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Review

Anandamide and other *N*-acylethanolamines: A class of signaling lipids with therapeutic opportunities

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A B S T R A C T

N-acylethanolamines (NAEs), including *N*-palmitoylethanolamine (PEA), *N*-oleoylethanolamine (OEA), *N*-arachidonoylethanolamine (AEA, anandamide), *N*-docosahexaenoylethanolamine (DHEA, synaptamide) and their oxygenated metabolites are a lipid messenger family with numerous functions in health and disease, including inflammation, anxiety and energy metabolism. The NAEs exert their signaling role through activation of various G protein-coupled receptors (cannabinoid CB_1 and CB_2 receptors, GPR55, GPR110, GPR119), ion channels (TRPV1) and nuclear receptors (PPAR-α and PPAR-γ) in the brain and periphery. The biological role of the oxygenated NAEs, such as prostamides, hydroxylated anandamide and DHEA derivatives, are less studied. Evidence is accumulating that NAEs and their oxidative metabolites may be aberrantly regulated or are associated with disease severity in obesity, metabolic syndrome, cancer, neuroinflammation and liver cirrhosis. Here, we comprehensively review NAE biosynthesis and degradation, their metabolism by lipoxygenases, cyclooxygenases and cytochrome P450s and the biological functions of these signaling lipids. We discuss the latest findings and therapeutic potential of modulating endogenous NAE levels by inhibition of their degradation, which is currently under clinical evaluation for neuropsychiatric disorders. We also highlight NAE biosynthesis inhibition as an emerging topic with therapeutic opportunities in endocannabinoid and NAE signaling.

1. Introduction

Over the past decades, lipids have emerged as important signaling molecules in health and disease. Lipids come in a range of shapes and sizes and are classified in eight different categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides [1]. Signaling lipids often exert their bioactivities through activation of various proteins, including G protein-coupled receptors (GPCRs), ion channels and nuclear receptors. Within the class of fatty acyl lipids, the *N*-acylethanolamines (NAEs) have garnered attention as a family of bioactive fatty acid amides with diverse roles in inflammation, neurotransmission, appetite, fertility, stress and anxiety. The NAEs incorporate saturated, mono- or polyunsaturated fatty acyl groups in their structures, which determine their signaling function. The most frequently occurring and studied NAEs are *N*-palmitoylethanolamine (PEA), *N*-stearoylethanolamine (SEA), *N*-oleoylethanolamine (OEA), *N*-linoleoylethanolamine (LEA), *N*-arachidonoylethanolamine (AEA) and *N*-docosahexaenoylethanolamine (DHEA) (Table 1). At present, many outstanding questions still exist with regard to their biological actions. Here, an overview is provided of NAE biosynthesis and degradation, current understanding of their physiological functions and potential therapeutic applications of modulating the NAE tone.

2. NAE metabolism

In 1979, Schmid and co-workers reported the accumulation of NAEs in infarcted dog heart [33]. Shortly hereafter, the same lab showed that *N*-acylphosphatidylethanolamines (NAPEs), a previously unknown lipid class, were equally upregulated [34]. Due to the structural similarities of NAPEs and NAEs, a precursor-product relationship was proposed [35]. Ensuing studies revealed that NAPEs are produced by the transfer of the sn-1 acyl group of phosphatidylcholine (PC) to the amine of phosphatidylethanolamine (PE), forming NAPE and 2-acyl-lysoPC (Fig. 1) [36]. Next, the phosphodiester bond of NAPE is hydrolyzed to generate NAE and phosphatidic acid (PA). Finally, the NAE is degraded to fatty acid (FA) and ethanolamine.

2.1. (p)NAPE biosynthesis

The canonical acyl transfer reaction that produces NAPEs is carried out by a Ca^{2+}-dependent *N*-acyltransferase (Ca-NAT). High Ca-NAT enzymatic activities have been found in heart, brain and testis tissues [35,38,39]. Although remaining elusive for decades, the serine

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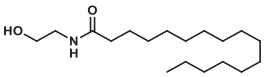
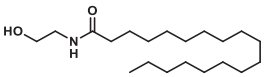
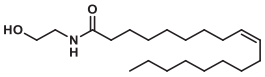
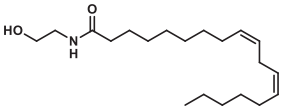
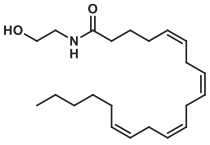
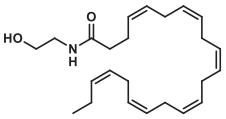
Table 1
N-acylethanolamine (NAE) family members and their reported biological activities.

Name	Structure	Receptor	Bioactivity
PEA (16:0)		PPAR-α [2] GPR55 [3] GPR119 [4]	Anti-inflammatory [5] Neuroprotective [6] Anti-epileptic [7] Analgesic [8] Anorectic [9]
SEA (18:0)		GPR119 [4]	Anti-inflammatory [10] Anorectic [11]
OEA (18:1-ω9)		PPAR-α [12] GPR119 [4]	Anti-inflammatory [13] Anorectic [9] Analgesic [14] Neuroprotective [15]
LEA (18:2-ω6)		PPAR-α [16] GPR119 [17]	Anorectic [18] Neuroprotective [19]
AEA (20:4-ω6)		CB_1 [20] CB_2 [21] TRPV1 [22]	Neurotransmission [23] Orexigenic [24] Analgesic [25] Anxiolytic [26] Memory formation [27] Neuroprotective [28] Fertility [29]
DHEA (22:6-ω3)		GPR110 [30]	Neurogenesis [31] Anti-inflammatory [32]

Abbreviations: PEA = *N*-palmitoylethanolamine, SEA = *N*-stearoylethanolamine, OEA = *N*-oleoylethanolamine, LEA = *N*-linoleoylethanolamine, AEA = *N*-arachidonoylethanolamine, DHEA = *N*-docosahexaenoylethanolamine, PPAR-α = peroxisome proliferator-activated receptor α, GPR = G protein coupled receptor 55/110/119, CB = cannabinoid receptor 1/2, TRPV1 = Transient receptor potential vanilloid 1.

hydrolase phospholipase A2 group IV E (PLA2G4E, $cPLA_2\varepsilon$) was recently identified by Cravatt and colleagues as a NAPE-generating Ca-NAT in cells, matching the reported expression and activity profile [40]. Also plasmalogen-type PEs – incorporating a vinyl ether at the sn-1 position – were found to be suitable substrates for PLA2G4E, thereby producing plasmalogen-NAPEs (pNAPEs) [41,42]. pNAPEs are considered to be an important source of NAEs in the brain, which is illustrated by the finding that in mouse brain total pNAPE content was 4-fold higher than NAPE [43]. In contrast, NAPEs were almost exclusively observed in the mucosal layer of rat jejunum, while in the serosal layer both NAPE and pNAPE species were abundant [44]. The activity of PLA2G4E was enhanced by various anionic lipids including phosphatidylserine (PS) *in vitro* and in living cells [41,45]. PLA2G4E is localized in lysosomal and endosomal compartments as well as the plasma membrane [42,46]. Interestingly, in rat brain lysate Ca-NAT activity preferably generated *N*-arachidonoyl-containing (p)NAPEs with polyunsaturated acyl groups at the sn-2 position [47]. This may indicate that the Ca^{2+}-dependent generation of AEA favors polyunsaturated (p)NAPEs as precursors [47]. Very recently, two PLA2G4E KO mice strains have been reported [48–50]. Murakami and co-workers confirmed that PLA2G4E is responsible for NAPE and NAE biosynthesis *in vivo*, with most striking reductions in skin, muscle and stomach [48,49]. Ueda and colleagues showed evidence that PLA2G4E is required for the post-mortem increase of brain NAPEs and NAEs upon ischemia [50]. Nonetheless, both models did not reveal significant reductions of endogenous brain NAPEs and NAEs, indicating that alternative pathways are able to maintain the basal levels of these lipids. Currently, no selective pharmacological tools are available to modulate PLA2G4E *in vivo*.

A second family of *N*-acyltransferases (NATs) – discovered and characterized by the Ueda lab – can produce NAPEs in a Ca^{2+}-independent manner and were termed phospholipase A and acyl transferase (PLAAT) 1-5 [51–56]. These enzymes belong to the cysteine hydrolase superfamily and show expression in the central nervous system (CNS) as well as in peripheral tissues. PLAAT family members exhibit NAT or phospholipase $PLA_{1/2}$ activity to a variable extent. In particular, PLAAT2 (HRASLS2) showed high NAT activity – comparable to PLA2G4E – followed by PLAAT5 (HRASLS5, iNAT), PLAAT1 (HRASLS, A-C1) and PLAAT4 (HRASLS4, TIG3, RARRES3), while PLAAT3 (HRASLS3, PLA2G16, AdPLA, H-REV107) displayed almost solely $PLA_{1/2}$ activity [56]. PLAAT2 preferably transferred the sn-1 acyl group of PC over the sn-2 group, suggesting that it is involved in the biosynthesis of saturated and mono-unsaturated NAEs [56]. Similar to PLA2G4E, PLAAT2 accepted both PE and plasmalogen-type PE as substrates [41,56]. Protein overexpression of PLAAT2 in human embryonic kidney (HEK293) and osteosarcoma (U2OS) cells afforded large increases in NAPE and NAE species, including ω-6 and ω-3 polyunsaturated NAEs such as AEA and DHEA [56,57]. Interestingly, a limited increase of pNAPEs was observed in the PLAAT2-HEK293 stable expressing cell line, suggesting that PLAAT2 prefers PE as an acceptor lipid in this cell type [56]. Gene expression of *PLAAT2* was high in the liver, kidney, small intestine, colon, testis and trachea [53,58]. This suggests that PLAAT2 may be involved in NAE biosynthesis in the gut, where it supplies a basal level of NAPE content. Notably, PLAAT2 and PLAAT4 expression is absent in rodents, which hampers the study of these enzymes in genetic models [53]. The Davies group developed a convenient way to study PLAAT2 *in vivo* by expressing the human enzyme in *E. coli* Nissle 1917 (EcN) and administering the derived plasmid pPLAAT2-EcN into mice drinking water [59,60]. Two week treatment of pPLAAT2-EcN afforded significant increases in plasma NAPE and NAE levels. When put on a high fat diet, pPLAAT2-EcN-treated mice showed less weight gain and food intake compared to their control littermates [60].

Compared to PLAAT2, PLAAT5 did not have any preference for the sn-1 or sn-2 acyl group of PC, suggesting that it could be involved in *N*-arachidonoyl-PE and thus AEA biosynthesis [51,52]. Overexpression of PLAAT5 in U2OS cells indeed revealed a strong increase of NAEs including AEA, except for DHEA [57]. Although all PLAAT members

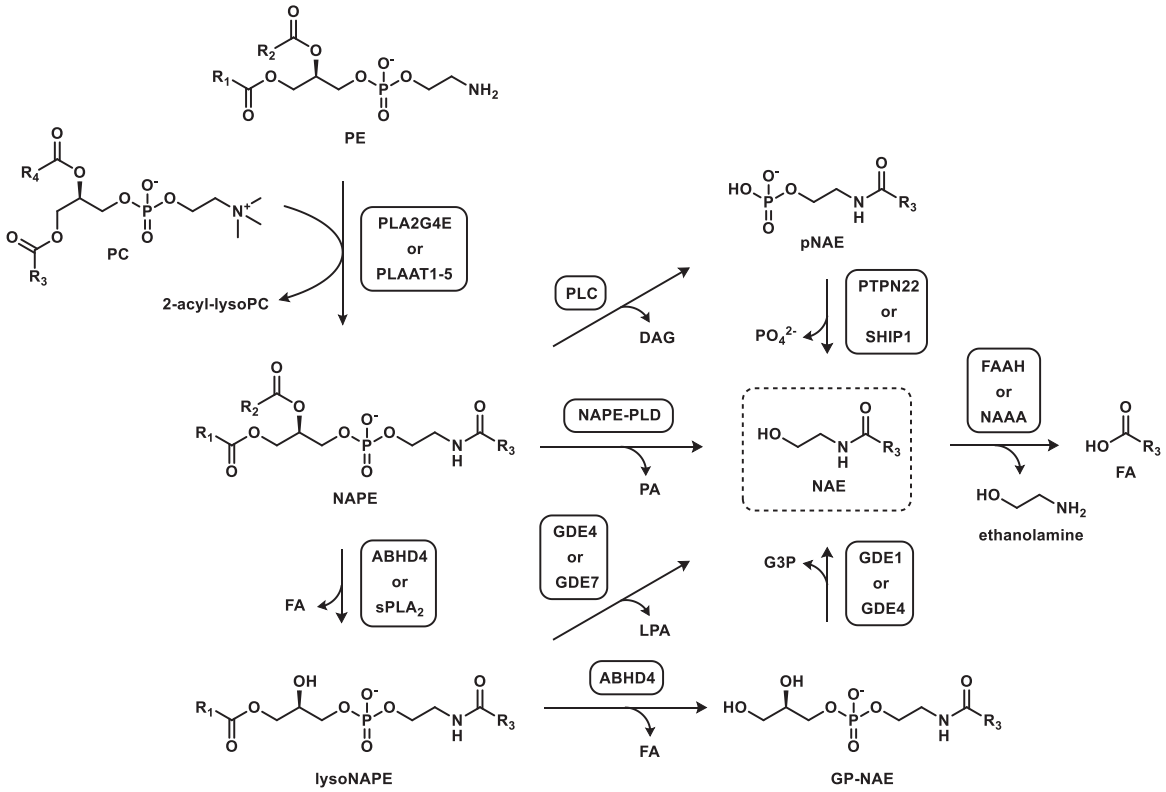


Fig. 1. Biosynthetic pathways of *N*-acylethanolamines (NAEs). In total, four different enzymatic routes have been reported that can produce NAEs [37]. In the canonical pathway, *N*-acylphosphatidylethanolamine (NAPE) is formed from phosphatidylethanolamine (PE) and phosphatidylcholine (PC) catalyzed by phospholipase A_2 group IV E (PLA2G4E). This is followed by NAPE phospholipase D (NAPE-PLD)-mediated hydrolysis to NAE. Fatty acid amide hydrolase (FAAH) catabolizes NAEs into fatty acids (FAs) and ethanolamine. Abbreviations: PLAAT1-5 = phospholipase/acyltransferase 1-5; ABHD4 = α,β-hydrolase domain 4; GDE1,4 or 7 = glycerophosphodiesterase 1,4 or 7; PLC = phospholipase C; PTPN22 = protein tyrosine phosphatase non receptor type 22; SHIP1 = phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1; NAAA = *N*-acylethanolamine acid amidase; GP-NAE = glycerophospho-*N*-acylethanolamine; LPA = lysophosphatidic acid; PA = phosphatidic acid; DAG = diacylglycerol; G3P = glycerol-3-phosphate; pNAE = phospho-*N*-acylethanolamine.

were initially described as tumor suppressors, this role has not been reported for PLAAT5. It is interesting to note that in humans and rodents *PLAAT5* shows specific gene expression in testis and pancreas, suggestive of a specialized role in these tissues [52,61].

Of the PLAAT members, PLAAT1 has high expression in brain of humans and rodents, suggesting it could play a role in brain NAPE biosynthesis [55]. Further tissue distribution was reported in testis, skeletal muscle and heart. Overexpression of PLAAT1 in COS-7 cells gave significant increases of NAPE, pNAPE and NAE levels [62]. Two isoforms of PLAAT1 have been identified; a longer isoform with a polybasic *N*-terminal domain that is found in the nucleus and cytoplasm and is expressed in humans and mice, and a shorter isoform that is expressed in mouse cytoplasm only [63]. Both isoforms showed predominantly NAT activity when purified and produced NAPEs and NAEs when expressed in cells [63]. So far, no genetic KO models have been described for PLAAT family members 1 and 5. To what extent the Ca^{2+}-independent pathway contributes to NAPE and pNAPE biosynthesis *in vivo*, is therefore still unclear [37].

2.2. NAE biosynthesis

In 2004, the enzyme that produces NAEs in a single step from NAPEs or pNAPEs was isolated and named *N*-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) (Fig. 1) [64]. A crystal structure revealed that NAPE-PLD forms a membrane-bound homodimer with two Zn^{2+}-ions in its active site [65]. NAPE-PLD is classified as a metallo-β-lactamase and is distinct from the PLD family [64]. Brain, kidney and testis tissues were found to abundantly express NAPE-PLD [64]. Interestingly, NAPE-PLD did not display any substrate preference *in vitro* [66]. Furthermore, PE increased the NAPE-PLD enzymatic activity, suggesting that the enzyme is constitutively active [67]. *In vitro*, NAPE-PLD activity was elevated by specific bile acids, as well as polyamines such as spermine and spermidine [65,68,69]. In the presence of deoxycholic acid, NAPE-PLD showed elevated activity for *N*-arachidonoyl-PE compared to *N*-palmitoyl-PE, suggesting that the production of specific NAEs may be regulated *in vivo* [68]. Multiple NAPE-PLD knockout (KO) studies described a significant reduction of saturated and unsaturated NAEs in the brains of mice [43,70,71]. In accordance, NAPE and pNAPE precursors were greatly enhanced [43,70]. However, levels of ω-6 and ω-3 polyunsaturated NAEs – AEA and DHEA, respectively – were decreased in some but not in all KO strains [70]. It was therefore proposed that genetic deletion of NAPE-PLD stimulated compensatory mechanisms which counteract the reduction of AEA and DHEA content [70]. In peripheral organs such as heart, kidney, liver and jejunum, NAPE-PLD KO mice did not present decreased NAE levels, although NAPE concentrations were highly elevated, except for jejunum [72]. Recently, we have described the first *in vivo* active NAPE-PLD inhibitor, LEI-401, which reduced brain AEA levels in mice [73]. LEI-401 activated the hypothalamus-pituitary-adrenal axis and impaired the fear extinction response, thereby mimicking the effects of a CB_1 receptor antagonist. The effects of LEI-401 were prevented by co-administration of a FAAH inhibitor. This indicates that modulation of NAPE-PLD activity with inhibitors in an acute and time-dependent manner is able to modulate AEA levels in the brain and suggests the presence of an endogenous NAE tone controlling emotional behavior.

Three additional pathways have been discovered that can also

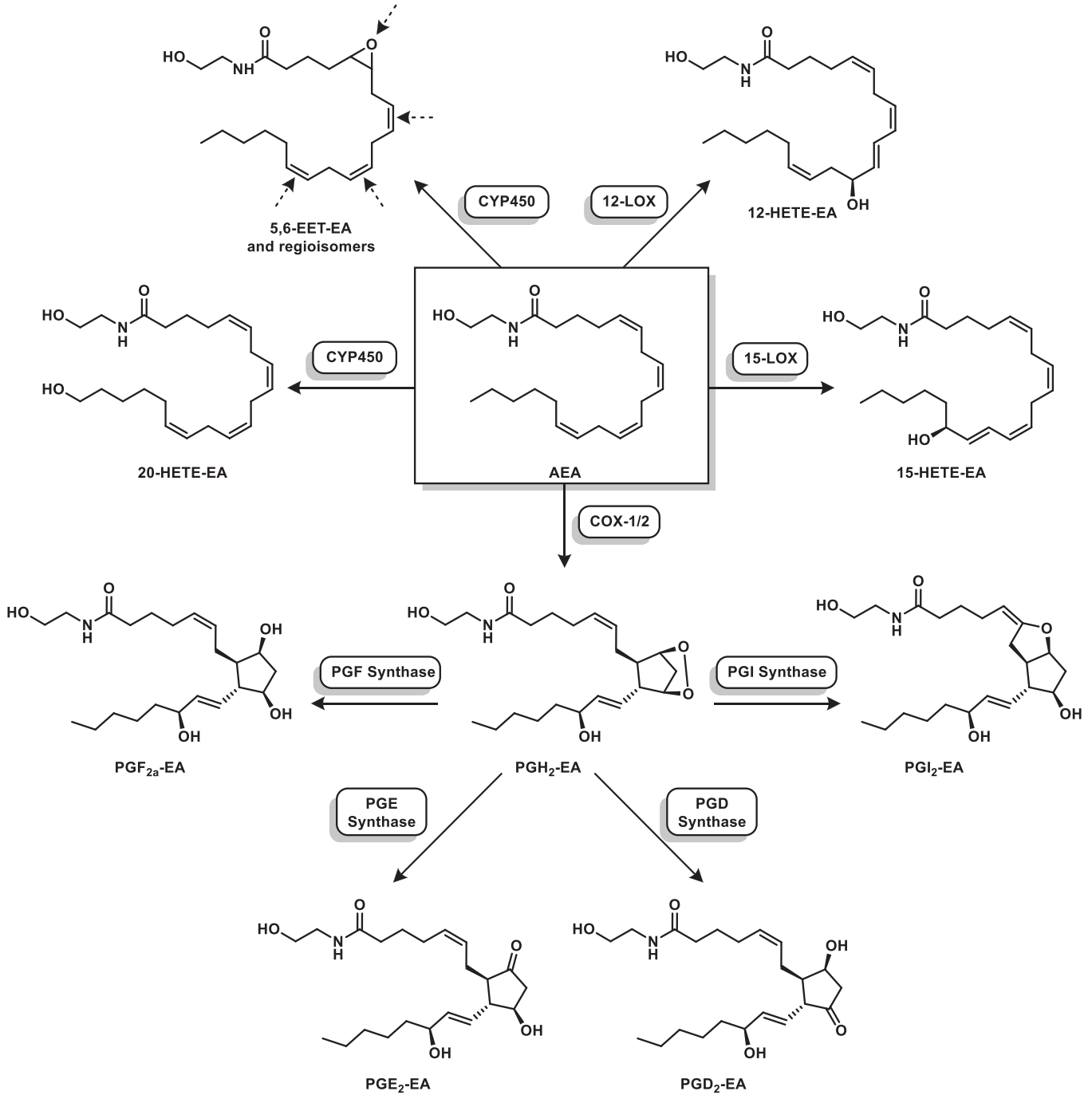


Fig. 2. Oxidative metabolic pathways for AEA. See text for details. Abbreviations: AEA, N-arachidonoylethanolamide (anandamide); COX, cyclooxygenase; CYP450, cytochrome P450; EET-EA, epoxyeicosatrienoyl ethanolamide; HETE-EA, hydroxyeicosatetraenoyl ethanolamide; LOX, lipoxygenase; PG-EA, prostaglandin ethanolamide.

produce NAEs (Fig. 1). Firstly, two phospholipases were reported that can hydrolyze the fatty acyl esters of NAPEs. Three isoforms of secretory phospholipase A2 (sPLA$_2$-IB, IIA and V) were described to exclusively cleave the NAPE sn-2 ester to form lysoNAPE and a fatty acid [74]. The serine hydrolase α,β-hydrolase domain 4 (ABHD4) performed the same reaction, but did not show any specificity towards the sn-1 or sn-2 ester [75]. In addition, ABHD4 could hydrolyze the fatty acyl ester of lyso-NAPE, generating glycerophospho-NAE (GP-NAE). Very recently, PLA2G4C (cPLA$_2\gamma$), a family member of PLA2G4E, was reported to have similar PLA$_{1/2}$ activity as ABHD4, acting on NAPE and lysoNAPE [76]. GP-NAE, in turn, is converted by glycerophosphodiesterase 1 (GDE1, MIR16) and GDE4 (GDPD1) to afford NAE and glycerol-3-phosphate (G3P) [77,78]. A second pathway involves cleavage of the lysoNAPE phosphodiester by GDE4 or GDE7 (GDPD3) in a lysoPLD-type reaction, producing NAE and lysophosphatidic acid (LPA) [78,79]. Expression of ABHD4 was high in brain and testis, but not in heart [75]. ABHD4 KO mice displayed decreased levels of GP-NAE and lyso-(p)NAPE in the brain, however NAE content, including AEA, was not reduced [80]. The activity of GDE1 was stimulated by Mg^{2+}-ions and high protein expression levels were found in brain, testis, liver and kidney tissues [77]. Genetic deletion of GDE1 in mice also did not afford a significant decrease of brain NAE levels, therefore the physiological importance of

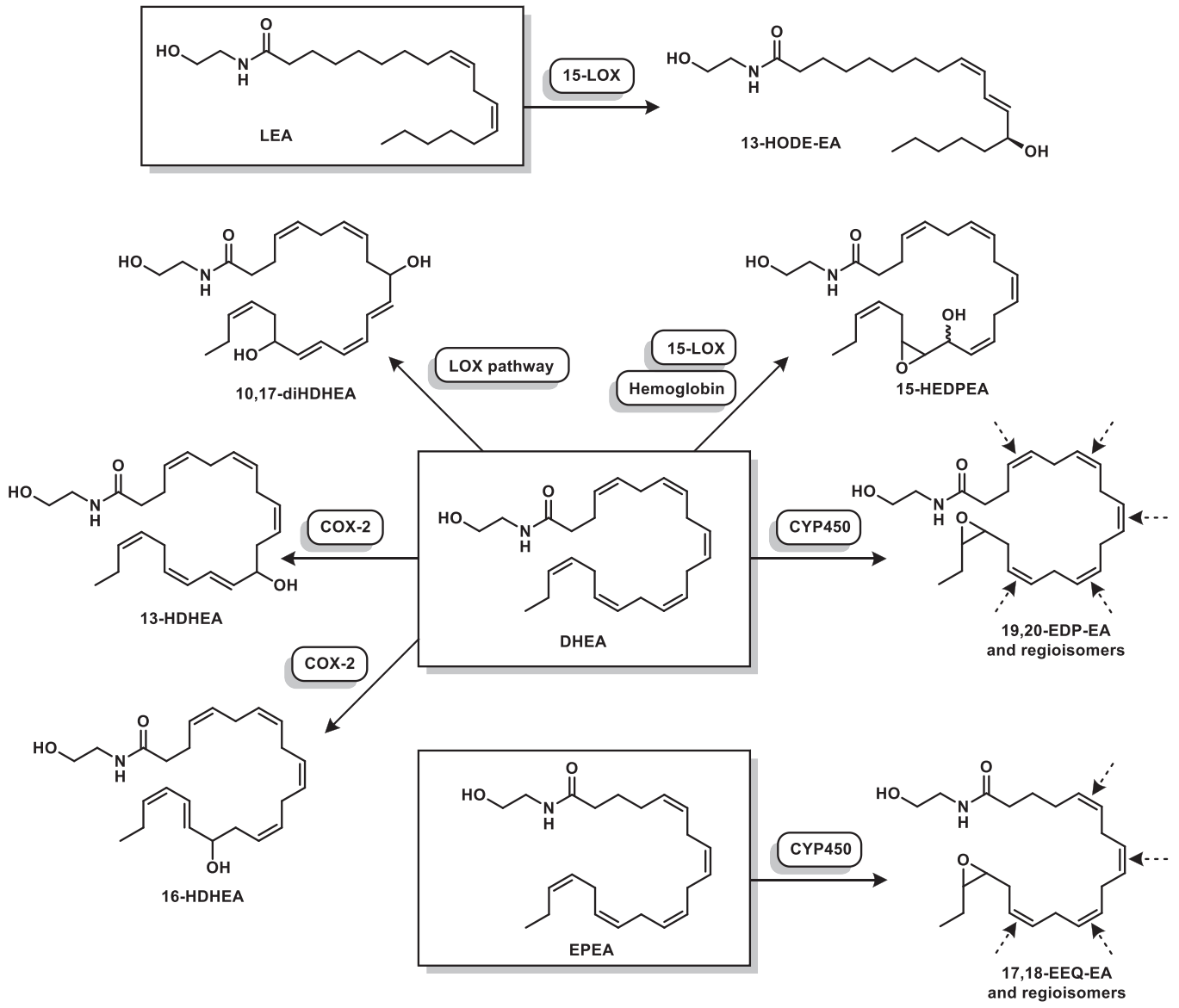


Fig. 3. Oxidative metabolic pathways for LEA, DHEA and EPEA. See text for details. Abbreviations: COX, cyclooxygenase; CYP450, cytochrome P450; DHEA, docosahexaenoyl ethanolamide; EDP-EA, epoxydocosapentaenoyl ethanolamide; EEQ-EA, epoxyeicosatetraenoyl ethanolamide; EPEA, eicosapentaenoyl ethanolamide; HDHEA, hydroxydocosahexaenoyl ethanolamide; HODE-EA, hydroxyoctadecadienoyl ethanolamine; LEA, N-linoleoyl ethanolamine; LOX, lipoxygenase.

this pathway for the formation of brain NAEs is still under debate [81]. The recently reported GDE4 and GDE7 enzymes, as well as the $sPLA_2$s have yet to be further characterized in KO models to establish their role in NAE biosynthesis *in vivo* [37]. It is interesting to note that the second product of the lysoPLD pathway is LPA, a bona fide signaling lipid in the CNS involved in cell proliferation and synaptic transmission [82].

A third NAE biosynthetic pathway was described to be important in macrophages, where lipopolysaccharide (LPS) induced elevation of AEA in a NAPE-PLD-independent manner [83]. It was proposed that a yet unknown PLC-type enzyme hydrolyzes the phosphodiester of NAPE to produce phosphoNAE and diacylglycerol (DAG). Two phosphatases were identified, protein tyrosine phosphatase non-receptor type 22 (PTPN22) and phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1 (SHIP1), that can catalyze the dephosphorylation of phosphoNAE to NAE and phosphate [83,84]. Both PTPN22 and SHIP1 were induced in macrophages upon LPS stimulation. Incubation of phosphoNAE with brain tissue from PTPN22 KO mice demonstrated reduced conversion to AEA compared to wild-type (WT), indicating a possible role *in vivo* [83]. The LPS-mediated downregulation of NAPE-PLD expression in macrophages has since been confirmed by a second group [85]. In contrast, mice that were administered with the proinflammatory stimuli LPS or carrageenan showed a NAPE-PLD-dependent decrease of PEA in peritoneal macrophages [85]. The levels of AEA were however not reported. Collectively, these results suggest that there may be specific NAE biosynthetic routes in macrophages that can be activated during inflammation.

2.3. NAE hydrolysis

The hydrolysis of NAE to fatty acid and ethanolamine can be performed by several enzymes (Fig. 1) [86]. Fatty acid amide hydrolase (FAAH) displays specificity towards AEA over saturated and monounsaturated NAEs and has high expression in human brain, but is absent in heart tissue [87]. Genetic or pharmacological blockade of FAAH resulted in a large increase of brain AEA levels in mice, as well as smaller but significant increases of PEA and OEA [88–90]. FAAH is therefore regarded as the primary AEA metabolizing enzyme in the brain. Surprisingly, in the liver, FAAH was found to catalyze the reverse reaction

Table 2
Oxidative metabolites of AEA, LEA, DHEA and EPEA and their reported biological activities.

Progenitor	Metabolite	Known receptor	Bioactivity
AEA	$PGF_{2\alpha}$-EA	FPAlt4 [110]	
	PGE_2-EA	Unknown	Anti-inflammatory [111,112] Pro-apoptotic [113,114]
	PGD_2-EA	Unknown	Pro-apoptotic [114]
	PGI_2-EA	Unknown	
	12-HETE-EA	CB_1/CB_2 [108,115]	
	15-HETE-EA	TRPV1 [116]	Anti-allodynic and antihyperalgesic [116]
	20-HETE-EA	Unknown	
	5,6-EET-EA	CB_2 [117,118] TRPV4 [119]	
LEA	13-HODE-EA	TRPV1 [120]	
DHEA	10,17-diHDHEA	CB_2 [121]	Reduction of PMN chemotaxis [121] Organ-protective [121]
	15-HEDPEA	CB_2 [121]	Reduction PMN chemotaxis [121] Organ-protective [121]
	13-HDHEA	Unknown	Anti-inflammatory [122]
	16-HDHEA	Unknown	Anti-inflammatory [122]
	19,10-EDP-EA	CB_1/CB_2 [123]	Anti-inflammatory [123] Vasodilatory and anti-angiogenic [123] Increased platelet aggregation [123]
	16,17-EDP-EA	CB_1/CB_2 [123]	
	10,11-EDP-EA	CB_1/CB_2 [124]	Pro-apoptotic and induced cell migration [124] (also for 7,8- and 13,14- regioisomers)
EPEA	17,18-EEQ-EA	CB_1/CB_2 [123]	Anti-inflammatory [123] Vasodilatory and anti-angiogenic [123] Reduction of platelet aggregation [123]
	14,15-EEQ-EA	CB_1/CB_2 [123]	

during liver regeneration which could be attributed to highly increased arachidonic acid levels, but not ethanolamine [91]. A second fatty acid amidase (FAAH-2) was identified and shares 20% sequence identity with FAAH [92]. FAAH-2 is specific for higher mammals including primates and marsupials and is not present in rodents. It is expressed in peripheral organs such as heart and ovary. Whereas FAAH localizes to the endoplasmic reticulum in cells, FAAH-2 was reported to be enriched in lipid droplets [93]. Contrary to FAAH, FAAH-2 preferred primary fatty acid amides (*e.g.* oleamide) over NAEs as substrates [92].

A third NAE-hydrolyzing enzyme was described to be active in cells of the immune system [94]. *N*-acylethanolamine acid amidase (NAAA) is located in lysosomes and preferentially hydrolyzes saturated NAE species [95]. NAAA is an *N*-terminal cysteine hydrolase and shares no homology with FAAH (a serine hydrolase). The biochemical activity of NAAA was enhanced by PE, PC and sphingomyelin (SM) phospholipids as well as dihydrolipoic acid [96]. The crystal structure of NAAA revealed that upon association to the lipid membrane due to electrostatic interactions, NAAA undergoes a conformational change, generating a cavity between two of its subunits so that substrates can bind [97]. Pharmacological inhibition of NAAA in mice produced significant elevations of brain PEA and OEA, but not AEA levels [98]. NAAA-deficient mice were first described by Piomelli and co-workers, who showed that KO mice had two-fold higher PEA levels in skin, but unchanged OEA compared to WT when treated with 1-fluoro-2,4-dinitrobenzene (DNFB), a model for atopic dermatitis [99]. No NAE levels were reported in naïve mice. A second NAAA KO mouse was recently described, where the mice lacked changes in PEA in most tissues studied including brain and skin, except for bone marrow, where a 2.5-fold PEA elevation was found [100]. Surprisingly, AEA was also increased in bone marrow as well as in lungs, while OEA saw no significant changes between genotypes. Primary macrophages extracted from these mice presented a two-fold increase of PEA and AEA, but not OEA. This suggests a possible role for NAAA in the degradation of AEA, although up or downregulation of alternative metabolic pathways should not be ruled out.

Very recently, acid ceramidase (AC), which shares 33% sequence identity with NAAA, was reported as a novel NAE hydrolase, preferring shorter aliphatic NAEs such as *N*-lauroylethanolamine [101]. In tissue homogenates of saposin D deficient-mice, a chaperone that activates AC in the lysosome, a reduction of NAE hydrolysis was observed.

2.4. NAE oxidation

Aside from the classic hydrolytic routes, polyunsaturated NAEs also serve as substrates for oxidative enzymes such as lipoxygenases (LOX), cyclooxygenases (COX) and cytochrome P450 enzymes (Fig.s 2, 3). The resulting oxygenated products bear structural similarity to the well-studied eicosanoids, but they have distinct signaling activities (Table 2). These metabolites reveal the existence of crossover between the endocannabinoid and eicosanoid signaling pathways, and their discovery has changed the view on NAE metabolism by LOX, COX and cytochrome P450 enzymes from a degradation pathway, to a bioactivation pathway [102].

Lipoxygenases (LOXs) are a family of non-heme iron-containing dioxygenases, which introduce oxygen to PUFAs in a free radical mechanism. This affords hydroperoxyl derivatives which are in turn metabolized into hydroxylated or epoxidized lipids [103]. The main mammalian LOX enzymes are 5-, 12- and 15-LOX, annotated by their preferential oxidation position on arachidonic acid [104]. Oxidation of AA and DHA is the most well-known role for LOX enzymes, which can produce a variety of immunomodulatory lipid signaling molecules, including leukotrienes (LTs), hydroxyeicosatetraenoids (HETEs), lipoxins (LXs), D-series resolvins and maresins [103,105]. AEA has been found to serve as a substrate for 12- and 15-LOX, resulting in 12-HETE-EA and 15-HETE-EA, respectively [106–108]. Interestingly, Forsell *et al.* found that human 15-LOX-1 can metabolize neutral lipids such as AEA to the same extent or better than arachidonic acid, suggesting that 15-LOX-1 oxidation of NAEs can be comparable to the metabolism of arachidonic acid in 15-LOX-1-expressing cells [109].

Cyclooxygenase enzymes consist of two isoforms COX-1 and COX-2 which are homodimeric enzymes responsible for the formation of several important bioactive derivatives of arachidonic acid. While COX-1 is constitutively expressed, COX-2 expression is either inducible or constitutive, depending on the tissue [125]. For example, in the brain and spinal cord, COX-2 is constitutively expressed in neurons and radial glia, but not in astrocytes, oligodendrocytes or microglia [126]. Induction of COX-2 can be triggered by a number of cytokines and inflammatory mediators, and is considered the main source of prostanoids that are formed during inflammation. COX enzymes mainly use arachidonic acid to produce prostaglandin H_2 (PGH_2) through concerted oxidation-reduction reactions. PGH_2, in turn, is used to form prostaglandins (PGs), prostacyclins and thromboxanes (TXs), collectively referred to as prostanoids [125]. Although COX-1 has substrate preference for fatty acyls with a free carboxyl group, COX-2 has been shown to oxygenate AEA, resulting in prostaglandin H_2 ethanolamide (PGH_2-EA) which is converted into prostaglandin ethanolamide derivatives by prostaglandin synthases, such as PGE_2-EA, PGD_2-EA, PGI_2-EA, and $PGF_2\alpha$-EA. [102,127–129] Although COX-2 oxidation of AEA is slower than for AA [130], it represents a significant metabolic pathway for AEA in the brain [131,132].

Another important oxidative pathway of NAEs is carried out by CYP450 enzymes, of which 57 genes have been identified in humans [133]. These enzymes play important roles in the biotransformation of

hormones and xenobiotics and have been well-described for their effect on drug metabolism and clearance. Whereas LOX and COX enzymes function as dioxygenases, CYP enzymes activate molecular oxygen and introduce one oxygen atom to their substrate, while the other O atom is reduced to water. Oxidation of lipids can occur in different ways: epoxidation of a double bond, terminal (ω-) hydroxylation or subterminal (ω-1) hydroxylation [134]. The different CYP450 isoforms can be classified under either hydroxylases or epoxygenases by their major reaction product using arachidonic acid, but this terminology has its limits as the major reaction changes depending on the PUFA substrate, and several CYP enzymes have no preference [135]. While epoxidation of ω-6 and ω-3 fatty acids result in established anti-inflammatory and cardioprotective metabolites [136–138], NAE metabolism by CYP450 enzymes has only recently gained attention [139].

3. Physiological functions of NAEs and (p)NAPEs

3.1. NAPE and pNAPE

NAPEs and pNAPEs are minor phospholipid species that constitute less than 0.1% of total phospholipid content in most tissues [140]. Classically, (p)NAPEs have been regarded as precursors of NAEs, however, recent overviews have highlighted that (p)NAPEs may have biological functions of their own [140,141]. These include putative roles in neuroprotection, anti-inflammation and satiety. During cellular injury, NAPEs accumulate significantly in damaged tissue by 10- to 50- fold, presumably due to an influx of calcium ions [142,143]. This phenomenon has been observed in ischemia of the brain, heart and testis in various mammals such as mice, rats, dogs and humans [34,144–150]. Also in plants NAPEs increase under cellular stress [141,151,152]. Importantly, NAPE levels are higher than their corresponding NAE congeners in brain ischemia, which may suggest a neuroprotective function [153]. Conversion of PE to NAPE has a proposed membrane stabilizing role, possibly due to intra- or intermolecular hydrogen bonding of the newly formed *N*-acyl amide, which embeds in the lipid bilayer [154,155]. The exact consequences of this event in cellular models has however not been well characterized. Speculatively, the acquired negative charge and conical shape of NAPEs may induce membrane curvature somewhat analogous to mitochondrial cardiolipins. NAPE-enriched liposomes were less prone to dye leakage [156]. Furthermore, NAPEs induced membrane fusion in the presence of Ca^{2+}-ions [157,158]. This effect was found to be NAPE-specific as other anionic phospholipids such as phosphatidylserine (PS) and phosphatidylglycerol (PG) did not stimulate membrane fusion [157]. The fusogenic properties of NAPE-liposomes have been exploited for drug delivery: Liposomes incorporating the neuroprotective ganglioside GM1 were enriched in the brains of treated rats [159]. *N*-palmitoyl-PE-enriched liposomes decreased phagocytosis in mouse macrophages, thereby contributing to the termination of inflammation [160]. In the rat jejunum, NAPE levels, specifically *N*-oleoyl-PE, were increased after feeding [44,161]. NAPE has been described as a lipid hormone that can decrease food intake, while exogenous NAPE was able to induce weight loss in mice [162]. However, following reports have contested this claim and point towards NAE metabolites as the cause of the observed anorectic effect [163,164]. Increased NAPE levels have also been found in a mouse model of Parkinson's disease [149,165]. A neuroprotective role for NAPEs was proposed, as deletion or silencing of NAPE-PLD was associated with improved survival of dopaminergic neurons [165]. NAPE-PLD was also found to modulate LRRK2 membrane localization in mouse brain, the most-prevalent risk gene in Parkinson's disease, possibly via regulation of NAPE levels [166]. Collectively, these studies provide evidence for a putative biological role of NAPEs in neurodegeneration and inflammation. The molecular mechanisms through which NAPEs exert their bioactivities should therefore be addressed. Genetic or pharmacological tools that enable modulation of NAPE metabolic enzymes may help to answer these questions.

3.2. PEA

In the 1950s, PEA was the first member of the NAE family to be identified in egg yolk, soybean lecithin and later in mammalian tissues [167,168]. It was immediately noted that PEA possessed anti-allergic and anti-inflammatory properties in a guinea pig model of anaphylactic arthritis [167]. Following reports revealed that PEA also produces anti-epileptic, neuroprotective, analgesic and anorectic effects [7,9,169–172]. During acute brain ischemia in rats, PEA levels increased 25-fold specifically in damaged brain areas [173]. Exogenous administration of PEA was neuroprotective in various disease models such as traumatic brain injury, Parkinson's and Alzheimer's disease [171,174,175]. Multiple biological targets have been identified for PEA that can explain its pharmacological effects [172,176]. The nuclear receptor peroxisome proliferator-activated receptor (PPAR)-α mediates its anti-inflammatory and analgesic effects [6,177]. Furthermore, PEA displays affinity for GPR119, a fat sensor in the gut, although OEA is regarded as a more potent agonist *in vivo* [4,178]. Another receptor through which PEA can exert its bioactive effects is GPR55, however, these findings have been questioned in later studies [3,179]. Today, PEA is marketed as a dietary supplement as well as a skin cream in many countries. Numerous clinical trials have been conducted with PEA for the treatment of pain, demonstrating that, overall, PEA produces few unwanted side effects and shows promise as an analgesic [8,180].

3.3. SEA

Although SEA and PEA differ just two methylene groups in chain length, SEA has been studied far less extensively. This may be due to the fact that unlike PEA, SEA did not present affinity for PPAR-α [2]. Nevertheless, SEA showed affinity for GPR119 and shares several bioactivities with PEA [4]. SEA produced anti-inflammatory effects in a mouse cutaneous anaphylaxis model [10]. In rat brain, SEA levels were similar to PEA and showed a comparable 25-fold increase upon brain ischemia [173]. Furthermore, oral administration of SEA in mice produced an anorectic effect, presumably through increase of liver stearoyl-CoA desaturase-1 (SCD-1) mRNA expression [11]. Recently, SEA showed neuroprotective effects against LPS-induced neuroinflammation in mice [181]. These findings indicate that SEA may have therapeutic properties and therefore the inclusion of this lipid species when determining NAE levels in cellular or tissue samples is justified.

3.4. OEA

OEA is a well-studied member of the NAE family, especially in the gastrointestinal system. Upon oral administration in mice, OEA demonstrated anorectic effects that were mediated by peripheral PPAR-α [12,182,183]. OEA has the highest potency for PPAR-α of all the NAEs [16]. Endogenous OEA levels in the small intestine were markedly reduced in starved mice and significantly increased after refeeding compared to free-feeding mice [9,44,184]. Accordingly, OEA is regarded as a satiety factor that is released upon food intake [161,185]. Both short-term and chronic high fat diets (HFDs) were found to decrease levels of OEA in rat jejunum, but not in other tissues such as brain and liver [16,186]. It was proposed that reduction in OEA levels may cause the reduced satiety and hyperphagia as witnessed in obesity [176,185]. Furthermore, intraperitoneal (i.p.) administration of OEA reversed striatal HFD-associated dopamine deficiency in mice in a PPAR-α-dependent manner [187]. Histamine originating from mast cells, a type of immune cell mostly known for its role in allergy, was found to enhance hepatic levels of OEA during fasting in mice via activation of the H_1 receptor [188]. This resulted in enhanced ketogenesis, a process providing energy during times of low nutrient availability. In accordance, OEA-induced ketogenesis was attenuated by deletion of NAPE-PLD [188].

OEA has also been implicated as a regulator of hedonic homeostasis,

dampening the self-administration of alcohol and nicotine in rodents and monkeys and acting through PPAR-α [189,190]. Multiple synthetic PPAR-α agonists have been evaluated in the clinic for the treatment of substance use disorders, however, this has produced mixed results [191]. The limited translation of animal to human studies may be explained by sex-related variability, the lack of drug potency or PPAR isoform selectivity and the absence of evidence of target engagement [191]. Besides PPAR-α, OEA showed *in vitro* affinity for GPR119, a receptor that modulates feeding behavior [4]. Nevertheless, OEA produced anorectic effects in both GPR119 WT and KO mice, indicating that *in vivo*, this activity is not required for satiety [192]. Similar to PEA, administration of OEA in rodents was reported to generate anti-inflammatory, neuroprotective and analgesic effects [13–15]. These are likely mediated by activation of PPAR-α, although also PPAR-α-independent mechanisms have been described [177,193–195]. In rat brain, OEA concentrations were found to be roughly one-third of PEA and SEA levels and showed a comparable 30-fold increase upon cerebral ischemia [173]. A putative neuroprotective role for the NAE family was therefore hypothesized, acting via multiple molecular mechanisms [171,196].

3.5. LEA

LEA has received less attention compared to the other NAE family members, even though it possesses similar bioactivities as OEA and PEA. Importantly, endogenous levels of LEA in rat jejunum were found to be 4 to 6-fold higher than OEA, PEA and SEA upon fasting and refeeding [184]. Intraperitoneal administration of LEA in rats elicited a reduction of food intake, which was dependent on PPAR-α activation and was comparable to OEA and PEA [9,18]. Because of the high intestinal levels of LEA, it was proposed that the anorectic effect could also in part be mediated through GPR119, for which LEA shows equal activity as OEA [17]. This has yet to be confirmed in genetically deleted GPR119 rodents. In a rat stroke model, treatment with exogenous LEA demonstrated a neuroprotective effect [19]. Although endogenous LEA levels in rat brain accumulated 30-fold upon brain ischemia similar to PEA and SEA, the absolute concentrations were just 1% to 5% of saturated NAEs, suggesting only a minor role in the brain *in vivo* [173]. Recently, also α-linolenoylethanolamide (ALEA, 18:3-ω3) was shown to suppress food intake in rats [197]. Interestingly, after i.p. administration ALEA levels were 20-fold enhanced in brain, but this was not the case for LEA, suggesting an active brain uptake mechanism [197]. Taken together, these studies highlight the need to further explore the biological role of these NAEs, which may have important roles in satiety and energy metabolism.

3.6. AEA

AEA or anandamide has been studied most extensively of all the NAE family members. In most tissues, AEA levels are 10 to 100 times lower than PEA, SEA and OEA [198,199]. However, unlike other NAEs, AEA can activate the cannabinoid (CB_1)-receptor [20]. The CB_1 receptor is one of the most abundant GPCRs in the mammalian brain and is activated by (-)-Δ [9]-tetrahydrocannabinol (THC), the psychoactive component of cannabis. As a result, AEA and 2-arachidonoylglycerol (2-AG), a second endogenous CB_1 receptor agonist, are termed endogenous cannabinoids or endocannabinoids. Both AEA and 2-AG are partial agonists for the CB_1 receptor with submicromolar potency [21]. Absolute levels of AEA are generally lower than 2-AG in brain, although large differences have been observed depending on the method of analysis [200]. In bulk extracts of whole or region-specific rat brain lysates, AEA levels were typically 100- to 1000-fold lower compared to 2-AG, but this difference was only 2- to 8-fold when measured via *in vivo* microdialysis [200]. It was proposed that this is caused by the more rapid post-mortem increase of 2-AG compared to AEA [200]. AEA is regarded as a tonic neuromodulator – *i.e.*, it continuously signals in the basal state – which is released by neurons upon Ca^{2+}-stimulation and is quickly degraded by FAAH [23,201–203]. Although AEA was initially described as a retrograde neurotransmitter, NAPE-PLD is localized presynaptically and FAAH postsynaptically, suggesting that AEA may function as an anterograde signaling lipid [204]. AEA can also act as an intracellular messenger, formed upon an influx of Ca^{2+}-ions via activation of the G_q-pathway [205].

The word '*ananda*' – meaning bliss in Sanskrit – was aptly chosen, as increased AEA signaling produces analgesic, anxiolytic and antidepressant effects through CB_1 receptor signaling in the brain [26,206,207]. Conversely, acute and repeated stress exposure in rats afforded a decrease in AEA content in the amygdala, mediated by enhanced FAAH activity [208]. Stressed rats showed an inverse correlation between amygdalar AEA and plasma stress hormone levels (corticosterone) [209]. Diminished brain AEA signaling upon repeated stress increased secretion of corticosterone [210]. In contrast, repeated stress elevated amygdalar 2-AG levels, which attenuated hypothalamic-pituitary-adrenal (HPA) axis activation. AEA and 2-AG are therefore hypothesized to be the effectors of HPA-axis signaling in the brain, while having functionally distinct roles [210,211]. In addition to its roles in modulating fear and stress behavior, AEA was reported to promote neuroprotection, memory formation and food intake via brain CB_1 receptor activation [27,28,212,213]. Pharmacological studies in mice indicated that exogenous AEA produces cannabimimetic responses, which are rapid in onset, but shorter and less potent than THC, presumably due to its fast catabolism [214]. Correspondingly, FAAH KO mice were supersensitive to AEA treatment [88]. Exogenous AEA administration in rats generated a central CB_1-receptor-dependent orexigenic (appetite-stimulating) effect similar to THC [24].

AEA has also been linked to CB_1 receptor signaling in the periphery, for instance in adipocytes, the female reproductive system and skin tissue where it is involved in energy expenditure, implantation and epidermal differentiation, respectively [215,216]. Interestingly, peripheral CB_1 receptor activation is implicated in food intake and intestinal AEA levels were found to be highly increased in starved mice [217]. The analgesic effects of AEA were also observed in the periphery, where peripheral blockade of FAAH produced antinociception via a CB_1 receptor-dependent mechanism [218]. Notably, the antinociceptive effect of AEA increased synergistically when combined with PEA in a mouse model of peripheral pain [25].

AEA has been described as a partial agonist with submicromolar potency for the CB_2 receptor, which is primarily expressed in the immune system and is involved in the inflammatory response [21,219–221]. Even though 2-AG has been suggested to be the true endogenous CB_2 receptor ligand because of its generally higher levels compared to AEA and its ability to act as a full agonist [21,222], also AEA was reported to modulate inflammation via activation of the CB_2 receptor by reducing pro-inflammatory cytokines in cells [215,223].

Besides the cannabinoid receptors, AEA can activate the transient receptor potential vanilloid 1 (TRPV1) ion channel [22]. AEA has therefore been termed an endovanilloid [224,225]. TRPV1, also known as the capsaicin receptor, is an important player in pain perception and is localized at peripheral sensory neurons [226]. Evidence is accumulating that TRPV1 is expressed in the CNS as well [227,228]. The activation of TPRV1 by AEA causes an cellular influx of Ca^{2+}-ions and has been linked to locomotor depression, hyperalgesia under inflammatory conditions, vasodilation and hypothermia [205,226].

3.7. DHEA

Over the past decade, DHEA has come into view as a member of the NAE family with unique properties in neuronal signaling [31]. As such, the name synaptamide was coined for its ability to induce neurogenesis [229]. DHEA was found to have nanomolar affinity for GPR110 (also known as ADGRF1), an adhesion-type GPCR highly expressed in the hippocampus [30]. DHEA generated neurite outgrowth and synapse

formation in neurons derived from WT mice, but not from GPR110 KO littermates. GPR110 KO mice showed reduced spatial memory and object recognition, but have yet to be profiled completely. Recent structural studies have provided further insight into how DHEA binds and activates the GPR110 receptor [230,231]. DHEA has also been reported to have anti-inflammatory properties [32,232]. In LPS-treated microglia and macrophage cells, DHEA reduced pro-inflammatory cytokines or eicosanoids, respectively [32]. In addition, LPS-induced neuroinflammation in mice was significantly decreased after i.p. administration of DHEA and was dependent on GPR110 [233]. In a mouse model of mild traumatic brain injury, DHEA attenuated disease-associated optic tract neuropathology and visual dysfunction in WT, but not in GPR110 KO mice [234]. Brain DHEA levels are generally 2- to 10-fold higher than AEA, while the opposite is true for plasma [70,71,198,235]. Furthermore, brain DHEA concentrations are linked directly with brain content of docosahexaenoic acid (DHA, 22:6), an ω-3 polyunsaturated fatty acid [236–238]. DHA is preferably acquired from the diet, but can also be synthesized from the essential fatty acid α-linolenic acid (18:3-ω3) [239]. The biosynthesis of DHEA is considered to follow the same route as other NAEs via formation of NAPE and hydrolysis by NAPE-PLD, which was confirmed in two NAPE-PLD KO mouse strains [43,71]. A third NAPE-PLD KO mouse strain did not show a reduction of brain DHEA and displayed elevated levels of brain DHEA upon administration of a fish oil diet rich in DHA [70,240]. Also inhibition of NAPE-PLD by a specific inhibitor LEI-401 did not reduce DHEA levels in mouse neuroblastoma cells [73]. This suggests that alternative pathways are involved in DHEA production in the brain.

Few studies have looked at the physiological role of DHEA in the periphery. It has been reported that under normal conditions, peripheral tissue levels of DHEA often exceed AEA, for example in the heart, kidney, jejunum and skin [72,241]. GPR110 was found to be expressed in various peripheral organs including kidney, prostate and lung, which points to a possible role of DHEA signaling in these tissues [242].

3.8. Oxidative metabolites of AEA, DHEA and EPEA

3.8.1. AEA

Metabolism of AEA by COX-2 affords prostamides $PGF_{2\alpha}$-EA, PGE_2-EA and PGD_2-EA, which were produced in FAAH knockout mice (Fig. 2) [243]. These oxygenated AEA derivatives generally lack affinity for the corresponding prostanoid or cannabinoid receptors, but have biological activities of their own. For instance, whereas $PGF_{2\alpha}$ activates the PGF_2 receptor, $PGF_{2\alpha}$-EA activates a heterodimer of the PGF_2 receptor and one of its splice variants [110]. This effect is emulated by a synthetic derivative of $PGF_{2\alpha}$-EA, bimatoprost, a drug used for the treatment of glaucoma and eyelash hypotrichosis [244].

PGE_2-EA reduced tumor necrosis factor alpha (TNFα) production in a cAMP-dependent pathway in LPS-stimulated human PBMCs [112], and inhibited the activity of the IL-12p40 promoter in LPS- and IFNγ-stimulated microglia cells [111]. PGE_2-EA is relatively stable, with a half-life of over 5 h in human and rat plasma. It is not hydrolyzed, but instead slowly dehydrated into its isomer PGB_2-EA. [245] PGD_2-EA and PGE_2-EA induced cell death in cancer cell lines [113,114].

Oxidation products 12-HETE-EA and 15-HETE-EA, formed by 12- and 15-LOX respectively, have not been detected as endogenous metabolites yet. However, LOX inhibitors abrogate some of the physiological effects of endocannabinoids, suggesting that the transformation by 12/15-LOX takes place and that these products have biological activity [116]. For example, 15-HETE-EA is a potent agonist of recombinant rat TRPV1 [116], and spinal administration of the FAAH inhibitor URB597 caused anti-allodynic and anti-hyperalgesic effects in neuropathic rats. These effects were blocked by a selective TRPV1 receptor antagonist and significantly attenuated by a 12/15-LOX inhibitor [116], which suggests that 12- and 15-HETE-EA may be partly responsible for the activity of AEA on the TRPV1 receptor [246,247]. In addition, 12-HETE-EA has been shown to reduce cytotoxic edema in an animal model of excitotoxicity, whereas 15-HETE-EA enhanced the neuroprotective effect of AEA [248].

Although 15-HETE-EA has lower affinity for the CB_1/CB_2 receptors, 12-HETE-EA has similar affinity as AEA and the hydroxylated metabolites are more resistant to FAAH hydrolysis [108,249,250]. Moreover, in an *in vitro* system, 15-HETE-EA was found to be an activator of NAPE-PLD and a competitive inhibitor of FAAH, indicating lipoxygenase metabolites of AEA are modulators of the endocannabinoid system (ECS) [249–251].

Bioactive metabolites of arachidonic acid include CYP450-produced epoxyeicosatrienoic acids (EETs), which are key players in vascular function and disease due to their protective vasodilatory, anti-inflammatory and pro-angiogenic effects [252,253]. EETs may act through the transient receptor potential vanilloid-4 (TRPV4) cation channel, the PPAR nuclear receptors as well as some putative GPCRs [254]. When AEA is used as a substrate by CYP450 enzymes various epoxide and hydroxylated derivatives are formed such as 20-HETE-EA. This process is mediated by CYP3A4, CYP2D6 or CYP4F2 [117,255,256], which are present in human liver or brain microsomes [256,257].

Interestingly, the CYP450-produced 5,6-epoxyeicosatrienoic acid ethanolamide (5,6-EET-EA) has a greatly increased affinity for the CB_2 receptor, as demonstrated by measuring cAMP levels in cells expressing CB_2 receptors [117] as well as direct binding assays [255]. Moreover, 5,6-EET-EA also rapidly activates TRPV4 at low nanomolar concentrations [119].

3.8.2. LEA

Soy bean lipoxygenase and human recombinant LOX-15 were found to oxidize LEA, resulting in 13-HODE-EA (Fig. 3) [258–260]. Human neutrophils and eosinophils converted LEA into 13-HODE-EA due to their high lipoxygenase expression. 13-HODE-EA was detected in human saliva and skin [120,260]. 13-HODE-EA lacked activity on the cannabinoid receptors as well as PPARα and γ, but was found to be a weak agonist for TRPV1 and an inhibitor of FAAH [120].

3.8.3. DHEA and EPEA

During inflammation, immune cells can be activated and directed to the site of inflammation by chemoattractants such as leukotriene B4 (LTB4), an oxygenated derivative of arachidonic acid. In contrast, lipoxygenase products of DHEA, 10,17-dihydroxydocosahexaenoyl ethanolamide (10,17-diHDHEA) and 15-hydroxy-16(17)-epoxydocosapentaenoyl ethanolamide (15-HEDPEA) reduced leukocyte motility and inhibited formation of platelet-leukocyte aggregates at concentrations as low as 10 pM (Fig. 3) [121]. The metabolites acted as CB_2 agonists with sub-nanomolar potency. Moreover, 15-HEDPEA was protective in a mouse model of organ reperfusion injury [121].

DHEA exerted some of its anti-inflammatory properties through competitive or uncompetitive inhibition of COX-2, but also served as a COX-2 substrate [232]. Oxidative metabolites of DHEA from COX-2 included 13- and 16-HDHEA, which had moderate anti-inflammatory effects by reducing production of TNFα, interleukin (IL)-1β and interleukin-1 receptor antagonist IL-1Ra, but did not affect nitric oxide (NO) and IL-6 release in RAW264.7 macrophages [122].

CYP450 enzymes metabolized DHEA and EPEA into bioactive metabolites. Epoxide derivatives of DHEA and *N*-eicosapentaenoylethanolamine (EPEA) were present in rat brain and peripheral organs. Moreover, the metabolites were produced by incubation of DHEA or EPEA in rat brain homogenates, as well as recombinant human CYP2J2 and activated BV-2 microglial cells [123]. Epoxidation of the terminal alkene of DHEA and EPEA was preferred and resulted in 19,20-epoxydocosapentaenoic acidethanolamide (EDP-EA) and 17,18-epoxyeicosatetraenoic acidethanolamide (EEQ-EA), respectively. These metabolites significantly reduced pro-inflammatory markers IL-6 and NO in BV-2 microglia, while inducing expression of anti-inflammatory marker IL-10. These effects could be partially blocked by inhibition of the CB_2

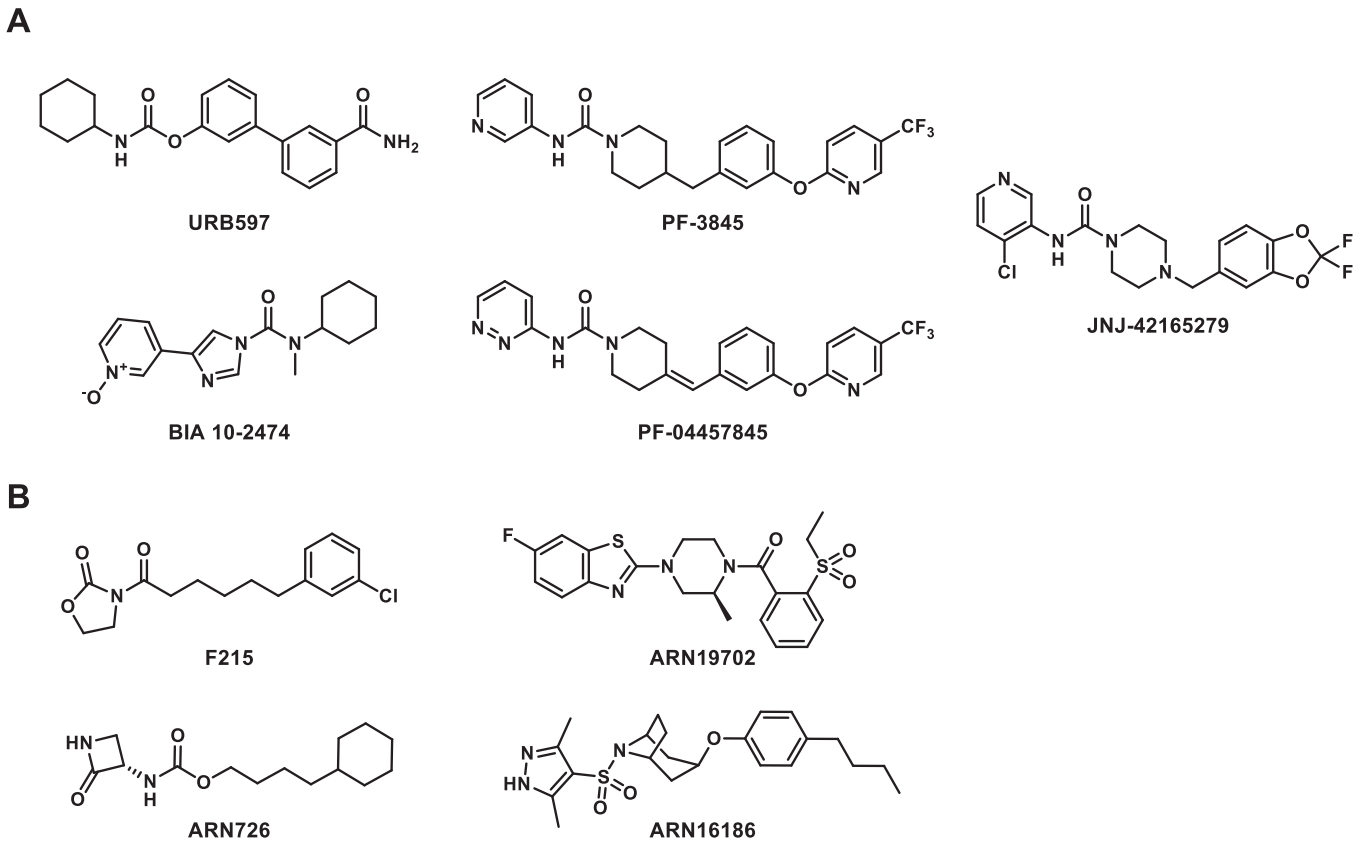


Fig. 4. Structures of selected *in vivo*-active inhibitors of A) fatty acid amide hydrolase (FAAH) or B) *N*-acylethanolamine acid amidase (NAAA).

receptor and PPARγ and several of the epoxide derivatives were shown to activate CB_1 and CB_2 receptors.

Moreover, 17,18-EEQ-EA and 19,10-EDP-EA mediated platelet aggregation, vasodilation in bovine coronary arteries and antiangiogenic effects in human microvascular endothelial cells. As these DHEA- and EPEA-derived epoxides were found at concentrations comparable to those of AEA and 14,15-EET, they are expected to play an important role in neuroinflammation and cerebrovascular diseases [123].

Endogenous DHEA epoxide production and anti-tumorigenic properties have been investigated in an osteosarcoma (OS) model [124]. Different regioisomers reduced cell viability and migration behavior of OS cell lines. Although 10,11-EDP-EA was found to activate both cannabinoid receptors, its anti-tumorigenic properties were only partially mediated by CB receptor signaling. Notably, the epoxides were detected in higher levels in the lungs of mice with metastatic lesions compared to healthy tissue, indicating that they might play a physiological role in tumorigenesis and cancer metastasis [124].

4. Pharmacological modulation of NAE metabolism

As outlined in the prior sections, NAEs possess bioactivities that may be used for therapeutic intervention. Moreover, in certain pathological conditions NAE levels are disrupted, for example in cancer, obesity and neurodegenerative diseases and have been linked to disease progression and severity [261–263]. Modulating the NAE tone could therefore be a viable treatment strategy for these pathologies. However, due to the polypharmacology of NAEs acting on multiple receptors that can have opposing outcomes, it is not always clear whether NAE levels should be enhanced or reduced [264]. In the following section, an overview will be provided of the therapeutic potential of blockade of NAE degradation as well as its biosynthesis. The main conclusions are depicted in Fig. 8.

4.1. Inhibition of NAE hydrolysis

4.1.1. FAAH

After the discovery of the NAE-hydrolyzing enzyme FAAH in 1995, it became apparent that increasing NAE levels by genetic or pharmacological disruption of FAAH had profound effects on ECS signaling [265]. To date, multiple research groups and pharmaceutical companies have developed *in vivo* active and brain penetrant FAAH inhibitors (Fig. 4A) [266,267]. Upon administration in rats or mice, the irreversible FAAH inhibitors URB597, PF-3845 and PF-04457845 increased AEA levels with 3- to 7-fold in brain and plasma, while PEA and OEA were also elevated with 8- to 20-fold in the same tissues [26,89,268]. Limited data are available with respect to other NAEs after FAAH inhibition, although one study reported that PF-3845 could similarly elevate SEA, LEA and DHEA levels with 5- to 20-fold in the brain, but in plasma only LEA and DHEA were increased [269]. Pre-clinical research in rodents revealed that inhibition of FAAH may be exploited for treatment of inflammatory or neuropathic pain, acting via central or peripheral CB_1 and CB_2 receptor activation [89,218,270,271]. Furthermore, pharmacological FAAH disruption has shown promise for treating anxiety [26], depression [206], post-traumatic stress disorder (PTSD) [272], Parkinson's disease [273], nausea [274], skin inflammation [275], pruritus [276], inflammatory bowel disease [276], glaucoma [277], hypertension [278], traumatic brain injury [279], HIV-associated neurocognitive disorders [280] and multiple sclerosis-associated spasticity [281].

Several FAAH inhibitors have been tested in Phase I and II clinical trials with mixed success [282,283]. The selective oral inhibitor PF-04457845 was found to be well tolerated in healthy volunteers, completely blocked FAAH activity in isolated leukocytes and increased plasma AEA (10-fold), LEA (9-fold), OEA (6-fold) and PEA (3.5-fold) concentrations [284]. However, in a subsequent Phase II clinical trial for osteoarthritic pain of the knee, PF-04457845 did not produce analgesia

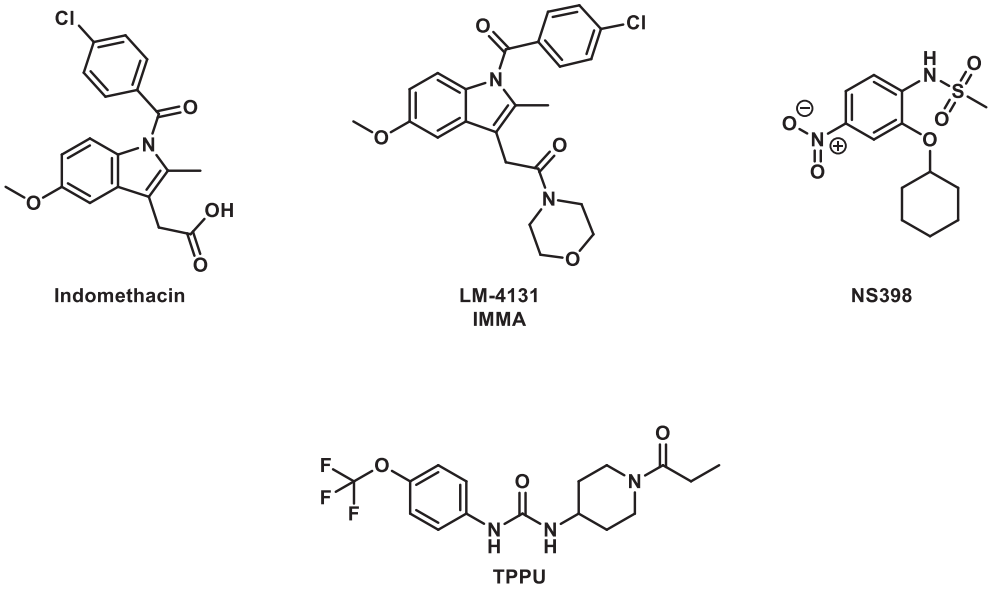


Fig. 5. Structures of COX-2 and sEH inhibitors.

[285]. In 2016, the oral covalent FAAH inhibitor BIA 10-2474 (Fig. 4A) was tested in healthy volunteers in a Phase I clinical study, which led to the tragic death of one individual and mild-to-severe neurological symptoms in four others [286]. It was later revealed that BIA 10-2474 displayed off-target activities against multiple serine hydrolases in the CNS, whereas PF-04457845 was highly selective for FAAH and did not present adverse effects in multiple clinical studies [287]. Accordingly, the observed neurotoxic side effects of BIA 10-2474 are presumed not to be caused by inhibition of FAAH [283].

In the last few years, a number of clinical successes with FAAH inhibitors, predominantly in the fields of substance use and psychiatric disorders, have sparked newfound interest [288]. In a Phase II clinical study, PF-04457845 was effective for the treatment of cannabis withdrawal symptoms [289]. More recently, PF-04457845 was found to enhance recall of fear extinction memory and reduced stress in healthy adults in a Phase I clinical trial [290]. Another oral FAAH inhibitor, JNJ-42165279, failed to increase fear extinction memory recall in healthy males, but did elicit anxiolytic effects in individuals with social anxiety disorder (SAD) in two separate clinical trials [291,292]. Currently, JNJ-42165279 is being evaluated in patients with PTSD in a Phase 1/2 trial (EudraCT 2020-001965-36), of which the results have yet to be reported [293]. Collectively, these results demonstrate the first successful clinical translation of FAAH inhibitors and provide encouraging evidence for their use in psychiatric disorders such as PTSD and SAD [293–296].

4.1.2. NAAA

In recent years, inhibitors of the other NAE-hydrolyzing enzyme NAAA have come to the foreground [297,298]. Several *in vivo* active NAAA inhibitors have been reported, showing encouraging results for the treatment of inflammatory and neuropathic pain, allergic dermatitis and multiple sclerosis [98,299–301]. Considerable evidence points towards a PPAR-α-mediated mechanism [282,300,302]. First-generation irreversible NAAA inhibitors ARN726 and F215 (Fig. 4B) were able to increase PEA and OEA concentrations 2- to 4-fold in lungs of mice after an inflammatory stimulus, but not in naïve mice [301,303]. It is possible that these compounds elicited an inflammation-specific effect, although their low plasma stability and fast clearance could also explain the observed results [266]. Importantly, the increase of OEA illustrates the difference between *in vivo* and *in vitro* NAAA activity, where high preference towards hydrolysis of PEA is seen [95]. A second-generation reversible NAAA inhibitor (ARN19702, Fig. 4B) presented improved drug-like properties and was able to elevate brain PEA and OEA levels (2-fold) of healthy mice, but not AEA [98]. Very recently, Piomelli and co-workers reported that NAAA regulates the transition of acute to chronic pain in mice, which could be alleviated by genetic deletion of NAAA or treatment with ARN19702 or ARN16186 – a third-generation reversible NAAA inhibitor (Fig. 4B) [304]. NAAA ablation or ARN16186 administration also reduced disease progression and preserved locomotor activity in a mouse model of multiple sclerosis [305,306]. A newly described NAAA inhibitor with oral bioavailability (ARN19689) will be a prime candidate to be tested in these inflammatory disease models [307].

4.2. Inhibition of NAE oxidative metabolism

Inhibition of FAAH does not only enhance the effects of NAEs by preventing their degradation, it also increases the available NAE pool for oxidative metabolism. This is exemplified by the increase of PG-EA in FAAH KO mice [243]. In addition, substantial evidence points to COX-2 as a regulator of endocannabinoid signaling and tone [131,308]. COX-2 KO mice had basally elevated brain AEA levels [309]. Moreover, pharmacological inhibition of COX-2, but not of FAAH, potentiated retrograde endocannabinoid synaptic signaling in the hippocampus [310]. NSAIDs inhibited the metabolism of AEA in rat cerebellar membrane preparations [311], extended the stability of exogenous AEA in mouse brain [131], and resulted in endocannabinoid-mediated spinal antinociception [312,313]. Notably, peripheral antinociceptive effects of AEA and NSAIDs were synergistic [314], and ibuprofen interacted with AEA in both acute and inflammatory pain [315].

These results indicate that COX-2 inhibitors are able to modulate AEA signaling, but their effects cannot be easily disconnected from inhibition of pro-inflammatory eicosanoids. An attempt to distinguish between the two pathways has been made by development of substrate-selective COX-2 inhibitors, which reduced AEA oxidation but did not prevent prostaglandin formation [308]. This led to the development of LM-4131, a morphole amide derivative of indomethacin= (Fig. 5) [309]. LM-4131 dose-dependently increased brain AEA concentrations in mice, as did parent compound indomethacin and the COX-2 selective inhibitor NS398. LM-4131 did not increase endocannabinoid concentrations in COX-2 KO mice. However, in a seperate study, LM-4131 did not elevate the endogenous levels of AEA and 2-AG, but did alleviate hyperalgesia and mechanical allodynia in a chronic constriction injury (CCI) mouse

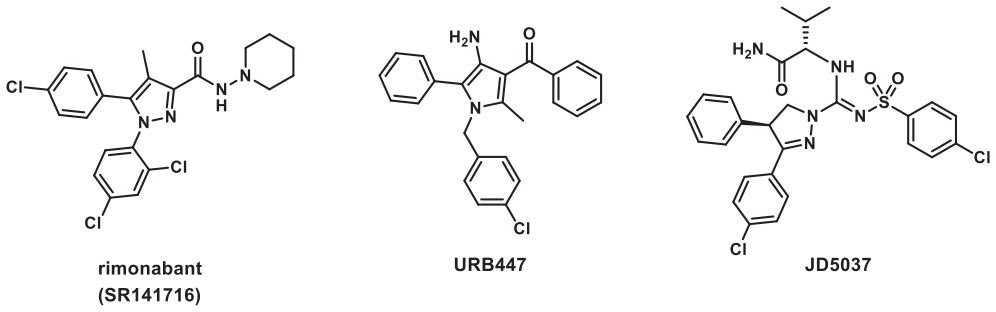


Fig. 6. Structures of selected central (rimonabant) or peripherally restricted (URB447, JD5037) CB_1 receptor antagonists.

model [316]. These effects were found to be partially dependent on the CB_2 receptor-using a CB_2 receptor antagonist. Overall, substrate-selective COX-2 inhibitors may function through modulation of the ECS, but it remains unclear whether this occur through an increase of AEA levels by inhibition of its oxidative metabolism [309,317].

Epoxide derivatives of fatty acids are mainly broken down by the enzyme soluble epoxide hydrolase (sEH) thereby producing their corresponding diols, which are generally less biologically active [318]. Recently, attention has focused on sEH inhibition in the CNS, where EETs exert neuroprotective and anti-inflammatory effects. Development towards sEH inhibitors has afforded orally bioavailable compounds such as 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl)urea (TPPU), a subnanomolar inhibitor with low to moderate blood-brain barrier penetration (Fig. 5) [319]. Highlighting the significance of lipid epoxides in the CNS, sEH inhibition by TPPU decreased reperfusion injury after focal cerebral ischemia in rats, significantly reduced infarction, the inflammatory response and improved sensorimotor function [320,321]. CYP450 metabolites of NAEs may be partly responsible for the neuroprotective effects of sEH blockade, as AEA and EPEA epoxidation increased CB_2 receptor affinity [117,255], and EPEA and DHEA epoxides were found to have CB-receptor-dependent anti-inflammatory effects. Interestingly, dual inhibition of sEH and FAAH gave a synergistic antinociceptive effect in animal models of inflammatory and neuropathic pain [322].

4.3. Inhibition of NAE biosynthesis

Blocking NAE biosynthesis with pharmacological agents is a strategy in endocannabinoid research with few precedent due to a lack of selective and *in vivo*-active inhibitors [323]. Nevertheless, there is substantial evidence that reducing the NAE tone could be beneficial in pathological conditions such as obesity, metabolic syndrome, cancer and liver cirrhosis [264]. The potential net effect of inhibiting NAE production would be indirect antagonism of the respective NAE receptors. Because the cannabinoid receptors, PPAR-α, TRPV1, GPR55, GPR110 and GPR119 have additional endogenous agonists besides the NAEs, this will likely lead to only partial receptor deactivation [283]. We and others have recently described the first generation NAE biosynthesis inhibitors that target NAPE-PLD and PLAAT enzymes (Fig. 7) [57,73,324–327]. Here, different conditions are outlined where decreasing NAE levels could be of therapeutic value.

4.3.1. Obesity and metabolic syndrome

The endocannabinoid system (ECS) is a key player in energy balance and food intake, both in the CNS and the periphery [328,329]. The centrally active CB_1 receptor antagonist rimonabant (Acomplia®, Fig. 6) was clinically approved for treatment of obesity and metabolic syndrome as it induced significant weight-loss, decreased food intake and improved insulin resistance [330–332]. Unfortunately, patients treated with rimonabant suffered from depression-like side effects leading to its withdrawal from the market [333,334]. Peripherally restricted CB_1 receptor antagonists have shown comparable pre-clinical efficacy and are currently being pursued as potential anti-obesity drugs without psychiatric side effects [335,336]. Alternatively, inhibiting NAE biosynthesis could be a possible therapeutic strategy. It has become increasingly clear from human studies and animal models that endocannabinoid and NAE signaling is disrupted during diet-induced obesity and metabolic disease [263,337]. Mice receiving a high fat diet for 18 weeks showed sustained elevation of plasma NAE levels including AEA, as well as increased expression of the NAE biosynthetic enzyme NAPE-PLD in brown adipose tissue [338]. Furthermore, obese mice expressed elevated levels of AEA, NAPE-PLD and the CB_1 receptor and decreased levels of FAAH in adipose tissues [339]. In adipocytes, CB_1 receptor activation is associated with energy storage by increasing fatty acid uptake and lipogenesis and decreasing mitochondrial biogenesis, resulting in attenuated browning of white adipose tissue [329,340]. Mice fed a high fat diet for 3 weeks developed liver steatosis and showed greatly increased hepatic AEA levels, but not 2-AG [341]. This was credited to reduced FAAH activity, although NAPE-PLD activity was not determined. In the small intestine of rodents administered a high fat diet for 1 week, normal OEA mobilization after feeding was disrupted, possibly explaining the diminished satiety and hyperphagia observed in diet-induced obesity [16,176,186]. Sham feeding of a lipid-based meal to rats for 5 days resulted in an increase of jejunal AEA and 2-AG levels, which was dependent on signaling of the vagus nerve [342]. Enhanced NAPE-PLD and reduced FAAH activities in the jejunum were reported, yet interestingly, OEA levels were not affected. Peripheral CB_1 receptor blockade (URB447, Fig. 6) attenuated fat sham feeding, which supports the hypothesis that endocannabinoids are released upon high fat food consumption and drive a positive feedback loop via CB_1 receptor signaling [342]. In pancreatic islets, AEA content and NAPE-PLD gene expression was enhanced in fatty diabetic versus lean rats [343]. The AEA-induced apoptosis of insulin-producing beta cells via peripheral CB_1 receptor activation was proposed to enable the progression of type II diabetes. Accordingly, chronic treatment with the peripherally restricted CB_1 receptor antagonist JD5037 (Fig. 6) reversed islet elevation of AEA levels and NAPE-PLD expression and restored blood glucose levels to normal in overweight diabetic rats, although they remained insulin resistant [343].

In humans, an analogous relationship between NAEs and obesity has been described. In a small human study (24 subjects), circulating AEA levels, but not 2-AG, peaked before a meal and significantly decreased postprandially in lean, but not in obese individuals [344]. A larger human study (328 subjects) revealed that obesity is associated with an increased AEA tone in plasma, as well as altered circulatory PEA/AEA and OEA/AEA ratios, indicative of enhanced appetite and diminished satiety [345]. In the same cohort, plasma 2-AG levels were not found to be upregulated in obese individuals [346]. In another large human trial (997 subjects), circulating AEA concentrations were also associated with BMI [347]. Furthermore, AEA correlated with non-alcoholic steatohepatitis (NASH) disease severity and was therefore proposed as a biomarker [347]. Also in subcutaneous white adipose tissue, AEA was

found to be elevated in obese individuals (75 subjects), while FAAH gene expression was reduced in this tissue [348]. Of note, two earlier reports did not observe the AEA increase in human visceral or subcutaneous fat tissue [349,350]. Three studies described that humans carrying the rs324420 variant of FAAH (C385A, p.Pro129Thr) were associated with increased obesity [351–353]. This mutant causes a reduction of FAAH activity due to an increased sensitivity for its proteolysis, thereby elevating levels of AEA [354]. These combined clinical and pre-clinical data suggest that lowering AEA concentrations may offer a therapeutic opportunity for treatment of obesity, metabolic syndrome, type II diabetes and liver steatosis. At the same time, it is not yet known which organs contribute to circulatory NAEs, which needs to be addressed [355].

Several studies have looked at the role of NAPE-PLD in energy metabolism. In a large human cohort, a common NAPE-PLD haplotype was described to be protective against severe obesity [356]. Mice with a genetic deletion of *NAPE-PLD* presented a reduced food intake and overall leaner phenotype than their WT littermates [240]. Of note, these effects were not observed in a different NAPE-PLD KO strain [357]. On the other hand, FAAH ablation in mice increased energy storage, body weight and adipose tissue and promoted the appetite-stimulating effect of AEA, rather than the OEA-induced satiety [358]. These studies suggest that inhibition of NAPE-PLD may constitute a potential treatment for metabolic syndrome and obesity. Conversely, when investigating the specific contribution of NAPE-PLD in various tissues the results were less clear. Mice with a specific deletion of *NAPE-PLD* in adipose tissue had a predisposition for obesity while receiving a normal diet [359]. When administered a high fat diet for 8 weeks, adipocyte NAPE-PLD KO mice showed increased body weight gain compared to WT. Notably, in both diets, levels of the anorectic OEA, PEA and SEA were decreased in NAPE-PLD KO adipose tissue, but not of orexigenic AEA. A similar NAE profile was observed in WT mice receiving a high fat versus a control diet [359]. Conditional KO of intestinal NAPE-PLD in mice induced hyperphagia upon initial high fat diet administration and exacerbated fat mass accumulation compared to WT mice [360]. When receiving a normal diet, intestinal NAPE-PLD KO mice displayed reduced intestinal levels of AEA, OEA, PEA and SEA. In contrast, after 16 weeks of high fat diet, jejunal NAE concentrations in WT and intestinal NAPE-PLD KO mice did not significantly differ [360]. Recently, a third KO mouse model looked at hepatocyte-specific deletion of *NAPE-PLD*, where mice fed a normal diet developed a high fat diet-like phenotype and trended towards increased total and lipid liver mass compared to WT. [361] A high fat diet partially exacerbated the obese phenotype in the KO mice. Under normal diet conditions OEA, LEA and DHEA were significantly decreased in the liver by 20% compared to WT mice, but not AEA and PEA. A more striking 50% depletion was observed for monoacylglycerols, including 1/2-AG, specific oxysterols and bile acids, suggesting that hepatocyte NAPE-PLD regulates multiple bioactive lipid families in the liver [361]. Collectively, these data indicate that NAPE-PLD functioning in the gut and adipose tissue is altered during obese conditions. It remains to be determined what the effect of global or peripheral pharmacological NAPE-PLD blockade will be on energy balance and food intake in metabolic syndrome and obesity.

4.3.2. Cancer

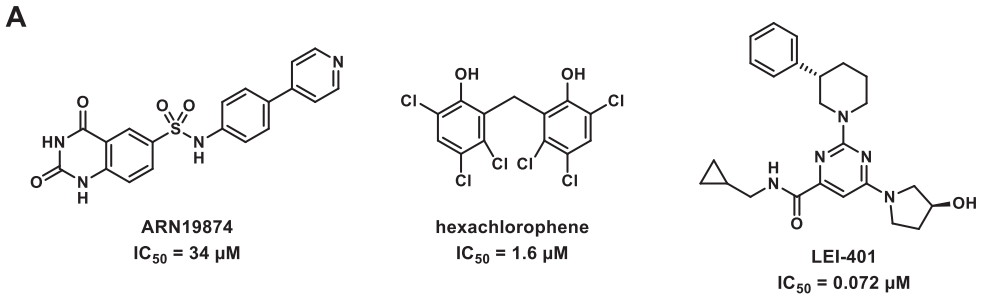
Multiple studies have reported disrupted NAE levels in various forms of cancer and associations between NAE receptors and tumor proliferation [362–364]. For example, elevated NAE levels have been observed in cancers of the bladder [365,366], prostate [365], endometrium [367], colon [368] and liver [369]. Heightened CB_1 receptor expression and an association with disease severity was described for colorectal [370,371], prostate [372], renal [373], hepatic [369] and ovarian [374] cancer. Furthermore, in human tissue samples of endometrial cancer NAPE-PLD protein expression was upregulated and FAAH downregulated correlating with advanced disease [354]. This was accompanied by a 3-fold increase in tissue levels of AEA and PEA, but not OEA, compared to benign tissue [367]. As such, the authors proposed AEA and PEA as putative biomarkers of endometrial cancer. It is yet to be determined whether these NAEs play a beneficial or detrimental role in this disease. Notably, the same authors recently reported that GPR55 expression was similarly enhanced in endometrial cancer, but not CB_1 and CB_2 receptors [375,376].

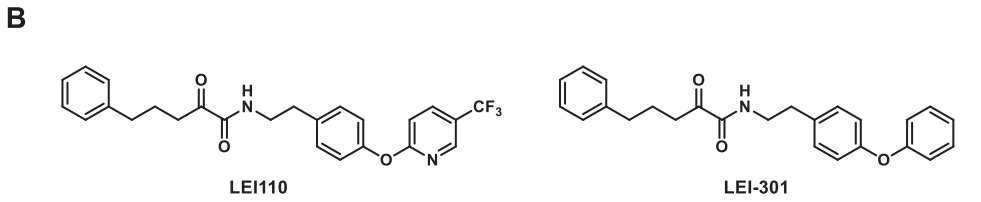
In hepatocellular carcinoma (HCC) hepatic CB_1 receptor and NAPE-PLD expression as well as AEA concentrations were found to be elevated both in humans and mice [369]. Treatment with the peripherally restricted CB_1 receptor antagonist JD5037 or CB_1 receptor KO mice suppressed tumor growth. These findings were underscored in a second study, showing that AEA acts as a tumor promoter in HCC via the CB_1 receptor [377]. Accordingly, FAAH KO mice displayed a worsened tumor progression. In addition, human hepatic tumor tissue exhibited reduced FAAH expression [377]. In chronic lymphocytic leukemia (CLL) patients, plasma levels of OEA were upregulated and correlated with the number of circulating tumor cells [378]. After treatment with the chemotherapy drug lenalidomide, patients in clinical remission presented significantly reduced plasma OEA. Patient derived CLL cells expressed NAPE-PLD and a role for overproduction of OEA by these cells was proposed [378]. Importantly, PPAR-α expression was found to be elevated in CLL patients and associated with an advanced disease stage [379]. Furthermore, a PPAR-α antagonist was able to reduce tumor burden in a mouse model of CLL [380]. Taken together, these studies suggest that targeting NAE biosynthetic enzymes, possibly NAPE-PLD, could have beneficial effects in leukemia or hepatic cancer.

4.3.3. Chronic liver disease

Besides hepatic cancer and steatosis, also cirrhosis has been implicated in aberrant NAE signaling [381–383]. Liver cirrhosis is most often caused by alcohol abuse, hepatitis or steatosis and has a high mortality rate. In monocytes derived from humans and rats with cirrhotic liver, AEA levels were found to be elevated [384,385]. Similar findings were observed in another study, reporting increased circulatory AEA, OEA and PEA levels in cirrhotic patients, which correlated with advanced disease stage [386]. Hypertension of the portal vein is a major complication of advanced cirrhosis as a result of intrahepatic vascular resistance due to excessive scarring (fibrosis) and vasodilation in mesenteric arteries [383]. AEA induced vasodilation in mesenteric vessels from cirrhotic rats, whereas controls samples were less sensitive to AEA [387]. Antagonists for the CB_1 receptor (rimonabant) or TRPV1 (capsazepine) blocked this effect [387]. Accordingly, administration of rimonabant in cirrhotic rats decreased mesenteric blood flow and portal hypertension [385]. CB_1 receptor expression is low in healthy human liver, but it was upregulated in fibrotic and cirrhotic samples [388]. Genetic deletion or pharmacological blockade of CB_1 receptors (rimonabant) reduced hepatic fibrogenesis in three different fibrotic rat models [388]. This was extended to advanced cirrhotic rats, where treatment with rimonabant for two weeks reversed fibrosis [389]. Also the peripherally restricted antagonist JD5037 attenuated fibrosis in mice [390].

The relevant biosynthetic pathway of circulatory AEA in cirrhosis is still unknown. It is well established that cirrhotic patients have elevated plasma levels of endotoxins and increased hepatic macrophages [391–393]. LPS was reported to induce AEA production in mouse macrophages, which was dependent on the PLC/phosphatase biosynthetic pathway [83,84]. Pro-inflammatory stimuli such as LPS were found to downregulate NAPE-PLD expression in mouse macrophages, thereby reducing anti-inflammatory PEA concentrations [85]. Of note, LPS injection in mice with a hepatocyte-specific deletion of *NAPE-PLD* gave increased sensitivity to liver inflammation [361]. These results suggest that NAPE-PLD is not responsible for the elevation of circulatory AEA in cirrhosis. To summarize, the described studies point towards pathological signaling of AEA in hepatic fibrosis and cirrhosis and suggest that blocking CB_1 receptor activation or AEA biosynthesis, possibly via the PLC/phosphatase pathway, could be of potential therapeutic





IC_{50} (μM) for:	PLAAT2	PLAAT3	PLAAT4	PLAAT5
LEI110	0.16	0.10	0.63	0.032
LEI301	0.050	0.25	0.050	0.040

Fig. 7. Structures of selected A) NAPE-PLD and B) PLAAT family inhibitors and their biochemical potencies.

benefit.

4.3.4. Reducing NAE levels in the brain

NAE signaling in the brain is involved in numerous processes such as memory formation, stress, anxiety and inflammation [211]. At present, different strategies are being investigated that activate the cannabinoid and PPAR receptors by enhancing the NAE tone (*e.g.* FAAH inhibition) or by using CB_1/CB_2 agonists as therapeutic treatment for neurological conditions [267,283]. The potential benefits of reducing NAE levels in the CNS are for the most part unclear [394]. This is primarily because of a lack of genetic or pharmacological tools to investigate brain NAE depletion. The neurophysiological properties and behavior of mice with a deletion in one of the NAE-producing enzymes such as NAPE-PLD, ABHD4 and GDE1 have not been profiled, since brain AEA concentrations were not unambiguously reduced [37]. Multiple studies have reported dysregulated brain NAE levels in neurological disorders, although more evidence is needed whether NAEs contribute to disease severity or relief. In neurodegenerative diseases such as multiple sclerosis and Parkinson's disease, AEA levels were found to be elevated in human cerebrospinal fluid [395–397]. It is proposed that increased AEA does not induce disease progression, but rather provides neuroprotection via CB_1 or CB_2 receptor activation as a result of the neuroinflammatory component of these diseases [262,398]. Substantial evidence has been collected for the beneficial effects of CB_1 and CB_2 receptor activation in CNS injury, but confoundingly, several studies also point to a positive effect of CB_1 receptor inhibition [398,399]. For example, CB_1 receptor blockade with rimonabant was neuroprotective in various rodent stroke models and enhanced AEA levels were harmful [173,400,401]. Combination of a CB_2 receptor agonist and a CB_1 receptor antagonist gave an additive reduction of infarction in a transient cerebral ischemia mouse model [402]. Unexpectedly, using KO mice the same authors reported that the neuroprotective effect of rimonabant was CB_1 receptor-independent, suggesting that this drug may possess a significant off-target [403]. In a notable recent study, double deletion of CB_1 and CB_2 receptors provided improved recovery after stroke [404]. Further research is needed to assess the role of AEA in stroke and whether inhibiting its biosynthesis may be beneficial. Importantly, it will be necessary to evaluate the effect of depletion of other NAEs such as OEA and PEA, which are more abundant in the brain and have neuroprotective or anti-inflammatory effects acting in part via PPAR-α [6,177,405].

Several recent reports have revealed potential biological functions of NAE biosynthetic enzymes in the brain. A frameshift variant of NAPE-PLD in several dog breeds was reported to be a risk factor for leukoencephalomyelopathy, a myelination disorder [406]. The impact of this NAPE-PLD variant on the enzymatic activity or brain NAE concentrations has yet to be determined. The (p)NAPE-producing enzyme PLA2G4E was described as a possible risk gene for panic disorder [407]. More recently, PLA2G4E was shown to modulate memory retrieval and provided cognitive resilience in a mouse model of Alzheimer's disease (AD) [408]. Notably, loss of PLA2G4E expression was associated with end-stage AD with dementia in humans, while PLA2G4E overexpression in APP/PS1 mice restored cognitive deficits without affecting amyloid or tau pathology [408]. It has long been known that Ca-NAT activity is depleted in aging rat brain, while NAPE-PLD activity increases with age, which may therefore be linked to cognitive impairment [140,145]. An important caveat that will need to be addressed is the specific contribution of PLA2G4E in the brain towards the generation of NAPEs and NAEs *in vivo*. Two recent KO mouse models did not show reductions of endogenous brain NAPE and NAE levels, suggesting that compensatory effects may be in place via alternative pathways [49,50]. Thus, it may be necessary to block PLA2G4E activity in an acute manner to reveal its contribution to NAPE and NAE biosynthesis, however, brain-active PLA2G4E inhibitors have not been described yet.

To be able to investigate NAE depletion in the brain, two studies looked at selective overexpression of the AEA-degrading enzyme FAAH in specific brain regions using a viral vector. In the hippocampus this afforded an elevation of anxiety-like behavior and a deficit in object recognition memory and in extinction of aversive memory [409]. Interestingly, reduced NAE levels were observed for AEA and PEA, but not OEA. In contrast, FAAH overexpression in the amygdala produced an anxiolytic effect and decreased conditioned fear responses [410]. These

studies indicate that depleting the brain NAE tone can have brain region-specific outcomes.

In recent years, the first inhibitors of NAPE-PLD have been described, such as ARN19874 and hexachlorophene (Fig. 7A) [326,327,411,412]. These molecules however lack potency for *in vivo* use. More recently, our lab reported the first centrally-active NAPE-PLD inhibitor LEI-401, which reduced brain AEA levels in mice, activated the HPA-axis and diminished fear extinction (Fig. 7A) [73]. These results, in combination with the known psychiatric side effects associated with brain CB_1 receptor antagonism, suggest that brain AEA depletion may be ill-suited as a therapeutic approach in patients with normal brain AEA levels. Conversely, the rs324420 variant of FAAH (C385A, p.Pro129Thr), which occurs in 20% of the global population and causes elevated brain AEA levels, was proposed to be involved in substance use disorders [413]. Decreasing brain AEA levels in this population could therefore potentially constitute an interesting pharmacological strategy.

4.3.5. Other indications

Besides the aforementioned conditions, other diseases may potentially be amenable to NAE level modulation such as inflammatory bowel disease (IBD) and skin disorders. Crohn's disease (CD) patients with the FAAH rs324420 variant were associated with a more severe disease phenotype, while variant carriers with ulcerative colitis (UC) showed an earlier disease onset than WT carriers [414]. Notably, PLA2G4E expression was significantly upregulated in a mouse model of colitis [415]. Multiple reports have shown perturbed, but also diverging NAE levels in IBD patients [416]. Colonic PEA and AEA, but not 2-AG levels were significantly elevated in biopsies from UC patients [417,418]. Another study saw increases of plasma NAEs in UC and CD patients, but decreased gene expression of *NAPE-PLD* in UC patient biopsies [419]. In contrast, a third report showed decreased levels of AEA, but not PEA, in biopsies of inflamed mucosa from UC and CD patients, alongside a reduced activity of NAPE-PLD and an increase of FAAH [420]. CB_1 receptor protein expression was elevated in inflamed tissues, while CB_1 receptor agonism exerted anti-inflammatory effects [420]. A recent study described that NAEs drive a pathological microbiome shift in IBD [421]. PEA, OEA, LEA and AEA were elevated in stool samples of UC and CD patients as well as in a mouse model of T-cell-induced colitis. A high dose of these NAEs shifted *ex vivo* grown microbial communities to an IBD-like state. It is important to note that the used NAE concentrations (50-100 μM) are 10-100 fold higher than the observed concentrations in stool, in particular for AEA [421]. Nevertheless, this study, together with the discovery that gut bacteria produce NAE-like lipid messengers that activate human host NAE receptors such as GPR119 [422], highlights the ongoing crosstalk between host and microbiome. More research is needed to determine whether downregulation of NAE signaling can be beneficial in IBD.

Also in skin disorders NAE levels and their metabolic enzymes were found to be perturbed. A recent report described elevated levels of NAEs and their NAPE precursors as well as enhanced *PLA2G4E* gene and protein expression in a mouse model of psoriasis [48]. Also in human psoriatic skins increased *PLA2G4E* expression was observed compared to healthy controls [48]. An anti-inflammatory role for PLA2G4E was proposed as KO mice displayed a more severe inflammatory phenotype compared to their WT littermates and topical NAE treatment attenuated psoriatic markers and skin swelling [48]. The NAE-mediated anti-inflammatory effects were partially recapitulated by a PPAR-α agonist [48]. A second study found similar increases of PLA2G4E and family members PLA2G4D and PLA2G4F in lesions from psoriatic patients and in skin from pityriasis rubra pilaris (PRP) patients [423]. Here, the authors suggested a pro-inflammatory role for PLA2G4E as IL17A and TNFα cytokines upregulated *PLA2G4E* expression in keratinocytes and a topical aspecific inhibitor for $cPLA_2$ (AACOCF3) alleviated the inflammatory phenotype in a psoriasis mouse model. However, it cannot be ruled out that the inhibitory activity of AACOCF3 on PLA2G4A ($cPLA_2\alpha$) or other phospholipases may account for the observed results. Besides psoriasis, also in skin necroptosis PLA2G4E was proposed to play a pro-inflammatory function [424]. PLA2G4E induced lysosomal membrane permeabilization after ischemia, thereby triggering necroptosis. Genetic inhibition of *PLA2G4E* with an adeno-associated virus vector (AAV) incorporating a *PLA2G4E*-targeting microRNA attenuated necroptosis in mice and promoted survival of random skin flaps [424]. Collectively, these studies point to possible anti- and pro-inflammatory effects of the NAE biosynthetic pathway, respectively, by the bioactivities of NAEs on their receptors or the biophysical changes caused by lipid remodeling during ischemia. Further investigations are needed using KO models and selective PLA2G4E inhibitors, although the latter have not been reported yet.

4.3.6. Targeting non-classical NAPE/NAE biosynthetic enzymes

The Ca^{2+}-independent NAE biosynthetic pathway, which involves the *N*-acylation of PE by a PLAAT family member and its conversion to NAE, has received increasing attention in the past decade [37]. The best characterized PLAAT member is PLAAT3, which has been proposed as a drug target in obesity, cancer and picorna- and rhinoviral infections [425–428]. Biochemical evaluation revealed that PLAAT3 has little *N*-acyltransferase activity in cells, suggesting a limited role in NAE biosynthesis [56]. Notably, PLAAT1, which possesses both $PLA_{1/2}$ and *N*-acyltransferase activity, and PLAAT3 were recently linked to a novel form of autophagy in the vertebrate eye [429]. In developing lens cells, Plaat1 in zebrafish and PLAAT3 in mice were recruited to intracellular membranes leading to the complete digestion of all organelles including mitochondria, ER, lysosomes and nuclei, resulting in fully cleared lenses [429]. The recruitment of Plaat1 and PLAAT3 was evoked by membrane damage and dependent on their C-terminal hydrophobic domain. Similar findings have been observed for human PLAAT3 in response to lysosomal damage [425]. Of note, cellular overexpression of PLAAT2 and PLAAT3 resulted in the disappearance of peroxisomes and led to a drastic decrease in plasmalogen-type lipids [56,430,431]. The C-terminal hydrophobic domain of PLAAT1 and PLAAT3 is shared by PLAAT2 and PLAAT4, which are present in humans but not in rodents, suggesting that these enzymes could have a similar role in organelle degradation. Furthermore, it will be interesting to explore the role of PLAAT family members in other cell types that are devoid of organelles such as keratinocytes and erythrocytes and whether NAPE and NAE lipids play a part in this form of autophagy [432].

To elucidate the biological functions of the PLAAT members we have recently described the first inhibitors for this family [57,324]. LEI-110 and LEI-301 make use of an α-ketoamide pharmacophore which forms a reversible covalent bond with the active site cysteine in PLAATs and have nanomolar to submicromolar potency for PLAAT2-5 in an *in vitro* activity-based protein profiling assay (Fig. 7B). Because of the high homology of PLAAT1 with its family members, it is expected that both compounds inhibit this enzyme as well, although it could not be overexpressed in our hands [57]. Cells overexpressing PLAAT2 or PLAAT5 showed large increases in NAE content, which was reduced by treatment with LEI-301 [57]. We anticipate that these compounds will guide the design of *in vivo* active and PLAAT family member-specific inhibitors.

5. Conclusions and future directions

Here we have sought to provide a comprehensive overview of the biosynthesis, oxidation and degradation of NAEs, their bioactivities and potential strategies to modulate the (oxidized) NAE tone in disease. It is a given that after the initial discovery of PEA over 60 years ago, the literature encompassing NAEs and their receptors has vastly expanded. Yet many open questions remain. Old (PLAAT1-5) and new (PLA2G4E) biosynthetic enzymes of NAPEs have been characterized in the past decade, but their contribution *in vivo* towards the production of NAEs needs further investigation, both in brain and peripheral tissues. Possible compensatory effects caused by redundancy in the NAE metabolic pathways have confounded the identification of brain AEA

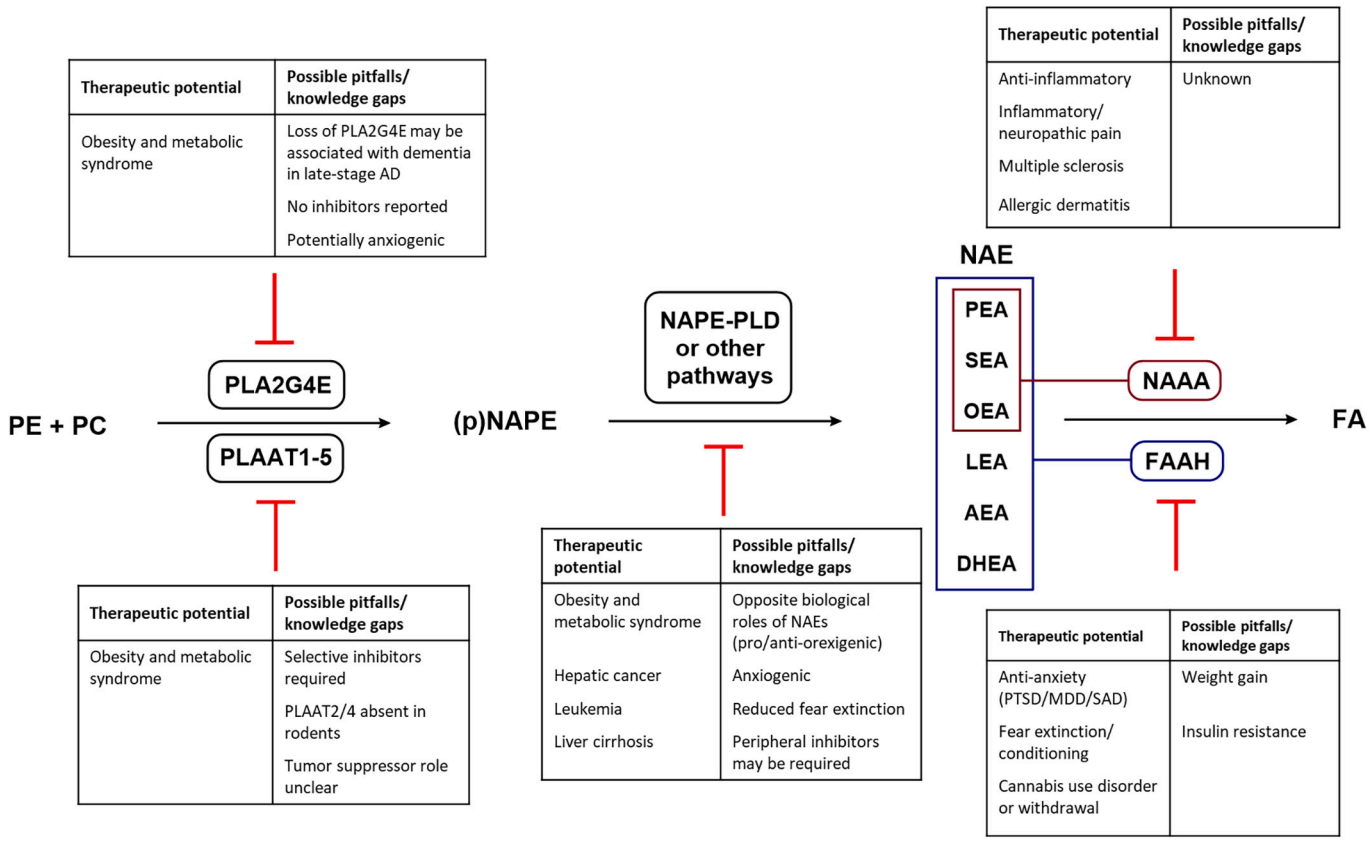


Fig. 8. Modulation of NAE metabolic enzymes may provide therapeutic benefit both by increasing or depleting NAE levels. FAAH inhibitors have seen the first recent clinical successes in psychiatric disorders, while NAAA inhibitors have gathered strong evidence for their implementation in inflammatory diseases. More work is needed to establish whether NAE depletion is a viable treatment option using novel genetic and (peripheral) pharmacological tools.

biosynthetic enzymes using genetic KO models, as AEA levels were not always reduced [37]. In contrast, pharmacological NAPE-PLD inhibition decreased brain AEA and modulated the stress and fear extinction response, suggesting that this signaling lipid is under tight control and requires acute interrogation [73]. Thus, centrally-active and selective inhibitors for all NAPE/NAE biosynthetic enzymes will be necessary to uncover the involvement of each protein in NAE production.

Many GPCRs, ion channels and nuclear receptors have been described through which NAEs and their oxidative metabolites exert their bioactivities including PPAR-α, PPARγ, CB_1, CB_2, GPR55, GPR110, GPR119 and TRPV1. Oftentimes these receptors can also be activated by other signaling lipids such as mono-acylglycerols, free fatty acids or eicosanoids, therefore the specific contribution of NAEs requires further exploration. Novel genetically encoded sensors such as $GRAB_{eCB2.0}$ for AEA in combination with a complete set of degradation and biosynthesis inhibitors may help to expose the role of specific NAE signaling lipids *in vivo* [433]. Furthermore, NAEs can have tissue specific distributions, which may provide clues towards their biological function. For example, LEA is the most abundant NAE in the gut, but highly depleted in the brain, while DHEA levels are an order of magnitude higher in brain compared to plasma. Therefore, as a recommendation, when performing NAE lipidomics measurements, the whole NAE family should be taken into account and not only a small subset of NAEs.

The therapeutic potential of modulating endogenous NAE levels has been extensively described in this work and is summarized in Fig. 8. With regard to blockade of NAE degradation, FAAH inhibitors have regained their place in the limelight after clinical successes in cannabis withdrawal, PTSD and SAD. The results of ongoing clinical trials with FAAH inhibitors in PTSD and other psychiatric disorders are eagerly awaited [293]. Accumulating pre-clinical evidence points to the therapeutic potential of NAAA inhibitors in inflammatory diseases such as multiple sclerosis, allergic dermatitis and neuropathic and inflammatory pain. Evaluation of newly developed orally available NAAA inhibitors in inflammatory disease models will be necessary to push these compounds forward towards the clinic. When using FAAH or NAAA inhibitors, it is pertinent to realize that oxidative metabolism of the unsaturated NAEs may be increased, especially under inflammatory conditions, which leads to the formation of new classes of bioactive signaling lipids. Conversely, inhibitors of oxidative metabolism may increase NAE levels, thereby enhancing CB_1 or CB_2 receptor signaling.

Lastly, in this work we have outlined pathological conditions in which NAE signaling is perturbed, such as obesity, metabolic syndrome, hepatocellular carcinoma, leukemia and liver cirrhosis. These diseases may therefore benefit from inhibition of the enzymes involved in NAE biosynthesis, which will result in a reduction of the endogenous NAE tone. With newly described pharmacological tools for the biosynthetic enzymes of NAEs, the helpful effects of NAE depletion in disease may now be uncovered. Peripheral restriction of the NAE biosynthetic inhibitors may prevent untoward CNS-side effects and may find, therefore, application in the treatment of these diseases.

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