New chemical tools to illuminate N-acylphosphatidylethanolamine biosynthesis

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1

General introduction

Lipids are a major type of biomolecules that are defined as small, hydrophobic or amphipathic molecules that (partially) originate from the condensations of ketoacyl thioesters and/or isoprene units.[1] This definition gives rise to a chemically diverse set of compounds, in which eight classes are distinguished: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides, sterol lipids and prenol lipids. These molecules exert a wide array of biological functions, including membrane building blocks, energy storage, vitamins, hormones, immunomodulatory agents and toxins.[1,2] Many signaling lipids are low-abundant, short-lived and chemically instable, which has complicated efforts to elucidate their physiological activities.[2]

N-acylphosphatidylethanolamines (NAPEs) are a family of glycerophospholipids with both structural and signaling activities (Figure 1.1). They are low-abundant, making up only 0.01% of the membrane phospholipids in animal brains under physiological conditions, corresponding to low nanomoles per gram tissue.[3] Nevertheless, they are widespread across different tissues, being found in mammalian central nervous system, gut, spleen, testes and plasma.[3,4] NAPEs are more abundant in plasma membranes than intracellular compartments and were found in erythrocytes, which do not have intracellular membranes.[5,6] They were first discovered in 1965[7], and until the 1990s they were regarded as nothing more than short-lived intermediates in the biosynthesis of phosphatidic acid (PA) or *N*-acylethanolamines (NAEs).[8–10] Only recently their specific biochemical and biophysical properties are being appreciated.[3,4] These properties include regulation of membrane dynamics, feeding and tissue degeneration (Figure 1.2).

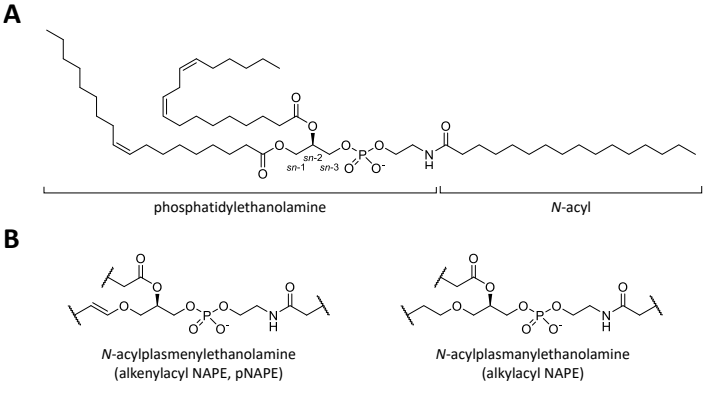

Figure 1.1. General structure of NAPE-type lipids. A) Structure of *N*-palmitoyl-1-oleoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine, a typical NAPE. Indicated are the phosphatidylethanolamine (PE) and *N*-acyl building blocks and the stereospecific numbering (*sn*) of the glycerol backbone. B) Structures of alternative NAPE-like species, with an *sn*-1 ether-linked substituent.

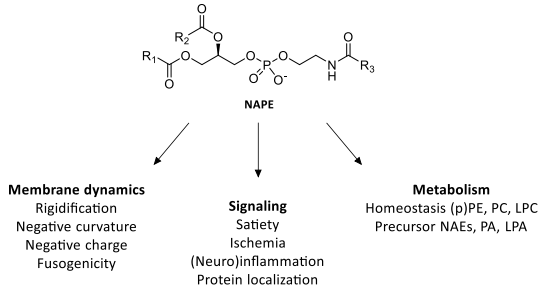

Figure 1.2. Overview of most important reported activities of NAPEs. Structural and signaling functions of NAPEs have been reported. In addition, they may exert functionalities through maintaining levels of up- or downstream bioactive metabolites. Functions are not mutually exclusive and may not be restricted to one classification.

Structural properties of NAPEs

NAPEs are triacylated glycerophospholipids synthesized from phosphatidylethanolamine (PE). In plants, NAPEs are formed by direct acylation of the PE amine with free fatty acids (FFAs)[11,12], whereas in mammals the acyl is transferred from a fatty acid donor.[13,14] This *N*-acyl transfer converts the zwitterionic PE into a negatively charged phospholipid (Figure 1.3). The composition of the three tails is highly diverse, but in general the *sn*-1 position is occupied by saturated or monounsaturated chains and the *sn*-2 position by (poly)unsaturated acyl groups, reflecting the composition of the PE precursor (Figure 1.1).[15,16] In rat brain, the *sn*-2 substituent was found to often be an oleoyl (18:1) ester, suggesting an enzymatic preference for 2-oleoyl-PE substrates.[17] The *N*-substituent composition appears to be dictated by acyl donor availability, as it mirrors the *sn*-1 composition of PC.[3,18] *N*-palmitoyl (16:0)-PE (NPPE) was the most abundant NAPE species found in human plasma, rat brain, rat testes (over 50%) and rat heart (~20%), with lower levels of *N*-stearoyl (18:0), *N*-oleoyl (18:1) and *N*-linoleoyl (18:2)-PE. Reported levels of polyunsaturated species such as *N*-arachidonoyl (20:4), *N*-docosapentaenoyl (22:5) and *N*-docosahexaenoyl (DHA, 22:6)-PE range from 0.6% to 11%.[16,18–21] NAPE concentrations are highly dependent on cell type and developmental state. For example, the concentration of *N*-arachidonoyl-PE (NArPE) in rat brainstem and striatum is two to three times higher than in hippocampus, hypothalamus or diencephalon and six times higher than in cortex.[22] In adult rats, brain NArPE levels are increased over three-fold compared to neonatals.[23]

Important NAPE-like classes include *N*-acylplasmanylethanolamines, in which the *sn*-1 substituent is an alkyl ether, and *N*-acylplasmenylethanolamines or plasmalogen NAPEs (pNAPEs), in which the *sn*-1 substituent is a vinyl ether (Figure 1.1). Compared to canonical NAPEs, they can be relatively high-abundant. For example, in dog heart and rat brain, levels of *sn*-1 vinyl ether NArPEs were found to be equal to or higher than their *sn*-1 acyl counterpart, respectively.[24,25] Rat intestinal mucosa, however, does not contain *sn*-1 alkyl nor alkenyl NAPEs, whereas serosa contains all classes.[26] Nevertheless, (vinyl) ether-linked NAPEs are underappreciated as a separate class and plasmanyl species are often neglected in studies on NAPEs.[27–29]

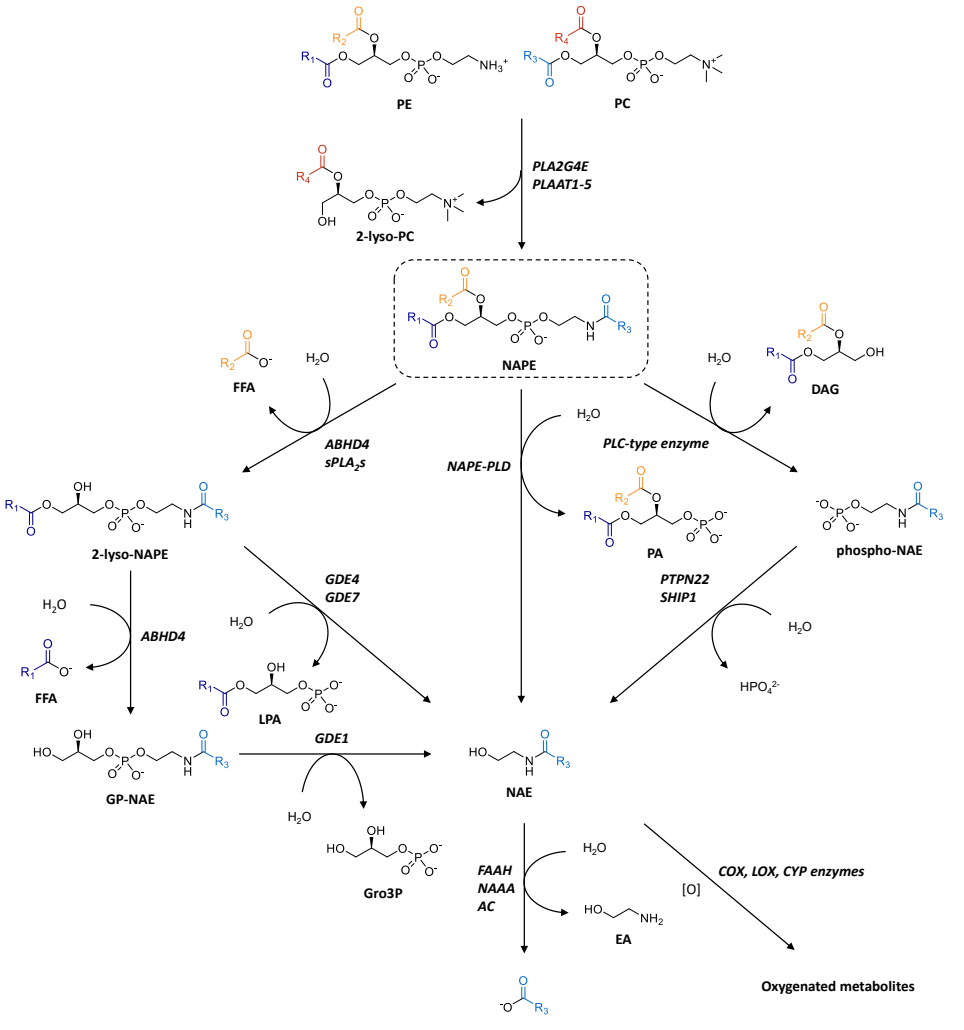

Figure 1.3. Metabolic pathways involved in biosynthesis and degradation of NAPEs. Shown are the transacylation reaction from canonical acyl donor phosphatidylcholine (PC) to phosphatidylethanolamine (PE), the direct hydrolysis of *N*-acylphosphatidylethanolamine (NAPE) to *N*-acylethanolamine (NAE) by NAPE-specific phospholipase D (NAPE-PLD), the three multistep pathways leading to NAE and the metabolism of NAE. Indicated are the responsible enzymes and secondary products formed. Ether-type NAPEs and alternative acyl donors are not explicitized. PLA2G4E: phospholipase A_2 group IV E, PLAAT: phospholipase A/acyltransferase, ABHD4: α/β hydrolase domain-containing protein 4, $sPLA_2$: secreted phospholipase A_2, GDE: glycerophosphodiesterase, PLC: phospholipase C, PTPN22: protein tyrosine phosphatase non-receptor type 22, SHIP1: Src homology 2 domain-containing inositol 5' phosphatase 1, FAAH: fatty acid amide hydrolase, NAAA: NAE-hydrolyzing acid amidase, AC: acid ceramidase, PA: phosphatidic acid, LPA: lysophosphatidic acid, GP-NAE: glycerophospho-NAE, Gro3P: glycerol-3-phosphate, DAG: diacylglycerol, EA: ethanolamine, FFA: free fatty acid.

The three tails of NAPEs give it extraordinary interactions in lipid membranes. Similar to other phospholipids, the *sn*-1 and *sn*-2 acyl groups are embedded in the lipid bilayer, but the conformation of the *N*-acyl chain depends on the number or carbons. Short-chain substituents (<10 carbons) appeared to be randomly oriented or to reside at the membrane interface, whereas longer-chain substituents (≥10 carbons) are oriented parallel to the other two substituents (Figure 1.4A, B).[30,31] Penetration of a third fatty acid chain into the membrane leaflet leads to increased steric bulk and consequently promotes a negative membrane curvature[32,33] and rigidifies the membrane.[34] In addition, acylation of the ethanolamine disrupts intermolecular hydrogen bonds between the amine and phosphate groups. However, this is probably compensated for by intermolecular hydrogen bonds between the N-H and carbonyls of the acylamide (Figure 1.4C).[34,35] The membrane-stabilizing effect of NAPEs has been exploited in liposome formulations. Liposomes with increased NAPE content were larger and less permeable, as determined by measuring leakage of its fluorescent cargo.[32] In a similar experiment, NPPE was shown to increase the stability of vesicles in the presence of human serum, an effect that was attributed to a combination of increased membrane rigidity, altered surface charge and reduced lipid exchange with serum lipoproteins.[36] In addition to a stabilizing function, *N*-dodecanoyl (12:0)-PE and NPPE-containing liposomes showed increased cation-dependent fusogenicity with each other, erythrocyte ghosts and U-937 cells.[37,38] The negative surface charge of NAPEs probably played a role, as low pH also stimulated fusion. However, no fusion was observed with phosphatidylglycerol (PG) or phosphatidylserine (PS)-containing liposomes, indicating a NAPE-specific effect.[37] In line with these results, NAPEs were enriched around division sites in mutant *Escherichia coli* lacking PG and cardiolipin, possibly fulfilling the natural role of these lipids in budding and cell division through their effects on membrane curvature and surface charge.[39] The combination of increased liposome stability in serum and fusogenicity make the incorporation of NAPEs into liposomal formulations interesting for drug delivery purposes.

NAPEs as feeding hormones

In fasted rats, plasma and lymph NAPE levels were found to increase following a high-fat diet.[20,40] These NAPEs are synthesized in the small intestine from the ingested lipids and their general composition reflects that of those. Of note, NArPE levels did not respond to feeding, but increased upon starvation.[41] *N*-palmitoyl-1,2-dioleoyl-*sn*-PE (*N*-palmitoyl-DOPE) administration was shown to have an anorectic effect, whereas DOPE and *N*-palmitoylethanolamine (PEA) did not.[20] The same effect was observed in *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) KO mice and with non-hydrolyzable *N*-palmitoyl-dialkyl-PE, indicating it was not mediated by NAPE metabolites.[42] Since administration of *N*-^{14}C-palmitoyl-DOPE led to an accumulation of the radiolabel in the hypothalamus and modulation of activity of hypothalamic neural networks involved in feeding behavior, it was suggested that NAPEs function as gut-

derived hormones sending satiety signals to the brain.[20] However, this view later was contested as other mechanisms could not be ruled out. For example, high concentrations of dipalmitoyl-PE and phosphatidic acid were shown to have a similar hypophagic effect, suggesting the satiety signaling is not specific for NAPEs.[42] Also, expression of NAPE-PLD, the enzyme responsible for the direct conversion of NAPEs to NAEs, in rat jejunum is postprandially increased, while expression of NAE-degrading enzyme fatty acid amide hydrolase (FAAH) is decreased, suggesting an important role for NAEs rather than NAPEs.[26] Indeed, NAPE-PLD activity was found to be required for the anorectic effects of NAPEs when they were administered orally instead of intraperitoneally.[43]

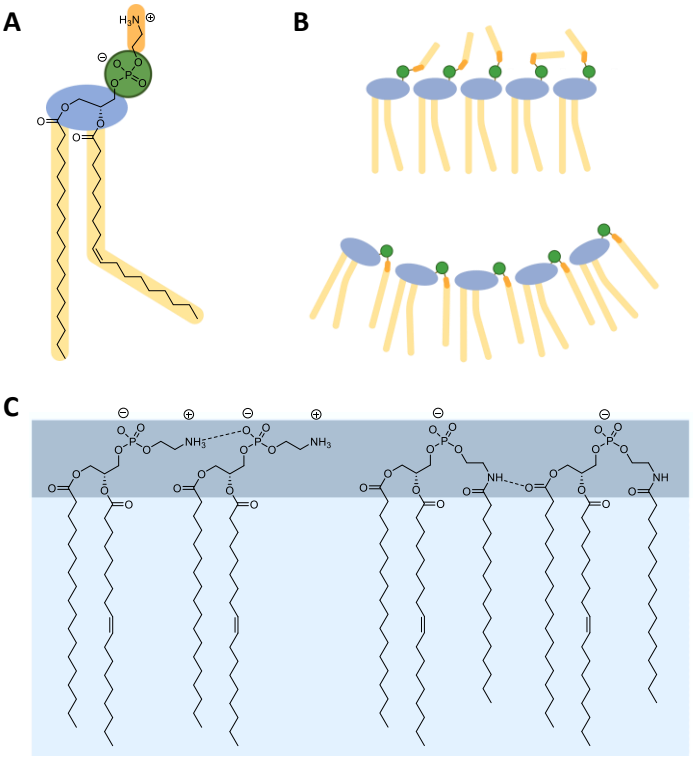

Figure 1.4. Schematic representation of NAPE conformations in lipid membranes. A) Cartoon representation of phosphatidylethanolamine (PE) as used in (B). B) Short-chain *N*-acyl substituents (top) are positioned randomly across the membrane interface and do not influence membrane curvature. Longer-chain *N*-acyls (bottom) are positioned parallel to the other two substituents and induce negative membrane curvature. C) PEs (left) make intermolecular hydrogen bonds between the amine and phosphate groups, whereas NAPEs (right) make hydrogen bonds between the carbonyl and NH of the amide.

NAPEs in degenerating tissue

Ischemia

One of the earliest recognized features of NAPE biology is their increased levels in degenerating tissue. In the 1980s, several studies reported an accumulation following ischemia. In myocardium of dogs, levels of diacyl NAPEs, their plasmalogens and *N*-acylplasmanylethanolamines were increased in central and peripheral infarcted areas to 4–6% of the phospholipid content, accompanied by a relative decrease in PE levels.[44] pNAPEs accounted for approximately 50% of the total NAPE-like species, the *sn*-2 *O*-acyls of which were highly enriched in arachidonoyl (75%). In contrast, diacyl NAPEs consisted for only 33% of 2-arachidonoyl species in central infarcted areas and were enriched in *sn*-2 linoleoyl esters instead.[44] Elevated levels of NAPEs were also found in infarcted myocardium of cats, but not of rabbits and humans.[9] In addition, NAPE accumulation was reported in ischemic rat brains following decapitation[8] or artery occlusion.[45,46] Here, diacyl NAPEs formed the majority (77%).[8] In striatum and cortex, the 2–3-fold NAPE accumulation was accompanied by a 30-fold increase in NAEs.[46] Models of chemical-induced anoxic toxicity were also shown to increase NAPE levels. During anoxia or NaN_3-induced anoxic stress, NAPEs accumulated in potato cells and cultured rat neocortical neurons.[47,48]

In general, the composition of the three lipids of the elevated NAPE species does not significantly differ between healthy and diseased tissue, with *sn*-1 *O*-acyl and *N*-acyl consisting mainly of palmitoyl, stearoyl or linoleoyl groups.[8,44–46,49] However, on the *sn*-2 position, NAPEs in ischemic tissue appeared to be enriched in linoleoyl or docosahexaenoyl esters.[8,44,47]

Neurodegeneration

Accumulation of NAPEs in neuronal cells has been observed in response to other stimuli than anoxic stress. Stimulation of cultured mouse neocortical neurons with *N*-methyl-D-aspartate (NMDA) or NMDA receptor agonist glutamate resulted in increased NAPE and NAE levels, preceding neuronal death.[50,51] In line, *in vivo* necrotic brain damage, induced by concussive traumatic injury or intrastriatal injection of NMDA in infant rats, provoked a 19- to 44-fold increase in NAPE levels within 24 h, respectively.[49] In contrast to other studies showing an ischemia-localized effect[44,46], NAPE levels were also elevated in the unaffected tissue of the contralateral cortex. Similarly, ethanol-induced neuronal apoptosis in infant mice led to an 4–6-fold accumulation of NAPEs, most notably in cortex, hippocampus and inferior colliculus.[52,53]

The neurotoxin 6-hydroxydopamine (6-OHDA), which is used in Parkinson's disease (PD) models for its ability to cause dopaminergic neuronal death[54–56], increased levels of specific NAPE species in the striatum, but not in the substantia nigra (SN).[29,57] (p)NAPEs with *sn*-2 *O*-arachidonoyl or docosahexaenoyl substituents and saturated *N*-acyls were significantly elevated, while *N*-arachidonoyl species were not affected. In mice lacking NAPE-PLD, NAPE levels were increased even further. In these mice, neuronal damage due to 6-OHDA was less pronounced, suggesting a protective role of NAPEs against 6-OHDA-

induced neurotoxicity.[29] 6-OHDA treatment led to an increase in activated Rac1 in the SN of wildtype mice, but not in NAPE-PLD KO mice. Rac1 is a GTPase implicated in dopaminergic neuronal survival and PD[58,59] that can be activated through interactions with membrane-bound leucine-rich repeat kinase 2 (LRRK2), also known as PARK8 and associated with both familial and sporadic PD.[60,61] LRRK2 was found to dissociate from the plasma membrane in NAPE-PLD KO mice.[62] It is tempting to speculate that elevated levels of NAPEs would exert a neuroprotective activity through alteration the plasma membrane architecture, leading to LRRK2 dissociation and subsequent reduced activation of Rac1, which has an anti-inflammatory effect. In line, *N*-palmitoyl-DOPE was shown to decrease Rac1 activation in macrophages, inhibiting phagocytosis.[63]

Moreover, (p)NAPE biosynthesis may provide an important means to maintain homeostasis of precursors PE and PC. These are important structural lipids in the brain, and deregulation of their levels is related to neurodegenerative diseases.[64] Both PE and PC are synthesized in the ER membrane from diacylglycerol (DAG) by specialized cytidylyltransferases. Expression of these enzymes was found to be increased in SN of PD patients.[65] PE can also be synthesized by decarboxylation of PS. Downregulation of this second pathway has been shown to cause accumulation of α-synuclein, a hallmark symptom of PD.[66] Plasmenylethanolamine composes over 50% of the PE content in neurons and even 80–90% of all phospholipids in myelin sheaths, where it provides structural rigidity.[67,68] Levels of pPE were found to drop dramatically during Alzheimer's disease (AD) onset and the decrease was correlated to the severity of the patient's dementia.[68]

Effects on NAPE levels were also observed in other forms of tissue degeneration. In a model system for inflammation, $CdCl_2$-treated rat testes showed increased NAPE levels enriched in saturated *N*-acyls. The NAEs levels were even more drastically increased.[69] Furthermore, NAPEs were able to activate lysosomal β-glucosidases from healthy individuals *in vitro*, but not those from Gaucher's disease patients, suggesting a possible link to the molecular basis of this disease.[70]

Metabolism of NAPEs

NAEs

Despite having physiological functions themselves, NAPEs are primarily known as the precursors to NAEs. NAEs have long been appreciated as a diverse class of signaling lipids, which exert a plethora of biological activities.[71–73] *N*-palmitoylethanolamine (PEA) was the first NAE to be identified.[71] It has anti-inflammatory[74], analgesic[75], retinoprotective[76,77], satietal[78] and anti-addictive[79,80] effects, which are mediated through activation of the peroxisome proliferator-activated receptor α (PPARα).[81,82] PEA levels are elevated in tissue surrounding stroke or ischemia[83], where it reduces infarct size through inhibiting astrocyte activation,[84] and it plays a neuroprotective role in Alzheimer's disease.[85–88] Affinity for

orphan receptors GPR55 and GPR119 and transient receptor potential cation channel V1 (TRPV1) has been reported, but the physiological relevance is unclear.[89–92]

N-oleoylethanolamine (OEA) is well-studied as a satiety factor produced intestinally from food.[26,93,94] It stimulates lipolysis[95] and reduces feeding[96,97] through its activation of peripheral PPARα and GPR119, and works as a satiety hormone in the brain.[98,99] Therefore it is considered an anti-obesity nutraceutical.[100] Like PEA, it has anti-addictive[101] and neuroprotective effects.[83] Furthermore, OEA is an antagonist of TRPV1 that alleviated L-DOPA-induced dyskinetic symptoms in a mouse model of PD.[102,103]

The most extensively studied NAE is *N*-arachidonoylethanolamine (AEA, or anandamide), because it targets the cannabinoid receptors CB_1 and CB_2.[104,105] It is relatively low-abundant (up to 100-times lower than PEA and OEA), but found in most tissues including the central nervous system (CNS).[106] Because CB_1 is activated by (-)-Δ^9-tetrahydrocannabinol (THC), the psychoactive component of marijuana, anandamide has gained much attention for its ability to function as an endogenous cannabinoid, or endocannabinoid. As such, it modulates emotional behavior, including stress[107,108], anxiety[109,110] and depression.[111] Indeed, anandamide was shown to reduce stress-induced corticosterone release[112], although a seemingly opposite effect was also reported.[113] Other cannabimimetic activities of AEA include orexigenia[114,115], anticonvulsion[116–118], hypotension[119], analgesia[107,120,121] and memory formation.[122,123] Furthermore, it is involved in neuroprotection[124–127], fertility[128,129], inflammation[130–133] and cancer.[134–136] Beside the cannabinoid receptors, AEA also activates the TRPV1 receptor and this way regulates vasodilation[137,138], nociception[139,140] and mood.[141,142]

Other NAEs have received less attention, despite their similar activities. *N*-stearoylethanolamine (SEA) has anti-inflammatory[143], anorexic[78,144] and pro-apoptotic[145] properties. *N*-linoleoylethanolamine (LEA) is the most abundant NAE in the jejunum, but has very low levels in the brain.[146] It was found to play a role in inflammatory bowel disease, stimulating bacterial growth.[147] *N*-docosahexaenoylethanolamine (DHEA, or synaptamide) stimulates neurogenesis, neuritogenesis and synaptogenesis through activation of GPR110[148,149] and also has anti-(neuro)inflammatory activity.[150,151] Some of the physiological roles of different NAPEs are likely related to the function of their respective NAE metabolites.

Metabolic pathways

Four metabolic pathways have been described for the conversion of NAPEs into NAEs. Of these four pathways, three go via one or more intermediate lipids, which have biological relevance on their own (Figure 1.3). The direct conversion of NAPEs and pNAPEs into NAEs is performed by NAPE-PLD.[10] NAPE-PLD is a metallo-β-lactamase, structurally unrelated to other human PLDs, that uses two active site Zn^{2+}-ions to hydrolyze the NAPE phosphate.[152,153] In its active conformation, it is a membrane-associated dimer that has a membrane-facing hydrophobic cavity to extract NAPEs from the membrane and allosteric pockets that accommodate activating bile acids and polyamines.[152,154–156] It is expressed in

several organs, most abundantly in brain, kidney and testes.[157] *In vitro*, NAPE-PLD is specific for (p)NAPEs (with modest lyso-NAPE-PLD activity), but has no selectivity for the *N*-acyl substituents, suggesting it is capable of synthesizing all NAEs.[157,158] Independent KO studies, however, have described conflicting results on AEA and DHEA levels, which might be attributed to compensatory effects.[159–161] Recently, the first *in vivo* active NAPE-PLD inhibitor was described, which decreased the levels of saturated, monounsaturated and ω-6-polyunsaturated NAEs, including AEA.[162] Beside NAEs, NAPE-PLD produces phosphatidic acid (PA). PA is a central precursor in the biosynthesis of many glycerolipids, including PE, PC and PS.[163] In addition, it has pH-dependent interactions with proteins involved in membrane component biosynthesis.[164]

The second pathway of NAPE degradation involves the sequential actions of α/β hydrolase domain containing protein 4 (ABHD4) and glycerophosphodiesterase 4 or 7 (GDE4, GDE7). First, the *sn*-2 ester is saponified by ABHD4, producing 2-lyso-NAPE, which is then hydrolyzed to NAE by lyso-NAPE-PLD activity of GDE4 or GDE7.[14] ABHD4 is a serine hydrolase that hydrolyzes NAPEs and *N*-acylphosphatidylserine (NAPS).[165] It is expressed in several tissues, most abundantly in CNS and testes.[166] GDE4 and GDE7 are lyso-PLDs that accept lyso-NAPEs, lyso-PE and lyso-PC and convert these to lysophosphatidic acid (LPA).[167,168] Little is known about the physiological importance of lyso-NAPEs, but LPA is regarded an important signaling lipid in (neuro)development, tissue regeneration and cancer.[169–172] Both GDE4 and GDE7 are ubiquitously expressed, but GDE4 is more abundant in brain and testes, whereas GDE7 is more abundant in kidney.[168] Both ABHD4 and GDE4 have a preference for plasmalogen substrates[165,167], suggesting this second pathway is used mainly for the degradation of pNAPEs. Alternatively, three secretory phospholipases, $sPLA_2IB$, $sPLA_2IIA$ and $sPLA_2V$, were shown to be able to hydrolyze the *sn*-2 ester of NAPEs.[173] As these enzymes are mainly present in the gastrointestinal system, they might be important for the local metabolism of NAPEs from food.

In the third metabolic pathway, ABHD4 sequentially hydrolyzes both NAPE *O*-acyls, producing glycerophospho-NAEs (GP-NAEs).[166] These intermediates are then converted to NAEs by GDE1 (also known as MIR16).[174] Like GDE4, GDE1 is a magnesium-dependent PLD predominantly expressed in CNS, kidney and testes.[174] It has shown activity on several glycerophospho metabolites, including GP-serine, GP-glycerate and GP-inositol.[175,176] Of note, GP-DHEA was found to be one of the most abundant peripheral GP-NAEs[161], while its levels in the brain were very low[174], suggesting distinct regulation of this pathway in different tissues. ABHD4 was recently shown to play a crucial role in anoikis in prostate cancer cells and embryonic brain development, but it is unclear which metabolites are involved in this process.[177,178] Because of the non-hydrolyzable *sn*-1 ether linkage in plasmanyl and plasmenyl NAPEs, these lipids cannot be metabolized via this third pathway.

The fourth NAPE metabolic pathway is the least characterized, and includes PLC-type hydrolysis to phospho-NAEs by an unknown enzyme followed by dephosphorylation to NAEs. Protein tyrosine phosphatase non-receptor type 22 (PTPN22) and Src homology 2 (SH2) domain-containing inositol 5′ phosphatase (SHIP1) have been identified to be able

to catalyze this final reaction. This pathway was discovered in macrophages and is responsive to lipopolysaccharide (LPS) stimulation.[179,180] Of note, PLC-type hydrolysis of NAPEs produces DAG, a key intermediate in the biosynthesis of many phospholipids.[67] It is unclear whether this pathway is used for the degradation of plasmanyl and plasmenyl NAPEs.

Bioactivity of NAEs is terminated by FAAH and *N*-acylethanolamine-hydrolyzing acid amidase (NAAA), that hydrolyze the amide bond to produce ethanolamine and FFAs. FAAH is a widely-expressed serine hydrolase that has been extensively studied for its central role in NAE metabolism, and specifically for AEA.[120,181,182] In higher mammals, including humans, but not rodents, a second enzyme named FAAH-2 was discovered that also hydrolyzes fatty acid amides, but without preference for NAEs.[183] Its physiological importance to NAE homeostasis has not been established. NAAA is a lysosomal cysteine hydrolase that belongs to the cholylglycine hydrolase family and is unrelated to FAAH.[184] It preferentially hydrolyses PEA and inhibition of NAAA was shown to have immunosuppressive effects.[185,186] Recently, the lysosomal cysteine hydrolase acid ceramidase (AC) was found to possess NAE hydrolytic activity.[187] AC is well-studied for its degradation of sphingolipids, and its concomitant role in lysosomal storage diseases.[188] The physiological relevance of its ability to hydrolyze NAEs remains unclear.

Polyunsaturated NAEs, such as AEA and DHEA, can additionally be converted to eicosanoid-type signaling lipids through oxygenation of their double bonds. Cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450 (CYP) enzymes form different types of eicosanoid-ethanolamines for which bioactivities have been reported, for example in immunomodulation and cytoprotection.[189,190] A detailed description of these functions is outside the scope of this thesis.

NAPE biosynthesis

In mammals, NAPEs are synthesized by transacylation reaction between PE and a fatty acid donor catalyzed by calcium-dependent or -independent *N*-acyltransferases (Figure 1.3). Phosphatidylcholine (PC) is considered the canonical donor, but PE and cardiolipin can also be used.[13,191,192] Calcium-dependent *N*-acyltransferase (Ca-NAT) activity was first described in ischemic dog heart in 1982[13], but the responsible enzyme remained elusive until in 2016, phospolipase A_2 Group IV E (PLA2G4E) was reported to be able to produce NAPEs from PE and PC (see below).[193]

PLAAT family

The second pathway of NAPE biosynthesis does not require calcium and is catalyzed by members of the phospholipase/acyltransferase (PLAAT) family.[194–196] PLAAT1–5 are small (162–279 amino acids) cysteine hydrolases encoded by the HRASLS1–5 genes that exert phospholipase A_1 (PLA_1), PLA_2 and acyltransferase activity.[17,197] They are structurally similar to lecithin-retinol acyl transferase (LRAT), but they cannot use retinol as an acyl acceptor.[196]

They are characterized by a conserved NCEHFV sequence, which contains the catalytic cysteine which is part of a Cys-His-His triad (except in PLAAT1), and a C-terminal hydrophobic domain (except in PLAAT5).[198] Whereas calcium-dependent NAT activity is regarded the canonical pathway of NAPE biosynthesis, the physiological importance of the PLAAT enzymes has not been fully elucidated.[14]

PLAAT1 (also known as A-C1) is highly expressed in testes and skeletal muscle and also in brain and heart, which is similar to the expression profile of PLA2G4E.[199] It has a Cys-His catalytic dyad and an N-terminal enrichment of basic residues, which is involved in its localization to the nucleus.[200] It displays higher *N*- and *O*-acyltransferase activity than $PLA_{1/2}$, with preferential cleavage of the *sn*-1 ester.[199] Its expression has been correlated to several forms of cancer, suggesting a role as tumor suppressor.[201,202] Recently, PLAAT1 was found to be responsible for developmental organelle degradation in the lens of zebrafish.[203] It is recruited to damaged organelle membranes dependent on its C-terminal hydrophobic domain.

PLAAT2 is expressed in gastrointestinal tissues, most abundantly in the small intestine and liver, and in kidney and trachea, but not in rodents.[195,204] Its C-terminal domain is important for its perinuclear localization.[204] PLAAT2 has the highest NAT activity of all PLAAT enzymes and also significant *O*-acyltransferase activity to 2-lysophospholipid acceptors.[27,195] Its anti-proliferative effect on several cancer cell lines may suggest a tumor suppressor function.[204]

PLAAT3 (also known as AdPLA, H-Rev107 or PLA2G16) is the most well-studied PLAAT. Its expression was found in many peripheral tissues, particularly in adipocytes.[195,205,206] It has a perinuclear localization, partially co-localizing with the endoplasmic reticulum (ER).[205] Of all PLAATs, PLAAT3 was found to have the highest phospholipase activity, with much lower transferase activity.[27] Contradictory results have been reported regarding preference towards the *sn*-1 or 2 position, as well as activation by calcium.[195,196,205] PLAAT3 has most extensively been studied as a tumor suppressor[207–213], but oncogenic activity has also been reported.[214,215] Similar to the activity of PLAAT1 in zebrafish, PLAAT3 is responsible for organelle degradation in the lens of mice.[203] Recently, it was found to be a host factor used by picornaviridae to prevent clearance.[216] In addition, overexpression of both PLAAT2 and PLAAT3 was found to cause peroxisomal dysfunction through their depletion of plasmenylethanolamines[27,217], which is known to be related to demyelinating diseases such as multiple sclerosis (MS).[67,218] PLAAT3 KO mice are resistant to diet-induced obesity due to increased lipolytic activity in adipocytes. It was suggested that PGE_2 derived from PLAAT3-produced AA inhibits lipolysis, implying PLAAT3 as an anti-obesity drug target.[206]

PLAAT4 (also referred to as RIG1, TIG3 or RARRES3) is ubiquitously found in human tissues, but not in rodents.[195] It has higher $PLA_{1/2}$ activity than NAT activity, but when it was overexpressed, NAPEs were shown to accumulate.[27,195] PLAAT4 expression is downregulated in several cancers[219–222], and its anti-proliferative effect was found to be dependent on its localization to the Golgi apparatus, mediated by the C-terminal hydrophobic segment.[223] In the epidermis it is involved in regulation of keratinocyte

proliferation and differentiation[224,225], and reduced expression is related to psoriasis and squamous cell carcinoma.[226]

PLAAT5 (also known as iNAT or RLP-1) is the largest of the PLAAT family.[14] It has low expression in brain and several peripheral organs, but is highly expressed in testes.[227,228] It is localized to the cytosol, possibly owing to the lack of a C-terminal hydrophobic domain.[227] PLAAT5 has higher NAT activity than $PLA_{1/2}$, and does not discriminate between *sn*-1 and *sn*-2 *O*-acyl cleavage.[227] No physiological function of PLAAT5 has been established yet.

Recently, the first PLAAT inhibitors have been published.[229,230] LEI-110 was able to inhibit PLAAT3-dependent arachidonic acid (AA) production in an overexpression system.[229] LEI-301 inhibited the accumulation of several NAEs by overexpressed PLAAT2 or PLAAT5.[230] These chemical tools will aid in elucidating the physiological role of these enzymes, including their relative importance in NAPE biosynthesis compared to Ca-NAT.

PLA2G4E

PLA2G4E is a serine hydrolase that belongs to the family of cytosolic phospholipases A_2 ($cPLA_2$), also known as the Group IV PLA_2s. This family of phospholipid-metabolizing enzymes consists of six known members (PLA2G4A–F, $cPLA_2\alpha$–ζ), which share low amino acid sequence identity (<40% on average).[231] They are, however, highly conserved throughout evolution, with human and mouse PLA2G4A sharing over 95% amino acid identity.[232] In both mice and humans, PLA2G4B, D, E and F form a gene cluster, suggesting evolutionary paralogy.[233] The PLA2G4 family is characterized by a catalytic Ser-Asp dyad. In PLA2G4A–C, an additional arginine residue is required for catalytic activity, and this Arg is conserved among all PLA2G4 enzymes.[234] All members except PLA2G4C have an N-terminal C2 lipid-binding domain with conserved residues involved in Ca^{2+} binding.[233] Each member has different preferential $PLA_{1/2}$, lyso-PLA and acyltransferase activity and expression pattern, suggesting different roles in phospholipid homeostasis.[231] For more information about PLA2G4A–D and PLA2G4F, see Box 1.

Early on it was recognized that PLA2G4E ($cPLA_2\varepsilon$) had only low PLA_1, PLA_2 and lyso-PLA activity, and in 2016 it was shown to be a principal *N*-acyltransferase that is strongly stimulated by calcium.[193,233] It was confirmed that this enzyme has the characteristics of the elusive Ca-NAT. The enzyme specifically transfers the *sn*-1 *O*-acyl from the acyl donor, which can be PC or PE, to the amine of (p)PE, but shows no preference for *sn*-1 lipid composition.[18,192,193] As such, PLA2G4E might be involved in the downstream production of NAEs.[192,235] When overexpressed, cellular *sn*-2-oleoyl-NAPE levels were increased most prominently, which is in line with the notion that these are the most abundant NAPE species in the brain.[17,192] Beside calcium, activity is stimulated by several anionic lipids, including PS, PA and PG, but not phosphatidiylinositol-4,5-bisphosphate ($PI(4,5)P_2$).[235,236]

Box 1 – PLA2G4 family

PLA2G4A is the first characterized and most-studied member of the PLA2G4 family.[257] It is a ubiquitously expressed cytosolic protein that translocates to intracellular membranes upon Ca^{2+} binding. From the elucidated X-ray crystal structure it was concluded that it possesses an N-terminal C2 domain featuring three calcium-binding loops (CBLs).[258] Upon calcium binding the negative charge of these is shielded, increasing the lipid binding properties of the domain.[259] Depending on the Ca^{2+} concentration, the C2 domain directs the enzyme to the membranes of the Golgi, ER or nuclear envelope.[243,260,261] The catalytic domain does not adopt a classical α/β hydrolase fold. Instead, the catalytic dyad is located in a deep funnel covered with a flexible, amphipathic lid that may confer substrate specificity, and a 'cap' region rich in basic residues that could interact with membrane phospholipids.[258] Translocation to intracellular membranes is required for catalytic activity, which can be enhanced through phosphorylation by various kinases.[262–264] A putative binding site for phosphatidylinositol phosphates (PIPs) is suggested to regulate intracellular localization as well as catalytic activity, but this has not been proven *in vivo*.[265–268] PLA2G4A has preferential PLA_2 activity and showed a strong preference for hydrolysis of *sn*-2 arachidonoyl esters from PC (regardless of the *sn*-1 linkage type), PE, PA and PS.[232,269] Indeed, it plays a central role in the production of arachidonic acid and its physiological importance has been established in mediating the release of immunomodulatory eicosanoids, which are oxidative products of arachidonic acid.[270–272] This way, PLA2G4A was found to be involved in the immune responses related to injury[271–274], ischemia[275], neurodegenerative diseases[276,277] and (auto)immune diseases[277–280], as well as in fertility[275] and cancer.[281,282] It contributes to trafficking of proteins within the Golgi apparatus and from the Golgi to the plasma membrane through the formation of membrane tubules.[244,283] This function appeared to require catalytic activity of PLA2G4A, but was independent of downstream production of eicosanoids, suggesting an additional function of PLA2G4A in mediating membrane dynamics.[244]

Less is known about the other members of the PLA2G4 family. In humans, three splicing isoforms of PLA2G4B ($cPLA_2\beta$) exist, of which only one (PLA2G4B3) was found to be endogenously translated.[284] It is found in most tissues, most abundantly in pancreas, liver and brain[234], and is constitutively localized to early endosomal and mitochondrial membranes.[284] It predominantly hydrolyzes 2-lysophospholipids in a calcium-independent manner.[284,285] The enzyme has an N-terminal truncated jumonji C (JmjC) domain upstream of the C2 domain, which lacks several conserved elements for histone lysine demethylation and therefore is of unknown relevance.[284,286] Recently, a read-through fusion gene formed by splicing of PLA2G4B with neighboring JmjD-containing protein 7 (JMJD7) was shown to play a role in cancer cell proliferation.[287]

PLA2G4B also has a C-terminal polybasic (PB) domain (-RRRQRR-) with unknown functionality.[284]

PLA2G4C (cPLA$_2$γ) is the smallest member of the family, as it lacks a C2 domain.[234] Consequently, its catalytic activity is calcium-independent.[234] PLA2G4C has low PLA$_1$ and PLA$_2$ activity, but higher lyso-PLA activity.[285,288] It has a preference for polyunsaturated *sn*-2 *O*-acyls, and overexpression increases arachidonic acid release.[289,290] In addition, it is able to transfer fatty acids from 1-lyso-PC and 1-lyso-PE to 2-lyso-PC and 2-lyso-PE.[291] Recently, it was shown to accept NAPEs as substrate for sequential *sn*-1 and *sn*-2 hydrolysis to GP-NAEs *in vitro*, similar to ABHD4.[292] It is highly expressed in human heart and skeletal muscle[290], and is tightly associated with the ER membrane and possibly the outer mitochondrial leaflet.[291,293–295] N- and C-terminal protein lipidation sites are potentially involved in the subcellular localization.[289–291,294,296] PLA2G4C is involved in lipid droplet formation and translocates to these upon formation.[295] Here, it plays a role as a host factor in hepatitis C viral genome replication and virion assembly.[297] The similarity of this function to that of PLAAT3 makes PLA2G4C an interesting subject for investigation.

PLA2G4D (cPLA$_2$δ) is predominantly a PLA$_1$, but also has relatively high PLA$_2$ activity, which can be slightly stimulated by calcium.[285] It has preference for PC and 2-lyso-PC over other phospholipids and preferentially cleaves linoleoyl esters.[298] After PLA2G4A, it is the only PLA2G4 for which the crystal structure was elucidated, which showed a remarkable tandem C2 domain.[240] The N-terminal of these was homologous to the C2 of PLA2G4A and showed conserved CBLs, whereas the second had similar overall topology but no CBLs. Of note, the second C2 showed significant sequence similarity with PLA2G4B, E and F, suggesting these might also have a double C2 domain.[240] Similar to PLA2G4A, PLA2G4D calcium-dependently translocates from the cytosol to ER/Golgi membranes.[233] In humans, it is found exclusively in stratified squamous epithelia of skin and cervix.[298] Its expression is increased in mast cells and keratinocytes in psoriatic lesions and atopic dermatitis, and the enzyme was found in mast cell-derived exosomes that could be taken up by antigen-presenting cells.[298,299]

Similar to PLA2G4B and C, mouse PLA2G4F (cPLA$_2$ζ) preferentially hydrolyzes 2-lyso-PLA esters, but it also contains PLA$_1$ and PLA$_2$ activity.[233,285] Its PLA$_1$ and PLA$_2$ activity were found to be stimulated by calcium, while lyso-PLA activity was not.[285] High expression was found in thyroid, and also some in stomach and intestinal tissue.[233] When overexpressed, mPLA2G4F localized to the cytosol and did not respond to calcium.[233] It appeared to be involved in arachidonic acid production in mouse lung fibroblasts.[300] Human PLA2G4F was reported to be the main phospholipase responsible for mitochondrial arachidonic acid production in healthy myocardium, providing the precursor for production of protective epoxyeicosatrienoic acids (EETs).[301]

Activity was increased most notably by PS, which also increased PLA2G4E's affinity for Ca^{2+}.[235] However, since these experiments were carried out on purified enzyme, the physiological relevance is not clear. The enzyme is expressed in brain, heart, testes, skeletal muscle and skin. Within the brain, only neuronal cells were found to express PLA2G4E.[193,237] Of note, the tissue distribution is highly similar to PLAAT1 and PLAAT5, both of which also have high acyltransferase activity.[199,227]

In humans, two isoforms of PLA2G4E exist, which differ in their N-terminal sequence. *In vitro*, these isoforms, consisting of 834 and 868 amino acids (97 and 100 kDa, respectively), showed similar activity and substrate preference.[235] Google's AlphaFold 2 predicts the N-terminus to be disordered (Figure 1.5)[238,239], and no physiological relevance for the two isoforms is known.[235] PLA2G4E has a C2 domain with three calcium-binding loops (CBLs), followed by a region with high sequence similarity to PLA2G4D's second C2 domain.[240] AlphaFold 2 predicts a C2-like topology for this region, but lacking CBLs, similar to PLA2G4D (Figure 1.5 and Box 1).[238] The lipase domain contains the conserved Arg^{384} and Ser^{412}-Asp^{700} catalytic dyad. How the topology of this domain prevents hydrolysis of substrate in favor of transacylation is not known.[241] Additionally, it has a C-terminal polybasic (PB) domain (-KKKRLK-), similar to PLA2G4B.[235] Removal of any of these domains was found to be detrimental for catalytic activity, but the mechanism through which the C2 and PB domains cooperate in substrate conversion was not elucidated.[236]

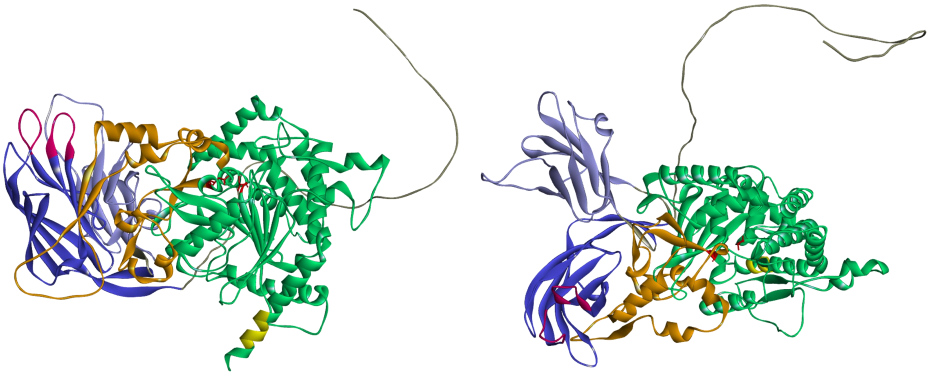

Figure 1.5. AlphaFold2 prediction of human PLA2G4E structure. Domains are color-coded based on sequence similarity with hPLA2G4A and hPLA2G4D. The disordered N-terminus (grey) is followed by the C2 domain (blue), which contains two calcium-binding loops (red). A second domain with C2 topology (purple) is predicted, but does not contain calcium-binding loops. The central lipase domain (green) contains the catalytic dyad (red, stick representation), a putative cap domain (orange) and the C-terminal polybasic stretch (yellow).

PLA2G4E is constitutively localized to perinuclear membranes.[233] In RAW264.7 macrophages, human PLA2G4E localized to lysosomes, which are rich in PS.[235] In HeLa cells, mouse PLA2G4E localized to vesicles and tubules associated with the clathrin-independent endocytic machinery. It was specifically present in early endosomes, but not in late endosomes, lysosomes or the Golgi apparatus. This localization appeared to be mediated

by interactions of the PB domain with PIPs, predominantly $PI(4,5)P_2$, which are enriched in clathrin-independent endocytic vesicles.[242] Removal of either the PB or the C2 domain abrogated the specific localization.[236] Additionally, in contrast to PLA2G4A and D[233,243], treatment with ionomycin was also shown to release the protein into the cytosol, which was suggested to be a consequence of calcium-dependent PIP degradation.[242] At these vesicles, PLA2G4E is involved in recycling internalized cargo, including MHC-I, CD55 and CD147, back to the plasma membrane. Tubule formation and trafficking were dependent on catalytic activity, but which metabolites are involved is not known.[242] As noted above, NAPEs have membrane- and vesicle-stabilizing properties, stimulate liposome fusion and might be involved in cellular division.[3,4] In addition, they can regulate the membrane association of intracellular proteins.[62] Lysophospholipids, which are a side product of NAPE production, can induce positive membrane curvature needed for vesicle formation and budding. PLA2G4A's regulation of Golgi trafficking might be mediated by lysophospholipids[244], suggesting that PLA2G4E could regulate endosomal recycling through production of either or both NAPEs and lysophospholipids (Figure 1.6).

One interesting class of clathrin-independent endocytic vesicles are caveolae. These vesicles, formed by assembly of caveolin and cavin, are thought to invaginate from the plasma membrane depending on the local lipid composition, particularly PS.[67,245] They are enriched in PS and $PI(4,5)P_2$[246], and have been shown to colocalize with Rac[247] and AEA.[248,249] Caveolae are not found in neurons, but high levels are present in endothelial and muscle cells.[250] It is tempting to speculate about the role of NAPEs and PLA2G4E in caveolae, but PLA2G4E depletion in cervical cells was shown to have no effect on caveolin-1.[242] However, a possible link in other cell types cannot be ruled out.

Recently, expression of PLA2G4E was linked to memory retention in Alzheimer's disease.[251] AD patients with clinical symptoms of cognitive deficits appeared to have lower brain PLA2G4E expression than early-stage AD patients and healthy controls. In a mouse model for AD, animals with impaired spatial memory showed downregulated cortical and hippocampal PLA2G4E compared to wild type mice. Resilient transgenic animals with intact memory, however, had significantly higher brain PLA2G4E expression than the impaired mice. Importantly, overexpression of PLA2G4E in the hippocampus of symptomatic mice restored their memory and increased neurogenesis, suggesting an important role for PLA2G4E in memory formation and retention.[251] Whether catalytic activity of PLA2G4E is required and which metabolites are involved in this process is not known. Furthermore, gene variants of PLA2G4E have been correlated to AD[252,253] and panic disorder[254], supporting a role in neurological diseases.

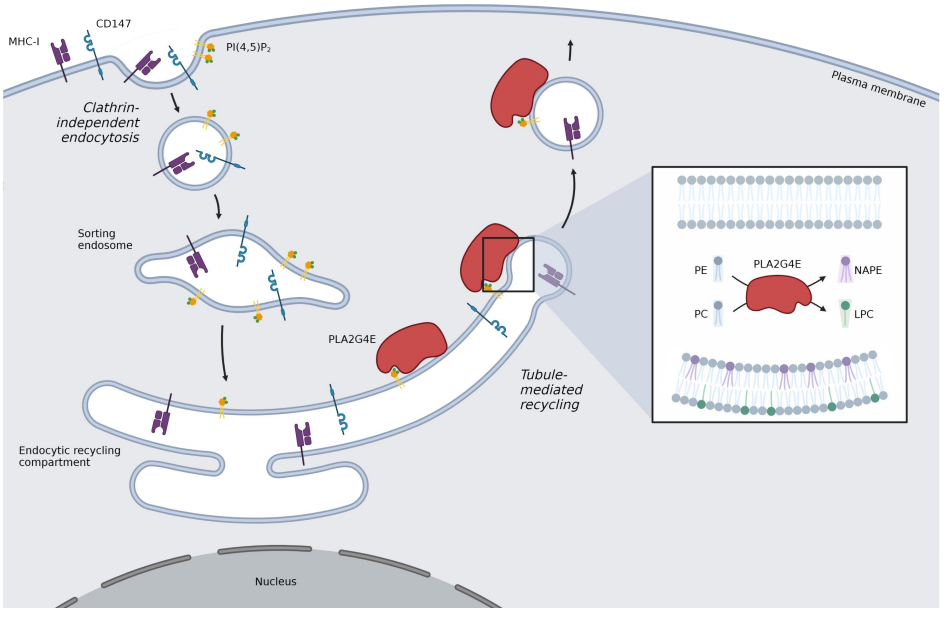

Figure 1.6. Schematic overview of the putative role of PLA2G4E in clathrin-independent endocytic recycling. Cargo internalized via clathrin-independent endocytosis (e.g. MHC-I and CD147) is transported to a sorting endosome to determine its fate. One pathway of recycling the cargo back to the plasma membrane involves tubular elements produced from the endocytic recycling compartment. Catalytic activity of PLA2G4E, recruited via interactions with $PI(4,5)P_2$, is necessary for tubule formation and transport to the plasma membrane. This may be dependent on modification of the membrane architecture by either or both NAPEs and lyso-PC (LPC) produced by PLA2G4E (see inset). Other possible endocytic pathways are omitted for clarity.

Aim and outline of this thesis

NAPEs are an underexplored class of phospholipids with diverse functionalities within the cell. Understanding their roles in membrane dynamics, signaling and metabolism and the regulation thereof will help to understand the molecular mechanisms underlying cellular (patho)physiology. The ability to selectively modulate the biosynthesis and degradation of NAPEs is pivotal to study their biology, and might lead to the development of drugs for the treatment of (neuro)degenerative diseases. So far, no potent and selective inhibitors of the calcium-dependent route of NAPE biosynthesis have been reported. The aim of this thesis, therefore, was to develop PLA2G4E inhibitors that could potently and selectively decrease cellular NAPE levels (see also Box 2).

Chapter 2 describes the optimization of the first PLA2G4E inhibitors previously identified.[255] Organic synthesis and activity-based protein profiling (ABPP) were applied to build a library of compounds and determine their structure-activity relationships on PLA2G4E. This led to the identification of **WEN091** as potent PLA2G4E inhibitor (Figure 1.8).

Box 2 – Inhibitors of PLA2G4 enzymes

The central role of PLA2G4A in immunomodulatory lipid production has stimulated the development of several inhibitors for this enzyme. The first inhibitor discovered was arachidonyl trifluoromethylketone ($AACOCF_3$), a reversible covalent derivative of arachidonic acid (Figure 1.7).[302] It showed some selectivity over the $sPLA_2$ family, but does inhibit cyclooxygenases and is not regarded a selective inhibitor.[303] Nevertheless, it has been applied in many studies as a selective PLA2G4A inhibitor.[304,305] Several pyrrolidine-based inhibitors were developed, which were reported to be more selective.[306,307] These compounds showed efficacy in suppressing cellular arachidonic acid and lysophospholipid production.[307–309] Pyrrolidine-2 was reported to also be an inhibitor of PLA2G4F, but not PLA2G4B.[300] Pharmaceutical companies, including AstraZeneca[310], Asubio Pharma[311,312] and Avexxin[313–315] have developed nanomolar potent PLA2G4A inhibitors that showed *in vivo* efficacy in lowering levels of pro-inflammatory lipids and alleviating symptoms of inflammation. Ziarco Pharma's ZPL-5212372 (previously Pfizer) was proceeded into Phase II human trials for the treatment of atopic dermatitis.[316] Wyeth (now Pfizer) has developed ecopladib, which was orally efficacious in reducing inflammatory edema in rats[317], and giripladib, which was tested in Phase II for the treatment of osteoarthritis, but failed due to adverse gastrointestinal events.[318] These compounds displayed selectivity over PLA2G4B, C and F and cyclooxygenases.[317,319] Whether the adverse side effects were due to PLA2G4A inhibition or an unidentified off-target is not known. Recently, AK106-001616, a PLA2G4A inhibitor based on a different scaffold, showed less gastrointestinal problems than generic anti-inflammatory drug naproxen.[320] These examples indicate the therapeutic potential of PLA2G4A inhibition, but knowledge about the selectivity profile of the compounds is limited. No selective inhibitors of the other members of the PLA2G4 family have been reported.

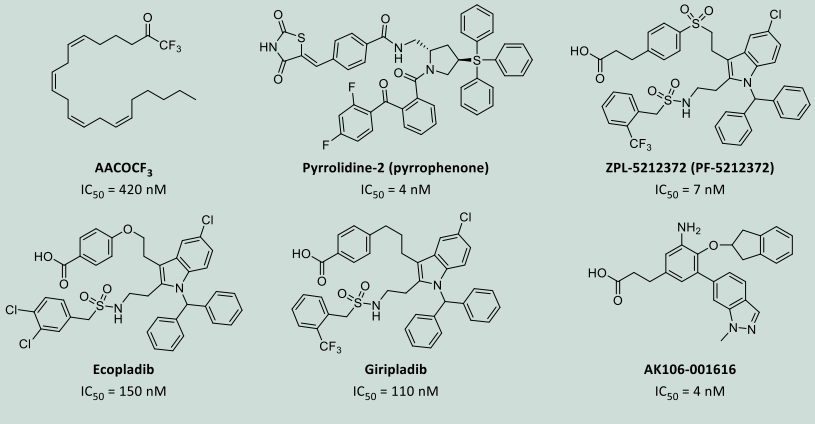

Figure 1.7. Structures of published inhibitors of PLA2G4 enzymes. Names and reported activities on PLA2G4A are indicated. Inhibitory values were determined in similar phospholipase assays measuring arachidonic acid release from PC-containing vesicles.[307,315,317,320]

Chapter 3 describes the biological evaluation of **WEN091**. Using ABPP and biochemical assays, the selectivity profile of **WEN091** was determined across PLA2G4 family members, NAPE-metabolic enzymes and other brain serine hydrolases. **WEN091** was found to be a potent and selective inhibitor of PLA2G4E *in vitro*, with important off-targets in Neuro-2a cells. Lipidomics analysis of PLA2G4E-overexpressing Neuro-2a cells showed that cellular NAPE levels can be decreased by treatment with **WEN091**.

Further optimization of the inhibitors is described in **Chapter 4**. Structural analogs of **WEN091** were synthesized with the aim of improving the cellular selectivity of the PLA2G4E inhibitors. ABPP and biochemical assays were used for the biological profiling of these inhibitors regarding their potency and selectivity. **IK015** and **WEN222** were identified as potent inhibitors of PLA2G4E *in vitro* with an improved selectivity profile in Neuro-2a cells. In addition, **WEN258** was identified as an inactive structural analog, which can be used as a control compound to distinguish on-target from off-target effects of the inhibitors (Figure 1.8).

In **Chapter 5** the development of a cellular target engagement assay for PLA2G4E is described. **WEN175**, an alkynylated inhibitor based on **IK015**, was synthesized, and was shown to activity-dependently label PLA2G4E (Figure 1.8). Using **WEN175**, the cellular activity of PLA2G4E was visualized and *in situ* target engagement of **WEN091** was confirmed. In addition, ABPP with **WEN175** confirmed PLA2G4E's requirement for both the C2 and PB domain for catalytic activity.

Chapter 6 describes the incorporation of caged hydrocarbons into novel inhibitors of lipid metabolic enzymes ABHD6, ABHD16a and diacylglycerol lipase (DAGL). These structures were combined with known chemical elements for target recognition, leading to the identification of **RED353** as a highly potent and selective ABHD6 inhibitor (Figure 1.8). In Neuro-2a cells, **RED353** showed better selectivity over AEA hydrolysis than widely-used ABHD6 inhibitor KT182, as determined using ABPP and targeted lipidomics. Furthermore, ABHD6 inhibition by **RED353** was shown to decrease basal levels of 2-arachidonoylglycerol, supporting previous reports that ABHD6 possesses DAGL activity.[256]

The findings of this thesis are summarized in **Chapter 7**. Here, concluding remarks are made and limitations of this study are noted. Suggestions are included for further research based on this thesis.

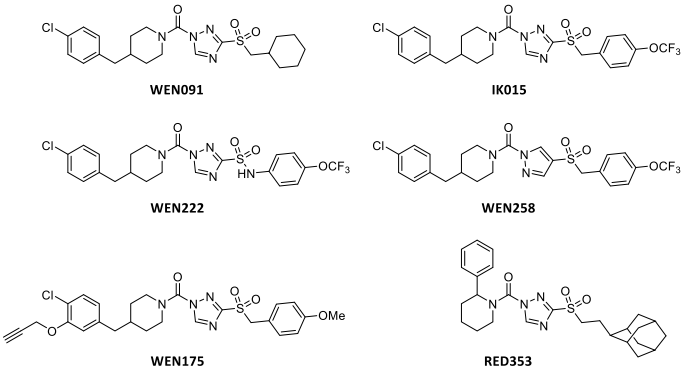

Figure 1.8. Structures of key inhibitors identified in this thesis. PLA2G4E inhibitor **WEN091** is identified in Chapter 2, PLA2G4E inhibitors **IK015**, **WEN222** and control compound **WEN258** are identified in Chapter 4, PLA2G4E ABP **WEN175** is identified in Chapter 5, and ABHD6 inhibitor **RED353** is identified in Chapter 6.

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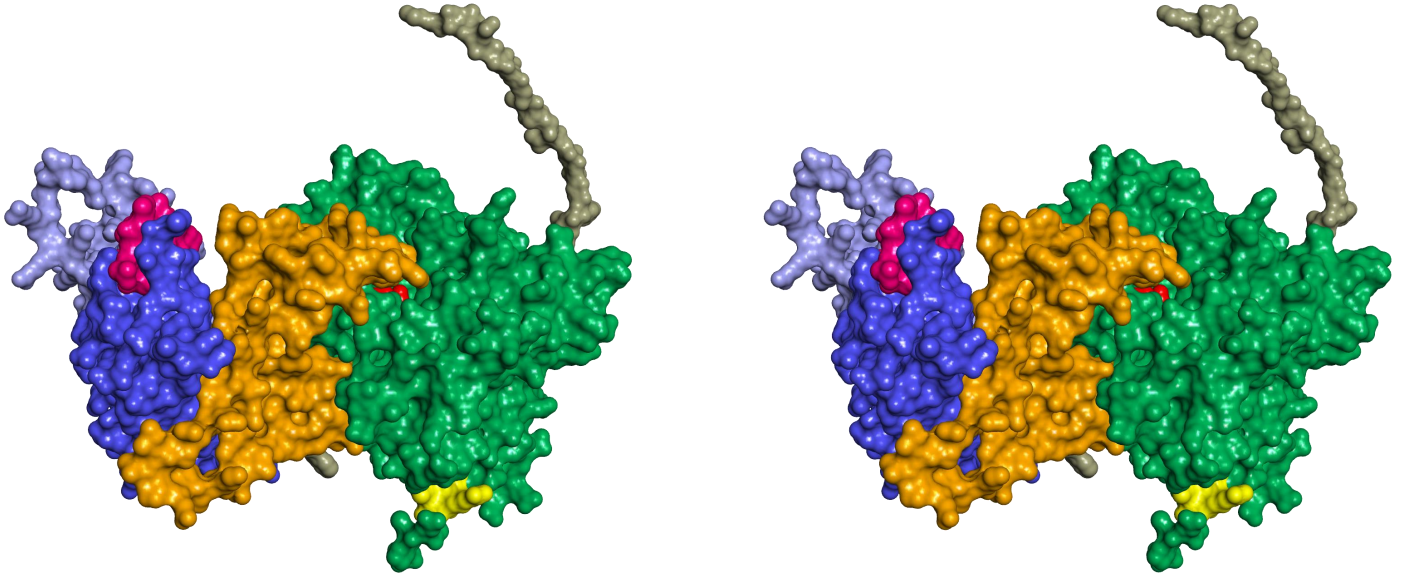

Stereoscopic image of the predicted structure of PLA2G4E (Alphafold2). To see the 3D image, hold the paper close to your face while looking cross-eyed. Slowly move the paper away from you. You should see a third image appearing in the middle. Try to get this one into focus by moving the paper further away or closer to your eyes. Grey: disordered N-terminus, blue: C2 domain with calcium-binding loops (magenta), purple: C2-like domain, green: lipase domain with active site residues (red), orange: putative cap region, yellow: polybasic domain.