Chapter 1

Therapeutic opportunities of modulating the endogenous N-acylethanolamine tone

Over the past decades, lipids have emerged as important signaling molecules in health and disease. Lipid messengers come in a range of shapes and sizes and are classified in seven different categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides. 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 determines their signaling function. The most frequently occurring 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 exist with regard to the biological actions of NAEs. In this chapter,
an overview is provided of NAE biosynthesis and degradation, current understanding of their physiological functions and potential therapeutic applications of modulating the NAE tone.

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Receptor</th>
<th>Bioactivity</th>
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<tr>
<td>PEA (16:0)</td>
<td><img src="image" alt="PEA Structure" /></td>
<td>PPAR-α&lt;sup&gt;2&lt;/sup&gt; GPR55&lt;sup&gt;3&lt;/sup&gt; GPR119&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Anti-inflammatory&lt;sup&gt;5&lt;/sup&gt; Neuroprotective&lt;sup&gt;6&lt;/sup&gt; Anti-epileptic&lt;sup&gt;7&lt;/sup&gt; Analgesic&lt;sup&gt;8&lt;/sup&gt; Anorectic&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>SEA (18:0)</td>
<td><img src="image" alt="SEA Structure" /></td>
<td>GPR119&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Anti-inflammatory&lt;sup&gt;10&lt;/sup&gt; Anorectic&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
<tr>
<td>OEA (18:1-ω9)</td>
<td><img src="image" alt="OEA Structure" /></td>
<td>PPAR-α&lt;sup&gt;12&lt;/sup&gt; GPR119&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Anti-inflammatory&lt;sup&gt;13&lt;/sup&gt; Anorectic&lt;sup&gt;9&lt;/sup&gt; Analgesic&lt;sup&gt;14&lt;/sup&gt; Neuroprotective&lt;sup&gt;15&lt;/sup&gt;</td>
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<tr>
<td>LEA (18:2-ω6)</td>
<td><img src="image" alt="LEA Structure" /></td>
<td>PPAR-α&lt;sup&gt;16&lt;/sup&gt; GPR119&lt;sup&gt;17&lt;/sup&gt;</td>
<td>Anorectic&lt;sup&gt;18&lt;/sup&gt; Neuroprotective&lt;sup&gt;19&lt;/sup&gt;</td>
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<tr>
<td>AEA (20:4-ω6)</td>
<td><img src="image" alt="AEA Structure" /></td>
<td>CB&lt;sub&gt;1&lt;/sub&gt;&lt;sup&gt;20&lt;/sup&gt; CB&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;21&lt;/sup&gt; TRPV1&lt;sup&gt;22&lt;/sup&gt;</td>
<td>Neurotransmission&lt;sup&gt;23&lt;/sup&gt; Orexigenic&lt;sup&gt;24&lt;/sup&gt; Analgesic&lt;sup&gt;25&lt;/sup&gt; Anxiolytic&lt;sup&gt;26&lt;/sup&gt; Memory formation&lt;sup&gt;27&lt;/sup&gt; Neuroprotective&lt;sup&gt;28&lt;/sup&gt; Fertility&lt;sup&gt;29&lt;/sup&gt;</td>
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<tr>
<td>DHEA (22:6-ω3)</td>
<td><img src="image" alt="DHEA Structure" /></td>
<td>GPR110&lt;sup&gt;30&lt;/sup&gt;</td>
<td>Neurogenesis&lt;sup&gt;31&lt;/sup&gt; Anti-inflammatory&lt;sup&gt;32&lt;/sup&gt;</td>
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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; GPR55, 110 or 119 = G-protein coupled receptor 55, 110 or 119; CB<sub>1</sub>/2 = cannabinoid receptor 1 or 2; TRPV1 = transient receptor potential vanilloid 1.
1.1 NAE metabolism

In 1979, Schmid and co-workers reported the accumulation of NAEs in infarcted dog heart. Shortly hereafter, the same lab showed that N-acylphosphatidylethanolamines (NAPEs), a previously unknown lipid class, were equally upregulated. Due to the structural similarities of NAPEs and NAEs, a precursor-product relationship was

![Figure 1](image-url)

**Figure 1.** Biosynthetic pathways of N-acylethanolamines (NAEs). In total, four different enzymatic routes have been reported that can produce NAEs. In the canonical pathway, N-acylphosphatidylethanolamine (NAPE) is formed from phosphatidylethanolamine (PE) and phosphatidylcholine (PC) catalyzed by phospholipase A2 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 and acyltransferase 1-5; ABHD4 = α,β-hydrolase domain 4; GDE4, 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; 1-LPC = 1-lysophosphatidylcholine; LPA = lysophosphatidic acid; PA = phosphatidic acid; DAG = diacylglycerol; G3P = glycerol-3-phosphate; pNAE = phospho-N-acylethanolamine.
proposed. \textsuperscript{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 1-lysoPC (Figure 1). \textsuperscript{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.

\subsection*{1.1.1 NAPE biosynthesis}

The canonical acyl transfer reaction that produces NAPEs, is carried out by a Ca\textsuperscript{2+}-dependent N-acyltransferase (Ca-NAT). High Ca-NAT enzymatic activities were found in heart, brain and testis tissues. \textsuperscript{35,38,39} Remaining elusive for more than two decades, the serine hydrolase phospholipase A2 group IV E (PLA2G4E) was recently identified as a NAPE-generating Ca-NAT in cells, matching the reported expression and activity profile. \textsuperscript{40} Also plasmalogen-type PEs, which incorporates a vinyl ether at the sn-1 position, were found to be suitable substrates for PLA2G4E, thereby producing plasmalogen-NAPEs (pNAPEs). \textsuperscript{41} pNAPEs are considered to be an important source of NAEs in the brain. In mouse brain, the total pNAPE amount was 4-fold higher than the NAPE content. \textsuperscript{42} 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. \textsuperscript{43} Interestingly, in rat brain lysate, Ca-NAT activity preferably generated N-arachidonoyl-containing (p)NAPEs with polyunsaturated acyl groups at the sn-2 position. \textsuperscript{44} This may indicate that the Ca\textsuperscript{2+}-dependent generation of AEA favors polyunsaturated (p)NAPEs as precursors. \textsuperscript{44}

A second family of NATs was discovered that can produce NAPEs in a Ca\textsuperscript{2+}-independent manner, termed phospholipase and acyl transferase (PLAAT) 1-5. \textsuperscript{45-50} These enzymes belong to the cysteine hydrolases and show expression in the central nervous system (CNS) as well in peripheral tissues. In particular, PLAAT2 showed high N-acyltransferase activity, comparable to PLA2G4E. \textsuperscript{41,50} Also PLAAT2 accepted both PE and plasmalogen-type PE as substrates. \textsuperscript{41} Expression of PLAAT2 was found to be high in the liver, kidney, small intestine, colon, testis and trachea. \textsuperscript{47,51} This suggests that PLAAT2 may be involved in NAE biosynthesis in the gut. Notably, PLAAT2 expression was absent in rodents. \textsuperscript{47} So far, no genetic or pharmacological tools have been described for the PLAAT family members. To what extend the Ca\textsuperscript{2+}-independent pathway contributes to NAPE and pNAPE biosynthesis \textit{in vivo}, is therefore still unclear. \textsuperscript{37}

\subsection*{1.1.2 NAE biosynthesis}

In 2004, the enzyme that produces NAEs in a single step from NAPEs or pNAPEs was identified as N-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) (Figure 1). \textsuperscript{52} A crystal structure revealed that NAPE-PLD forms a membrane-bound homodimer with two Zn\textsuperscript{2+}-ions in its active site. \textsuperscript{53} NAPE-PLD is classified as a metallo-\beta-lactamase and is distinct
Therapeutic opportunities of modulating the endogenous N-acylethanolamine tone

from the PLD family.\textsuperscript{52} Brain, kidney and testis tissues were found to abundantly express NAPE-PLD.\textsuperscript{52} Interestingly, NAPE-PLD did not display any substrate preference \textit{in vitro}.\textsuperscript{54} Furthermore, PE increased the NAPE-PLD enzymatic activity, suggesting that the enzyme is constitutively active.\textsuperscript{55} \textit{In vitro}, NAPE-PLD activity was found to be elevated by specific bile acids, as well as polyamines such as spermine and spermidine.\textsuperscript{53,56,57} Multiple NAPE-PLD knockout (KO) studies described a significant reduction of saturated and unsaturated NAEs in the brains of mice.\textsuperscript{42,58,59} In accordance, NAPE and pNAPE precursors were greatly enhanced.\textsuperscript{42,58} However, levels of \(\omega\)-6 and \(\omega\)-3 polyunsaturated NAEs – AEA and DHEA, respectively – were not decreased in all KO strains.\textsuperscript{58} It was therefore proposed that genetic deletion of NAPE-PLD stimulated compensatory mechanisms which counteract the reduction of AEA and DHEA content.\textsuperscript{58} 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.\textsuperscript{60} At present, the study of NAPE-PLD is hampered by a lack of \textit{in vivo} active inhibitors which are needed to elucidate its role in NAE biosynthesis.

Three additional pathways have been discovered that can also produce NAEs (Figure 1). Firstly, two phospholipases were reported that can hydrolyze the fatty acyl esters of NAPEs. Three isoforms of secretory phospholipase A2 (sPLA\textsubscript{2}-IB, IIA and V) were described to exclusively cleave the NAPE sn-2 ester to form lysoNAPE and a fatty acid.\textsuperscript{61} The serine hydrolase \(\alpha,\beta\)-hydrolase domain 4 (ABHD4) performed the same reaction, but did not show any specificity towards the sn-1 or sn-2 ester.\textsuperscript{62} In addition, ABHD4 could hydrolyze the fatty acyl ester of lysoNAPE, generating glycerophospho-NAE (GP-NAE). This lipid species is converted by glycerophosphodiesterase 1 and 4 (GDE1/4) to afford NAE and glycerol-3-phosphate (G3P).\textsuperscript{63,64} A second pathway involves cleavage of the lysoNAPE phosphodiester by GDE4 or GDE7 in a lysoPLD-type reaction, producing NAE and lysophosphatidic acid (LPA).\textsuperscript{64,65} Expression of ABHD4 was found to be high in brain and testis, but not in heart.\textsuperscript{52} ABHD4 KO mice displayed decreased levels of GP-NAE and lyso-(p)NAPE in the brain, however NAE content, including AEA, was not reduced.\textsuperscript{66} The activity of GDE1 was stimulated by Mg\textsuperscript{2+}-ions and high protein expression levels were found in brain, testis, liver and kidney tissues.\textsuperscript{63} Genetic deletion of GDE1 in mice also did not afford a significant decrease of brain NAE levels, therefore the physiological importance of this pathway for the formation of brain NAEs is still under debate.\textsuperscript{67} The recently reported GDE4 and GDE7 enzymes, as well as the sPLA\textsubscript{2}s have yet to be further characterized in KO models to establish their role in NAE biosynthesis \textit{in vivo}.\textsuperscript{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.\textsuperscript{68}
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. 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. 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), that could indicate a possible role in vivo.

1.1.3 NAE degradation

The hydrolysis of NAE to fatty acid and ethanolamine can be performed by several enzymes (Figure 2). Fatty acid amide hydrolase (FAAH) displays specificity towards AEA over saturated and mono-unsaturated NAEs and has high expression in human brain, but is absent in heart tissue. 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. 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 during liver regeneration which could be attributed to highly increased arachidonic acid levels, but not ethanolamine. A second fatty acid amidase (FAAH-2) was identified that shares 20% sequence identity with FAAH. FAAH-2 is specific for higher mammals including primates and marsupials and does not occur in rats or mice. 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. Contrary to FAAH, FAAH-2 preferred primary fatty acid amides (e.g. oleamide) over NAEs as substrates. A third NAE-hydrolyzing enzyme was described to be active in cells of the immune system. N-acylethanolamine acid amidase (NAAA) is lysosomally located and preferentially hydrolyzes saturated NAE species. NAAA is an N-terminal cysteine hydrolase and shares no homology with FAAH (a serine hydrolase). Pharmacological inhibition of NAAA in mice induced significant elevations of brain PEA and OEA, but not AEA levels. The development of NAAA KO mice is necessary to confirm these findings.

Besides hydrolysis of the amide bond, polyunsaturated NAEs such as AEA and DHEA can undergo oxygenation of the double bonds which produces eicosanoid-type lipids (Figure 2). Each of these oxygenated products have reported lipid signaling functions of their own. Cyclooxygenase (COX)-2 was described to convert AEA to various prostaglandin-ethanolamides (PG-EA), a lipid class designated as prostamides. For
example, AEA cycloxygenation by COX-2 followed by consecutive action of a PGF synthase produces PGF$_{2\alpha}$-EA. Both these enzymes occur in the CNS and it is suggested that PGF$_{2\alpha}$-EA is involved in inflammatory pain in vivo.$^{87,88}$ Also lipoxygenases (LOX) are able to use AEA as a substrate, generating for example 12-hydroxyeicosatetraenoic acid-ethanolamide (12-HETE-EA).$^{89,90}$ These oxygenated AEA derivatives inhibit FAAH and could therefore prolong NAE signaling.$^{91}$ Lastly, epoxidation of AEA by cytochrome P450 enzymes can produce different epoxides such as 5,6-epoxyeicosatrienoic acid-ethanolamide (5,6-EET-EA).$^{92,93}$ Since AEA is primarily hydrolyzed by FAAH and generally has low endogenous concentrations in most tissues, the biological importance of many of these oxygenated products is still unknown.$^{84}$

![Figure 2. Oxidative degradation pathways of anandamide showing representative products. Cycloxygenase 2 (COX-2) and prostaglandin synthases (e.g. PGF synthase) can convert AEA into prostamide-type lipids such as prostaglandin-F$_{2\alpha}$-ethanolamide (PGF$_{2\alpha}$-EA). Lipoxygenases (LOX) enzymes can hydroxylate AEA to form for example 12-hydroxyeicosatetraenoic acid-ethanolamide (12-HETE-EA). Cytochrome P450-type enzymes can produce various epoxygenated AEA derivatives such as 5,6-epoxyeicosatrienoic acid-ethanolamide (5,6-EET-EA).](image-url)

**1.2 Physiological functions of NAEs and (p)NAPEs**

**1.2.1 NAPE and pNAPE**

NAPEs and pNAPEs are primarily considered to be precursors of NAEs. However, recent overviews have highlighted that (p)NAPEs may have biological functions of their own.$^{94,95}$ These include putative roles in neuroprotection, anti-inflammation and satiety. During
cellular injury, NAPEs accumulate in damaged tissue, presumably due to an influx of calcium ions.\textsuperscript{96,97} This phenomenon has been observed in ischemia of the brain, heart and testis in various mammals such as mice, rats, dogs and humans.\textsuperscript{34,98-103} Also in plants NAPEs increase under cellular stress.\textsuperscript{94,104,105} Importantly, NAPE levels are higher than their corresponding NAE congeners in brain ischemia, which may suggest a neuroprotective function.\textsuperscript{106} Conversion of PE to NAPE has a proposed membrane stabilizing role, possibly due to hydrogen bonding of the newly formed amide.\textsuperscript{107,108} NAPE-enriched liposomes were found to be less prone to dye leakage.\textsuperscript{109} Furthermore, NAPEs induced membrane fusion in the presence of Ca\textsuperscript{2+}-ions.\textsuperscript{110,111} This effect was found to be NAPE-specific as other anionic phospholipids such as phosphatidylserine (PS) and phosphatidylglycerol (PG) did not stimulate membrane fusion.\textsuperscript{110} 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.\textsuperscript{112} N-palmitoyl-PE-enriched liposomes decreased phagocytosis in mouse macrophages, thereby contributing to the termination of inflammation.\textsuperscript{113} In the rat jejunum, NAPE levels, specifically N-oleoyl-PE, were increased after feeding.\textsuperscript{43,114} NAPE has been described as a lipid hormone that can decrease food intake, while exogenous NAPE was able to induce weight loss in mice.\textsuperscript{115} However, following reports have contested this claim and point towards NAE metabolites as the cause of the observed anorectic effect.\textsuperscript{116,117} Collectively, these studies provide evidence for a putative biological role of NAPEs in neurodegeneration and inflammation. The molecular mechanisms through which NAPEs exert its bioactivities should therefore be addressed. Genetic or pharmacological tools that enable modulation of NAPE metabolic enzymes may help to answer these questions.

1.2.2 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.\textsuperscript{118,119} It was immediately noted that PEA possessed anti-allergic and anti-inflammatory properties in a guinea pig model of anaphylactic arthritis.\textsuperscript{118} Following reports revealed that PEA also produces anti-epileptic, neuroprotective, analgesic and anorectic effects.\textsuperscript{7,9,120-123} During acute brain ischemia in rats, PEA levels increased 30-fold specifically in damaged brain areas.\textsuperscript{124} Exogenous administration of PEA showed to be neuroprotective in various disease models such as traumatic brain injury, Parkinson’s and Alzheimer’s disease.\textsuperscript{122} Multiple biological targets have been identified for PEA that can explain its pharmacological effects.\textsuperscript{123,125} The nuclear receptor peroxisome proliferator-activated receptor (PPAR)-\(\alpha\) was found to mediate the anti-inflammatory and analgesic effects of PEA.\textsuperscript{6,126} Furthermore, PEA displayed affinity for GPR119, a fat sensor in the gut, although OEA is regarded as a more potent agonist \textit{in vivo}.\textsuperscript{4,127} Another receptor through which PEA can exert its bioactive
Therapeutic opportunities of modulating the endogenous N-acyl ethanolamine tone

effects is GPR55, however these findings have been questioned in later studies.\textsuperscript{3,128} 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.\textsuperscript{8}

1.2.3 SEA

Although SEA and PEA differ just two carbons 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-\(\alpha\).\textsuperscript{2} Nevertheless, SEA showed affinity for GPR119 and shares several bioactivities with PEA.\textsuperscript{4} SEA produced anti-inflammatory effects in a mouse cutaneous anaphylaxis model.\textsuperscript{10} In rat brain, SEA levels were similar to PEA and showed a comparable 30-fold increase upon brain ischemia.\textsuperscript{124} Furthermore, oral administration of SEA in mice produced an anorectic effect, presumably through increase of liver stearoyl-CoA desaturase-1 (SCD-1) mRNA expression.\textsuperscript{11} These findings indicate that SEA may have therapeutic properties and the exclusion of this lipid species from NAE studies is unjustified. It is therefore recommended to include SEA in the standard NAE lipid panel to elucidate its biological role.

1.2.4 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 are mediated by peripheral PPAR-\(\alpha\).\textsuperscript{12,129,130} Of the NAE members, OEA showed the highest potency for PPAR-\(\alpha\).\textsuperscript{16} Endogenous OEA levels in the small intestine were markedly reduced in starved mice and significantly increased after refeeding compared to free-feeding mice.\textsuperscript{9,43,131} As such, OEA is regarded as a satiety factor that is released upon food intake.\textsuperscript{114,132} However, both short-term and chronic high fat diets were found to decrease levels of OEA in rat jejunum, but not in other tissues such as brain and liver.\textsuperscript{16,133} It was proposed that reduction in OEA levels may cause the reduced satiety and hyperphagia as seen in obesity.\textsuperscript{125,132} OEA also showed \textit{in vitro} affinity for GPR119, a receptor that modulates feeding behavior.\textsuperscript{4} Nevertheless, OEA produced anorectic effects in both GPR119 WT and KO mice, indicating that \textit{in vivo}, this activity is not required for satiety.\textsuperscript{134} Similar to PEA, administration of OEA in rodents was reported to generate anti-inflammatory, neuroprotective and analgesic effects.\textsuperscript{13-15} These are likely mediated by activation of PPAR-\(\alpha\), although also PPAR-\(\alpha\)-independent mechanisms have been described.\textsuperscript{126,135,136} 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.\textsuperscript{124} A putative
neuroprotective role for the NAE family was therefore hypothesized, acting via multiple molecular mechanisms.\textsuperscript{122,137}

1.2.5 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.\textsuperscript{131} Intraperitoneal (i.p.) 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.\textsuperscript{9,16} 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.\textsuperscript{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.\textsuperscript{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 \textit{in vivo}.\textsuperscript{124}

1.2.6 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.\textsuperscript{138,139} However, unlike other NAEs, AEA can activate the cannabinoid (CB\textsubscript{2})-receptor.\textsuperscript{20} The CB\textsubscript{2} receptor is one of the most abundant GPCRs in the mammalian brain and is activated by \textit{(-)-Δ\textsuperscript{9}-tetrahydrocannabinol} (THC), the psychoactive component of cannabis. As a result, anandamide and 2-arachidonoylglycerol (2-AG), a second endogenous CB\textsubscript{1} receptor agonist, are termed endogenous cannabinoids or endocannabinoids. AEA is regarded as a tonic neuromodulator – \textit{i.e.}, it continuously signals in the basal state – which is released by neurons upon Ca\textsuperscript{2+}-stimulation and is quickly degraded by FAAH.\textsuperscript{23,140-142} 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.\textsuperscript{143} AEA can also act as an intracellular messenger, formed upon an influx of Ca\textsuperscript{2+}-ions via activation of the G\textsubscript{i}-pathway.\textsuperscript{144}

The word \textit{‘ananda’} – meaning bliss in Sanskrit – was aptly chosen, as increased AEA signaling produces analgesic, anxiolytic and anti-depressant effects through CB\textsubscript{1} receptor signaling in the brain.\textsuperscript{26,145,146} Conversely, acute and repeated stress exposure in rats afforded a decrease in AEA content in the amygdala, mediated by enhanced FAAH activity.\textsuperscript{147} Stressed rats showed an inverse correlation between amygdalar AEA and plasma stress hormone levels (corticosterone).\textsuperscript{148} Diminished brain AEA signaling upon repeated stress increased secretion of corticosterone.\textsuperscript{149} In contrast, repeated stress
Therapeutic opportunities of modulating the endogenous N-acylethanolamine tone

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.\textsuperscript{149,150} 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\textsubscript{1} receptor activation.\textsuperscript{27,28,151,152} Pharmacological studies in mice showed that exogenous AEA produces cannabimimetic responses, which are rapid in onset, but shorter and less potent than THC, presumably due to its fast metabolism.\textsuperscript{153} Correspondingly, FAAH KO mice were supersensitive to AEA treatment.\textsuperscript{74} Exogenous AEA administration in rats generated a central CB\textsubscript{1}-receptor-dependent orexigenic (appetite-stimulating) effect similar to THC.\textsuperscript{24}

Anandamide has also been linked to CB\textsubscript{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 (Figure 3).\textsuperscript{154,155} Interestingly, peripheral CB\textsubscript{1} receptor activation is implicated in food intake as well, and intestinal AEA levels were found to be highly increased in starved mice.\textsuperscript{156} Also

\textbf{Figure 3.} AEA is involved in cannabinoid receptor 1 and 2 (CB\textsubscript{1} and CB\textsubscript{2}) signaling in both central and peripheral organs, of which several are depicted.\textsuperscript{154}
the analgesic effects of AEA were observed in the periphery, as peripheral blockade of FAAH produced antinociception via a CB₁ receptor-dependent mechanism.\textsuperscript{157} Notably, the antinociceptive effect of AEA increased synergistically when combined with PEA in a mouse model of peripheral pain.\textsuperscript{25}

AEA has additionally been described as a partial agonist for the CB₂ receptor, which is primarily expressed in the immune system and is involved in the inflammatory response.\textsuperscript{21,158-160} Typically, AEA levels are 10- to 1000-fold lower compared to 2-AG in most tissues.\textsuperscript{138} 2-AG has therefore been suggested to be the true endogenous CB₂ receptor ligand.\textsuperscript{161} Nevertheless, AEA was reported to modulate inflammation via activation of the CB₂ receptor by reducing pro-inflammatory cytokines in cells.\textsuperscript{154,162}

Besides the cannabinoid receptors, AEA also activates the transient receptor potential vanilloid 1 (TRPV1) ion channel.\textsuperscript{22} AEA has therefore been termed an endovanilloid.\textsuperscript{163,164} TRPV1, also known as the capsaicin receptor, is an important player in pain perception and is localized at peripheral sensory neurons.\textsuperscript{165} Evidence is accumulating that TRPV1 is expressed in the CNS as well.\textsuperscript{166,167} The activation of TRPV1 by AEA causes a cellular influx of Ca\textsuperscript{2+}-ions and has been linked to locomotor depression, hyperalgesia under inflammatory conditions, vasodilation and hypothermia.\textsuperscript{144,165}

\subsection*{1.2.7 DHEA}

Over the past ten years, DHEA has come into view as a member of the NAE family with unique properties in neuronal signaling.\textsuperscript{31} As such, the name synaptamide was coined for its ability to induce neurogenesis.\textsuperscript{168} Recently, DHEA was found to have nanomolar affinity for GPR110, an adhesion-type GPCR highly expressed in the hippocampus.\textsuperscript{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. DHEA has also been reported to have anti-inflammatory properties.\textsuperscript{32,169} In LPS-treated microglia and macrophage cells, DHEA reduced pro-inflammatory cytokines or eicosanoids, respectively.\textsuperscript{32,139} In addition, LPS-induced neuroinflammation in mice was significantly decreased after i.p. administration of DHEA. Brain DHEA levels are generally 2- to 10-fold higher than AEA, while the opposite is true for plasma.\textsuperscript{58,59,138,170} Furthermore, brain DHEA concentrations are linked directly with brain content of docosahexaenoic acid (DHA, 22:6), an \( \omega \)-3 polyunsaturated fatty acid.\textsuperscript{171-173} DHA is preferably acquired from the diet, but can also be synthesized from the essential fatty acid \( \alpha \)-linolenic acid (18:3-\( \omega \)3).\textsuperscript{174} 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.\textsuperscript{42,59} 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.\textsuperscript{58,175} This suggests that alternative pathways are also 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.\textsuperscript{60,176} 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.\textsuperscript{177}

1.3 Pharmacological modulation of NAE metabolism

As outlined in the prior section, NAEs possess desirable 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.\textsuperscript{178-180} 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.\textsuperscript{181} In the following section, a brief overview will be provided of the therapeutic potential of blockade of NAE degradation as well as its biosynthesis.

1.3.1 Inhibition of NAE degradation

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.\textsuperscript{182} To date, multiple research groups and pharmaceutical companies have developed \textit{in vivo} active and brain penetrant FAAH inhibitors (Figure 4A).\textsuperscript{183} 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 enhanced with 8- to 20-fold in the same tissues.\textsuperscript{26,75,184} Limited data is available of other NAE levels 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.\textsuperscript{185} 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\textsubscript{1} and CB\textsubscript{2} receptor activation.\textsuperscript{75,157,186,187} Furthermore, pharmacological FAAH disruption has shown promise for treating anxiety\textsuperscript{26}, depression\textsuperscript{145}, post-traumatic stress disorder\textsuperscript{188}, Parkinson’s
Chapter 1

disease\textsuperscript{189}, nausea\textsuperscript{190}, skin inflammation\textsuperscript{191}, pruritus\textsuperscript{192}, inflammatory bowel disease\textsuperscript{192}, glaucoma\textsuperscript{193}, hypertension\textsuperscript{194}, traumatic brain injury\textsuperscript{195}, HIV-associated neurocognitive disorders\textsuperscript{196} and multiple sclerosis-associated spasticity\textsuperscript{197}. Several FAAH inhibitors have been tested in Phase I and II clinical trials with mixed success.\textsuperscript{198,199} The selective inhibitor PF-04457845 was found to be well tolerated in healthy volunteers, completely blocked plasma FAAH activity and increased plasma AEA (10-fold), LEA (9-fold), OEA (6-fold) and PEA (3.5-fold) concentrations.\textsuperscript{200} However, in a subsequent Phase II clinical trial for osteoarthritic pain of the knee, PF-04457845 did not produce analgesia.\textsuperscript{201} Recently, PF-04457845 was reported to be efficacious for the treatment of cannabis withdrawal symptoms in a Phase II clinical study.\textsuperscript{202}

\begin{center}
\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{structures.png}
\caption{Figure 4. Structures of selected \textit{in vivo} active inhibitors of A) fatty acid amide hydrolase (FAAH) or B) \textit{N}-acylethanolamine acid amidase (NAAA).}
\end{figure}
\end{center}

In 2016, the covalent FAAH inhibitor BIA 10-2474 (Figure 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.\textsuperscript{203} 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. Accordingly, the observed neurotoxic side effects of BIA 10-2474 are presumed not to be caused by inhibition of FAAH.

Due to the limited success of FAAH inhibitors in the clinic, in recent years, inhibitors of the other NAE-hydrolyzing enzyme NAAA have come to the foreground. 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. Considerable evidence point towards a PPAR-α-mediated mechanism. First generation irreversible NAAA inhibitors ARN276 and F215 (Figure 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. It is possible that these compounds elicit an inflammation-specific effect, although their low plasma stability and fast clearance could also explain the observed results. Importantly, the increase of OEA illustrates the difference between in vivo and in vitro NAAA activity, since in the latter case NAAA showed high preference towards hydrolysis of PEA. A second generation reversible NAAA inhibitor (1, Figure 4B) presented improved drug-like properties and was able to elevate brain PEA and OEA levels (2-fold) of healthy mice, but not AEA. It is anticipated that the newly reported crystal structure of NAAA will aide future inhibitor design. In addition, the therapeutic exploitation of NAAA blockade will require KO mice to confirm the effects observed with pharmacological inhibitors.

1.3.2 Inhibition of NAE biosynthesis

Blocking NAE biosynthesis by pharmacological agents is an underdeveloped strategy in endocannabinoid research and so far no selective and in vivo active inhibitors have been described. 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. 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. Here, different conditions are outlined where decreasing NAE levels could be of therapeutic value.

Obesity and metabolic syndrome

The endocannabinoid system is a key player in energy balance and food intake, both in the CNS and the periphery. The centrally active CB1 receptor antagonist rimonabant (Acomplia®, Figure 5) was clinically approved for treatment of obesity and metabolic syndrome, as it induced significant weight-loss, decreased food intake and improved insulin resistance. Unfortunately, patients treated with rimonabant suffered from depression-like side effects leading to its withdrawal from the market. Peripherally
restricted CB₁ receptor antagonists have shown comparable pre-clinical efficacy and are currently being pursued as potential anti-obesity drugs without psychiatric side effects.  

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. 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. In adipocytes, CB₁ 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. In the liver, mice fed a high fat diet for 3 weeks developed steatosis and showed greatly increased hepatic AEA levels, but not 2-AG. 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. 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. Enhanced NAPE-PLD and reduced FAAH activities in the jejunum were reported, yet interestingly, OEA levels were not affected. Peripheral CB₁ receptor blockade (URB447, Figure 5) attenuated fat sham feeding, which supports the hypothesis that endocannabinoids are released upon high fat food consumption and drive a positive feedback loop via CB₁ receptor signaling. In pancreatic islets, AEA content and NAPE-PLD gene expression was enhanced in fatty diabetic versus lean rats. It was shown that AEA induced apoptosis of insulin producing beta cells via peripheral CB₁ receptor activation, thereby enabling the progression of type II diabetes. Accordingly, chronic treatment with the peripherally restricted CB₁ receptor antagonist

![Figure 5. Structures of selected central (rimonabant) or peripherally restricted (URB447, JD5037) CB₁ receptor antagonists.](image-url)
JD5037 (Figure 5) 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.225

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.226 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.227 In the same cohort, plasma 2-AG levels were not found to be upregulated in obese individuals.228 In another large human trial (997 subjects), circulating AEA concentrations were also associated with BMI.229 Furthermore, AEA correlated with non-alcoholic steatohepatitis (NASH) disease severity and was therefore proposed as a biomarker.229 These combined clinical and pre-clinical data suggest that lowering plasma 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.230

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.231 Mice with a genetic deletion of NAPE-PLD presented a reduced food intake and overall leaner phenotype than their WT littermates.175 Of note, these effects were not observed in a different NAPE-PLD KO strain.232 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.233 These studies suggest that inhibition of NAPE-PLD may constitute as a potential treatment for metabolic syndrome. However, mice with a specific deletion of NAPE-PLD in adipose tissue had a predisposition for obesity while receiving a normal diet.234 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.234 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.235 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.235 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.

**Cancer**

Multiple studies have reported disrupted NAE levels in cancer and associations between NAE receptors and tumor proliferation. Hepatic CB$_1$ receptor and NAPE-PLD expression as well as AEA concentrations were found to be elevated in hepatocellular carcinoma (HCC) both in humans and mice. Treatment with the peripherally restricted CB$_1$ receptor antagonist JD5037 or CB$_1$ receptor KO mice demonstrated 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. Accordingly, FAAH KO mice displayed a worsened tumor progression. In addition, human hepatic tumor tissue exhibited reduced FAAH expression. In chronic lymphocytic leukemia (CLL) patients, plasma levels of OEA were upregulated and correlated with the number of circulating tumor cells. 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. Importantly, PPAR-α expression was found to be elevated in CLL patients and associated with an advanced disease stage. Furthermore, a PPAR-α antagonist was able to reduce tumor burden in a mouse model of CLL. Taken together, these studies suggest that targeting NAE biosynthetic enzymes, in particular NAPE-PLD, could have beneficial therapeutic effects in leukemia or hepatic cancer.

**Chronic liver disease**

Besides hepatic cancer and steatosis, also cirrhosis has been implicated in aberrant NAE signaling. 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. Similar findings were observed in another study, reporting increased circulatory AEA, OEA and PEA levels in cirrhotic patients, which correlated with advanced disease stage. 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. AEA induced vasodilation in mesenteric vessels from cirrhotic rats, whereas control samples were less sensitive to AEA. Antagonists for the CB$_1$ receptor (rimonabant) or TRPV1 (capsazepine) blocked this effect. Accordingly, administration of rimonabant in cirrhotic rats decreased mesenteric blood flow and portal hypertension. CB$_1$ receptor expression is
Therapeutic opportunities of modulating the endogenous N-acyl ethanolamine tone

low in healthy human liver, but it was upregulated in fibrotic and cirrhotic samples. Genetic deletion or pharmacological blockade of CB1 receptors (rimonabant) reduced hepatic fibrogenesis in three different fibrotic rat models. This was extended to advanced cirrhotic rats, where treatment with rimonabant for two weeks reversed fibrosis.

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. LPS was reported to induce AEA production in mouse macrophages, which was dependent on the PLC/phosphatase biosynthetic pathway. In addition, pro-inflammatory stimuli such as LPS were found to downregulate NAPE-PLD expression in mouse macrophages, thereby reducing anti-inflammatory PEA concentrations. To summarize, the described studies point towards pathological signaling of AEA in hepatic fibrosis and cirrhosis and suggest that blocking CB1 receptor activation or AEA biosynthesis, possibly via the PLC/phosphatase pathway, could be of potential therapeutic benefit.

Reducing NAE levels in the brain?

In neurodegenerative diseases, for example multiple sclerosis and Parkinson’s disease, AEA levels were found to be elevated in human cerebrospinal fluid. It is proposed that the AEA increase does not induce disease progression, but rather provides neuroprotection via CB1 or CB2 receptor activation as a result of the neuroinflammatory component of these diseases. Substantial evidence has been collected for the beneficial effects of CB1 and CB2 receptor signaling in CNS injury, however, several studies also point to a positive effect of CB1 receptor inhibition. For example, CB1 receptor blockade with rimonabant was neuroprotective in various rodent models of brain injury and enhanced AEA levels were harmful. OEA and PEA, which are more abundant in the brain, have neuroprotective or anti-inflammatory effects acting in part via PPAR-α. In a mouse model of cerebral ischemia, activation of brain PPAR-α by OEA reduced infarct volume. Currently, different strategies are being investigated that activate the cannabinoid and PPAR receptors by enhancing the NAE tone (e.g. FAAH inhibition) or by using CB1/CB2 agonists as therapeutic treatment for neurological conditions. Recently, a frameshift variant of NAPE-PLD in several dog breeds was reported to be a risk factor for leukoencephalomyelopathy, a myelination disorder. The impact of this NAPE-PLD variant on the enzymatic activity or brain NAE concentrations, has yet to be determined.

NAE signaling in the brain is involved in numerous physiological processes, such as memory formation, stress and anxiety. At present, the potential benefits of reducing NAE levels in the CNS are unclear. The depressive side effects associated with brain CB1...
receptor antagonism, suggest that depletion of tonic AEA signaling could have a similar negative outcome. Recently, two reports 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.\textsuperscript{261} 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.\textsuperscript{262} These studies indicate that depleting the brain NAE tone can have brain region-specific outcomes. The neurophysiological behavior of mice with a genetic deletion in one of the NAE-producing enzymes such as NAPE-PLD, ABHD4 and GDE1 have not yet been profiled, since brain AEA concentrations were not unambiguously reduced.\textsuperscript{37} This highlights the need for centrally active NAE biosynthesis inhibitors, to expose the primary pathway of NAE and AEA generation, and to establish the effect of decreased NAE signaling.

1.4 Aim and outline of this thesis:

Selective and \textit{in vivo} active pharmacological tools that modulate NAE biosynthetic enzymes are necessary to elucidate the importance of these pathways. Furthermore, reducing the NAE tone may hold promise for the treatment of several pathological conditions. So far, no inhibitors have been described that can decrease NAE levels in cells or live animals. The aim of this thesis work is the discovery and application of new chemical tools for two NAE-generating enzymes: NAPE-PLD and PLAAT2.

To obtain new molecules that can inhibit NAPE-PLD, in \textbf{Chapter 2}, a fluorescence-based activity assay for NAPE-PLD was optimized to enable high-throughput screening for hit identification. A library of \textasciitilde350,000 compounds was screened. After multiple deselection rounds, five hit compounds were obtained with (sub)micromolar potency and reasonable physicochemical properties. Resynthesis and testing of the most promising hit – a pyrimidine-4-carboxamide – confirmed its activity for NAPE-PLD and provided a suitable starting point for the development of \textit{in vivo} active NAPE-PLD inhibitors.

In \textbf{Chapter 3}, a library of pyrimidine-4-carboxamides was generated to increase the potency of the HTS-hit compound for NAPE-PLD and to improve its physicochemical properties. By modifying different substituents one at a time, a structure-activity relationship map was created. This afforded the optimized NAPE-PLD inhibitor LEI-401 with nanomolar potency and favorable physicochemical features.
Therapeutic opportunities of modulating the endogenous \( N \)-acylethanolamine tone

In drug discovery and development, establishing target engagement of a drug candidate and its intended protein target is an essential step for success in pre-clinical and clinical research. To assess whether \textbf{LEI-401} can bind to NAPE-PLD in live cells, in \textbf{Chapter 4}, a photoaffinity labeling approach was investigated. First, photoaffinity probes were synthesized that allowed visualization of NAPE-PLD using gel-based fluorescent labeling or chemical proteomics. Finally, cellular target engagement of \textbf{LEI-401} with NAPE-PLD was confirmed by performing competition experiments in the photoaffinity assay.

In \textbf{Chapter 5}, the NAPE-PLD inhibitor \textbf{LEI-401} was profiled in cellular and \textit{in vivo} models to characterize its effect on NAE biosynthesis. In neuronal cells, \textbf{LEI-401} produced a marked reduction of multiple NAEs, including AEA, among a broad lipid panel. This effect was dependent on NAPE-PLD protein expression. Intraperitoneal administration in mice showed that \textbf{LEI-401} exhibited a good pharmacokinetic profile and passed the blood-brain barrier. A significant time- and dose-dependent decrease of AEA was observed in the brain, but not of other NAEs. Behavioral profiling in mice indicated that \textbf{LEI-401} produced hypomotility, antinociception and hypothermia. Also in a mouse model of inflammatory pain \textbf{LEI-401} elicited an analgesic effect. In short, \textbf{LEI-401} was identified as an \textit{in vivo} active NAPE-PLD inhibitor, capable of decreasing brain AEA levels.

\textbf{PLAAT2} is a \( \mathrm{Ca}^{2+} \)-independent \( N \)-acyltransferase that was reported to produce high levels of NAEs in cells. In \textbf{Chapter 6}, \( \alpha \)-ketoamide inhibitors were identified as PLAAT2 inhibitors through library screening with an activity-based probe. A structure-activity relationship analysis was performed, which yielded \textbf{LEI-301} as a nanomolar potent PLAAT2 inhibitor. \textbf{LEI-301} was able to significantly reduce NAE levels including AEA after PLAAT2 overexpression in cells.

\textbf{Chapter 7} summarizes the work described in this thesis and provides new avenues for future research.
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Therapeutic opportunities of modulating the endogenous N-acylethanolamine tone


Therapeutic opportunities of modulating the endogenous N-acylethanolamine tone


Chapter 1


Chapter 1


Therapeutic opportunities of modulating the endogenous N-acylethanolamine tone


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Therapeutic opportunities of modulating the endogenous N-acylethanolamine tone


