Localization of N-Acyl Phosphatidylethanolamine Phospholipase D (NAPE-PLD) Expression in Mouse Brain: A New Perspective on N-Acylethanolamines as Neural Signaling Molecules

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ABSTRACT

N-acylethanolamines (NAEs) are membrane-derived lipids that are utilized as signaling molecules in the nervous system (e.g., the endocannabinoid anandamide). An N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) that catalyzes formation of NAEs was recently identified as a member of the zinc metallohydrolase family of enzymes. NAPE-PLD /−/ mice have greatly reduced brain levels of long-chain saturated NAEs but wild-type levels of polyunsaturated NAEs (e.g., anandamide), suggesting an important role for NAPE-PLD in the biosynthesis of at least a subset of endogenous NAEs in the mammalian nervous system. To provide a neuroanatomical basis for investigation of NAPE-PLD function, here we have analyzed expression of NAPE-PLD in the mouse brain using mRNA in situ hybridization and immunocytochemistry. NAPE-PLD /−/ mice were utilized to establish the specificity of probes/antibodies used. The most striking feature of NAPE-PLD expression in the brain was in the dentate gyrus, where a strong mRNA signal was detected in granule cells. Accordingly, immunocytochemical analysis revealed intense NAPE-PLD immunoreactivity in the axons of granule cells (mossy fibers). Intense NAPE-PLD immunoreactivity was also detected in axons of the vomeronasal nerve that project to the accessory olfactory bulb. NAPE-PLD expression was detected in other brain regions (e.g., hippocampus, cortex, thalamus, hypothalamus), but the intensity of immunostaining was weaker than in mossy fibers. Collectively, the data obtained indicate that NAPE-PLD is expressed by specific populations of neurons in the brain and targeted to axonal processes. We suggest that NAEs generated by NAPE-PLD in axons may act as anterograde synaptic signaling molecules that regulate the activity of postsynaptic neurons. J. Comp. Neurol. 506:604 – 615, 2008.

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N-acylethanolamines (NAEs) are a class of lipids that are utilized as signaling molecules in the nervous system and other tissues/organisms (Schmid et al., 1990; Schmid, 2000). Probably the most widely known NAE is N-arachidonoyl ethanolamine or “anandamide,” which is an endogenous ligand for CB1 cannabinoid receptors in the central nervous system (Devane et al., 1992). Other physiologically active NAEs include the anti-inflammatory lipid N-palmitoyl ethanolamine (PEA) and the appetite-suppressing substance N-oleoyl ethanolamine (OEA) (Calignano et al., 1998; Jaggar et al., 1998; Rodriguez de Fonseca et al., 2001; Lambert et al., 2002).

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The discovery of anandamide as an endocannabinoid has stimulated interest in the mechanisms of biosynthesis of NAEs in mammals. NAE biosynthesis has been proposed to occur via a two-step enzymatic process. First, a Ca\(^{2+}\)-activated N-acyltransferase transfers the sn-1 acyl chain of a phospholipid onto the amine of phosphatidylethanolamine (PE) to generate an N-acyl PE (NAPE). Then NAPE is converted by a phospholipase D (PLD) into an NAE and phosphatidic acid (Schmid et al., 1990; Cadas et al., 1996; Schmid, 2000). A Ca\(^{2+}\)-independent N-acyltransferase that is highly expressed in testis has been discovered recently (Okamoto et al., 2004), but the molecular identity of a Ca\(^{2+}\)-activated N-acyltransferase is currently unknown. However, an N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) that is expressed in the brain has been identified (Okamoto et al., 2004). NAPE-PLD is a 396-amino acid residue protein in mouse and rat and a member of the zinc metallohydrolase family with a \(\beta\)-lactamase fold. Analysis of the occurrence of NAPE-PLD mRNA/protein in mouse organs/tissues revealed widespread expression but particularly high levels in brain and testis, consistent with the distribution of NAPE-PLD enzymatic activity (Okamoto et al., 2004).

Recently, knockout mice with the NAPE-PLD gene deleted have been generated to investigate the in vivo contribution of NAPE-PLD to NAE biosynthesis (Leung et al., 2006). Consistent with its proposed role in biosynthesis of NAEs, in brain tissue from NAPE-PLD mice there was a fivefold reduction in Ca\(^{2+}\)-dependent conversion of NAPEs to NAEs comprising both saturated and polyunsaturated N-acyl chains. However, when the levels of NAPE-PLD mRNA in NAPE-PLD\(^{-/-}\) mice were used to validate the specificity of the immunostaining observed in wild-type mouse brain. The data presented here provide both the first detailed analysis of the distribution of NAPE-PLD expression in the brain and a basis for further investigation of the physiological roles of NAPE-PLD in brain function.

**MATERIALS AND METHODS**

**NAPE-PLD mRNA in situ hybridization**

Mouse NAPE-PLD cDNA was subcloned from a pcDNA3.1(+) vector into pBlueScript SK(+) vector, after it was established that double digestion with KpnI and ApaI liberated cDNA inserts. Then digoxigenin (DIG)-labeled antisense and sense RNA probes for NAPE-PLD mRNA were generated using DIG RNA labeling mix (Roche Applied Science, Burgess Hill, UK) and T3 and T7 RNA polymerases (Promega, Southampton, UK; NEB, Hitchin, UK). Mouse brains dissected from mice perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; pH 7.4) were embedded in paraffin wax, and serial sections (8–10 \(\mu\)m) were cut using a Leica RM 2146 microtome and mounted on Superfrost Plus Micro Slides (VWR, Lutterworth, UK). Following hydration, slides were incubated with proteinase K (20 \(\mu\)g/ml in a buffer comprising 100 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% Tween 20) for 20 minutes at room temperature. Sections were postfixed with 4% PFA in PBS for 20 minutes at room temperature and washed in 5X SSC buffer (20X SSC = 3 M NaCl, 0.3 M Na\(_2\)H\(_5\)O\(_7\), pH 6.0) followed by 5X SSC buffer with 0.1% Tween 20 added. Then sections were incubated for 2 hours at 60°C with hybridization buffer (50% formamide in 5X SSC, 50 \(\mu\)g/ml yeast RNA, 50 \(\mu\)g/ml heparin, 0.1% Tween 20; 300 \(\mu\)l per slide).

Hybridization was performed overnight at 60°C in hybridization buffer containing 800 ng/ml of antisense or sense probes (150 \(\mu\)l per slide covered by a Parafilm “M” coverslip). Parafilm coverslips were floated off in 5X SSC buffer, and then slides were washed at 60°C in four 30-minute steps: 1) 3 parts 50% formamide in 5X SSC and 1
part 2X SSC; 2) 1 part 50% formalin in 5X SSC and 1 part 2X SSC; 3) 1 part 50% formalin in 5X SSC and 3 parts 2X SSC; and 4) 2X SSC. Slides were then washed at room temperature in 0.2X SSC buffer, followed by two washes in maleic acid buffer (MAB; 0.1 M maleic acid, 0.15 M NaCl, pH 7.5) with 0.1% Tween 20 added. Sections were blocked in 0.5% Blocking Reagent (Roche Applied Science) in MAB/0.1% Tween 20 for 1 hour at room temperature in a moist chamber. After the blocking solution was drained from slides, 500 μl of anti-DIG alkaline phosphatase-Fab fragments (Roche Applied Science) was added, diluted 1:5,000 with 0.5% Blocking Reagent in MAB/0.1% Tween 20, and incubated overnight at 4°C in a moist chamber. Slides were washed twice for 10 minutes in PBS buffer, followed by 10 minutes in BCL buffer (100 mM Tris, pH 9.5; 100 mM NaCl, 50 mM MgCl2, 0.1% Tween 20), and then covered with a chromogen mixture consisting of 175 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (VWR), 337.5 μg/ml nitroblue tetrazolium (VWR), and lavig Boise (Vector, Burlingame, CA) in BCL buffer.

When staining had developed (typically after 6–8 hours), slides were washed twice in PBS and then post-fixed with 4% PFA in PBS for 30 minutes at room temperature. Finally, slides were dehydrated, cleared in xylen, and prepared for microscopy by mounting coverslips over VectaMount (Vector). Sections were viewed under a Leica DMR2A compound microscope (Leica, Nussloch, Germany), and digital images were captured using a Retiga 1300 monochrome 12-bit camera (QImaging, Burnaby, BC, Canada) and QCapture 1.1.6 software (QImaging) running on a Macintosh G4 computer. Images were then processed to remove dust and assembled into figures using Photoshop 7.0 (Adobe Systems, San Jose, CA) running on a MacBook Pro computer. Images were interpreted and labeled with reference to a mouse brain atlas (Paxinos and Franklin, 2001).

Production and characterization of antibodies to NAPE-PLD

Antibodies to mouse NAPE-PLD were generated at QMUL using a synthetic peptide antigen (KHGES-RYLNDTDDFAEEFT) comprising the C-terminal region of the protein (Okamoto et al., 2004), which was custom synthesized and purified by the Advanced Biotechnology Centre at Imperial College London. The antigen peptide (NAPE-PLDc) was coupled to thyroglobulin as a carrier protein via the N-terminal lysine residue using (2282.3G, 2282.3T, 2283.3G, 2283.3T, 2284.3G, and affinity-purified NAPE-PLD antibody fractions collected with antisera (1:500) or with one of the six fractions of reducing conditions, with material from NAPE-PLDc and NAPE-PLDc′ mice in alternating lanes.

Gels were transblotted to nitrocellulose membrane, which was then cut into strips corresponding to two adjacent lanes. The nitrocellulose membrane strips were incubated with 5% Marvel in PBS for 1 hour at room temperature and then incubated individually overnight at 4°C with antisera (1:500) or with one of the six fractions of affinity-purified NAPE-PLD antibody fractions collected (2282.3G, 2282.3T, 2283.3G, 2283.3T, 2284.3G, and 2284.3T) diluted 1:5 or 1:10 or 1:15 in MPBST. After washing in MPBST, strips were incubated for 2 hours with alkali phosphatase-labeled goat anti-rabbit immunoglobulins (Vector) diluted 1:1,000 in MPBST; then, after washing with 0.05% Tween 20 in PBS (PBST), bound antibodies were revealed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) alkaline phosphatase substrate kit IV (Vector).

Affinity purification of NAPE-PLD antibodies

Dot-blot analysis revealed the presence of anti-peptide antibodies in sera collected after the first, second, and third boosts of the NAPE-PLDc-immunized rabbits. Samples of antiserum obtained from rabbits after the third and final boost (2282.3, 2283.3, 2284.3) were subjected to affinity purification using a column of NAPE-PLDc antigen peptide coupled to AminoLink® Gel (Pierce, Rockford, IL), prepared according to the manufacturer’s instructions. Bound antibodies were eluted with 15 ml 100 mM glycine (G; pH 2.5) and then with 15 ml 100 mM triethylamine (T; pH 11.5), which were collected separately in 1 ml fractions.

Western blotting

Brains dissected from both wild-type (NAPE-PLDc′) and knockout (NAPE-PLDc−/−) mice generated in the Crlant lab (Leung et al., 2006) were snap frozen on dry ice and sent to the Elphick lab for Western blot analysis. Following homogenization on ice in a buffer comprising 2 mM Tris-EDTA, 5 mM MgCl2, 320 mM sucrose, and protease inhibitors (Complete Mini; Roche Diagnostics, Lewes, E. Sussex, UK), particulate material was removed by centrifugation (5,500 rpm) for 15 minutes at 4°C in an Eppendorf 5804R centrifuge. Samples of brain homogenate supernatants (50 or 75 μg protein per lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% polyacrylamide Tris-glycine gel under reducing conditions, with material from NAPE-PLDc−/− and NAPE-PLDc′/− mice in alternating lanes.

Immunocytchemistry

Preliminary immunocytochemical analysis of NAPE-PLD expression was performed by using brains from mice of the BALB/c strain to establish methodology prior to analysis of brains from male NAPE-PLD-knockout mice (NAPE-PLDc−/−) and male wild-type littermates (NAPE-
PLD(*+*)). Two different methods for preparation of brain sections were tested: 1) Sectioning of paraffin wax-embedded brains fixed with 4% PFA in PBS and postfixed in Bouin’s fixative, as described previously (Egertová et al., 2003); or 2) Sectioning of frozen brains fixed with 4% PFA in PBS. The latter method was found to be preferable for immunocytochemical visualization of NAPE-PLD expression using the antibodies developed in this study and therefore this method is described in detail below.

Mice were asphyxiated with CO2 and perfused through the heart with 25 ml of 4% PFA in PBS (pH 7.4). Then brains were removed and transferred to fresh fixative for 1–4 days. After washing in PBS (2X 1 hour), brains were cryoprotected overnight with 20% sucrose in PBS. Serial sets of coronal sections (20 μm) of brain were prepared using a Leica freezing microtome (CM3050S) and collected as free-floating sections in PBS containing 0.05% sodium azide. Sections were washed in PBS and incubated with ~3% hydrogen peroxide in PBS for 30 minutes at room temperature to quench endogenous peroxidase activity. After washing in PBST, sections were blocked with 5% normal goat serum (NGS) in PBS with 0.2% Triton X-100 (PBSTx) for 2 hours at room temperature. Sections were then incubated overnight at 4°C with affinity-purified antibodies to mouse NAPE-PLD (2282.3T) diluted 1:7–1:10 in PBSTx with 5% NGS and 0.05% sodium azide added. After washing in PBSTx, slides were incubated for 4 hours at room temperature with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Jackson ImmunoResearch, West Grove, PA). After washing in PBS, bound antibodies were revealed using diaminobenzidine (with nickel) as a substrate (Vector).

Finally, free-floating sections were collected on polylysine slides (VWR), dehydrated, cleared in xylene, and prepared for microscopy by mounting coverslips over DPX. Images of stained sections were then captured as described above for mRNA in situ hybridization.

RESULTS
Localization of NAPE-PLD mRNA in mouse brain

The most striking feature of NAPE-PLD expression in mouse brain revealed by mRNA in situ hybridization with DIG-labeled antisense RNA probes was in the hippocampal formation, where intense staining was evident in the granule cell layer of the dentate gyrus (Fig. 1A). No staining was observed in the dentate gyrus in control experiments in which a DIG-labeled sense RNA probe was tested on sections from wild-type mouse brains (Fig. 1B) or in which the DIG-labeled antisense RNA probe was tested on sections of NAPE-PLD knockout mice (Fig. 1C), demonstrating the specificity of our methods for visualizing NAPE-PLD mRNA. NAPE-PLD expression was also detected in other brain regions, but staining was less intense than in the granule cell layer of the dentate gyrus (Fig. 2A–B). For example, in Figure 2A, compare the staining in the granule cell layer of the dentate gyrus (DG) with staining in the pyramidal cell layer of the hippocampus (Hi) and staining of cells in the medial geniculate thalamic nucleus (MG). Expression of NAPE-PLD in other brain regions is described below and illustrated in Figure 2.

In the hippocampus, staining was present in pyramidal cells throughout all three fields (CA1–CA3), as illustrated in Figure 2C (CA1; low magnification) and Figure 2D (CA3; high magnification). In Figure 2C, note again the higher intensity of staining in the granule cell layer of the dentate gyrus compared with staining in the CA1 pyramidal cell layer.

NAPE-PLD mRNA expression was detected in several regions of the olfactory system. In the olfactory bulb, staining was present in granule cells (Fig. 2E) and peri- and supragranular layers (not shown). In the olfactory cortex, staining was present in neuronal cell body layers in the olfactory tubercle (Fig. 2F) and the piriform cortex (Fig. 2G). In the amygdaloid complex, staining was associated with cells in the cortical and medial amygdaloid nuclei (Fig. 2H), whereas the basal (Fig. 2H) and lateral nuclei (not shown) had less staining.

Staining was present in several thalamic nuclei, but at quite low intensity; for examples, see Figure 1A showing lateral posterior nuclei and Figure 2A showing the medial geniculate nucleus in the hypothalamus. Staining was evident in cells of the ventromedial nucleus (Fig. 2I). In the neocortical staining was widespread but typically at a low intensity; for example, see Fig. 2J showing an area of visual cortex. In some neocortical areas, however, more intense staining was evident in superficial neuronal cell body layers; for example, see Figure 2K showing retrosplenial cortex. In the cerebellar cortex, staining was present in the granule cell layer and Purkinje cell layer, but not in the molecular layer or white matter (Fig. 2L).

Collectively, the results obtained from mRNA in situ hybridization experiments indicate that NAPE-PLD is expressed by neurons in several brain regions but with variations between neuronal types in the levels of NAPE-PLD expression. However, for these observations to be interpreted from a functional perspective, immunocytochemical analysis of the distribution of NAPE-PLD protein in brain is necessary.

Western blot analysis of brain homogenates from NAPE-PLD(*+*) and NAPE-PLD(*−*) mice with antisera and affinity-purified antibodies to the C-terminal region of mouse NAPE-PLD

Western blot analysis of brain homogenates from NAPE-PLD(*+*) mice using antisera (2282.3, 2283.3, 2284.3) revealed several immunoreactive bands, ranging in molecular mass from ~25 kDa to ~150 kDa (Fig. 3A–C, respectively). The most intensely stained band revealed by the 2282.3 antisera (Fig. 3A) and the 2284.3 antisera (Fig. 3C) was located between the 37-kDa and 50-kDa markers, which is consistent with the expected molecular mass for NAPE-PLD (~46 kDa). However, this band was also detected in brain homogenates from NAPE-PLD(*−*) mice, as indeed were other immunoreactive bands in brain homogenates from NAPE-PLD(*−*) mice (Fig. 3A–C). Thus, the strongly immunoreactive band located between the 37-kDa and 50-kDa markers is not in fact NAPE-PLD but is another protein with a molecular mass very similar to NAPE-PLD that is recognized by antibodies present in the 2282.3 and 2284.3 antisera. Hence, it was necessary to affinity-purify antibodies to the NAPE-PLD C-terminal peptide antigen.

Immunoglobulins eluted with glycine (G) and triethylamine (T) were collected separately and then tested in Western blots of brain homogenates from NAPE-PLD(*+*) and NAPE-PLD(*−*) mice (Fig. 3D–F). 2282.3(G) labeled a
band in NAPE-PLD+/+ brain homogenates with a molecular mass of ~46 kDa, which was not detected in NAPE-PLD−/− brain homogenates (Fig. 3D). However, with 2282.3(G) two strongly immunostained bands located between the 25-kDa and 37-kDa markers were also detected in both NAPE-PLD+/+ and NAPE-PLD−/− brain homogenates. 2282.3(T) revealed a single band in NAPE-PLD+/+ brain homogenates with the expected molecular mass for NAPE-PLD (~46 kDa), which was absent in NAPE-PLD−/− mouse brain homogenates (Fig. 3D). Therefore, the 2282.3(T) fraction appeared to contain antibodies that selectively recognize NAPE-PLD and that we therefore used for immunocytochemical analysis of NAPE-PLD expression in mouse brain (see below). In contrast to 2282.3(T), affinity-purified antibodies derived from the 2283.3 and 2284.3 antisera exhibited cross-reactivity with multiple protein bands that were present in both NAPE-PLD+/+ and NAPE-PLD−/− brain homogenates (Fig. 3E,F), and therefore these antibodies were not used for subsequent immunocytochemical studies.

**Immunocytochemical localization of NAPE-PLD in mouse brain**

Immunocytochemical analysis of brain sections from NAPE-PLD+/+ mice using the 2282.3(T) antibodies revealed a widespread pattern of immunostaining. For example, in Figure 4A, immunoreactivity can be seen in the dentate gyrus (DG), the hippocampus (Hi), and the ventral (VTN) and mediodorsal (MD) thalamic nuclei. To assess the specificity of the immunostaining observed in brains from NAPE-PLD+/+ mice, the 2282.3(T) antibodies were also tested on sections of brains from NAPE-PLD−/− mice. The majority of immunostaining observed in brains from NAPE-PLD+/+ mice, which is described in more detail below, was not seen in brains from NAPE-PLD−/− mice and therefore can be attributed specifically to expression of NAPE-PLD.

For example, Figure 4B shows a coronal brain section from a NAPE-PLD−/− mouse that is in an intermediate position with respect to the wild-type brain sections shown in Figure 4A and C; note that the staining of the dentate gyrus, hippocampus, and thalamus that can be seen in Figure 4A is absent in Figure 4B, demonstrating that this staining can be specifically attributed to NAPE-PLD expression. However, there are structures that are stained in both Figure 4A and B, including the ependyma lining the lateral ventricles (see arrowheads) and the cell bodies and processes of hypothalamic magnocellular neurosecretory cells (see asterisks). This staining is therefore not attributable to NAPE-PLD expression and must be due to

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**Fig. 1.** Validation of the specificity of mRNA in situ hybridization methods used to visualize NAPE-PLD expression in mouse brain. **A:** DIG-labeled antisense RNA probes reveal intense staining in the granule cell layer of the dentate gyrus (GrDG) in wild-type mouse brain. Weakly stained cells can also be seen in the lateral posterior thalamic nucleus (LPTN). **B:** No staining is observed when wild-type mouse brain sections are incubated with DIG-labeled sense RNA probes, indicating that the staining observed in A is specific and attributable to NAPE-PLD expression. **C:** No staining is observed when brain sections from NAPE-PLD knockout mice are incubated with DIG-labeled antisense RNA probes, demonstrating that the staining observed in A is specific and attributable to NAPE-PLD expression. Abbreviations: GrDG, granule cell layer of dentate gyrus; LPTN, lateral posterior thalamic nucleus; Mol, molecular layer of the dentate gyrus; PoDG, polymorphic layer of the dentate gyrus. Scale bar = 200 μm in C (applies to A–C).
Fig. 2. NAPE-PLD expression in mouse brain as revealed by mRNA in situ hybridization with DIG-labeled antisense RNA probes. **A**: The intense staining of the granule cell layer of the dentate gyrus (DG) can be seen here in contrast to less intense staining in the CA3 pyramidal cell layer of the hippocampus (Hi) and weakly stained cells in the medial geniculate nucleus (MG). **B**: High-magnification image of the dentate gyrus showing stained granule cells (GrDG). **C**: The intense staining in the granule cell layer of the dentate gyrus (GrDG) is contrasted here with less intense staining in the CA1 pyramidal cell layer (Py) of the hippocampus. **D**: High-magnification image of the CA3 region of the hippocampus showing stained pyramidal cells (Py). **E**: In the olfactory bulb stained granule cells can be seen here in the granule cell layer (GrO) and in the mitral cell layer (Mi). **F**: Stained cells in the olfactory tubercle. **G**: Stained cells in the piriform cortex. **H**: Stained cells in the piriform cortex (Pir) can be seen here in contrast to less intense staining in the cortical (CA) and medial (MA) amygdaloid nuclei and very weak staining in the basomedial amygdaloid nucleus (BMA). **I**: Stained cells in the ventromedial hypothalamic nucleus (VM). **J**: Weakly stained cells in the visual neocortex; the asterisk labels layer I of the cortex. **K**: Retrosplenial neocortex, with staining strongest in the superficial cellular layers; the asterisk labels layer I of the cortex. **L**: Cerebellar cortex, with staining evident in both the granule cell layer (GCL) and Purkinje cell layer (PCL) but not in the molecular layer (ML) or white matter (WM). Abbreviations: BMA, basomedial amygdaloid nuclei; CA, cortical amygdaloid nuclei; DG, dentate gyrus; EPI, external plexiform layer of the olfactory bulb; GrDG, granule cell layer of the dentate gyrus; GrO, granule cell layer of the olfactory bulb; Hi, hippocampus; LMol, lacunosum moleculare layer; IPI, internal plexiform layer of the olfactory bulb; Mi, mitral cell layer of the olfactory bulb; MA, medial amygdaloid nuclei; MG, medial geniculate thalamic nucleus; ML, molecular layer of the cerebellar cortex; Or, stratum oriens of the hippocampus; Ov, olfactory ventricle; PoDG, polymorphic layer of the dentate gyrus; Pir, piriform cortex; PCL, Purkinje cell layer; Py, pyramidal cell layer of the hippocampus; Rad, stratum radiatum of the hippocampus; Tu, olfactory tubercle; VP, ventral pallidum; WM, white matter. Scale bar = 50 μm in B,D,L; 100 μm in C,E,G,I,J,K; 200 μm in A,H.
Fig. 3. Characterization of antisera (A–C) and affinity-purified antibodies (D–F) to mouse NAPE-PLD by Western blot analysis of brain homogenates from NAPE-PLD+/— and NAPE-PLD—/— mice. NAPE-PLD antisera [A: 2282.3(AS), B: 2283.3(AS), C: 2284.3(AS)] label bands in wild-type (+/+) brains with a wide range of molecular masses, including an intensely stained band between the 37-kDa and 50-kDa markers with a molecular mass similar to that expected for mouse NAPE-PLD (~46 kDa). However, all the stained bands evident in wild-type brains, including the intensely stained band between the 37-kDa and 50-kDa markers, are also detected in brains from knockout animals (—/—) and therefore are not NAPE-PLD, demonstrating the necessity for affinity purification of antibodies to the NAPE-PLD C-terminal peptide antigen. D: Affinity-purified antibodies derived from the 2282.3 antiserum and eluted with glycine (G) or triethylamine (T) label a ~46-kDa band in wild-type (+/+) brain homogenates that is not present in knockout (—/—) brain homogenates and that therefore must be NAPE-PLD (see arrow). The 2282.3(G) antibodies also label two bands between the 25-kDa and 37-kDa markers that are present in both wild-type and knockout brain homogenates. However, 2282.3(T) antibodies only stain the ~46-kDa band corresponding to NAPE-PLD, and therefore these antibodies were selected for immunocytochemical analysis of NAPE-PLD expression in mouse brain. E,F: Affinity-purified antibodies derived from the 2283.3 and 2284.3 antisera also label a ~46-kDa band corresponding to NAPE-PLD (see arrows). However, these antibodies also cross-react with other proteins and therefore were not used for immunocytochemical analysis of NAPE-PLD expression in mouse brain.
Fig. 4. Immunoreactivity in coronal sections of mouse forebrain (A–F) attributable to NAPE-PLD expression by comparative immunocytochemical analysis of NAPE-PLD/* mice (B). A: Immunostaining in anterior regions of the dentate gyrus (DG), hippocampus (Hi), and thalamus (MD, VTN). B: Immunostaining is almost completely absent in this coronal section of NAPE-PLD knockout mouse brain positioned intermediate with respect to the sections of wild-type brain shown in A and C. The only staining present is in ventricular ependyma (arrowhead) and in hypothalamic magnocellular neurons (*) and, with the exception of these features, all of the staining in wild-type brain (A, C–F) is specifically attributable to NAPE-PLD expression. C–F: Here, in addition to immunostaining in the hippocampal formation (DG, Hi) and thalamus (Gus, LG, MD, MG, Po, VP), NAPE-PLD immunoreactivity is also present in the neocortex (NC), piriform cortex (Pir), amygdaloid complex (AmC), and hypothalamus (Hy). Abbreviations: AmC, amygdaloid complex; CPu, caudate putamen; DG, dentate gyrus; Gus, gustatory thalamic nucleus; Hi, hippocampus; ic, internal capsule; LG, lateral geniculate thalamic nucleus; MD, mediodorsal thalamic nucleus; MG, medial geniculate thalamic nucleus; NC, neocortex; Pir, piriform cortex; Po, posterior thalamic nucleus; VP, ventroposterior thalamic nucleus; VTN, ventral thalamic nuclei. Scale bar = 500 μm in A–F.
cross-reaction of the NAPE-PLD antibodies with other proteins. Likewise, the staining of ventricular ependyma and choroid plexus that can be seen in Figure 4C–F is also not attributable to expression of NAPE-PLD.

The images of NAPE-PLD immunoreactivity (ir) in wild-type mouse brain sections shown in Figure 4 provide an overview of the most prominent features. In addition to staining associated with the dentate gyrus, hippocampus, and thalamus, which was highlighted above, immunoreactivity was also evident in the neocortex, olfactory (piriform) cortex, hypothalamus, and amygdaloid complex. More detailed interpretation of the immunostaining, however, requires images of higher magnification than those shown in Figure 4. Therefore, selected higher magnification images of several regions of the brain are shown in Figure 5 and described below. In all brain regions in which immunostaining was detected, the staining was not evident in neuronal somata but appeared to be localized in stained fibers surrounding unstained cell bodies and dendrites.

The most striking feature of NAPE-PLD expression in the mouse brain was intense NAPE-PLD-ir present in the polymorphic layer of the dentate gyrus (Fig. 5A), where the pattern of staining was consistent with localization of NAPE-PLD in mossy fibers, the axons of dentate gyrus granule cells (Longo et al., 2003). Accordingly, analysis of immunostained sections at high magnification revealed that NAPE-PLD-ir was localized in mossy fiber varicosities surrounding unstained cells in the polymorphic layer (Fig. 5B). Immunolabeled mossy fibers were also evident in the stratum lucidum of the CA3 region of the hippocampus (Fig. 5C). Weaker immunostaining was evident in the stratum radiatum and stratum oriens of the hippocampal CA1–CA3 fields (Fig. 5A,D), surrounding unstained somata and dendrites of pyramidal cells (Fig. 5E). In the neocortex, immunostaining was present in superficial layers, but layer VI was only very weakly stained.

In addition to the polymorphic layer of the dentate gyrus, a second region of the brain with very intense immunostaining was the accessory olfactory bulb. Here NAPE-PLD-ir was localized in glomeruli and in axons that project into the accessory olfactory bulb from the vomeronasal nerve (Fig. 5G,H). Immunostaining was also present in the granule cell layer and glomerular layer of the main olfactory bulb, but this staining was less intense than staining in the vomeronasal nerve and the accessory olfactory bulb (Fig. 5H).

Other regions of the forebrain where NAPE-PLD-ir was present included the caudate putamen and the lateral nucleus of the septum (Fig. 5L,J). The majority of thalamic nuclei were immunostained, as can be seen in the low-magnification images of Figure 4. A higher magnification image of immunostaining in the medial and lateral geniculate nuclei is shown in Figure 5K. Compared with the forebrain, relatively little staining was evident in the midbrain and hindbrain. For example, in the cerebellar cortex very weak immunostaining was present in the molecular layer (Fig. 5L) at an intensity close to the threshold for detection.

**DISCUSSION**

Here we describe the distribution of NAPE-PLD expression in the mouse brain, utilizing mRNA in situ hybridization methods and novel antibodies to a NAPE-PLD C-terminal peptide antigen. Importantly, we have employed use of NAPE-PLD knockout mice (NAPE-PLD−/−) for unequivocal evaluation of the specificity of NAPE-PLD antibodies and the immunostaining observed in brains from wild-type mice (NAPE-PLD+/−). NAPE-PLD mRNA was detected in the somata of identified neuronal populations in several regions of the brain, including granule cells in the dentate gyrus, olfactory bulb, and cerebellar cortex and pyramidal cells in the hippocampus and cortex.
NAPE-PLD immunoreactivity was not, however, detected in the somata of these or any other neuronal populations, and the patterns of immunostaining revealed by antibodies to NAPE-PLD indicate that this enzyme is targeted to axons. Although we cannot exclude the possibility that NAPE-PLD is also expressed by glial cells in the brain, the immunostaining obtained provided no evidence of non-neuronal expression.

Importantly, the overall regional distribution of NAPE-PLD mRNA in mouse brain correlated with the distribution of NAPE-PLD protein. For example, both NAPE-PLD mRNA and protein are very abundant in the hippocampal formation but are present at relatively low levels in the cerebellum. Previously, the relative abundance of NAPE-PLD in different regions of the rat brain has been investigated by measurement of NAPE-PLD enzyme activity,
Western blotting, and real-time PCR (Morishita et al., 2005). NAPE-PLD was detected in all rat brain regions analyzed, but it was most abundant in the thalamus. Our data also indicate that NAPE-PLD is widely expressed in the thalamus, but based on the intensity of immunostaining it appears that in mouse brain NAPE-PLD is more abundant in the hippocampal formation. There may therefore be species differences in the relative abundance of NAPE-PLD in brain regions.

Within regions of the mouse brain mRNA in situ hybridization and immunocytochemistry typically yielded complementary patterns of staining, which can be attributed to the targeting of NAPE-PLD protein to the axons of neuronal somata that express the NAPE-PLD gene. There was also correspondence in the relative intensity of staining revealed by mRNA and protein labeling methods in different neuronal populations. Thus, neurons strongly labeled for NAPE-PLD mRNA had intense NAPE-PLD-ir in their axons (e.g., dentate gyrus granule cells). Conversely, neuronal populations with a weaker mRNA signal (e.g., olfactory bulb granule cells) had correspondingly lower levels of axonal NAPE-PLD-ir.

The brain region with the most striking pattern of NAPE-PLD expression was the dentate gyrus, where a strong NAPE-PLD mRNA signal was detected in granule cells and intense NAPE-PLD immunoreactivity was present in the varicose axons of these cells (mossy fibers). This high level of NAPE-PLD expression in granule cells is of particular interest from a functional perspective because the dentate gyrus is part of the hippocampal formation, which has a fundamental role in some forms of learning and memory as well as having high seizure susceptibility. In hippocampal circuitry, the dendrites of granule cells in the molecular layer of the dentate gyrus receive excitatory input from the entorhinal cortex via the perforant pathway. Then axons of granule cells (mossy fibers) form en passant synapses with neurons such as mossy cells in the polymorphic layer of the dentate gyrus before projecting to the CA3 region of the hippocampus, where they synapse onto the proximal dendrites of pyramidal cells. The presence of intense NAPE-PLD immunoreactivity in the axons of mossy fibers both in the polymorphic layer of the dentate gyrus and in the stratum lucidum of the CA3 field of the hippocampus suggests, therefore, that NAPE-PLD may be involved in regulation of hippocampal synaptic transmission. However, there is at present no direct evidence of such a role and testing this hypothesis may require the development of novel selective inhibitors of NAPE-PLD and/or comparison of hippocampal synaptic transmission in NAPE-PLD−/− mice.

The discovery that NAPE-PLD appears to be principally involved in biosynthesis of saturated, long-chain (e.g., C20:0 and C22:0) NAEs in the brain (Leung et al., 2006) focuses attention on the physiological roles of these lipids in the brain. Based on the observations reported here, it would be of particular interest to analyze the effects of these molecules on synaptic transmission between mossy fibers and mossy cells or CA3 pyramidal cells. We speculate that NAEs generated by NAPE-PLD in the presynaptic axon terminals of mossy fibers may function as anterograde synaptic signaling molecules, acting postsynaptically and influencing the activity of mossy cells and CA3 pyramidal cells. Furthermore, although whole-brain levels of anandamide are unaltered in NAPE-PLD−/− mice, it is still possible that NAPE-PLD contributes to anandamide/endocannabinoid signaling, particularly in brain regions where its expression is high (e.g., hippocampal formation).

In animal models of temporal lobe epilepsy, sprouting of mossy fibers is observed, with the formation of new synapses between mossy fiber terminals and dendrites of granule cells and inhibitory interneurons occurring in the inner third of the molecular layer of the dentate gyrus (Longo et al., 2003). NAPE-PLD-ir is more intense in the inner third of the molecular layer (IML) than in more superficial layers (Fig. 5D), which may reflect the presence of NAPE-PLD in mossy fiber terminals in the IML. Therefore, an increase in the intensity of NAPE-PLD-ir in the IML may accompany sprouting of mossy fibers in temporal lobe epilepsy. Moreover, the abundance of NAPE-PLD in mossy fibers provides a rationale for investigation of a potential role for NAPE-PLD and the NAEs that it generates in regulation of hippocampal seizure activity.

A second region of the brain with particularly intense NAPE-PLD-ir was the accessory olfactory bulb, where immunostaining was localized in axons projecting from the vomeronasal nerve. These axons originate from the vomeronasal organ, which contains pheromone-sensitive receptors (Brennan, 2001). The presence of NAPE-PLD in vomeronasal axons suggests, therefore, that this enzyme and the NAEs it generates may participate in synaptic mechanisms associated with neural processing of pheromone-stimulated electrical activity in the accessory olfactory bulb. Interestingly, NAPE-PLD-ir is also present in the glomerular layer of the main olfactory bulb and is associated with axons derived from olfactory nerve, but this immunostaining is less intense than in the vomeronasal axons associated with the accessory olfactory bulb.

The expression of NAPE-PLD by other neuronal populations in the forebrain is not as striking as in dentate gyrus mossy fibers or vomeronasal axons but is nevertheless important to consider. However, detailed discussion of NAPE-PLD expression in all regions of the brain where it is detected would be excessive, and therefore we will focus on a few selected brain regions.

In the hippocampus, NAPE-PLD mRNA is present in pyramidal cells of the CA1–CA3 fields, and the pattern of NAPE-PLD immunoreactivity in the hippocampus, with immunostaining surrounding but not localized within pyramidal cell somata or dendrites, is consistent with targeting of the NAPE-PLD protein to the axons of hippocampal pyramidal cells. Thus, NAEs generated by NAPE-PLD may be involved in regulation of excitatory synaptic transmission throughout the hippocampal formation.

In the cortex, NAPE-PLD mRNA expression was clearly evident in pyramidal cells of the olfactory cortex, but in the neocortex the intensity of staining for NAPE-PLD mRNA expression was typically rather low. Nevertheless, analysis of NAPE-PLD immunoreactivity in the cortex revealed a pattern of staining consistent with axonal targeting of NAPE-PLD in cortical pyramidal cells. Interestingly, the intensity of NAPE-PLD immunostaining was higher in superficial layers of the neocortex than in layer VI, which may reflect axonal targeting of NAPE-PLD in superficial pyramidal cells that project within the cortex.

In the cerebellar cortex NAPE-PLD mRNA expression was detected in the granule cell layer, and weak NAPE-PLD-ir was detected in the molecular layer. These data indicate that the NAPE-PLD gene is expressed by cerebellar...
granule cells and that, consistent with evidence from other brain regions, NAPE-PLD protein is targeted to the axons of these neurons (parallel fibers) in the molecular layer. Furthermore, if NAEs generated by NAPE-PLD act as anterograde synaptic signaling molecules, as suggested above, then NAEs generated presynaptically in parallel fibers may influence the activity of postsynaptic Purkinje cells.

NAEs generated by NAPE-PLD in the brain are potential substrates for the degradative enzyme fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996, 2001; Patricelli and Cravatt, 2001). Thus, the physiological half-life of NAEs generated by NAPE-PLD in the brain may be influenced by the proximity and relative abundance of FAAH. It is of interest, therefore, to compare the distribution of NAPE-PLD and FAAH in the mouse brain. The distribution of FAAH expression in the rat and mouse brain has been analyzed previously by using immunocytochemical techniques (Egertová et al., 1998, 2003, 2004; Tsou et al., 1998). Like NAPE-PLD, FAAH is widely expressed but with regional and cellular variation in its relative abundance. FAAH is expressed by many neuronal populations and is also expressed in ventricular ependymal cells and oligodendrocytes. Interestingly, in neurons that express the FAAH gene the FAAH protein is typically targeted to the somatodendritic neuronal compartment (Egertová et al., 2003; Gulyas et al., 2004). Thus, it appears that NAPE-PLD and FAAH are targeted to different subcellular compartments in neurons, giving rise to complementary distribution patterns. For example, in the hippocampus FAAH is expressed by pyramidal cells and targeted to their somatodendritic compartment (Egertová et al., 2003; Gulyas et al., 2004), whereas the data presented in this paper indicate that NAPE-PLD is targeted to the axons of pyramidal cells. Likewise, both FAAH and NAPE-PLD are widely expressed in thalamic nuclei, but FAAH-ir is present in neuronal somata (Egertová et al., 2003) and NAPE-PLD-ir surrounds unstained neuronal somata. This suggests that if NAEs generated by NAPE-PLD in the brain are utilized as anterograde synaptic signaling molecules, then the presence of FAAH in neuronal somata and dendrites may facilitate rapid postsynaptic inactivation of these molecules.

In conclusion, the data presented in this paper provide the first insight into the neuroanatomy of NAPE-PLD expression in the brain and a new perspective on the neural functions of this enzyme and NAEs that it generates. Our finding that NAPE-PLD is targeted to the axons and axon terminals of identified neurons provides a basis for investigation of a potential role for NAEs as mediators of anterograde signaling at synapses.

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LITERATURE CITED