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N-Acetylated-α-linked acidic dipeptidase (NAALADase), is a membrane-bound peptidase which hydrolyzes the endogenous neuropeptide N-acetyl-aspartyl-glutamate (NAAG) to N-acetyl-aspartate (NAA) and the excitatory amino acid, glutamate (GLU). Although there is evidence that NAAG might be a neurotransmitter, this dipeptide could also function as a precursor form of GLU, which is liberated by the dipeptidase. We found that the activity of this NAAG hydrolyzing enzyme in genetically epilepsy-prone rats was 11-26% greater than control in brain regions, including the amygdala, hippocampus and cerebellum, as well as the pyriform, entorhinal and frontal cortices. This is consistent with possible increased availability of GLU in certain CNS synapses in these rats, which are reported to have increased susceptibility to audiogenically, electrically and chemically induced convulsions.

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# Genetically epilepsy-prone rats have increased brain regional activity of an enzyme which liberates glutamate from *N*-acetyl-aspartyl-glutamate [1]

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*N*-Acetylated-α-linked acidic dipeptidase (NAALADase) is a membrane-bound peptidase which hydrolyzes the endogenous neuropeptide *N*-acetylaspartylglutamate (NAAG) to *N*-acetylaspartate (NAA) and the excitatory amino acid, glutamate (Glu). Although there is evidence that NAAG might be a neurotransmitter, this dipeptide could also function as a precursor form of Glu, which is liberated by the dipeptidase. We found that the activity of this NAAG hydrolyzing enzyme in genetically epilepsy-prone rats was 11–26% greater than control in brain regions, including the amygdala, hippocampus and cerebellum, as well as the pyriform, entorhinal and frontal cortices. This is consistent with possible increased availability of Glu in certain CNS synapses in these rats, which are reported to have increased susceptibility to audiogenically, electrically and chemically induced convulsions.

Injection of the dipeptide *N*-acetylaspartylglutamate (NAAG) into rat hippocampus causes prolonged seizure activity, similar to that seen following infusion of quisqualic acid, a glutamate agonist[23]. NAAG is a dipeptide found in the CNS in high concentrations[4,10,14]. NAAG is hydrolyzed to glutamate (Glu) and *N*-acetylaspartate (NAA) by *N*-acetylated-α-linked acidic dipeptidase (NAALADase), a chloride-dependent peptidase, enriched in synaptic membranes[17]. Despite evidence supporting the view of NAAG as a putative neurotransmitter, the identification, characterization and distribution of NAALADase suggest an alternative hypothesis: NAAG may function as a storage form of Glu, which could be liberated by this peptidase and released into the synaptic cleft[2,12,19,20]. We previously reported that non-kindled convulsions are as effective as kindled seizures in decreasing the activity of this enzyme in the amygdala, pyriform cortex, frontal cortex and entorhinal cortex[12]. This indicates that the decreases seen in the kindled group should be attributed to the aftereffects of convulsive activity rather than to the kindling process. Such decreases could possibly serve as a homeostatic mechanism, limiting CNS excitability by restricting the availability of Glu at synapses where NAAG might be the precursor. Extending this hypothesis, we asked whether the activity of this dipeptidase might be elevated in conditions associated with increased excitability, such as genetic epilepsy. In a pilot investigation of this question, we found elevated NAALADase activity in homogenates from brain regions of adult progeny inbred from severe-seizure genetically epilepsy-prone rats (GEPR-9s), compared to

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[1] Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NIH publication 86-23, 1985 edition. The views of the author(s) do not purport to reflect the position of the Department of the Army or the Department of Defense, (para 4-3, AR 360-5).

controls (unpublished data). The present experiment is a replication of the pilot study with two refinements: (1) to ensure positive phenotype in 100% of the GEPR-9s delivered, rats were screened by the supplier for tonic extensor convulsive responses to audiogenic challenge; (2) NAALADase assays were carried out in membrane preparations, which yield higher activity of this membrane-bound enzyme than do homogenates[2,19].

Adult male GEPR-9[5,15] and control[15] rats (12–18 weeks of age, 350–500 g, obtained from Dr. John Dailey, Department of Basic Sciences, University of Illinois College of Medicine, Peoria, IL 61656) were maintained in a sound-attenuating animal enclosure. The development, testing and screening of these colonies is detailed by Reigel et al.[15]. GEPR-9s were derived from Sprague–Dawley rats, inbred and screened to select for reliable tonic convulsive responses to audiogenic challenge. Ninety-five percent of audiogenically screened GEPR-9 progeny meet the criteria for susceptibility to severe audiogenic seizures[5,15], and only these rats are shipped as screened GEPR-9s. Control rats were derived from Sprague–Dawley rats, inbred and screened to select for reliable resistance to audiogenic challenge[15], and all animals shipped were seizure-free on three separate audiogenic challenges (Dr. John Dailey, personal communication). After shipment to the Walter Reed Army Institute of Research, the rats were individually housed for over 80 days in a temperature-controlled room with a 12 h/12 h light/dark cycle, and food and water were freely available.

Rats were killed by decapitation, alternating GEPR-9s and controls. The brains were removed and immediately dissected on ice. The dissection was derived from a method previously published[11]. The regional samples were weighed, rapidly frozen on dry ice and stored at −70°C until assayed. Samples were identified as to brain region, but treatment groups were coded, and samples were assayed without identification of treatment group.

To assay for NAALADase activity, tissue samples were homogenized (six strokes, 600 r.p.m.) in ice-cold 50 mM Tris-HCl buffer (pH 7.4 at 37°C) at a dilution of 1:100 (wet weight of tissue (g)/volume of buffer (ml)). An aliquot of the homogenate was sonicated for 5 s to disrupt cellular membranes and centrifuged for 20 min at 4°C at 35,000 *g*. The entire pellet was then resuspended in 50 mM Tris-HCl + 0.5% Triton X-100 by sonication. The rest of the radioenzymatic assay[17] was performed as modified by Slusher et al.[19]. Due to the ionic sensitivity of enzyme activity we substituted triple-distilled, deionized water (purchased from J.T. Baker, Inc., Phillipsburg, NJ), for double-distilled, deionized, tap water in all assay reagents. In brief, the assay measured the amount of [$^3$H]Glu liberated from [$^3$H]NAAG during a 15 min incubation at 37°C, in 50 mM Tris-HCl buffer, pH 7.4, with Triton X-100 in a final concentration of 0.05%. Substrate and product were separated (95% resolution) via anion-exchange liquid chromatography. Samples were assayed in triplicate. NAALADase activity was calculated based on picomoles of [$^3$H]Glu produced from [$^3$H]NAAG per mg protein per min. The data were decoded and analyzed by a one-way analysis of variance.

As shown in Table I, NAALADase activity was greater in GEPR-9s compared to controls in membrane preparations from each of the brain regions examined. Activity was higher in the pyriform (19%), entorhinal (24%) and frontal (26%) cortices, as well as in the hippocampus (18%), amygdala (17%) and cerebellum (11%). Several of these regions are involved in limbic seizures. It is unlikely that spontaneous seizures affected these results, as they rarely occur in the audiogenically seizure-sensitive GEPR-9[15]. Moreover, since convulsions decrease NAALADase activity[12], if they did occur, they would lead to an underestimation of the increase we find in GEPRs. GEPR-9s and controls in this study had been screened at the University of Illinois on three occasions between 45–65 days of age for the presence (GEPR-9) or absence (control) of

TABLE I

*NAALADase activity in GEPR-9 vs control rat brain membranes*

| *Region* | *Control (pmol per mg protein per min ± SEM)* | *GEPR-9* | *F* | *P* | *N* | *Increase (%)* |
|---|---|---|---|---|---|---|
| Amygdala | 25.6 ± 1.8 | 30.0 ± 1.4 | 6.8 | < 0.05 | 10 | 17 |
| Pyriform Cortex | 20.1 ± 1.3 | 23.8 ± 0.9 | 19.0 | < 0.002 | 10 | 19 |
| Entorhinal Cortex | 22.0 ± 0.9 | 27.3 ± 1.3 | 44.3 | < 0.001 | 10 | 24 |
| Frontal Cortex | 18.0 ± 1.1 | 22.7 ± 0.9 | 47.0 | < 0.001 | 10 | 26 |
| Hippocampus | 23.8 ± 1.0 | 28.0 ± 1.0 | 41.8 | < 0.001 | 10 | 18 |
| Cerebellum | 30.4 ± 1.1 | 33.8 ± 0.8 | 33.4 | < 0.001 | 10 | 11 |

tonic convulsive responses to audiogenic challenge[15]. But it is unlikely that the convulsive responses of GEPR-9s during screening would have affected the results, because almost 3 months elapsed between the audiogenic testing and the sacrifice of the animals. Furthermore, we statistically compared the percent differences from the present study with those seen in the pilot study done in regional brain homogenates from unscreened GEPR-9s (which never had convulsions) and controls. It may be inferred that only 5% of the unscreened GEPR progeny would have failed to meet the criteria for GEPR-9 phenotype, had they been tested[15]. In the pilot study, NAAG-hydrolyzing activity was significantly elevated in the pyriform (21%), entorhinal (27%) and frontal (19%), cortices, as well as in the hippocampus (29%), amygdala (8%) and cerebellum (15%). These percent elevations, in homogenates from unscreened GEPRs in the pilot study, are not distinguishable by ANOVA from the percent elevations seen in membrane preparations from screened GEPR-9s in the present study (Table I).

GEPR-9s have been found to be hypothyroid, with abnormally low serum levels of thyroxine up to day 22 and low levels of triiodothyronine throughout life[13]. Abnormalities of the auditory system have also been reported in GEPRs, but Reigel, et al.[16], point out that seizure susceptibility is associated with neural structures outside that system, as well. Moreover, GEPRs also have increased susceptibility to electrically and chemically induced seizures, and the latter susceptibility appears developmentally prior to audiogenic susceptibility[16]. Numerous studies of neurochemical abnormalities in the GEPR-9 have described deficiencies in central noradrenergic[6,22] and serotonergic[9] activity. In recent years, however, epilepsy research has increasingly focussed on the possible role of putative excitatory amino acid neurotransmitters such as Glu[8] and aspartate (ASP)[7]. NAAG has been proposed to be a neurotransmitter/neuromodulator at a subpopulation of Glu/ASP synapses. A significant number of studies, employing neurochemical, immunocytochemical, stimulation and lesioning techniques, as well as demonstrations of calcium-dependent, stimulated release of NAAG have supported this hypothesis (see review by Coyle et al.[3]). Of particular relevance to epilepsy is the description of NAAG-immunoreactive neurons in the entorhinal cortex[1], a region in which NAAG levels were increased by kindling[11].

NAALADase appears to be the primary mechanism for catabolism of NAAG in brain[19]. It has been shown to hydrolize NAAG into NAA and Glu both *in vitro*[17] and *in vivo*[2,21]. Activity is weakly inhibited by the reaction products Glu and NAA[17,19]. This peptidase has been solubilized from synaptosomal membranes and purified to apparent homogeneity[19]. The purified enzyme migrates as a single band on an SDS gel with an apparent MW of 94 kDa, and displays a remarkably high apparent affinity for its putative substrate, NAAG, with a $K_m$ of 140 nM[19]. The enzyme has been immunocytochemically localized in the CNS[20], where it is selectively enriched in many structures known to contain NAAG. Developmentally, the activity of this peptidase in the cerebral cortex of normal rats reaches 80% of maximum at 21 days of age[2], a developmental milestone which coincides with the period of synaptogenesis in normal rats, as well as the age at which seizure incidence in response to audiogenic challenge first reaches 100% in GEPR-9s[16]. Although some evidence suggests that NAAG might be a neurotransmitter, more recently it has been proposed that the function of NAALADase *in vivo* may be to liberate a neuroactive fragment, such as Glu from its dipeptide precursor[2,12,19]. Therefore, assuming access to substrate, the elevated activity of this enzyme seen in the GEPR-9 might increase availability of NAA and Glu in certain synapses of the brain. While the increase in Glu is expected to be excitatory, there is no indication that NAA would have any effect on neurotransmission[2].

Further experiments are required to fully assess the implications of the present study. We anticipate conducting studies to determine the effect of thyroidectomy on the ontogeny of NAAG hydrolyzing activity, as well as an expanded regional analysis of the activity of this peptidase in strains of seizure-prone and seizure-resistant rats and mice to determine whether abnormally high activity of this enzyme is always associated with genetically determined seizure susceptibility[18].

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