Aspartoacylase deficiency does not affect N-acetylaspartylglutamate level or glutamate carboxypeptidase II activity in the knockout mouse brain

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Abstract

Aspartoacylase (ASPA)-deficient patients [Canavan disease (CD)] reportedly have increased urinary excretion of N-acetylaspartylglutamate (NAAG), a neuropeptide abundant in the brain. Whether elevated excretion of urinary NAAG is due to ASPA deficiency, resulting in an abnormal level of brain NAAG, is examined using ASPA-deficient mouse brain. The level of NAAG in the knockout mouse brain was similar to that in the wild type. The NAAG hydrolyzing enzyme, glutamate carboxypeptidase II (GCP II), activity was normal in the knockout mouse brain. These data suggest that ASPA deficiency does not affect the NAAG or GCP II level in the knockout mouse brain, if documented also in patients with CD.

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Canavan disease (CD) is an autosomal recessive disorder caused by aspartoacylase (ASPA) deficiency [16]. This enzyme hydrolyzes N-acetylaspartic acid (NAA) to acetate and aspartate [7]. While ASPA is localized in oligodendrocytes [4], the substrate, NAA, is restricted to neurons in the gray matter of the brain [14,26]. Deficiency of the enzyme results in the accumulation of NAA in the brain and elevated NAA excretion in the urine of patients with CD [16,17]. Abnormalities in the brain of patients with CD include spongy degeneration with swollen astrocytes and elongated mitochondria [1–3,10,13]. Patients with CD show mental retardation, macrocephaly, hypotonia and head lag [29].

The knockout mouse for CD shows neurological impairment and neuropathology similar to what has been observed in patients with CD [18]. The knockout mouse brain has vacuolation throughout the white matter in the deep cortex and the white matter bundles in the corpus striatum [18]. The hippocampus has vacuolation in the pyramidal area and sparing of the dentate bundles. The Purkinje cell layer, granular layer and white matter of the cerebellum are also affected in the knockout mouse [18].

Deficiency of ASPA results in the accumulation of NAA in the brain of knockout mice as observed in patients with CD [18]. The levels of glutamate and glutamate-GABA pathway-associated enzymes aspartate aminotransferase, glutamate dehydrogenase and α-ketoglutarate dehydrogenase [21–23,25] are lower in the knockout mouse brain. In neurons, NAA and glutamate are ligated into N-acetylaspartylglutamate (NAAG) in the presence of N-acetylaspartate-1-glutamate ligase [28]. The acidic peptide NAAG is most prevalent in the brain, with a high affinity for metabotropic glutamate receptors, mGluR3 [12,15]. NAAG is cleaved into NAA and glutamate by glutamate carboxypep-
tidase II (GCPII)/N-acetylated-α-linked acidic dipeptidase (NAALADase) \[6,11,20\]. While GCP II is located in astrocytes, NAAG is restricted to neurons\[15,19\]. Patients with CD showed elevated excretion of urinary NAAG \[8,9\]. Because ASPA deficiency is the basic cause of CD \[16\], it is unclear whether the enzyme defect results in the accumulation or the depletion of brain NAAG to cause elevated excretion of urinary NAAG. To investigate the possibility, the NAAG level was estimated in the brain of ASPA-deficient mouse. In addition, the NAAG hydrolyzing enzyme, GCPII, activity was also measured.

The brain content of NAAG was determined using nuclear magnetic resonance (NMR) spectral analysis as followed earlier\[21,25\]. All animal procedures were approved by the Institution’s Animal Care and Use Committee. Brain samples from wild type and knockout mice were frozen in liquid nitrogen, crushed and then were placed in a 12% solution of perchloric acid for overnight extraction at 4 °C. Following perchloric extraction, samples were centrifuged at 10,000 g for 10 min at 4 °C. Supernatants were neutralized, lyophilized and redissolved in D2O. High resolution, 400 or 750 MHz proton NMR spectra were run on the supernatants. Measurements were performed on Varian Unity-plus spectrometers using water-suppressed proton NMR spectroscopy. The NMR parameters for the single pulse experiments were: TR = 10 s; acquisition time = 3 s; saturation delay = 2 s; and signal averages = 128. The residual water peak was set to 4.70 ppm. The peak integral for the NAAG methyl protons (singlet, 2.04 ppm) was compared to the creatine methyl (singlet, 3.04 ppm) to calculate relative metabolite concentrations.

To perform the GCP II assay, wild type and knockout mice were sacrificed, the brain was removed and brain parts (cerebrum, hypothalamus, cerebellum and brainstem) were separated as followed earlier \[24,25\]. The brain was sonicated in ice-cold 50 mM Tris–HCl buffer (pH 7.4) and centrifuged at 30,000 × g for 30 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 50 mM Tris–HCl containing 0.5% Triton X100. After centrifugation at 30,000 × g for 30 min, the supernatant containing membrane was collected and the protein in the supernatant was assayed using a BCA protein assay reagent (Pierce, Illinois). Glutamate carboxypeptidase II activity was determined by analyzing the hydrolysis of [3H]NAAG radio-labelled at the glutamate residue.

Glutamate carboxypeptidase II activity was measured as described earlier \[5,19\]. The reaction mixture for the assay contained 50 mM Tris–HCl (pH 7.4), 0.05% Triton X100, 0.3 μCi N-acetyl-L-aspartyl-L-[3,4-3H]glutamate (Perkin Elmer, MA), 119.17 nM non-radiolabelled NAAG (Sigma, Michigan), 1 mM cobalt chloride (Sigma, MI) and 25 μg membrane protein in a total volume of 250 μl. Assay reactions were incubated at 37 °C water bath and were quenched after 60 min with 250 μl of ice-cold sodium phosphate buffer (0.1 M, pH 7.4). The assay mixture was then applied to a distilled water-prewashed mini column of AG 1-X8 anion-exchange resin [200–400 mesh, formate form (Bio-Rad Laboratories, California)], and glutamate was selectively eluted with 1 ml of scintillation flour (Sigma). Radioactivity was determined by LS 6000 IC scintillation spectrometry (Beckman, California).

Glutamate carboxypeptidase II activity is expressed as nmol of formed glutamate/mg protein/h, and the calculations were performed as followed earlier \[5\]. Data were analyzed using ANOVA.

To examine whether the ASPA gene mutation affects the NAAG content of the brain, the level of NAAG was measured. NMR spectral integral analysis indicated that NAAG was not affected in the knockout mouse brain compared to the wild type (Fig. 1). The ratio of NAAG/Cr. in the knockout mouse brain was 0.032 ± 0.003 (n = 5 ± S.E.) compared to that in the wild type, 0.028 ± 0.004 (n = 4 ± S.E.).

![Fig. 1. A representative 400 MHz proton NMR spectrum from a knockout mouse brain showing the spectral region where the NAA and NAAG methyl protons resonate. The NAAG methyl peak is resolved from the NAA methyl.](image)

![Fig. 2. Measurement of glutamate carboxypeptidase II activity in the brain of knockout mouse for CD. Glutamate carboxypeptidase II activity in the whole brain, cerebrum, hypothalamus, cerebellum and brainstem of knockout mouse did not have significant difference compared to that in the wild type. (n = 10 ± S.E.).](image)
The hydrolyzing enzyme, GCP II, activity was normal in the whole brain of the knockout mouse as observed in the wild type (Fig. 2). The activity of GCP II in different parts of the brain (cerebrum, hypothalamus, cerebellum and brainstem) of the knockout mouse was similar to that observed in the wild type (Fig. 2).

ASPA deficiency is the basic cause of CD [16]. Elevated excretion of urinary NAAG was reported in ASPA-deficient patients [8,9]. Because the neuropeptide NAAG is abundant in the brain [15,19], whether ASPA deficiency affects brain NAAG to result in an increased excretion of urinary NAAG is not known. Brain NAAG may contribute to lead elevated urinary NAAG by two possible ways. Either depletion of NAAG by nerve cell rupture or accumulation of NAAG in the brain due to a defect in the hydrolysis of the enzyme, GCP II. Subsequently, the resulting NAAG will be removed through urine as waste. Therefore, brain NAAG was determined in the mouse model for CD using NMR spectroscopy.

The amount of NAAG in the knockout mouse brain was found to be similar to that in the wild type. This data suggests that ASPA deficiency does not affect the normal level of NAAG in the mouse brain. The amount of NAAG in the brain can also be interpreted by measuring NAAG hydrolyzing enzyme, GCP II, activity [11,20,27]. Elevated or reduced levels of NAAG in the brain affect the normal activity of GCP II [11,20,27]. Therefore, GCP II activity is also to be studied in the mouse brain. The normal activity of GCP II in the ASPA-deficient mouse brain observed in the present study suggests that ASPA deficiency does not affect the hydrolyzing enzyme activity and this is likely due to the normal level of brain NAAG seen in the mouse.

These observations in the knockout mouse brain suggest that ASPA deficiency does not affect the level of brain NAAG or the hydrolyzing enzyme, GCP II. Presumably, brain NAAG is not likely involved to result in an increased excretion of urinary NAAG in the knockout mouse, if documented also in patients with CD.

References


