynaptically by NAPE-PLD and in contrast to the retrograde release of 2-AG (Gulyas *et al.*, 2004; Nyilas *et al.*, 2008).

In mammals, but not rodents, a second FAAH (FAAH-2) has been found, with a preferential localisation within lipid rafts rather than the endoplasmic reticulum (the location of FAAH) (Kaczocha *et al.*, 2010; Wei *et al.*, 2006). The two FAAH species have different relative hydrolysis rates for substrates. Thus at pH 9 (in the region of the pH optimum for both FAAH enzymes), the rate of hydrolysis of 100 μ M AEA, OEA, PEA, and *N*-oleoyl taurine, respectively, relative to that of oleamide was 1.75, 0.58, 0.22 and 0.77 for human FAAH transfected into COS-7 cells, and 0.055, 0.23, 0.024 and not detected for *N*-terminal FLAG-tagged FAAH-2 transfected into these cells (Wei *et al.*, 2006). The sensitivity of the two enzymes to inhibition is also different, with FAAH-2 being more sensitive than FAAH(-1) to inhibition by the carbamate inhibitor URB597 and the α -ketoheterocycle OL-135, whilst the reverse is true for the piperazinyl phenyl urea compound JNJ-1661010 and the isoflavone biochanin A (Karbarz *et al.*, 2009; Thors *et al.*, 2010b; Wei *et al.*, 2006) (Table 1).

In addition to FAAH, NAEs are hydrolysed by NAAA, an enzyme with a pH optimum around pH 5, and with a preference for the unsaturated substrates over the saturated substrates (PEA > myristoylethanolamide > stearoylethanolamide (SEA) $\simeq$ OEA > linoleoylethanolamide > AEA (Ueda *et al.*, 2001). The enzyme has its highest distribution in the lung where it is localised to the lysosomes of macrophages (Tsuboi *et al.*, 2007; Ueda *et al.*, 2001). NAAA is a glycoprotein with sequence homology to acid ceramidase rather than FAAH (Tsuboi *et al.*, 2005).

9. Enzymatic hydrolysis of 2-AG and related monoacylglycerols

Multiple enzyme activities have been identified to be capable of hydrolysing 2-AG in vitro, including monoacylglycerol lipase (MAGL) (Dinh *et al.*, 2002a), ABHD2 (Miller *et al.*, 2016), ABHD6 (Blankman *et al.*, 2007; Navia-Paldanius *et al.*, 2012), ABHD12 (Navia-Paldanius *et al.*, 2012), neuropathy target

esterase (van Tienhoven *et al.*, 2002) and carboxylesterase 1 (Xie *et al.*, 2010). However, it's clear that MAGL performs the bulk of 2-AG hydrolysis in most tissues given the impact of gene disruption on 2-AG levels (Pan *et al.*, 2011; Zhong *et al.*, 2011). In addition, a functional, activity-based protein profiling approach using mouse brain membranes indicated that MAGL accounted for the majority of 2AG hydrolysis (~85 %), while ABHD6 and ABHD12 accounted for the remainder (Blankman *et al.*, 2007). Despite this predominance of MAGL, it's quite likely that some of these enzymes have influential roles on 2-AG turnover, albeit in niche locations, both at tissue and cellular levels.

Aside from a common substrate, many of these enzymes have a common structure in that they are alpha/beta hydrolases. These are a large family of enzymes characterised by a primary sequence motif known as an α/β hydrolase fold domain – an extended organisation of α -helices and β -sheets – where the most recognisable family member is acetylcholinesterase (Thomas *et al.*, 2014). This family also includes the diacylglycerol lipases (DAGL- α and β), epoxide hydrolases, and lipoprotein and endothelial lipases.

MAGL: MAGL is a 303-aa protein (~33 kDa) serine hydrolase, with a characteristic triad of a charge relay of Asp²³⁹-His²⁶⁹ around the catalytic nucleophile Ser¹²². There are no identifiable transmembrane (TM) domains, but the enzyme is found associated with both cytosolic and particulate compartments (Ghafouri *et al.*, 2004). In adipose tissue, MAGL performs the final step in lipolysis, hydrolysing monoacylglycerols to produce free fatty acid and glycerol; this role was identified over 30 years before a role in endocannabinoid turnover was suggested (Vaughan *et al.*, 1964). In some tissues, such as the brain, it appears that MAGL-evoked arachidonic acid is the predominant route for prostaglandin production (Nomura *et al.*, 2011) and above). MAGL shows hydrolytic preference for 2-AG, but not AEA (Dinh *et al.*, 2002a; Dinh *et al.*, 2002b), but with little specificity between acylglycerols (Ghafouri *et al.*, 2004; Vandevoorde *et al.*, 2005).

ABHD2: The *ABHD2* gene encodes a 425-aa protein (~48 kDa) serine hydrolase (Ser²⁰⁷-Asp³⁴⁵-His³⁷⁶).

A single TM is predicted at 10-30, with an extracellular catalytic domain. Glycosylation is predicted at

Asn¹³⁶ and Asn⁴¹⁰, while a single nucleotide polymorphism has been described (Arg²⁵³Gln). ABHD2 was initially cloned as one of three α/β -hydrolases from mouse lung (Edgar and Polak, 2002). The mRNA was expressed in mouse brain, heart, kidney, liver, lungs, skeletal muscle and spleen, but was highest in testes (Edgar and Polak, 2002). Gene trap analysis in mice suggested an expression in smooth muscle (vascular and non-vascular), but not skeletal muscle (Miyata *et al.*, 2005). The mice lacking *abhd2* appeared to have normal vasculature, but explants were more proliferative and cuff placement resulted in greater intimal hyperplasia. A variety of links with potential therapeutic indications have been reported. For example, in unstable angina (Miyata *et al.*, 2008), hepatitis B virus propagation (Ding *et al.*, 2008), age-related pulmonary emphysema (Jin *et al.*, 2009), ovarian (Yamanoi *et al.*, 2016) and prostate cancer (Obinata *et al.*, 2016). ABHD2 is reported to have progesterone-dependent monoacylglycerol lipase activity and to be inhibited by MAFP (Miller *et al.*, 2016). In addition, ABHD2 appears to be able to hydrolyse triacylglycerols (Naresh Kumar *et al.*, 2016).

ABHD6: ABHD6 is a 337-aa protein (~38 kDa) serine hydrolase (Ser¹⁴⁸-Asp²⁷⁸-His³⁰⁶). A single TM is predicted at 9-29, with an intracellular catalytic domain. ABHD6 is a monoacylglycerol hydrolase (Navia-Paldanius *et al.*, 2012), with little selectivity for 1- compared to 2-acylglycerols (Thomas *et al.*, 2013), and possibly bis(monoacylglycerol)phosphate hydrolase (Pribasnig *et al.*, 2015). Antisense knockdown of ABHD6 led to accumulation of numerous phospholipids and lysophospholipids in mouse liver implying a role in glycerophospholipid turnover in the liver (Thomas *et al.*, 2013). ABHD6 has been implicated in diabetes/metabolic disorder (Zhao *et al.*, 2016; Zhao *et al.*, 2015), inflammation (Alhouayek *et al.*, 2013) and epilepsy (Naydenov *et al.*, 2014).

ABHD12: ABHD12 is a 398-aa protein (~45 kDa) serine hydrolase (Ser²⁴⁶-Asp³³³-His³⁷²). A single TM is predicted at 75-95, with an extracellular catalytic domain. ABHD12 is a monoacylglycerol hydrolase (Navia-Paldanius *et al.*, 2012), but may also regulate lysophosphatidylserine levels (Kamat *et al.*,

2015). Loss-of-function mutations in ABHD12 are associated with a disorder known as PHARC (polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataracts) (Fiskerstrand *et al.*, 2010).

10 Pharmacological inhibitors of monoacylglycerol hydrolase activities

There is a tantalising indication for potential therapeutic exploitation of these enzymes, but the realisation of this potential is absolutely dependent on the provision of new information on the distribution of these enzymes and the identification of their ‘natural’ substrates. In addition, generation of cheap, reproducible high-throughput screening assays will facilitate novel drug discovery to allow future pharmacological investigation, thereby enhancing an understanding of the role/s of these enzymes in pathological circumstances, leading ultimately to their therapeutic exploitation.

MAGL inhibitors: For MAGL activity, a number of useful tools are commercially available. JZL184 is a relatively selective (certainly compared to FAAH) irreversible MAGL inhibitor with a pIC₅₀ value of 8.1-8.4 (Chang *et al.*, 2012; Long *et al.*, 2009) in human and mouse. JZL184 is slightly less potent against rat MAGL, with a pIC₅₀ value of 6.5-7.1 (Woodhams *et al.*, 2012). In a separate study, a similar discrepancy between mouse and rat potency of JZL184 was also noted, although with slightly lower potencies (Aaltonen *et al.*, 2013). It’s likely that these lab-to-lab differences reflect the irreversible nature of the inhibitor, where conditions such as substrate concentration, pH, protein content and (probably most influential) pre-incubation period are major sources of variation.

JJKB048 is even more potent and selective as a MAGL inhibitor, with a sub-nanomolar potency (pIC₅₀ value of 9.4-9.7), with essentially equivalent potency at mouse, rat or human enzymes (Aaltonen *et al.*, 2013). Although substantially less potent (pIC₅₀ 6.6), Compound 21 (Hernandez-Torres *et al.*, 2014) represents a reversible MAGL inhibitor, which is active *in vivo*.

The use of JZL184 *in vivo* produced effects similar to those obtained using THC or synthetic CB₁ cannabinoid agonists (Long *et al.*, 2009), indicating that there may be psychoactive limiting factors

for therapeutic application of MAGL inhibitors. Despite this, there are reports of anxiolysis (Sciolino *et al.*, 2011), reduction in pain behaviours (Aaltonen *et al.*, 2016; Woodhams *et al.*, 2012) and nausea-like behaviours (Sticht *et al.*, 2012), as well as neuroprotection in a model of Parkinson's disease (Fernandez-Suarez *et al.*, 2014).

ABHD6 inhibitors: WWL70 was the first described selective ABHD6 inhibitor; screening mouse brain membranes using the activity-based protein profiling system generated a pIC₅₀ value of ~7.2 (Li *et al.*, 2007). From the same group, WWL123 is a similarly structured inhibitor with a slightly less potent profile (Bachovchin *et al.*, 2010). In addition, KT185 has been described as an orally-active ABHD6-selective inhibitor (Hsu *et al.*, 2013).

In vivo administration of WWL70 was neuroprotective in a mouse model of traumatic brain injury (Tchantchou and Zhang, 2013), and also alleviated symptoms in a model of multiple sclerosis in a manner dependent on CB₂ cannabinoid receptors (Wen *et al.*, 2015). WWL70 also protected mice from the weight gain associated with a high fat diet (Thomas *et al.*, 2013), in a manner consistent with the involvement of the nuclear hormone receptors PPARs (Zhao *et al.*, 2016) (see O'Sullivan and Pistis, this volume). WWL123 has also been shown to be effective in vivo, by reducing seizures in an epilepsy model (Naydenov *et al.*, 2014).

ABHD12 inhibitors: Currently, there is no selective inhibitor that targets ABHD12. However, it is possible to use a process of elimination to identify involvement of ABHD12 in monoacylglycerol hydrolase activity. Thus, ABHD12 is relatively insensitive to the MAGL inhibitors JZL184 or JKK-048, the ABHD6 inhibitor WWL70 or the DAGL inhibitor RHC80267, but may be inhibited by the non-selective serine hydrolase inhibitor MAFP and THL (more widely known as an inhibitor of DAGL, see above, and pancreatic lipase) (Blankman *et al.*, 2007).

11 Oxidative metabolism of endocannabinoids

Given that AEA and 2-AG have an arachidonoyl side-chain, it is not surprising that they share some of the metabolic pathways of arachidonic acid, or that many of the products of these pathways have been shown to have biological actions of their own. The main enzymes involved are summarised below. Aficionados of chemical structures are referred to Urquhart *et al.* (2015), where these catabolic pathways are well illustrated.

Cyclooxygenase (COX) -2 pathway. Both AEA and 2-AG are metabolised by COX-2, but not by COX-1 (due to a narrower active site), to yield prostaglandin ethanolamides (PG-EAs) and prostaglandin glyceryl esters (PG-GEs), respectively (Kozak *et al.*, 2000; Yu *et al.*, 1997). COX-2 acts as a functional heterodimer, whereby allosteric binding to one site affects the catalytic activity at the other, and crystallographic studies of COX-2 have indicated that 1-AG binds slightly differently to the two sites (Mitchener *et al.*, 2015; Vecchio and Malkowski, 2011). PGF_{2α}-EA formation from AEA proceeds via the PGF-EA synthase catalysed reduction of PGH₂-EA (Moriuchi *et al.*, 2008; Yang *et al.*, 2005), and produces its biological effects, including an increased firing of spinal cord nociceptive neurons, as a result of an interaction with a heterodimeric receptor that is not responsive to PGF_{2α} (Gatta *et al.*, 2012; Woodward *et al.*, 2013). Most of the work on the properties of PG-GEs have been undertaken using the more stable 1,3-regioisomers, but a picture is emerging whereby both PG-EA and PG-GE compounds can affect inflammation, with both pro- and anti-inflammatory effects being reported (review, see (Alhouayek and Muccioli, 2014)). The stable PG-EA analogue bimatoprost is clinically used for the treatment of glaucoma (see (Woodward *et al.*, 2013)). Relatively little is known about the metabolism of PG-EAs, but a major metabolic pathway for PG-GEs is hydrolysis to PG catalysed by several enzymes, lysophospholipase A₂ playing a major role in this respect (Manna *et al.*, 2014).

Lipoxygenase (LOX) pathways. Less work has been undertaken on LOX-catalysed hydroperoxidation of the endocannabinoids, but both AEA and 2-AG are substrates of 12- and 15-LOX to produce products that are biologically active either at CB receptors or at peroxisome proliferator-active

receptor α (Edgemond *et al.*, 1998; Hampson *et al.*, 1995; Kozak *et al.*, 2002; Moody *et al.*, 2001; Ueda *et al.*, 1995; van der Stelt *et al.*, 2002). In addition, the 15-LOX product of AEA can be metabolised by glutathione transferases to form cysteinyl-containing metabolites (“eoxamides”, in analogy with the eoxins produced from arachidonic acid) (Forsell *et al.*, 2012). The biological properties of the eoxamides is not known.

CYP450 pathways. In 1995, Bornheim and colleagues reported that the incubation of AEA with liver microsomes in the presence of NADPH resulted in the production of several compounds, the levels of which could be induced following pretreatment of the mice with CYP450 enzyme inducers (Bornheim *et al.*, 1995). Subsequent work mainly by Hollenberg and colleagues have identified CYP3A4, CYP2D6, CYP4F2 and CYP2J2-derived AEA metabolites, and shown that the 5,6-epoxide metabolite of AEA is a reasonably potent and selective CB₂ receptor agonist (McDougle *et al.*, 2014; Snider *et al.*, 2007; Snider *et al.*, 2009; Snider *et al.*, 2008; Walker *et al.*, 2016). CYP-derived metabolites of 2-AG with activity towards CB receptors have also been described (Chen *et al.*, 2008; McDougle *et al.*, 2014).

12 Integrating the catabolism of the endocannabinoids and related NAEs in health and disease

From the above, it is clear that there are a multiplicity of synthetic and degradative pathways for the endocannabinoids. At first sight, this would suggest that a “point block” (i.e. a selective inhibition or genetic deletion of one of the metabolic enzymes) would be expected to have a limited effect (since the endocannabinoid would merely find another metabolic route), unless the enzyme in question was predominant. Indeed, under extreme conditions (such as genetic deletion), additional metabolic pathways in addition to those described above may come into play (Mulder and Cravatt, 2006). As pointed out previously, deletion of NAPE-PLD reduces, but does not completely block, AEA production in the brain (Leishman *et al.*, 2016; Leung *et al.*, 2006; Tsuboi *et al.*, 2011). In this section, the effects of point blocks of the catabolic enzyme FAAH is considered.

Early studies using FAAH inhibitors and FAAH^{-/-} mice demonstrated that, providing enzyme activity was sufficiently inhibited, brain levels of AEA were dramatically increased (Cravatt *et al.*, 2001; Kathuria *et al.*, 2003). This is not a universal finding: in lipopolysaccharide + interferon γ -activated macrophages, for example, hydrolysis of exogenous AEA is totally blocked by the FAAH inhibitor URB597, but the endogenous levels of AEA are, at best, only modestly affected (Gouveia-Figueira *et al.*, 2015a). Studies investigating the effects of FAAH inhibition and/or genetic deletion on AEA and related NAE levels either in different brain regions or in different parts of the body have been undertaken, but these tend to suffer from statistical issues such as small sample sizes, lack of consideration of unequal variances between groups and, not least, lack of compensation for multiple significance testing. This does not impact upon the large-scale changes reported, but more modest changes may be either over-interpreted or missed completely.

An alternative approach is to look at the organism as a whole. In a study from 2014, a three compartment model (brain, plasma and rest of body) was used to simulate the changes over time in plasma AEA, PEA, OEA and linoleoyl ethanolamide levels in humans treated with the FAAH inhibitor PF-04457845. They could simulate the observed data well, but only if the model included an FAAH-independent clearance (Benson *et al.*, 2014). The authors suggested that NAAA may be a suitable candidate for this additional clearance, on the basis that PEA was also encompassed by this finding. Certainly, inhibition of NAAA increases PEA and OEA levels in mouse lungs (Ribeiro *et al.*, 2015), but it is unclear as to whether the more limited organ distribution of this enzyme is sufficient to explain the data.

This somewhat complex system becomes even more convoluted when patients with diseases potentially tractable to treatment with inhibitors of AEA / NAE hydrolysis are considered, such as patients with pain and inflammation (for review for FAAH and pain, see (Fowler, 2015); for inflammation and NAAA, see (Alhouayek *et al.*, 2015; Solorzano *et al.*, 2009) for examples in experimental animals). In this case, the situation is complicated by the presence and influence of

cytokines and other factors that can dramatically affect the relative expression of the catabolic enzymes. Thus, in addition to a large induction in COX-2 activity, cytokines like the interleukins IL-4 and IL-10 increase FAAH activity, whilst IL-12 and interferon- γ reduce it (Maccarrone *et al.*, 2001b; Thors *et al.*, 2010a). In unstimulated RAW264.7 macrophages, the mRNA expression of NAAA is about 160-fold higher than that of FAAH. However, upon activation with 24 h of treatment with lipopolysaccharide + interferon- γ , NAAA mRNA is reduced five-fold, whilst FAAH mRNA is increased fourfold (Gabrielsson *et al.*, submitted; see Figure 3). Whilst these changes are less than the 1400-fold induction of COX-2 in the cells, the treatment clearly changes the balance of AEA catabolic enzymes in the cells. Further, changes are likely to be cell-specific: in lymphocytes, for example, lipopolysaccharide reduces rather than increases FAAH expression (Maccarrone *et al.*, 2001a). Such changes in the relative expression of the catabolic enzymes may provide an explanation for the loss of effect of the FAAH inhibitor URB597 towards increasing hindpaw AEA, OEA and 2-AG levels in rats with neuropathic pain following spinal nerve ligation (Jhaveri *et al.*, 2006), and even could be a contributing factor to the failure of the FAAH inhibitor PF-04457845 in osteoarthritis (Huggins *et al.*, 2012). Modelling such changes is by no means easy, but a simple model can be constructed from the known K_m and k_{cat} values for FAAH and COX-2 for given AEA concentrations in the vicinity of the enzymes (Figure 3). Add to this a) the ability of endogenous lipids such as cholesterol, phosphatidylcholine, dihydrolipoic acid and AEA-derived LOX metabolites to influence the activity of FAAH and/or NAAA (Dainese *et al.*, 2014; Tai *et al.*, 2012; van der Stelt *et al.*, 2002) and b) differences in CYP3A4, 2B6 and 2D6 polymorphisms with respect to their ability to metabolise AEA (Pratt-Hyatt *et al.*, 2010; Sridar *et al.*, 2011) and it becomes clear that the potential for differences in catabolism of endocannabinoids between cells, organs, individuals and between healthy volunteers and patients, becomes considerable.

The aim of this review was to discuss the turnover of endocannabinoids rather than the pharmacology of the system, but it is clear from the above discussion that “point block” may not be optimal in cases where alternative catabolic pathways are present. An interesting alternative is the

design of compounds attacking more than one catabolic pathway. One possibility is to target both COX-2 and FAAH. Two approaches have been taken: one is to start from the modest FAAH inhibitory potency of COX inhibitors such as ibuprofen and flurbiprofen, and to optimise the former without compromising the latter. This has led to the identification of (*R*)-2-(2-fluorobiphenyl-4-yl)-*N*-(3-methylpyridin-2-yl)propanamide, a mixed-type reversible inhibitor of FAAH and substrate-selective inhibitor of COX-2 (Gouveia-Figueira *et al.*, 2015b; Karlsson *et al.*, 2015). An alternative approach is to link a COX inhibitor (flurbiprofen) to a carbamate FAAH inhibitor with a chemical bridge (Migliore *et al.*, 2016; Sasso *et al.*, 2015). It will be interesting to see whether such compounds prove to be useful in conditions where both COX-2 and FAAH play central roles in AEA catabolism.

13 Conclusion

There is an attractive complexity associated with the network of enzymes involved in the synthesis, hydrolysis and transformation of endocannabinoids. The various genetic models and pharmacological tools are beginning to allow dissection of the patho/physiological roles of these enzymes, which paves the way for future exploitation in a clinical environment.

Acknowledgments

CJF thanks the Swedish Science Research council (Grant no. 12158) and the Research Funds of the Medical Faculty, Umeå University for research support.

Conflict of interest

The authors have no conflict of interest to relate.

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Table 1: Pharmacological tools used to investigate endocannabinoid turnover

Compound	Comment
Biochanin A	A reversible inhibitor with a modest selectivity for FAAH-2
DH376	A selective DAGL inhibitor
DO34	A selective DAGL inhibitor
JJKB048	A potent, selective MAGL inhibitor
JNJ-1661010	An irreversible inhibitor with a modest selectivity for FAAH-2
JZL184	A selective MAGL inhibitor, with reduced potency at the rat enzyme compared to human and mouse
KT185	An orally-active inhibitor of ABHD6
LEI015	An orally-active, reversible DAGL inhibitor
MAFP	A non-selective irreversible inhibitor of many eCB-metabolising enzymes, including FAAH, MAGL, ABHD6, ABHD12
OL-135	A reversible inhibitor with a modest selectivity for FAAH compared to FAAH-2
RHC80267	A DAGL inhibitor, which fails to inhibit ABHD12
Tetrahydrolipstatin	A DAGL inhibitor, which also inhibits ABHD12 and pancreatic lipase
URB597	An irreversible inhibitor with a modest selectivity for FAAH compared to FAAH-2
WWL123	An ABHD6 inhibitor
WWL70	An ABHD6 inhibitor

For further information and the appropriate citations, please refer to the text

Legends to figures

Figure 1. Parallel and convergent pathways of endocannabinoid metabolism

The graphic illustrates three phases of endocannabinoid turnover: synthesis, hydrolysis and transformation. For clarity, a number of alternative, apparently minor pathways of endocannabinoid metabolism are not shown.

Figure 2. Regulation of NAE synthesis.

Panels A and B. Levels of AEA (yellow), palmitoylethanolamide (PEA, blue), stearoylethanolamide (SEA, orange), oleoylethanolamide (OEA, green) and linoleoylethanolamide (purple) in relation to their corresponding NAPEs. In panel A, the values (means $\pm$ SEM, when not enclosed by the symbols, N= 3-6) are for cerebral cortices from rat pups 24 h after intrastriatal injection of either saline or 25 nmol of NMDA, and are drawn from the data of Hansen et al. (2001a and b). The regression line was calculated from the mean logged values and has a slope of 0.99 ± 0.06 . In panel B, the values (means $\pm$ SD, N=3) are for uterine implantation and inter-implantation sites for female mice on pregnancy day 7, and are drawn from the data of Schmid et al. (1997). The regression line, calculated from the mean logged values for all NAEs except the two AEA values, has a slope of 1.04 ± 0.34 . The highest mean value of AEA (1345 pmol / μ mol lipid phosphorous) corresponds to 20 pmol / mg tissue.

Figure 3. Relative expression of the AEA catabolic enzymes: regulation and consequences.

Panel A. Changes in the mRNA expression of *Faah*, *Naaa*, *Cox2* and *Lox15* in RAW264.7 cells following 24 h incubation with lipopolysaccharide and interferon- γ . The data are shown as mean $\pm$ 95% bootstrapped percentile confidence limits of the difference in the ΔCt values between untreated and treated cells, $n=7-16$. A $\Delta\Delta Ct$ of x corresponds to a 2^{-x} fold change, and this is shown on the right axis. The bootstrapped percentile confidence limits of the difference were calculated using 100000 iterations with the package Hmisc for the R computer programme. (drawn from data recalculated from the study of L. Gabrielsson, S. Gouveia-Figueira, M. Alhouayek & C.J. Fowler, submitted for publication). Panel B shows the relative activities for two enzymes A and B at different relative concentrations (shown as enz B : enz A in the figure) of the two enzymes, calculated assuming both enzymes have the same k_{cat} , while the K_m for enzyme A is four times lower than that for enzyme B. To our knowledge, such kinetic constants for FAAH and COX-2 have not been presented where the enzymes have been assayed under the same conditions (i.e. pH, presence or absence of detergent etc.). The family of curves demonstrate that the metabolic pathway of the substrate, i.e. via enzyme A or B, clearly depends on the ambient concentration and relative expression of the enzymes.