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Aspartoacylase gene knockout results in severe vacuolation in the white matter and gray matter of the spinal cord in the mouse

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Canavan disease (CD) is a neurodegenerative disorder characterized by the spongy degeneration of the white matter of the brain. Aspartoacylase (ASPA) gene mutation resulting enzyme deficiency is the basic cause of CD. Whether the ASPA defect in CD affects the spinal cord has been investigated using the ASPA gene knockout mouse. Luxol fast blue-hematoxylin and eosin staining in the spinal cord of the knockout mouse showed vacuolation in both white matter and gray matter areas of cervical, thoracic, lumbar, and sacral segments of the spinal cord. However, more vacuoles were seen in the gray matter than the white matter of the spinal cord. ASPA activity in the cervical, thoracic, lumbar, and sacrococcygeal regions of the spinal cord was significantly lower in the knockout mouse compared to the wild type. The enzyme defect in the knockout mouse was also confirmed using the Western blot method. These observations suggest that the ASPA gene defect in the mouse leads to spinal cord pathology, and that these changes may be partly involved in the cause of the physiological/behavioral abnormalities seen in the knockout mouse, if documented also in patients with CD.

Keywords: Canavan disease; Spinal cord vacuolation; Aspartoacylase; Knockout mouse; Gray matter; White matter; Spongiform degeneration

Introduction

Canavan disease (CD) is an autosomal recessive leukodystrophy, leading to spongy degeneration of the brain (Adachi et al., 1972; Adornato et al., 1972; Canavan, 1931; Globus and Strauss, 1928). The clinical features of the disease include developmental delay, megalencephaly, hypotonia, and early death (Von Bogaert and Bertrand, 1949).

Canavan disease is caused by aspartoacylase (ASPA) gene mutation (Kaul et al., 1993) resulting enzyme deficiency (Matalon et al., 1988). The enzyme hydrolyzes NAA to aspartate and acetate (Birnbaum, 1955; Birnbaum et al., 1952; Tallen et al., 1956). Deficiency of the aspartoacylase leads to accumulation of N-acetylaspartic acid (NAA) in the brain and an increase of NAA in the urine of patients with Canavan disease (Grodd et al., 1990; Matalon et al., 1989). Children with CD do not develop the skills for sitting, standing, walking, or talking. Hypotonia is replaced by spasticity later in life. How a single gene mutation resulting in enzyme deficiency causes all these abnormalities seen in CD is under investigation.

ASPA gene knockout in the mouse showed symptoms similar to patients with CD (Matalon et al., 2000). The deficiency of ASPA activity and elevated excretion of NAA were also observed in the mouse model (Matalon et al., 2000). The brain of the mouse with CD demonstrates spongiform degeneration in the white matter of the brain (Matalon et al., 2000), a feature also typical of patients with CD (Adachi et al., 1972; Canavan, 1931; Globus and Strauss, 1928). Therefore, this mouse model is useful to understand the events involved in their human counterpart, patients with CD.

Although ASPA gene knockout resulting brain pathology has been previously studied (Matalon et al., 2000; Surendran and Matalon, 2004; Surendran et al., 2003a,b, 2004a), involvement of spinal cord in CD has not been previously investigated. Therefore, the present study specifically targets changes in the spinal cord of the knockout mouse.

Abbreviations: BCD, Basis cornus dorsalis; CACD, Caput cornus dorsalis; CC, Central canalis; CD, Cornu dorsalis; CCD, Cervix cornus dorsalis; CL, cornu laterale; CV, Cornu ventrale; FD, Funiculus dorsalis; FL, Funiculus lateralis; FV, Funiculus ventralis; ND, Nuc. Dorsalis (Clarke); NCD, Nuc. Cornucommissuralis dorsalis; NCV, Nuc. Cornucommisuralis ventralis; NDL, Nuc. Dorsolateralis; NDM, Nuc. Dorsomedialis; NML, Nuc. Intermediolateralis; NPD, Nuc. Proprius cornus dorsalis; NPM, Nuc. Posteromarginalis; NVL, Nuc. Ventrolateralis; NVM, Nuc. Ventromedialis; NR, Nuc. Reticularisv; SG, Substantia gelatinosa.

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Materials and methods

Histochemical processing of the spinal cord with luxol fast blue-hematoxylin and eosin

All animal procedures were approved by the Institution's Animal Care and Use Committee. Nine each of wild type and knockout mice were perfused in 10% neutral phosphate-buffered formalin. The spinal cord was dissected out by spinal laminectomy and different regions of the spinal cord (cervical, thoracic, lumbar, and sacral) were separated and stored in 10% buffered formalin for histological processing. Spinal cord regions were dehydrated through graded alcohol, cleared with xylene and embedded in paraffin. Transverse sections were cut at 7 μm thickness, deparaffinized and rehydrated. Sections were stained with preheated luxol fast blue (LFB) at 80°C for 30 min. Slides were rinsed in 95% alcohol, washed in water, and differentiated in 0.05% lithium carbonate followed by in 70% alcohol. These sections were rinsed in deionized water and counterstained with hematoxylin and eosin. These sections were counterstained with hematoxylin and eosin (H&E), dehydrated through 95% and 100% alcohol series, cleared in xylene and coverslipped with synthetic resin. The result of the staining showed myelin in blue color, Nissl substance in violet color and nuclei in violet color (Carson, 1997; Luna, 1968). Different areas in the cervical, thoracic, and lumbar segments of the spinal cord were photographed by Olympus microscope to show the distribution of vacuolation. Spinal cord figure was labeled as shown in the atlas of the mouse brain and spinal cord (Sidman et al., 1971).

Aspartoacylase activity assay

Aspartoacylase assay was performed as followed earlier (Surendran et al., 2003a). Six each of the wild type and homozygous knockout mice were sacrificed and the spinal cords were removed. Briefly, the spinal cord was separated into cervical, thoracic, lumbar, and sacral-coccygeal regions, then homogenized and the homogenate was used as a source of enzyme. The assay was carried out in a total volume of 600 μl with 50 mM Tris–HCl (pH 8.0), 0.5% (w/v) NP-40, 50 mM sodium chloride, 1 mM calcium chloride, 2.8mM N-acetylaspartic acid and the enzyme at 370C for 3 h. The reaction was coupled with malic dehydrogenase, glutamic oxalacetic transaminase and β-nicotinamide adenine dinucleotide reduced form and the amount of L-aspartate released in the aspartoacylase reaction was quantified by spectrophotometry at 340 nm. One milliunit of aspartoacylase activity is equivalent to 1 nano mole of aspartate released in 1 min. Values were calculated using analysis of variance. The value $P < 0.05$ was considered significant.

Western blotting detection of ASPA protein

Five each of the wild type and knockout mice were sacrificed and spinal cords from both groups of animals were pooled separately. Western blotting was performed as followed earlier (Surendran et al., 2001). Protein from wild type and CD spinal cord in equal quantities was loaded into a 12% Tris–HCL gel well (Bio-Rad Laboratories, Hercules, CA). After electrophoresis, protein was transferred to a nitrocellulose membrane (Bio-Rad). The membrane was incubated with ASPA polyclonal antibody. After a wash, incubation was done with anti-rabbit IgG-HRP antibody (Amersham Pharmacia Biotech, New Jersey). The incubated membrane was washed with PBS and stained with Supersignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, Illinois). The stained membrane was exposed on a Kodak XOmat film (Kodak, USA) and developed in a Kodak RP X-OMAT processor.

Results

The histology of the spinal cord in the homozygous knockout mouse showed severe vacuolation in the white matter and gray matter present throughout all levels of the spinal cord. Transverse sections obtained from the C5, T1, and L6 areas of the spinal cord are photographed and shown in Fig. 1. Vacuoles are more numerous in the gray matter than the white matter of the spinal cord. Since pathology in the right and left sides of the spinal cord was identical in the knockout mouse, half part of the spinal cord is shown in the figure.

Several ascending pathways are projected and congregated in the posterior funiculus, anterolateral system, and lateral margin of lateral funiculus. The anterolateral system includes lateral and anterior funiculi (Clark, 1984; Martin, 1989). In the cervical spinal cord of the homozygous knockout mouse, the white matter and gray matter were severely affected (Figs. 1A–B) compared to no change in the wild type (Figs. 1 C–D). Vacuolation was observed in the funiculus dorsalis (FD), funiculus ventralis (FV) and funiculus lateralis (FL) in the cervical region of the knockout mouse (Figs. 1A–B), while no vacuoles were seen in the same regions in the wild type (Figs. 1C–D). Extensive vacuolation was also observed in the dorsal horn, intermediate zone and ventral horn areas, nucleus posteromarginalis (NPM), substantia gelatinosa (SG), nucleus cornucommissuralis dorsalis (NCD), nucleus proprius cornus dorsalis (NPD), nucleus cornucommisuralis ventralis (NCV), nucleus reticularis (NR), nucleus dorsomedialis (NDM), and nucleus ventromedialis (NVM) in the knockout mouse (Figs. 1A–B) compared to the wild type (Figs. 1C–D). The ventral horn area is the descending place of lateral corticospinal tract, rubrospinal tract, vestibulospinal tract, and medial reticulospinal tract. Reticular area is the termination place for a large number of the anterolateral system axons (Clark, 1984; Martin, 1989).

The thoracic region of the homozygous knockout mouse showed vacuolation throughout white matter and gray matter (Figs. 1E–F), as observed in the cervical region of the spinal cord. White matter was affected by vacuolation in the FD, FV and FL areas in the knockout mouse (Figs. 1E–F), while the same regions in the wild type spinal cord showed no vacuoles (Figs. 1G–H). Similar changes were also observed throughout gray matter areas, including nucleus intermediolateralis (NML), cornu ventrale (CV), nucleus dorsolateralis (NDL), NVM, SG, NPM, cornu dorsale (CD), NPD, nucleus dorsalis (Clarke; ND), and cornu lateralis (CL) of the knockout mouse spinal cord (Figs. 1E–F), while no vacuoles were seen in the wild type (Figs. 1G–H).

Extensive vacuolation was observed throughout the lumbar region of the spinal cord. The gray matter areas of the spinal cord, including caput cornus dorsalis (CACD), cervix cornus dorsalis (CCD), basis cornus dorsalis (BCD), NVM and ND were affected in the knockout mouse (Figs. 1I–J). Clarke's nucleus (ND) is the arising place of dorsal spinocerebellar tract. ND goes to the dorsal and lateral parts of the funiculus (Clark, 1984; Martin, 1989). The white matter regions of the spinal cord, FL, FV, and FD in the