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

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

RUTH LUTHI-CARTER asterisk plus plus superscript, URSA V. BERGER superscript, AMY K. BARCZAK superscript, MATTHEW ENNA asterisk, and JOSEPH T. COYLE asterisk plus plus superscript

*Department of Psychiatry, Massachusetts General Hospital-East, Charlestown, MA 02129; †The Consolidated Department of Psychiatry, Harvard Medical School, Boston, MA 02115; ‡Graduate Program in Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21205; and §Renal Division, Brigham and Women’s Hospital, Boston, MA 02115

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ABSTRACT N-acetylated alpha-linked acidic dipeptidase (NAALADase) hydrolyzes acidic peptides, such as the abundant neuropeptide N-acetyl-alpha-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-alpha-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 agonist at the mGluR3 subtype of metabotropic receptor, 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

Addition of recombinant RNasin (Promega, Fisher Scientific) or Amplitaq (Perkin–Elmer) polymerase according to the supplier’s recommendations by using a GenAmp 480 thermal cycler (Perkin–Elmer). Thermal cycling parameters varied with the manufacturer’s recommended conditions with the addition of 3.33 mM dimethyl sulfoxide in the RNA denaturation step and 40 units/25 µl 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) followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension step of 72°C for 10 min.

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

To whom reprint requests should be addressed at: McLean Hospital, 115 Mill Street, Belmont, MA 02178. e-mail: jcoyle@warren.med.harvard.edu.

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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, GAAGTCTGAGACATCAAGA and 3′ primer, TACGGTGAAGCCCGACG; primer pair II (R19): 5′ primer, GCAGTACGGCGCATGAAAC and 3′ primer, TAGGACACAGGACCATATA; primer pair III (R70): 5′ primer GCAGTAGAAGACGTTGACAG and 3′ primer, TACGGTGAAGCCCGACG.

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), ATAGTTAACCACCTGAGTCTG and B (PCR primer) TAGGAACACAGGACCATATA, 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 Colon/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 × 10^6 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 (Amer sham) 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 PEPIDERSTRUCTURE 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), pDNA5CAT (negative) and PSMA2 (positive) plasmids were transfected with 1 μg of plasmid DNA. Transfections were performed in parallel with control transfections, 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 rat or cell lines was prepared by using 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 × 10^6 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 Schraen-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 using an 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
end of the W6 sequence (position 921 of the mRNA); the
together using the endogenous
cDNA was constructed by splicing the R70 and W6 clones
full coding region (at the 3 end). Thus, the dissimilarity in the 5' untranslated sequence falls dramatically, except for a few conserved motifs flanking the start codon. These sequences

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 XhoI 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 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
The cDNA contains a 2,256-base ORF; its translation appears under the cDNA sequence. Numerical index for the cDNA sequence is in italics. Start and stop codons are indicated by bold lettering. The putative transmembrane domain is underlined. Glycosylation consensus sites are indicated by roman type; index for the amino acid sequences is in italics.

**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 a 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.
cells in registry. (c) In a coronal brain section (arrows indicate examples of dual-labeled mRNA to GFAP-positive astroglia in a field of rat hippocampal cells of the cerebellum. The colocalization of NAALADase-like mRNA visualized with cRNA probes appears to be widespread in 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 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. (e) 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. (c) Bright-field photomicrograph of an adjacent section of tissue to which a control sense cRNA of NAALADase was applied shows no labeling. [Bars = 50 μm (a) and 5 μm (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 mechanism 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 folyly-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-
timate 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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