

Isolation and expression of a rat brain cDNA encoding glutamate carboxypeptidase II

(astrocytes/excitatory neurotransmission/*N*-acetyl- α -L-aspartyl-L-glutamate/prostate-specific membrane antigen)

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ABSTRACT *N*-acetylated alpha-linked acidic dipeptidase (NAALADase) hydrolyzes acidic peptides, such as the abundant neuropeptide *N*-acetyl- α -L-aspartyl-L-glutamate (NAAG), thereby generating glutamate. Previous cDNA cloning efforts have identified a candidate rat brain NAALADase partial cDNA, and Northern analyses have identified a family of related RNA species that are found only in brain and other NAALADase-expressing cells. In this report, we describe the cloning of a set of rat brain cDNAs that describe a full-length NAALADase mRNA. Transient transfection of a full-length cDNA into the PC3 cell line confers NAAG-hydrolyzing activity that is sensitive to the NAALADase inhibitors quisqualic acid and 2-(phosphonomethyl)glutaric acid. Northern hybridization detects the expression of three similar brain RNAs approximately 3,900, 3,000, and 2,800 nucleotides in length. *In situ* hybridization histochemistry shows that NAALADase-related mRNAs have an uneven regional distribution in rat brain and are expressed predominantly by astrocytes as demonstrated by their colocalization with the astrocyte-specific marker glial fibrillary acidic protein.

The enzymatic activity of NAALADase (*N*-acetylated alpha-linked acidic dipeptidase or glutamate carboxypeptidase II) was first described in rat brain as a quisqualate-sensitive peptidase activity that released glutamate from the neuropeptide *N*-acetyl- α -L-aspartyl-L-glutamate (NAAG) (1). Since that time, much has been learned about the enzyme, its substrate, and their relationships to excitatory neurotransmission. The intact NAAG peptide serves as a negative modulator of glutamatergic neurotransmission through two distinct actions. First, the peptide is a weak partial agonist at the *N*-methyl-D-aspartate (NMDA) subclass of ionotropic receptors and thereby inhibits the gating of the channel by its endogenous effector glutamate (2–5). Second, NAAG is an agonist at the mGluR3 subtype of metabotropic receptor, which is negatively coupled to adenylyl cyclase (6). Adding another level of complexity to the effects of NAAG on glutamatergic systems is its hydrolysis by NAALADase to glutamate and *N*-acetylaspartate. Although the intact peptide has predominantly inhibitory actions, its NAALADase-mediated hydrolysis to yield glutamate may activate all glutamate receptor subtypes.

One form of the NAALADase enzyme, a 94-kDa plasma membrane glycoprotein, was purified to homogeneity from rat brain by Slusher *et al.* (7). Antisera against this protein have been used in immunohistochemical studies to localize the expression of NAALADase in rat brain, peripheral nerves, and kidney (8, 9). Using these same antisera to screen a rat brain

cDNA expression library, Carter *et al.* (10) identified a partial cDNA that exhibited substantial sequence similarity to a cDNA encoding an investigational human prostate cancer marker antigen known as the prostate-specific membrane antigen (PSM) (11, 12). Subsequent transfection studies demonstrated that PSM hydrolyzes NAAG, thereby showing the first functional expression of a NAALADase cDNA clone (10).

To further our characterization of NAALADase mRNAs expressed in the nervous system, we have used reverse transcription-PCR and nucleic acid hybridization to isolate a set of PSM-related cDNAs from rat brain. The present report provides a description of a complete rat brain NAALADase mRNA coding sequence and the characterization of its functional expression. We also have examined the spatial and cellular expression of NAALADase-like mRNAs in the brain by using *in situ* hybridization histochemistry.

MATERIALS AND METHODS

Chemicals. General chemical reagents were obtained from Fisher Scientific or Sigma. Promega brand restriction endonucleases were purchased from Fisher Scientific.

Cell Lines and Tissues. The PC3 tumor cell line was obtained from the American Type Culture Collection and grown in DMEM supplemented with 2 mM glutamine and 10% fetal bovine serum (GIBCO/BRL). Adult Sprague-Dawley rats (Taconic Farms) were sacrificed with an overdose of pentobarbital, and tissues were removed and either processed immediately or frozen on dry ice (RNA preparations) or frozen in isopentane on dry ice (for *in situ* hybridization and immunohistochemistry).

Reverse Transcription-PCR Cloning. Reverse transcription reactions were conducted at 47–50°C for 2 hr by using Super-script II reverse transcriptase (BRL) according to the manufacturer's recommended conditions with the addition of 3.33 mM dimethyl sulfoxide in the RNA denaturation step and 40 units/25 μ l of recombinant RNasin (Promega, Fisher Scientific). First-strand cDNA was purified by using PCR Purification Cartridges (Advanced Genetic Technologies, Gaithersburg, MD). PCRs were performed with native Pfu (Stratagene) or Amplitaq (Perkin-Elmer) polymerase according to the supplier's recommendations by using a GenAmp 480 thermal cycler (Perkin-Elmer). Thermal cycling parameters consisted of an initial denaturation step (94°C for 4 min)

Abbreviations: GFAP, glial fibrillary acidic protein; NAALADase, *N*-acetylated alpha-linked acidic dipeptidase; NAAG, *N*-acetyl- α -L-aspartyl-L-glutamate; PSM, prostate-specific membrane; NMDA, *N*-methyl-D-aspartate.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF040256).

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followed by 30–35 cycles of amplification (94°C for 1 min, 60–68°C for 1 min, 72°C for 3 min), and ending in a final extension step (72°C for 7 min). Primer sequences are as follows: primer pair I (R1–2): 5' primer, GAAAGCTGAGAACATCAAGA and 3' primer, TACTTGGGAAGACCGACAG; primer pair II (R19): 5' primer, GCAGTAGAGCCGCAGTAGAAC and 3' primer, TAGGACAACAGGACATCATAA; primer pair III (R70): 5' primer GCAGTAGAGCCGCAGTAGAAC and 3' primer, TACTTGGGAAGACCGACAG. The R11 clone was isolated by using a 5' rapid amplification of cDNA ends kit (BRL) with modifications described above in the reverse transcriptase and first-strand cDNA purification steps, and gene-specific primers A (RT primer): ATAGTTAACATACACTAGATC and B (PCR primer) TAGGACAACAGGACATCATAA, together with the anchor primer supplied by BRL.

Screening of Recombinant cDNA Libraries. Rat brain cDNA libraries were obtained from Rachael Neve (McLean Hospital, Harvard Medical School, Boston, MA) and Stratagene (catalog no. 936518). For nucleic acid hybridization screening, recombinant plaque lysates (approximately 50,000 plaque-forming units/15-cm plate) were transferred to nitrocellulose or nylon discs (BA80, Schleicher & Schuell or Colony/Plaque Screen membranes, NEN/DuPont), alkali denatured, and neutralized per ref. 13. Dried filters then were hybridized to a random-primed ³²P-radiolabeled cDNA probe (specific activity = 1.5–6.0 × 10⁹ dpm/μg) prepared by using a Prime-It kit (Stratagene) at 65°C overnight in an aqueous hybridization medium (14). Low-stringency washes were performed at room temperature in 2× standard saline citrate (SSC) + 0.1% SDS, followed by high-stringency washes with 0.2× SSC + 0.1% SDS at 65°C.

DNA Sequencing and Analysis. Dideoxy sequencing reactions were performed by using Sequenase kit 70770 (Amersham) or a Pfu (exo-) Cyclist system (Stratagene) according to the manufacturer's instructions. Sequence analyses were conducted by using the programs BLAST, FASTA, BESTFIT, MAP, FITCONSENSUS, MOTIFS, and PEPTIDESTRUCTURE from the GCG Package, Version 7, Genetics Computer Group (Madison, WI).

Transient Transfections. R72 plasmid DNA was prepared by using a Qiagen Endotoxin-free Maxiprep system (Qiagen, Chatsworth, CA). Monolayer cultures of PC3 cells in 100-mm dishes were transfected with 25 μg of plasmid DNA by using the calcium phosphate-mediated method of Graham and van der Eb (15). pcDNA3CAT (negative) and PSMA2 (positive) control transfections were performed in parallel with experimental transfections. Cells were harvested 48 hr posttransfection for enzymatic assays, and protein concentrations were determined by using the enhanced protocol BCA assay with BSA as the standard (Pierce, Rockford, IL).

Enzyme Assays. Monolayer cultures of the transfected PC3 cell lines were scraped into 5 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4 at 37°C) containing 0.5% Triton X-100 and solubilized by sonication. NAALADase radioenzymatic assays were conducted in triplicate as described by Robinson *et al.* (1) by using *N*-acetylaspartyl[³H]glutamate (NEN/DuPont). The specificity of NAAG-hydrolyzing activity was assessed by using the NAALADase inhibitors quisqualic acid [ref. 1; Research Biochemicals, and 2-(phosphonomethyl)glutaric acid (16), a generous gift of Barbara Slusher (Guilford Pharmaceuticals, Baltimore, MD)]. Activity is reported as the mean of three assays ± SEM.

Northern Blotting. Total RNA from Sprague–Dawley rats or cell lines was prepared by the method of Chirgwin *et al.* (17) or obtained commercially from CLONTECH. RNA was separated by electrophoresis through a 1.2% agarose gel containing 3% formaldehyde, electrophoretically transferred to a nylon membrane (GeneScreen, NEN/DuPont), and hybridized to a random-primed ³²P-radiolabeled cDNA probe (specific activity = 1.5–6.0 × 10⁹ dpm/μg) prepared by using a

Prime-It kit (Stratagene) at 42°C overnight. Final high-stringency washes were performed with 0.1× SSC + 0.1% SDS at 45°C. Hybridization was detected by autoradiography using a Molecular Dynamics PhosphorImager.

Immunoblotting. The characteristics of the anti-NAALADase antiserum that was used for Western blots have been reported previously (7). Ten-centimeter SDS-polyacrylamide minigels (ref. 18; 7.5% acrylamide) were used to electrophoretically separate the protein samples, which included a molecular weight standard. These subsequently were electrophoretically transferred to nitrocellulose membranes in 3-cyclohexylamino-1-propanesulfonic acid buffer, pH 11.0, 1% methanol. Membranes were blocked overnight at 4°C with 1% BSA in 20 mM Tris-buffered saline (TBS, pH 7.2). After washing twice for 15 min with TBS + 0.1% Tween-20, membranes were probed with anti-NAALADase antiserum (1:1,000) for 1 hr at room temperature. Membranes then were washed and incubated with horseradish peroxidase-conjugated anti-guinea pig secondary antibody (1:10,000 dilution, Jackson ImmunoResearch) for 1 hr. Immunoreactive signal was visualized with a chemiluminescent detection system (ECL and Hyperfilm-ECL, Amersham).

In Situ Hybridization Histochemistry. Digoxigenin-labeled antisense and sense runoff transcripts were synthesized from linearized plasmids W6 or R1–2 by using the Genius Kit (Boehringer-Mannheim) according to the manufacturer's instructions. Transcripts were alkali-hydrolyzed to an average length of 200–400 bp. *In situ* hybridization of a mixture of W6 and R1–2 probes (approx. 200 ng/ml) was performed on 12-μm cryosections of fresh frozen brain based on the protocol by Schaeren-Wiemers and Gerfin-Moser (19). Hybridization proceeded at 68°C for 18 hr. After hybridization, sections were washed briefly three times in 2× SSC and twice for 30 min in 0.2× SSC at 68°C. The hybridized digoxigenin-labeled probes were visualized by using anti-digoxigenin Fab fragments (Boehringer Mannheim) and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP)/nitroblue tetrazolium (NBT) substrate and developed for 42 hr. To determine the extent of distribution of the NAALADase hybridization signal in astrocytes, some sections were coimmunostained for glial fibrillary acidic protein (GFAP). Sections were incubated for 1 hr in rabbit anti-GFAP primary antiserum (Dako, 1:250 dilution) in the presence of 2% normal goat serum. Anti-GFAP immunoreactivity was visualized with biotinylated anti-rabbit secondary antibodies complexed to avidin-fluorescein isothiocyanate (Vector Laboratories; 1:100 dilution).

RESULTS

Cloning and Sequence Analysis of New Candidate NAALADase cDNAs. A candidate NAALADase rat brain cDNA clone (W6) had been isolated previously by immunoscreening using anti-NAALADase antiserum (10). Sequence similarity subsequently permitted the cloning of a full-length cDNA from a NAALADase-expressing human prostatic tumor cell line. The W6 clone contains a 1,407-base ORF, but no initiation codon (Fig. 1). To extend the characterization of the putative rat brain NAALADase mRNA in the 5' direction, a reverse transcription-PCR cloning strategy was used. For these experiments, combinations of PSM-derived 5' primers and W6-derived 3' primers, or a 5' rapid amplification of cDNA ends approach with rat cDNA-derived 3' primers and a nonspecific 5' primer were used. The resultant products are described below and schematically presented in Fig. 1.

R1–2. This species was generated by using a PSM-derived 5' primer and a W6-derived 3' primer. The resultant cDNA, 680 bases in length, is 88% identical to bases 456–1135 of the PSM/NAALADase cDNA sequence (GenBank entry M99487). Subsequent cloning of other cDNAs that overlap this

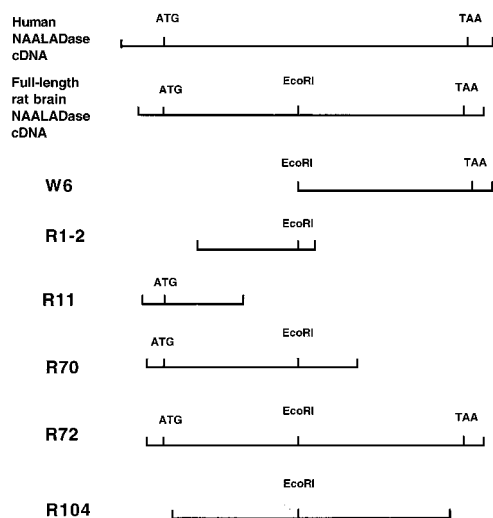


FIG. 1. Comparison of rat cDNAs. Six rat cDNAs are aligned with the full-length human cell line and rat brain NAALADase cDNAs. The partial clones are comprised for the following bases relative to the full rat sequence: W6, 921-2347; R1-2, 2720951; R11, 1-433; R70, 24-951; R72, 24-2347; and R104, 77-2286.

one indicate that the human NAALADase-derived 5' primer was not a faithful match to the rat brain mRNA.

R11. The use of primers derived from the human 5' untranslated sequence in combination with rat-derived 3' primers was unsuccessful in amplifying the remainder of the ORF. Therefore, a 5' rapid amplification of cDNA ends strategy was undertaken to complete the cDNA coding sequence. First-strand cDNA was synthesized from a gene-specific primer, polydeoxycytidine-tailed, and PCR-amplified with a nonspecific (polyguanosine/inosine-tailed) 5' primer and a specific 3' primer. Repeated analysis of rat brain mRNA by this method indicates that the 425-base R11 clone is an accurate representation of the mRNA 5' end. Comparison of this species to the M99487 human sequence shows conservation of a small region around the putative start methionine codon, but less similarity in the 5' untranslated region. Thus, the dissimilarity in the 5' untranslated sequences explained our inability to amplify rat brain cDNAs by using human NAALADase/PSM-derived 5' primers.

R70. After collecting a series of cDNAs that apparently spanned the full length of the coding region, long stretches of cDNA were amplified for expression studies and for defining the relationships of the partial clones to each other. The R70 clone includes the R11, R1-2, and W6 junctions and thus indicates that these partial clones all represent portions of the same mRNA.

R104. In parallel with the reverse transcription-PCR cloning, rat brain cDNA libraries were screened using the previously isolated cDNAs as probes. The R104 cDNA, isolated from a rat hippocampal library, spans all but 42 bases of the full coding region (at the 3' end). Thus, although formally possible, it is highly improbable that the characterized sequence is a hybrid representing more than one mRNA.

Transient Transfection of the Full-Length cDNA Confers NAAG-Hydrolyzing Activity. A full-length NAALADase cDNA was constructed by splicing the R70 and W6 clones together using the endogenous *EcoRI* restriction site at the 5' end of the W6 sequence (position 921 of the mRNA); the full-length cDNA was placed into the *KpnI* and *XbaI* restriction sites of the pcDNA3 mammalian expression vector, creating plasmid R72. The R72 plasmid was transiently transfected into the NAALADase-negative PC3 cell line (10). Lysates of the R72-transfected cells demonstrated hydrolytic activity against NAAG that was sensitive to the NAALADase

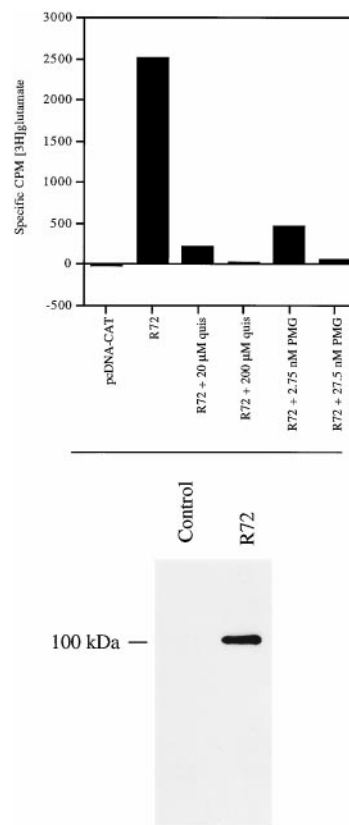


FIG. 2. Transfection of R72 confers NAALADase activity and immunoreactivity. (Upper) Samples of R72-transfected and pcDNA3CAT-transfected (control) cell lysates were assayed for NAALADase activity; activity is expressed as total [³H]Glu liberated from *N*-Ac-Asp-[³H]Glu ([³H]NAAG) after subtraction of protein-free blank. SEM is contained within the outlines of the bars in the graph. NAALADase inhibitors quisqualate (1, 20) and 2-(phosphonomethyl)glutaric acid (PMG) (16) were included at concentrations of 10 and 100 times their reported *K*_{is}. Expressed in terms of percent inhibition, the data translate as follows: 20 μM quisqualate, 91.5%; 200 μM quisqualate, 98.5%; 2.75 nM PMG, 80%; 27.5 nM PMG, 97.4%. Control cell lysates demonstrated no NAAG hydrolytic activity. (Lower) Twenty micrograms of total protein from control or R72-transfected cells was immunoblotted with anti-NAALADase antiserum. A specific immunoreactive band with an apparent molecular mass of approximately 100 kDa is observed.

inhibitors quisqualic acid and 2-(phosphonomethyl)glutaric acid in a concentration-dependent fashion (Fig. 2). Control-transfected cells showed no activity. This confirmed that the R72 insert was indeed a full-length NAALADase cDNA clone. Further, immunoblotting of the R72-transfected lysate with anti-rat brain NAALADase antisera revealed expression of a 100-kDa protein (Fig. 2), which is not present in untransfected lysates and is similar in size to the NAALADase purified from rat brain (7).

Structural Analysis of the Rat Brain cDNA and its Encoded Protein. The rat brain NAALADase cDNA and its predicted polypeptide (Fig. 3) share characteristics with the previously cloned human NAALADase/PSM cDNA. The coding regions display 85% identity at the cDNA level, 85% identity (89% similarity) at the amino acid level, and share the position of start and stop codons. The coding regions have slightly different sizes, however, with the rat's being slightly larger (2,256 versus 2,250 nucleotides, corresponding to 752 versus 750 amino acids). The two species diverge most considerably in the 5' end of the coding region, where the identities drop to 70% in the first 173 bases of the ORF. Further, the identity of the 5' untranslated sequence falls dramatically, except for a few conserved motifs flanking the start codon. These sequences

1 AAATATTGAGCTGCGACTCTCTGCTGAGAGGCGGCGGAGGACTGCTGAGAAAGGCGAAAGTTCGCGAGG 71
 72 ATG TGG AAG CCG CAG CAG GAG GAC TCC GCG GAG CCG CTG GCA CCG CCG CAA CCG TCG TTC CCG CCG GCA ACA CTG CCG CCG TTC 161
 I M W H A O Q D S D S B A E A L G R R Q R W F C A G T L V L L A F 30
 162 ACT GAA ACC TTC ACC ACT GCG TTC CTT TTC GCG TGG ATT ATA AAA CCG TCC AAT GAT ACT ACT GAG AGT GGT TCC TCC CCG AGG AAG 251
 III... 25
 252 AAG CCA TTG TTG CCA TGG AAG CCG CCG GAG ACC ATC AAA AAA TTG TTA TAC TTC ACA CCG ACA CCA CCG TTG CCA GAG ACG CAG CAG 341
 41 K E F L Q E L K A E N I K R F L Y N P T R T P H L A G A T Q H 30
 342 AAT TTT GCA CCG CTT GCA AAG CAA ATT GAT CCG CCG TCG AAA GAA TTG GAC CCG GAT TTT GGT GAG TTA TCG GAT TAT GAT CCG CCG TCC 431
 2E N P F L A K Q I H A Q W K E F G L D L L V E L S D Y D V L L S 239
 432 TTC ACA AAT AAG ACC CCG ACC TCC ACC TTC ATA ATT AAT GAA GAT GAT GCG ACC TTC ACA ACA TTA CTA CCG GAA CCG CCG ACC 521
 251 Y F H L P F M L A V L D G L A G A F S L D W E K R P S T E P L Q 150
 522 CCG CAG TCC GAG AAC ACA TCA GAG CTA CCG CCA CAC ACC CCG TTC TCC TCA CCA CAG CCA CCA GCG GCG GAT CCA GTA TTG GGT ACC 611
 151 P G Y F M I S D V V P P Y S A F S P Q G T P E G G D L V V V N H 189
 612 TAT GCA GCA ACT GAA CCG TTC TTT AAA CTG GCA CCG CCG ACC ATG ATC ATC TGT TGT CCG GAG AGT ACC ACC ACC ACC GAG TAT GCG CAA CCG 701
 181 Y A R T E D F F K L E R V V H X I N C S G K I V I A R Y G Q V 219
 702 TTC ACG ACG AAT AAG GGT AAG AAA AAT CCG CCG CCG CCG CCG AAA GAA ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC 791
 221 F R G E K V L W N A G L A G A R S I I L Y G D P A D Y P V F G 269
 792 CCG AAG TCC TAT CCG CCG CCG ACC 881
 241 V K S Y P D D G W N L P G G G V V Q R G N V L H L N G A G D P L 279
 882 ACG ACA CCG TAT CCA CCA AAT GAA TAT CCG CCG ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC 971
 271 T P G Y P A N E Y A Y R H E F T E A V Q L F S I P V P I G 309
 972 TTC GAT GAT CAG CAG ACA TTA GCA CAT AAT ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC 1061
 302 Y D D R O K L L Q E H M G G S A P D S S W R G Q L K W P Y N 130
 1062 GTC ACA CCG CCG TTC CCG CCG ACC TTC TCA AAA CAG ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC 1151
 331 V G P G F G F A G N P F E R K Q K V K L H I H S Y N K V T R I Y N V 369
 1152 AAT ACC ACC CCG AAG ACA CCG TTG GAA CCA GCA AAG TAT GGT ATT CTT GCA GCG CAG AAG GAT CCG CCG TTC GGT GCG ATT CCG CCG 1241
 361 I G T L K G A V E P D R Y V I L G G H R D A W V F G G G I D P 389
 1242 CAG ACC CCA GCA CCG CCG GGT 1331
 381 Q S G L P S P D E G F E R T P L K X K G W R F R T P L F 429
 1332 GCA ACC TCA GAT CCG GAA TTT CCG 1421
 421 A S W D A E E F G L L G S T S W A R E H S R L L Q E R G V A 459
 1422 TTC ACC ACC CCG GGT 1511
 451 Y I N A D S S I E G N Y T L K V D C T P L M H S L V Y M L T 489
 1512 AAA GCG CCG CCG ACC 1601
 481 K H L P S P D E G F E R T P L K X K G W R F R T P L F 529
 1602 GTC ACA CCG CCG TTC CCG CCG ACC TTC TCA AAA CAG ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC 1691
 511 M P R I S K L G S G N D F E V F P Q R L G I A S G R A R Y T 549
 1692 AAA AAT TCG AAA AAC AAC AAA CCG ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC 1781
 541 K N W K N N K V S S Y P L Y H S V Y E T Y E L V E K F Y D P 579
 1782 ACG TTC AAG TTT GAG ACC CCG 1871
 571 T F K Y F E G L A D V R G A M V F E L A B S I V L P F D C Q 629
 1872 GTC ACC CCG 1961
 602 S Y A V L L K K H A E T I Y M I S M N H P P O B M K K A Y M I S 639
 1962 TTT GAT TCC CCG TTC CCG CCA CCG AAT AAT TTT CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG 2051
 631 F G S L F S A V N M P T A D V A S K F N Q F L O D L D K S N P 669
 2052 ATA TTC AAG ATT TTT AAT GAT GAT CTA AAT TTT CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG 2141
 661 I H L L P L V L D G V R G A M V F E L A B S I V L P F D C Q 699
 2142 CCG ACC ACC TTT CCG CCG ACC 2231
 691 H I I Y A P S S H N X Y A G E S F P G I Y D A L F D I N N K 739
 2232 CCG ACC ACC TTT AAG CCG TCG AGA GAA CCG AAT TTT CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG 2321
 721 V D T S K A M R E V K R Q I S I A A F T V Q A A A E T L R E G 759
 2322 GTC GCA TAA GACTTCCTGCAAGT 2347
 751 V D

FIG. 3. Full sequence of rat brain NAALADase cDNA (2,347 bp). The cDNA contains a 2,256-base ORF; its translation appears under the cDNA sequence. Numerical index for the cDNA sequence is in roman type; index for the amino acid sequences is in italics. Start and stop codons are indicated by bold lettering. The putative transmembrane domain is underlined. Glycosylation consensus sites are indicated by + (weak, NXT or NXS for X = D, W, or P) or * (strong, NXT or NXS for X ≠ D, W, or P).

may be important for translation of the mRNA. The rat polypeptide, like the human, has an approximate molecular mass of 84 kDa, and possesses glycosylation consensus sites in its large putative extracellular domain (Fig. 3). Also like the human enzyme, rat brain NAALADase appears to be a type II membrane protein with conservation of basic arginine residues at positions 16, 17, and 19, which are predicted to anchor its amino terminal cytoplasmic domain. This predicted topology is in accord with previous evidence of NAALADase's plasma membrane localization and extracellular hydrolysis of NAAG (9, 21, 22). The hydrophobic membrane-spanning domain of the rat enzyme (residues 20–44) is predicted to be one amino acid longer than the human (residues 20–43). Whereas human NAALADase/PSM has a consensus protein kinase C sequence (position 14), this is not present in the predicted amino acid sequence of rat brain NAALADase. Two other hydroxyl-containing residues, which might serve as phosphorylation acceptor sites, are conserved between the rat and human enzymes at positions 8 and 10, also within the predicted intracellular domain. These are not in the context of known kinase consensus sequences, however.

Northern Analyses with the Full-Length NAALADase Clone. To examine the expression of NAALADase-like RNAs, the R72 cDNA was used as a hybridization probe for Northern analyses. Rat brain and kidney were positive for three RNA bands of approximately 3,900, 3,000, and 2,800 nucleotides (Fig. 4). In support of their encoding NAALADase proteins, the observed set of transcripts are found in NAALADase-positive, but not in NAALADase-negative, tissues and cell lines (Fig. 4; ref. 10). Whether these three species arise by expression from different genes (23, 24) or from differential splicing (25) or consist of heteronuclear RNAs remains to be determined. In previous Northern blot experiments, three additional smaller bands of hybridization had been detected in

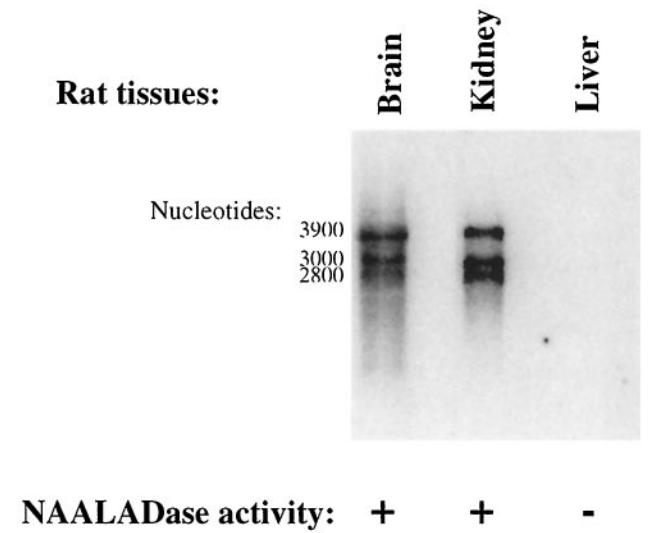


FIG. 4. Northern hybridization to the R72 cDNA. Electroblots of rat brain RNA (10 µg total RNA), rat kidney RNA [1 µg of poly(A) RNA], and rat liver RNA [1 µg of poly(A) RNA] were hybridized to a radiolabeled R72 cDNA probe. The same three transcripts of approximately 3.9, 3.0, and 2.8 kb in length are observed in both NAALADase-positive tissues (brain and kidney) whereas no hybridization is seen to RNA from the liver, which is NAALADase-negative. Hybridization to the poly(A) RNA (kidney) shows that the 3.9-kb band does not represent artifactual hybridization to 28S ribosomal RNA.

rat brain and kidney with estimated sizes of 2,100, 750, and 500 nucleotides (10). However, these smaller species were not evident under current conditions nor in Northern blots of poly(A) RNA isolated from rat kidney, suggesting that they may have represented degradation products.

In Situ Hybridization Histochemistry of Rat Brain Sections. *In situ* hybridization with NAALADase antisense cRNA probes yielded specific hybridization signals in rat brain. No signal was observed with sense cRNA probes (Fig. 5e). In general, NAALADase is expressed in brain cells in low abundance, as compared with the message levels of the NMDAR1 receptor or the astrocytic glutamate transporters (not shown). The expression of NAALADase-like mRNAs is widespread in the brain, with subcortical structures generally exhibiting higher levels of expression than forebrain regions (Fig. 5a). The exception to this trend is the olfactory bulb, which contains relatively high levels of NAALADase-like RNAs. Thus, the rostrocaudal gradient of mRNA expression detected by the NAALADase probe parallels the expression of NAALADase activity (21) and immunoreactivity (8) and the distribution of NAAG (26).

The profiles of cells labeled with the NAALADase cRNA probes were examined under higher magnification. In the cerebellar cortex, the large Purkinje cell bodies were devoid of labeling, whereas labeling was observed in the region immediately surrounding them. This distribution is consistent with labeling of Bergmann glia, an astrocytic subtype, which resides in this location in the mature cerebellar cortex (27). The possible expression of NAALADase by astrocytes then was explored more definitively by determining the extent of colocalization of NAALADase-like mRNAs with immunostaining for the astrocytic marker GFAP. The NAALADase mRNA is visualized in the perinuclear region by using a chromagenic detection method that can be observed under visual light (Fig. 5b) and astrocytic processes were immunostained for GFAP with an fluorescein isothiocyanate-labeled (fluorescent) secondary antibody complex (Fig. 5d). The dual-exposure photomicrograph (Fig. 5c) demonstrates the colocalization of the two markers in the same cells. NAALADase-positive cell profiles were GFAP-positive in approximately 90% of cells

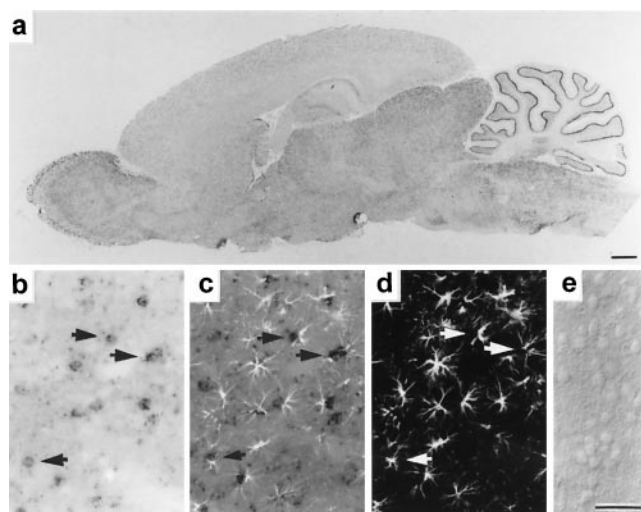


FIG. 5. *In situ* hybridization histochemistry for NAALADase-like mRNAs and colocalization with GFAP. Expression of NAALADase-like mRNA visualized with cRNA probes appears to be widespread in the brain, with midbrain-brainstem structures generally exhibiting higher hybridization than forebrain areas, except for the olfactory bulb, which shows a relatively high level of expression (*a*). Note particularly intense labeling of a narrow band near the Purkinje cell layer of the cerebellum. The colocalization of NAALADase-like mRNA to GFAP-positive astroglia in a field of rat hippocampal cells in a coronal brain section (arrows indicate examples of dual-labeled cells in registry). (*b*) Labeling with a NAALADase cRNA probe shows that small cell bodies in the hippocampus are encircled with dark staining for NAALADase-like message. (*d*) Immunostaining of this same group of hippocampal cells for the astrocyte-specific marker GFAP is shown, indicated by fluorescent fluorescein isothiocyanate-labeling of fibers within the cell processes. (*c*) A double-exposure photomicrograph shows that the two signals colocalize to the same cells, with fluorescent GFAP-positive processes extending from dark perinuclear halos of NAALADase labeling. (*e*) Bright-field photomicrograph of an adjacent section of tissue to which a control sense NAALADase cRNA was applied shows no labeling. [Bars = 50 μ m (*a*) and 5 μ m (*b-e*).]

examined. The subtype(s) of the NAALADase-positive/GFAP-negative cells remain to be determined.

DISCUSSION

Molecular Characterization of a Rat Brain NAALADase mRNA and Its Expression in the Central Nervous System. In this report, we describe the cloning of a set of rat brain cDNAs that represent a full-length NAALADase mRNA. The rat brain clone shows a high degree of sequence similarity to the human cDNA throughout the length of its ORF, and the two predicted proteins also share many structural features. Three NAALADase-like RNA species are observed in Northern blots only in rat tissues that also express NAALADase activity. These mRNAs require further characterization to determine their individual relationships to activity.

To understand the mechanisms by which NAAG and NAALADase may cooperate in intracellular signaling, their distinct spatial relationships must be determined. The localization of NAALADase immunoreactivity to synapse-dense neuropil (8) and the enrichment of its activity in the synaptosomal fraction (21) previously had suggested a neuronal localization. As shown in Fig. 5, *in situ* hybridization experiments now have demonstrated that at least 90% of NAALADase-expressing cells in brain are astrocytes. This localization is consistent with previous reports of immunohistochemical localization of NAALADase in nonmyelinating Schwann cells in the peripheral nervous system (9), increases of NAALADase activity after decortication or kainate lesions to the

striatum (21), and the presence of NAAG-hydrolytic activity in murine astrocyte cultures (ref. 22 and R.L.-C., L. Passani, and J.T.C., unpublished data). Astrocytes are known participants in glutamatergic signaling (28–35). Because astrocytic endfeet wrap around neuronal synaptic contacts in close proximity to the synaptic cleft (36), astrocytic NAALADase is in the position to regulate perisynaptic NAAG levels.

Although our present data demonstrate a prominent astrocytic component of NAALADase expression, neurons also may express the peptidase, but perhaps at lower levels or in subpopulations that have not yet been identified. There is suggestive evidence for neuronal expression of the enzyme, because NAALADase activity has been found in purified neuronal populations in culture (22).

NAAG, NAALADase, and Glutamate in Excitatory Signaling. There is considerable evidence that NAAG serves a role in neuronal communication and interacts with glutamatergic systems. NAAG is present in brain in high concentrations (26), is localized primarily to neurons (37, 38), is present in synaptic vesicles (39), and is released from neurons by a calcium-dependent mechanism on depolarization (40–42). Intact NAAG inhibits glutamate's activation of NMDA receptors (2). Consistent with this finding, NAAG has been shown to inhibit the development of long-term potentiation in the rat hippocampus (5), to diminish NMDA-receptor-dependent survival of cultured neurons (43), and to inhibit NMDA-receptor-mediated norepinephrine release from hippocampal slices (3). NAAG is also an agonist at metabotropic mGluR3 receptors (6). Activation of class II inhibitory metabotropic receptors (mGluR2/3) attenuates the neurotoxic consequences of NMDA receptor overstimulation *in vivo* (44) and *in vitro* (45). Thus, NAAG's concerted actions negatively modulate NMDA receptor function both directly and indirectly through mGluR3.

In vitro, NAALADase demonstrates glutamate carboxypeptidase activity against two classes of substrates, alpha-linked acidic peptides such as NAAG, α -aspartylglutamate, and α -glutamylglutamate (1, 20) and gamma-linked peptides such as γ -glutamylglutamate and folyl-poly- γ -glutamates (20, 46). Of NAALADase's known substrates, only NAAG, γ -glutamylglutamate, and folates have been detected in neural tissue, and the apparent affinity of γ -glutamylglutamate for NAALADase and concentrations of folates in the extracellular space of the central nervous system are considerably lower than those of NAAG (1, 20, 47, 48). NAALADase's regional and developmental expression in brain correlate with levels of NAAG, suggesting that the two are functionally related (8, 21, 49). Moreover, the catabolism of NAAG *in vivo* in the rat striatum is pharmacologically consistent with hydrolysis by NAALADase (50, 51). Thus, NAAG appears to be the most likely substrate for the enzyme in the central nervous system.

The demonstration that NAALADase-like mRNAs are expressed predominantly by astrocytes may lend insight into NAAG's roles in modulating glutamatergic neurotransmission. Depolarization of presynaptic terminals containing NAAG results in its release into the synaptic cleft. Because our *in situ* hybridization data argue against the expression of NAALADase by most neurons, NAAG is likely to remain intact in the cleft where it inhibits the action of glutamate at NMDA receptors. As neuropeptides are preferentially released during conditions of high presynaptic activity (52), this inhibitory action of NAAG would come into play under conditions in which the risk for NMDA-receptor-mediated excitotoxicity would be greatest. Because NAAG does not inhibit α -amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors, fast excitatory neurotransmission would not be impeded. In addition to inhibiting synaptic NMDA receptors, NAAG likely diffuses out of the synapse, given the low velocity of NAAG transport (53). Under these circumstances, NAAG might activate extrasynaptic glu-

tamate receptors either intact or after conversion to glutamate by astrocytic NAALADase.

NAALADase activity has been demonstrated to be altered in animal models of epilepsy (54), amyotrophic lateral sclerosis (55), and schizophrenia (56), consistent with an important role in regulating glutamate receptor activity in pathologic conditions. The characterization of a brain NAALADase mRNA and the initial elucidation of its cellular expression greatly facilitate further research on the role of NAAG and NAALADase in normal synaptic transmission and in disorders of the nervous system.

Note Added in Proof. After submission of this article, an independent report of the cloning of rat brain NAALADase appeared (57).

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