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Biosynthetic Pathways of Bioactive *N*-Acylethanolamines in Brain

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Abstract: Ethanolamides of long-chain fatty acids are a class of endogenous lipid mediators generally referred to as *N*-acylethanolamines (NAEs). NAEs include anti-inflammatory and analgesic palmitoylethanolamide, anorexic oleoylethanolamide, and the endocannabinoid anandamide. Since the endogenous levels of NAEs are principally regulated by enzymes responsible for their biosynthesis and degradation, these enzymes are expected as targets for the development of therapeutic agents. Thus, a better understanding of these enzymes is indispensable. The classic "*N*-acylation-phosphodiesterase pathway" for NAE biosynthesis is composed of two steps; the formation of *N*-acylphosphatidylethanolamine (NAPE) by *N*-acyltransferase and the release of NAE from NAPE by NAPE-hydrolyzing phospholipase D (NAPE-PLD). However, recent studies, including the analysis of NAPE-PLD-deficient (NAPE-PLD$^{-/-}$) mice, revealed the presence of NAPE-PLD-independent multi-step pathways to form NAEs from NAPE in animal tissues. Our recent studies using NAPE-PLD$^{-/-}$ mice also suggest that NAE is formed not only from NAPE, but also from *N*-acylated plasmalogen-type ethanolamine phospholipid (*N*-acyl-plasmenylethanolamine) through both NAPE-PLD-dependent and -independent pathways. Here, we present recent findings on NAE biosynthetic pathways mainly occurring in the brain.

Keywords: *N*-Acylethanolamine, anandamide, biosynthesis, NAPE-PLD, oleoylethanolamide, palmitoylethanolamide, phospholipid, plasmalogen.

INTRODUCTION

Ethanolamides of long-chain fatty acids are generally referred to as *N*-acylethanolamines (NAEs) and exist in a variety of organisms, including mammals [1]. Quantitatively major NAEs in brain tissues comprise palmitoylethanolamide (*N*-palmitoylethanolamine, PEA), oleoylethanolamide (*N*-oleoylethanolamine, OEA), and stearoylethanolamide (*N*-stearoylethanolamine, SEA) [2] (Fig. **1A**). PEA has recently attracted much attention due to its agonist activity toward peroxisome proliferator-activated receptor (PPAR)-α as well as its pharmacological activities such as anti-inflammatory, analgesic, and neuroprotective actions [2-4]. OEA is known to exert an anorexic action, which appears to be mediated by its binding to PPAR-α and transient receptor potential vanilloid 1 (TRPV1) or GPR119 [5]. In accordance with the appetite-suppressing effect of OEA, intestinal levels of NAEs, including OEA, are altered by food intake [6, 7]. The relationship between NAE levels and diets was also observed in *Caenorhabditis elegans* [8]. NAE abundance in *C. elegans* was reduced under dietary restriction and NAE deficiency was sufficient to extend lifespan through a dietary restriction mechanism. Among quantitatively minor species of NAEs, only arachidonoylethanolamide (*N*-arachidonoylethanolamine, anandamide) (Fig. **1A**) has been extensively studied because of its behavior as an endocannabinoid (endogenous agonist of the cannabinoid receptors CB1 and CB2) [9] and an endovanilloid (endogenous agonist of TRPV1) [10]. Anandamide exhibits cannabimimetic activities such as analgesia, neuroprotection, hypotension, and appetite stimulation [11]. TRPV1 has been suggested to participate in inflammatory heat hyperalgesia, vasodilatation, neuromotor disorders, and respiratory, gastrointestinal, and urinary functions. Experimental data are now accumulating in favor of anandamide acting at TRPV1 in some of these processes [10]. Recently, postsynaptic TRPV1 was shown to affect α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (also known as AMPA) receptor endocytosis to mediate anandamide-induced long-term depression in the hippocampus and nucleus accumbens [12, 13].

NAEs are biosynthesized in cells "on demand", and their endogenous levels are principally regulated by enzymes responsible for their formation and degradation (Fig. **1B**) [14, 15]. Thus, full characterization of the enzymes involved in the NAE metabolism is indispensable for a better understanding of the physiological and pathological roles of bioactive NAEs. In addition, specific inhibitors of these enzymes are expected to be therapeutic agents. In particular, two NAE-degrading enzymes, fatty acid amide hydrolase (FAAH) [16, 17] and NAE-hydrolyzing acid amidase (NAAA) [15, 18] are promising targets to potentiate the biological activities of endogenous NAEs [19, 20]. The cDNA cloning of *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD), from human,

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A

Chemical Structure			
Name	Palmitoylethanolamide (PEA)	Oleoylethanolamide (OEA)	Arachidonoylethanolamide (Anandamide)
Receptors	PPARα	PPARα TRPV1 GPR119	CB1 CB2 TRPV1
Major Biological Actions	Anti-inflammation Analgesia Neuroprotection	Anorexia	Analgesia Hypotension Long-term Depression

B

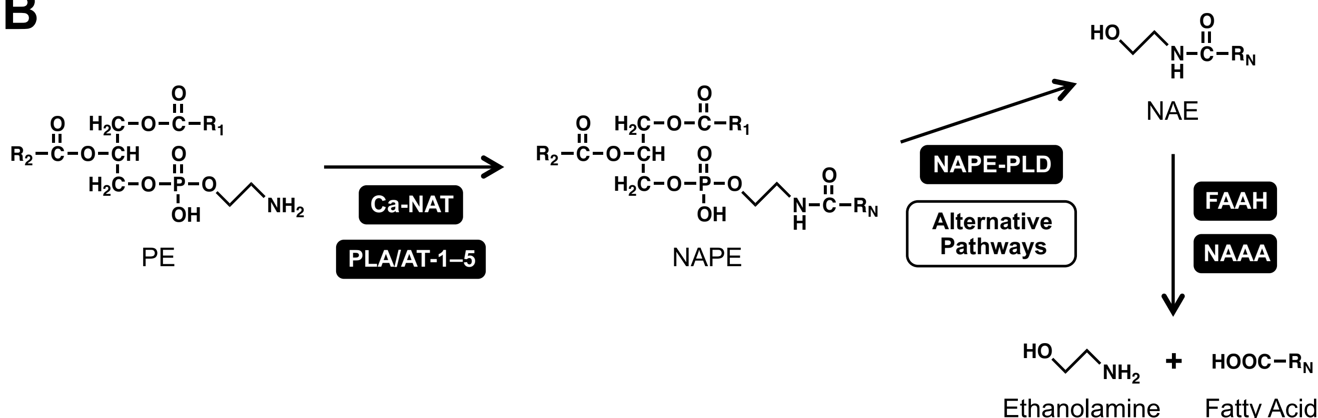

Fig. (1). Representative biological activities (**A**) and metabolic pathways (**B**) of NAEs.

mouse, and rat, facilitated molecular biological approaches to NAE biosynthetic pathways [21]. However, later studies demonstrated that NAEs are produced from *N*-acylphosphatidylethanolamine (NAPE) not only by the direct release by NAPE-PLD, but also through multi-step pathways involving multiple enzymes. In this minireview, we outline recent progress made on the NAE-biosynthesizing pathways, mainly in the brain. We emphasize results obtained from analysis of our NAPE-PLD-deficient (NAPE-PLD$^{-/-}$) mice [22].

BIOSYNTHETIC PATHWAYS OF NAES

The classic NAE-generating pathway in animal tissues is referred to as "transacylation-phosphodiesterase pathway" or "*N*-acylation-phosphodiesterase pathway" and is composed of two-step enzyme reactions (Fig. **1B**) [1]. The first step is the transfer of a fatty acyl chain of a glycerophospholipid molecule such as phosphatidylcholine (PC) to the amino group of phosphatidylethanolamine (PE), leading to the generation of NAPE. Membrane-bound Ca^{2+}-dependent *N*-acyltransferase (Ca-NAT) is believed to be the principal enzyme catalyzing this reaction [14, 23]. This enzyme abstracts an acyl chain exclusively from *sn*-1 position, but not from *sn*-2 position of the glycerol backbone of donor glycerophospholipids. In spite of the characterization in brain and other animal tissues [24, 25], its cDNA has not been cloned to date. The increase in intracellular Ca^{2+} concentration is thought to activate Ca-NAT, leading to the increase in endogenous NAPE levels, although submillimolar concentrations of Ca^{2+} are required for its activation *in vitro*. A recent imaging technique with mass spectrometry revealed that ischemia-reperfusion causes a remarkable increase in the levels of many species of NAPEs with a minor increase in NAEs in the whole injured area of neonatal rat brain [26]. This result suggests that NAPE formation is stimulated in dead cells.

Apart from Ca-NAT, we found that the gene product of *HRAS-like suppressor (HRASLS)5* functions as a cytosolic Ca^{2+}-independent *N*-acyltransferase, which forms NAPE from the acyl donor PC and acyl acceptor PE [25].

Furthermore, our studies revealed that other members (HRASLS1-4) of the tumor suppressor HRASLS family also possess NAPE-forming *N*-acyltransferase activities [27, 28]. It should be noted that these proteins also act as phospholipase (PL) A_1/A_2 and lysoglycerophospholipid *O*-acyltransferase utilizing PC as an acyl donor. We thus proposed to designate HRASLS1-5 as PLA/acyltransferase (PLA/AT)-1-5 [28]. HRASLS1 (also known as A-C1), HRASLS2, and HRASLS5 showed relatively high *N*-acyltransferase activities over PLA_1/A_2 activities. However, further studies are required to elucidate the physiological importance of HRASLS1-5 in NAPE generation. Unlike animals, NAPE formation in plants occurs by *N*-acylation of PE using free fatty acid as an acyl donor [29]. cDNA of NAPE synthase was cloned from *Arabidopsis thaliana* and the recombinant enzyme was shown to utilize acyl-CoA rather than free fatty acid [30]. However, another group recently reported that this gene encodes lysoglycerophospholipid *O*-acyltransferase, but not NAPE synthase [31].

In addition to being a precursor of various NAEs, it has been suggested that NAPE itself exerts several biological actions such as membrane-stabilizing effect [32]. Moreover, NAPE has been reported to be a circulating hormone, which is released from the intestine and mediates an anorexic effect [33]. However, this finding has been questioned since intraperitoneal injection of PE as well as NAPE caused a decrease in food intake [34].

In the second step of the classic pathway, NAPE is directly hydrolyzed to NAE and phosphatidic acid by NAPE-PLD (Fig. **1B**) [21]. NAPE-PLD is a membrane-associated protein belonging to the metallo-β-lactamase family, and its primary structure shows no homology to other PLDs. The enzyme specifically hydrolyzes NAPE and is essentially inactive for other glycerophospholipids [35]. Recent studies exhibited that lipopolysaccharide lowers the expression of NAPE-PLD mRNA as well as endogenous PEA level in macrophage cells, which may attenuate anti-inflammatory action of PEA in the lipopolysaccharide-induced inflammatory response [36]. This suppression of NAPE-PLD mRNA occurred at transcriptional level by changing the acetylation state of histone proteins bound to the NAPE-PLD promoter. More interestingly, a common haplotype in *NAPE-PLD* was found to be protective against severe obesity in a Norwegian population [37]. Instead of NAPE-PLD, which is not found in plants, β and γ isoforms of PLD, showing broad substrate specificity for various glycerophospholipids, are responsible for the NAE formation in plants [38].

In addition to the classic NAPE-PLD-dependent pathway, Natarajan *et al.* [39] earlier suggested that NAE can be formed from NAPE through “alternative pathways” composed of multi-step enzyme reactions (Fig. **2A**). To date, at least three alternative pathways have been proposed in mammalian tissues: (1) *O*-deacylation of NAPE to form *N*-acyl-lyso-PE (lyso-NAPE), followed by lysophospholipase D (lyso-PLD)-type hydrolysis to release NAE [40], (2) double *O*-deacylation to form glycerophospho-NAE (GP-NAE), followed by phosphodiesterase reaction to release NAE [41-43], and (3) PLC-type hydrolysis to form NAE-phosphate, followed by phosphatase reaction to release NAE [44, 45]. Apart from these pathways initiated by NAPE, NAE-forming routes, in which NAPE is not involved, have been reported. The routes comprise energy-independent condensation of free fatty acid and ethanolamine, which occurs in the reverse reaction of FAAH [46-48], and spontaneous formation of anandamide from arachidonoyl-CoA and ethanolamine [49].

BIOSYNTHESIS OF NAE FROM NAPE

In order to analyze NAPE-PLD-independent alternative pathways of NAE biosynthesis, we generated NAPE-PLD$^{-/-}$ mice [22]. The mutant mice were born at the expected Mendelian frequency, viable, and apparently healthy, as reported previously by Leung *et al.* [41] with NAPE-PLD$^{-/-}$ mice in which a different exon of *Nape-pld* gene was deleted. First, we assayed brain homogenates of wild-type and NAPE-PLD$^{-/-}$ mice for the formation of [^{14}C]PEA from *N*-[^{14}C]palmitoyl-PE (Fig. **2B**). The result showed that both brain homogenates have the PEA-generating activity, although the activity of NAPE-PLD$^{-/-}$ mice was lower than that of wild-type mice by 39%. The radioactive band corresponding to *N*-[^{14}C]palmitoyl-lyso-PE was also produced by both homogenates, suggesting the presence of NAPE-PLD-independent alternative pathways *via* lyso-NAPE as an intermediate metabolite.

We next measured brain levels of NAPE species as well as NAE species in wild-type and NAPE-PLD$^{-/-}$ mice by liquid chromatography-tandem mass spectrometry (LC-MS/MS). For analysis of the NAPE species the total numbers of carbon atoms and double bonds of *N*-acyl and *sn*-1 *O*-acyl chains are combined since we could not distinguish between these two acyl chains (Fig. **2C**). Most of the different molecular species of NAPE were much more abundant in NAPE-PLD$^{-/-}$ mice than in wild-type mice. The total number of the double bonds of *N*-acyl and *sn*-1 *O*-acyl chains in the major species was 0 or 1, showing that *N*-acyl chains are saturated or monounsaturated. Interestingly, the *sn*-2 *O*-acyl chains were mostly 18:1. NAPE-PLD$^{-/-}$ mice exhibited decreased brain levels of various NAEs, although statistical significances were not seen in several molecular species (Fig. **2D**). In agreement with *N*-acyl chains of NAPE, major NAE species were OEA, PEA, and SEA. These results were similar to those reported by Leung *et al.* [41], except that we observed significant reductions in the levels of polyunsaturated NAEs such as anandamide and docosahexaenoylethanolamide. Thus, NAPE-PLD was suggested to be at least partly responsible for the *in vivo* formation of all NAE species including anandamide. As for lyso-NAPE, all species remarkably increased in NAPE-PLD$^{-/-}$ mice, as compared with wild-type mice (Fig. **2E**). Both of *N*-acyl and *O*-acyl species were mostly palmitoyl, oleoyl, and stearoyl. In the double-*O*-deacylation pathway, lyso-NAPE is further deacylated to GP-NAE prior to the release of NAE. In fact, a significant increase in brain levels of GP-NAE species was observed in NAPE-PLD$^{-/-}$ mice [22]. Major *N*-acyl species of GP-NAE were again palmitoyl, oleoyl, and stearoyl.

Although PE serves as a precursor of NAPE, the brain levels of PE species were not generally different between

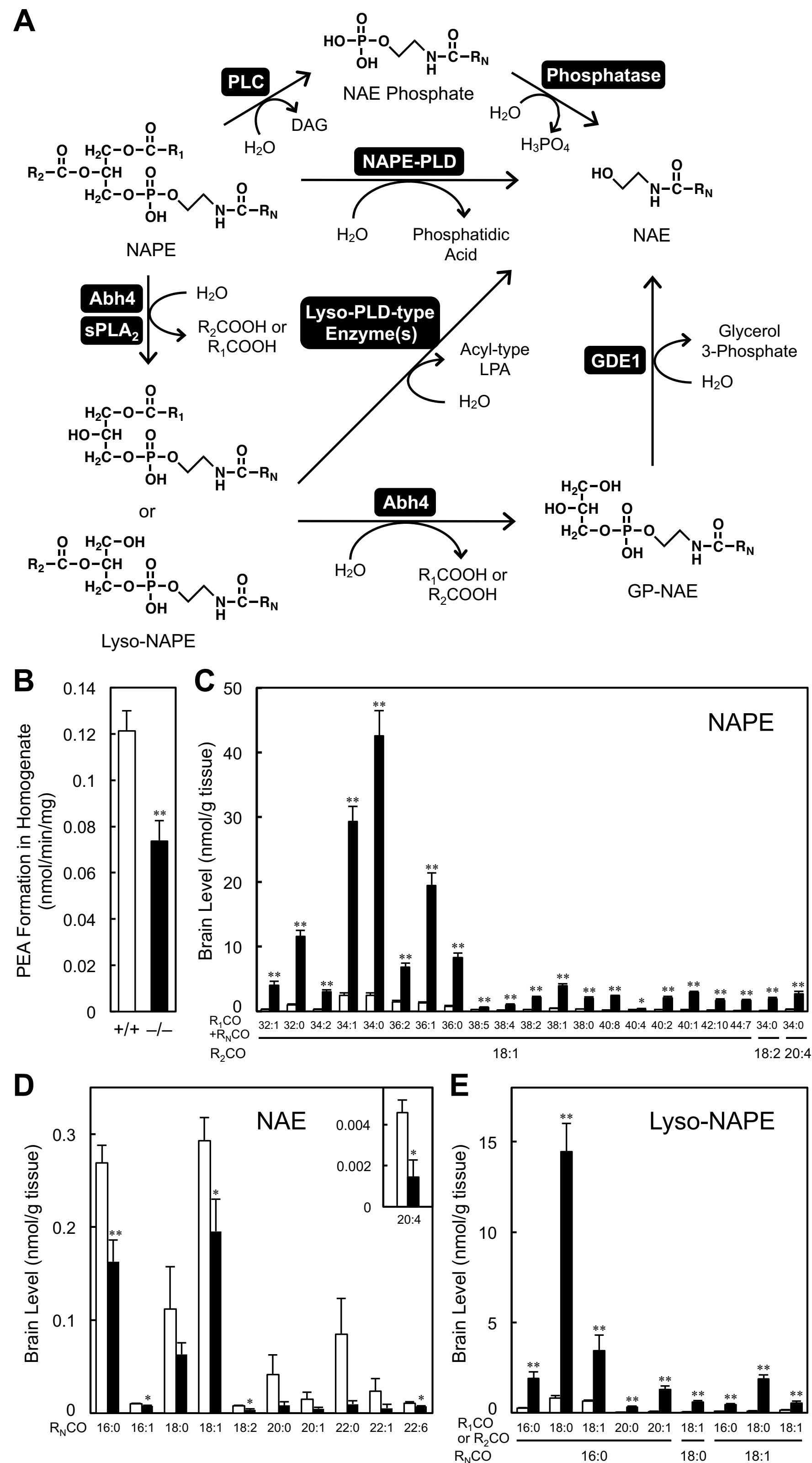

Fig. (2). Biosynthetic pathways for NAE from NAPE in mouse brain. (**A**) Proposed NAPE-PLD-dependent and -independent pathways. (**B**) NAE-forming activity from NAPE in brain homogenates. The brain homogenates of wild-type (open columns) and NAPE-PLD$^{-/-}$ (closed columns) mice were allowed to react with 100 μM *N*-[^{14}C]palmitoyl-PE, and [^{14}C]PEA-forming activities were examined (n = 3, means ± S.D.). **p < 0.01 *vs* wild-type mice. (**C**-**E**) Brain levels of NAPE (**C**), NAE (**D**), and lyso-NAPE (**E**) in wild-type (open columns) and NAPE-PLD$^{-/-}$ (closed columns) mice were determined by LC-MS/MS (n = 6, means ± S.E.). *p < 0.05 and **p < 0.01 *vs* wild-type mice. Reproduced from ref. [22], with permission from Elsevier. DAG, diacylglycerol.

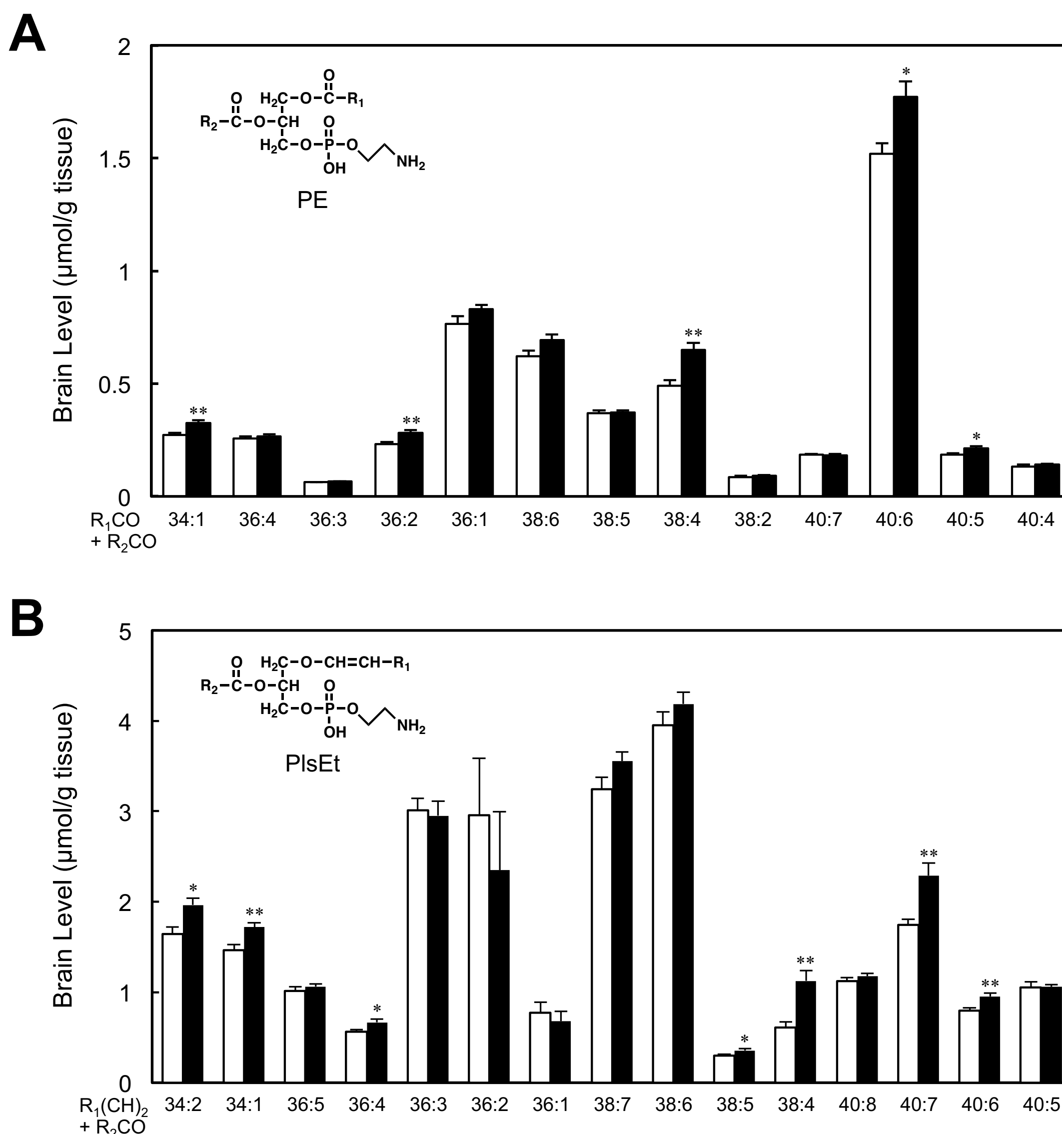

Fig. (3). Brain levels of PE (A) and PlsEt (B) in wild-type (open columns) and NAPE-PLD$^{-/-}$ (closed columns) mice were determined by LC-MS/MS on 4000 Q TRAP (Applied Biosystems/MDS Sciex) with an Imtact Cadenza CD-C18 column (100 mm x 1 mm, 3 µm) developed with acetonitrile/methanol (95:5) mixture containing 5 mM ammonium formate (n = 6, means ± S.E.). The brain lipid extract was analyzed for the molecular species of PE and PlsEt by positive ion multiple reaction monitoring (Q1 $[M + H]^+$, Q3 $[M - 141]^+$) against dimyristoyl-PE, an internal standard. *$p < 0.05$ and **$p < 0.01$ *vs* wild-type mice.

wild-type and NAPE-PLD$^{-/-}$ mice (Fig. **3A**). It is well known that PE contains polyunsaturated acyl chains at the *sn*-2 position in abundance. In agreement with this fact, more than half of major PE species possessed 5 or more double bonds in two *O*-acyl chains. Thus, the acyl composition of PE was clearly different from that of NAPE which mostly contained oleoyl group at *sn*-2 position and a saturated or monounsaturated acyl group at *sn*-1 position. Although the reason for this difference is unclear, one possibility is that NAPE-generating *N*-acyltransferase may preferentially use 2-oleoyl-PE as an acceptor substrate. Alternatively, enzymatic remodeling of the existing NAPE may enrich oleoyl group at *sn*-2 position, or NAPE containing a polyunsaturated acyl chain at *sn*-2 position may decompose faster.

Several enzymes involved in the NAPE-PLD-independent pathways have been reported. The deacylation of NAPE to lyso-NAPE is catalyzed by group IB, IIA, and V of secretory PLA_2s [40] and α/β hydrolase 4 (Abh4) (α/β hydrolase domain containing 4, ABHD4) [42]. Abh4 also catalyzes further deacylation of lyso-NAPE to form GP-NAE. Abh4 belongs to the α/β-hydrolase fold superfamily, which was named for a highly conserved tertiary fold consisting of alternating α helices and β sheets [50]. Abh4 is expressed in various mouse tissues, including brain, spinal cord, and testis [42]. Hydrolysis of GP-NAE to release NAE is catalyzed by glycerophosphodiesterase 1 (GDE1) [43]. GDE1 was originally identified in a yeast two-hybrid screen for proteins that interact with RGS16, a regulator of G-protein signaling [51]. GDE1 is one of mammalian

homologs of bacterial glycerophosphodiester phosphodiesterases, GlpQ and UgpQ [52]. These bacterial enzymes play critical roles in the hydrolysis of deacylated glycerophospholipids to glycerol phosphate and alcohol, which are utilized as major sources of carbon and phosphate. GDE1 was also shown to cleave glycerophosphoinositol *in vitro* [53] and its lack in mice elevated brain levels of glycerophosphoinositol, glycerophosphoserine, and glycerophosphoglycerate [54]. GDE1 is widely distributed in mouse tissues and its mRNA level is the highest in brain and spinal cord, followed by kidney, testis, and liver [43]. Double knockout mice of NAPE-PLD and GDE1 were generated and their brain levels of NAEs were compared with those of NAPE-PLD$^{-/-}$ mice [55]. Interestingly, a significant difference between both mice was not found, suggesting the involvement of unknown enzymes in the NAPE-PLD-independent pathways. We examined whether deficiency of NAPE-PLD compensatory increases the brain levels of Abh4 and GDE1 mRNAs (Fig. **4**). However, we could not see obvious differences in their levels between wild-type and NAPE-PLD$^{-/-}$ mice. Very recently, knockdown of Abh4 was reported to inhibit anoikis (cell death in response to loss of cell-cell and cell-matrix interactions) in prostate epithelial cells [56]. However, knockdown of GDE1 in the same cells did not exhibit this effect. Moreover, the addition of 10 μM glycerophospho-*N*-arachidonoylethanolamine to the Abh4 knockdown cells did not restore sensitivity to anoikis. Thus, the effect of Abh4 appears to be unrelated to anandamide and glycerophospho-*N*-arachidonoylethanolamine which are formed through the Abh4-GDE1 pathway.

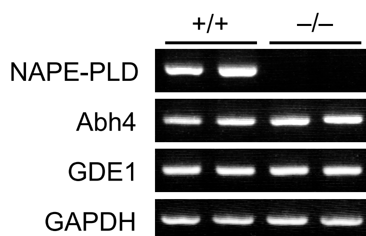

Fig. (4). mRNA levels of the enzymes involved in the NAPE-PLD-independent pathways. Total RNA was isolated from the brains of wild-type (+/+) and NAPE-PLD$^{-/-}$ (-/-), and mRNA levels of the indicated enzymes were analyzed by reverse transcription-PCR. The housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as the control. PCR for NAPE-PLD [21], GDE1 [43], and GAPDH [21] was performed as described previously, except that 27 and 23 cycles were used for NAPE-PLD and GDE1, respectively. PCR for Abh4 was performed under the following condition with the primers reported previously [42]: denaturation at 94°C for 48 s, annealing at 60°C for 48 s, and extension at 72°C for 48 s (27 cycles).

BIOSYNTHESIS OF NAE FROM *N*-ACYLATED PLASMALOGEN PHOSPHOLIPID

Plasmalogen-type phospholipids are a major class of glycerophospholipids, in which an alkenyl group is linked to the *sn*-1 position *via* a vinyl ether bond (α, β-unsaturated ether bond). Plasmenylethanolamine (PlsEt) represents plasmalogen-type ethanolamine phospholipid and is known to be abundant in the brain. We confirmed the presence of high amounts of PlsEt species in mouse brain (Fig. **3B**). The levels of major PlsEt species were not largely different between wild-type and NAPE-PLD$^{-/-}$ mice. Like PE species, more than half of major PlsEt species possessed 5 or more double bonds in *sn*-1 alkenyl and *sn*-2 acyl chains.

The presence of PlsEt in the brain suggests that *N*-acylated plasmalogen-type ethanolamine phospholipid (*N*-acyl-PlsEt) also exists in the same tissue and may serve as another precursor of NAE (Fig. **5A**). Accordingly, Astarita *et al*. [57] reported that 65% of the *N*-arachidonoyl-ethanolamine phospholipids in rat brain are plasmalogen-type. Schmid *et al*. [58] earlier reported that *N*-acyl-PlsEt is utilized by NAPE-PLD in rat heart microsome. We found that recombinant NAPE-PLD hydrolyzes *N*-[^{14}C]palmitoyl-PlsEt to [^{14}C]PEA at 70% of the rate of *N*-[^{14}C]palmitoyl-PE hydrolysis [22].

Due to the presence of a vinyl ether bond rather than an ester bond at *sn*-1 position of *N*-acyl-PlsEt, lysophospholipases, including Abh4, cannot release the hydrocarbon chain from the *sn*-1 position of *N*-acyl-lyso-PlsEt (Fig. **5A**). Therefore, if NAEs are formed in a NAPE-PLD-independent manner, a lyso-PLD-type phosphodiesterase, which directly releases NAE from *N*-acyl-lyso-PlsEt, is indispensable. We examined if this hypothetical route is present in the brain of NAPE-PLD$^{-/-}$ mice [22]. First, like wild-type mice, the brain homogenate of NAPE-PLD$^{-/-}$ mice converted *N*-[^{14}C]palmitoyl-PlsEt to [^{14}C]PEA (Fig. **5B**). The radioactive band corresponding to *N*-[^{14}C]palmitoyl-lyso-PlsEt was also detected. We next measured brain levels of *N*-acyl-PlsEt species (Fig. **5C**). In this figure, total numbers of carbon atoms and double bonds of *N*-acyl and *sn*-1 *O*-alkenyl chains are shown. As a result, a variety of molecular species of *N*-acyl-PlsEt were detected in the brain and most species were much more abundant in NAPE-PLD$^{-/-}$ mice than in wild-type mice. The total number of the double bonds of *N*-acyl and *sn*-1 *O*-alkenyl chains in the major species was 1 or 2. Considering that the double bond number of *sn*-1 *O*-alkenyl chain contains the double bond in the vinyl ether linkage, the results show that their *N*-acyl chains are saturated or monounsaturated. Like NAPE species, the *sn*-2 *O*-acyl chains were mostly 18:1. We also detected *N*-acyl-lyso-PlsEt, which was greatly increased in NAPE-PLD$^{-/-}$ mice (Fig. **5D**). Their major *N*-acyl species were palmitoyl and oleoyl, and *O*-alkenyl species were principally 18:1, 18:2, and 16:1. The PLA_2-type esterase that hydrolyzes *N*-acyl-PlsEt to *N*-acyl-lyso-PlsEt remains unidentified.

We found that the brain homogenate of NAPE-PLD$^{-/-}$ mice could release [^{14}C]PEA from *N*-[^{14}C]palmitoyl-lyso-PlsEt [22]. We then characterized this lyso-PLD-type enzyme. The reaction occurred between pH 6 and 9, with optimal pH around 7-7.4. The enzyme converted *N*-palmitoyl-, *N*-oleoyl-, and *N*-arachidonoyl-lyso-PlsEts to their corresponding NAEs at similar rates, showing wide substrate specificity in terms of *N*-acyl species of *N*-acyl-lyso-PlsEt. The activity was stimulated by 2 mM Mg^{2+} and inhibited by 0.1% Triton X-100. We noticed that these catalytic properties are shared with GDE1. As expected, we found that recombinant GDE1 exhibits the lyso-PLD activity to generate [^{14}C]PEA from *N*-[^{14}C]palmitoyl-lyso-PlsEt,

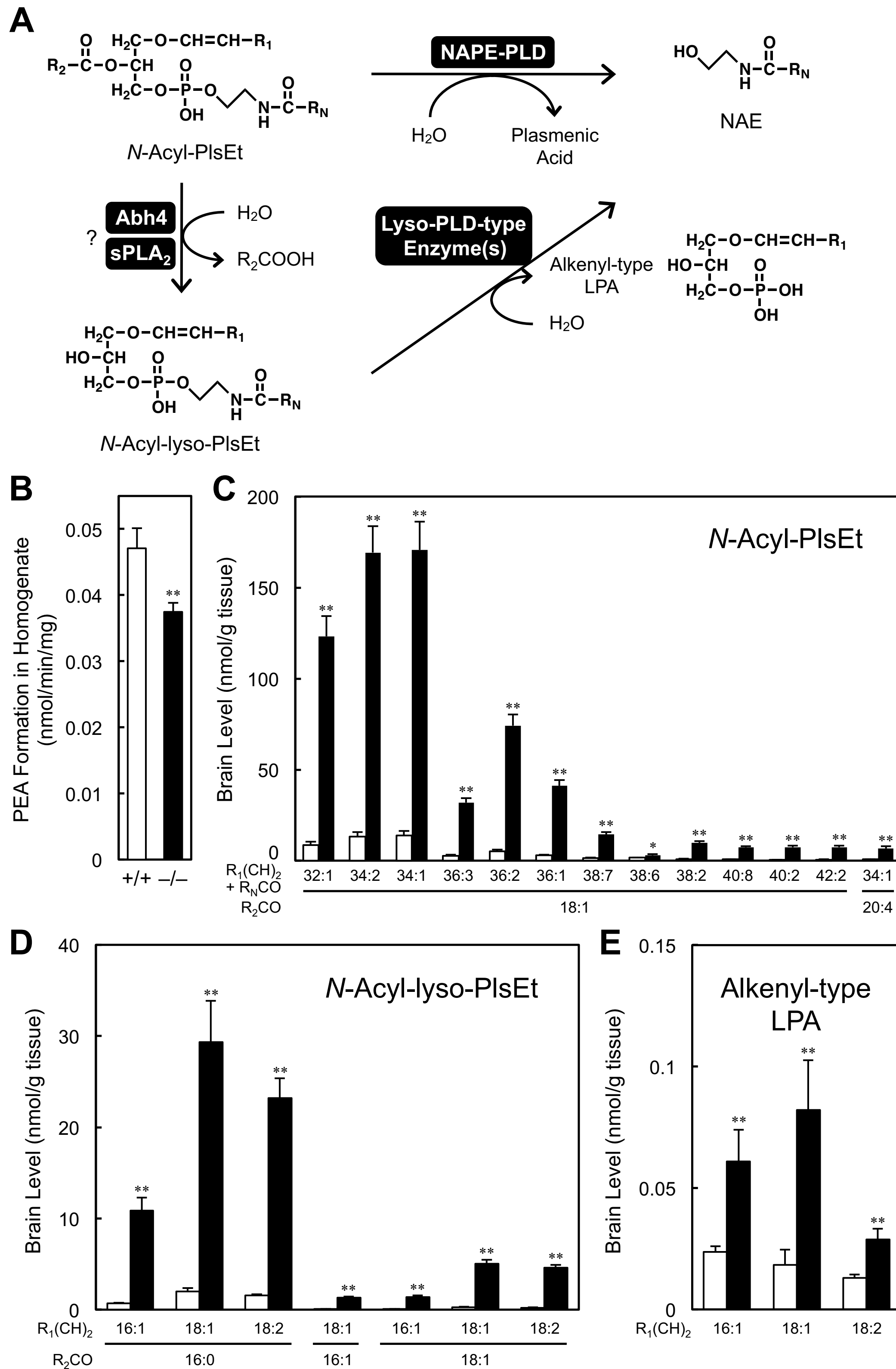

Fig. (5). Biosynthetic pathways for NAE from *N*-acyl-PlsEt in mouse brain. (**A**) Proposed NAPE-PLD-dependent and -independent pathways. (**B**) NAE-forming activity from *N*-acyl-PlsEt in brain homogenates. The brain homogenates of wild-type (open columns) and NAPE-PLD$^{-/-}$ (closed columns) mice were allowed to react with 100 µM *N*-[^{14}C]palmitoyl-PlsEt, and [^{14}C]PEA-forming activities were examined (n = 3, means ± S.D.). **p < 0.01 *vs* wild-type mice. (**C-E**) Brain levels of *N*-acyl-PlsEt (**C**), *N*-acyl-lyso-PlsEt (**D**), and alkenyl-type LPA (**E**) in wild-type (open columns) and NAPE-PLD$^{-/-}$ (closed columns) mice were determined by LC-MS/MS (n = 6, means ± S.E.). *p < 0.05 and **p < 0.01 *vs* wild-type mice. Reproduced from ref. 22, with permission from Elsevier.

although this activity was only 7.5% of the hydrolysis of glycerophospho-[^{14}C]PEA to [^{14}C]PEA by GDE1. Thus, GDE1 may be at least in part responsible for the lyso-PLD activity in the brain. The brain levels of alkenyl-type lysophosphatidic acid (LPA) species were also higher in NAPE-PLD$^{-/-}$ mice (Fig. **5E**). Since alkenyl-type LPA is produced together with NAE in the lyso-PLD reaction, this finding suggests that NAEs are at least partly derived from *N*-acyl-lyso-PlsEt accumulating in the brain of NAPE-PLD$^{-/-}$ mice. Overall, the results shown here strongly suggest that *N*-acyl-PlsEt species, contained in the brain tissue, are metabolized to their corresponding NAEs through the NAPE-PLD-independent pathway as well as by direct release by NAPE-PLD.

PERSPECTIVES

In this mini-review, we outlined recent results on biosynthetic pathways of bioactive NAEs mainly in the brain. Analysis of NAPE-PLD$^{-/-}$ mice revealed the existence of NAPE-PLD-independent pathways *in vivo*, and suggested that NAE biosynthesis is more complex than previously considered. However, it remains unsolved whether the NAPE-PLD-independent pathways play unique physiological roles or are simply alternatives of NAPE-PLD for the NAE formation. Thus it will be of interest to explore biological activities of the intermediate metabolites in the pathways such as lyso-NAPE and GP-NAE. Moreover, we should develop selective inhibitors of NAPE-PLD, Abh4, and GDE1, which are expected as useful tools to distinguish between NAPE-PLD-dependent and -independent pathways. We will also need to identify additional enzymes that may be involved in NAPE-PLD-independent pathways, and elucidate NAE-forming pathways in animal tissues other than brain.

ABBREVIATIONS

Abh4 = α/β hydrolase 4
Ca-NAT = Ca^{2+}-dependent *N*-acyltransferase
CB = Cannabinoid receptor
FAAH = Fatty acid amide hydrolase
GDE1 = Glycerophosphodiesterase 1
GP-NAE = Glycerophospho-*N*-acylethanolamine
HRASLS = HRAS-like suppressor
LC-MS/MS = Liquid chromatography-tandem mass spectrometry
LPA = Lysophosphatidic acid
NAAA = *N*-Acylethanolamine-hydrolyzing acid amidase
NAE = *N*-Acylethanolamine
NAPE = *N*-Acylphosphatidylethanolamine
NAPE-PLD = *N*-Acylphosphatidylethanolamine-hydrolyzing phospholipase D
OEA = Oleoylethanolamide
PC = Phosphatidylcholine
PE = Phosphatidylethanolamine
PEA = Palmitoylethanolamide
PL = Phospholipase
PlsEt = Plasmenylethanolamine
PPAR = Peroxisome proliferator-activated receptor
SEA = Stearoylethanolamide
TRPV1 = Transient receptor potential vanilloid 1

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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