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Transport of *N*-acetylaspartate via murine sodium/dicarboxylate cotransporter NaDC3 and expression of this transporter and aspartoacylase II in ocular tissues in mouse

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Abstract

Canavan disease is a genetic disorder associated with optic neuropathy and the metabolism of *N*-acetylaspartate is defective in this disorder due to mutations in the gene coding for the enzyme aspartoacylase II. Here we show that the plasma membrane transporter NaDC3, a Na^+-coupled transporter for dicarboxylates, is able to transport *N*-acetylaspartate, suggesting that the transporter may function in concert with aspartoacylase II in the metabolism of *N*-acetylaspartate. Since Canavan disease is associated with ocular complications, we investigated the expression pattern of NaDC3 and aspartoacylase II in ocular tissues in mouse by in situ hybridization. These studies show that NaDC3 mRNA is expressed in the optic nerve, most layers of the retina, retinal pigment epithelium, ciliary body, iris, and lens. Aspartoacylase II mRNA is coexpressed in most of these cell types. We conclude that transport of *N*-acetylaspartate into ocular tissues via NaDC3 and its subsequent hydrolysis by aspartoacylase II play an essential role in the maintenance of visual function.

Keywords: *N*-Acetylaspartate; Sodium/dicarboxylate transporter; NaDC3; Aspartoacyalse II; Retina; RPE; Canavan disease; Optic neuropathy

1. Introduction

N-Acetylaspartate (NAA) levels in the brain have been shown to provide a sensitive measure of neuronal survival [1,2]. As the second most abundant free amino acid in the brain, NAA plays an essential role in many cellular processes [1–3]. NAA functions in the protection of cells against osmotic stress, is implicated in cell-specific signaling during development and synthesis of the neuromodulator *N*-acetylaspartylglutamate (NAAG), and is considered as an intracellular source of acetyl groups essential for the synthesis of lipids [4–7]. This compound is found predominantly in neurons of the mature brain and is continually released from the neurons during excitation. The concentration of NAA in neurons has been found to be as high as 20 mM, but this compound is barely detectable in glia [4,5,8,9]. Consistent with the findings that NAA is present at very low levels in glia, the enzyme L-aspartate-*N*-acetyl transferase responsible for the synthesis of NAA is found only in neurons whereas the enzyme responsible for the breakdown of NAA, namely aspartoacylase II, is found predominantly in glia [10,11]. Since aspartoacylase II is a cytosolic enzyme, NAA must enter into glial cells for hydrolysis by the enzyme. Hydrolysis of NAA by aspartoacylase II releases the acetyl group that is used in the synthesis of lipids associated with myelination. The functional significance of this process is evident from the clinical consequences of Canavan disease, a debilitating and fatal genetic disorder caused by aspartoacylase II deficiency [12,13].

The uptake of NAA from the extracellular space by glia has been described by Sager et al. [14]. Studies from our laboratory have recently shown that the Na^+-coupled high-affinity dicarboxylate transporter NaDC3 is responsible for this uptake process [15]. These studies have also demonstrated the expression of NaDC3 in the nervous system by Northern blot analysis, in situ hybridization, and RT-PCR [15–17]. NaDC3 is expressed in the meningeal layers of

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supporting tissue that surround the brain, and also in the cortex where expression was detectable within the astrocytes scattered among the neuronal cells [16]. The expression pattern of NaDC3 in the brain is similar to that of aspartoacylase II [1–3]. This agrees with the suggested role of NaDC3 in delivering extracellular NAA to intracellular sites in glia for subsequent hydrolysis by aspartoacylase II [15]. Since transport by NaDC3 is as obligatory as the function of aspartoacylase II in the hydrolysis of NAA within the glial cells, we hypothesize that genetic defects in NaDC3 will have the similar clinical consequences as aspartoacylase II deficiency.

Patients suffering from Canavan disease often present with optic neuropathy [12,13]. NAA is found at high levels in neural retina [1–3]. Apparently, disruption of NAA metabolism due to aspartoacylase II deficiency interferes with retinal function. However, the expression pattern of this enzyme in ocular tissues has not been investigated. Since NaDC3 is essential for the entry of NAA into cells prior to hydrolysis by aspartoacylase II, we hypothesized that the transporter might also be expressed in ocular tissues where aspartoacylase II is expressed. In the present paper, we tested this hypothesis by examining the ability of murine NaDC3 to transport NAA and investigating the expression pattern of NaDC3 and aspartoacylase II in murine ocular tissues.

2. Materials and methods

2.1. Materials

[2,3-^{3}H]-Succinic acid (specific radioactivity, 37.5 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA). Cell culture supplies, Trizol reagent, and Lipofectin were purchased from Life Technologies (Gaithersburg, MD, USA). Restriction enzymes were from New England Biolabs (Beverly, MA, USA). *N*-Acetylaspartate was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cloning of mouse NaDC3

A mouse kidney cDNA library [18] was screened using a rat NaDC3 cDNA fragment as the probe. The fragment was generated from the parent rat NaDC3 cDNA [16] by digestion with *Eco*RI and *Bam*HI and labeled with [α-^{32}P]-dCTP. Positive clones were identified on the primary screening and the colonies were purified by secondary/tertiary screening. Both sense and antisense strands of the cDNA were sequenced using an automated Perkin-Elmer Applied Biosystems (Farmingham, MA) 377 Prism DNA sequencer.

2.3. Heterologous functional expression of mouse NaDC3 in mammalian cells

The function of the cloned mouse NaDC3 was characterized in HRPE cells (a human retinal pigment epithelial cell line) following heterologous expression of the cDNA using the vaccinia virus expression technique [16,17]. The transport function of the heterologously expressed mouse NaDC3 was measured as the uptake of [^{3}H]-succinate in the presence of Na^{+}. Uptake measurements were made at room temperature with an incubation period of 2 min. The composition of the buffer used in uptake measurements was 25 mM HEPES/Tris (pH 7.5), containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. Under no experimental conditions employed in these studies, the uptake of [^{3}H]succinate exceeded 5% of [^{3}H]succinate added to the extracellular medium at the start of the uptake measurement. Therefore, the concentration of radiolabel in the medium was never a limiting factor for uptake in any of the experiments described in this study. The experiments were repeated two or three times with independent transfections, each done in duplicate or triplicate. Data are presented as means ± S.E. of these replicate measurements.

2.4. RT-PCR and restriction analysis

Total RNA samples isolated from mouse eyes and kidney (positive control) were used for RT-PCR. The mouse NaDC3-specific primers used in the analysis were 5′-AGGGCTGTGAGGAGTCTGGGCTATC-3′ (sense) and 5′-GCCTGCTGCGAACAACTGTAACATC-3′ (antisense). These primers correspond to the nucleotide positions 1343–1367 and 2267–2291 in the full-length mouse NaDC3 cDNA isolated from the mouse kidney cDNA library. The expected size of the RT-PCR product was 949 bp. The resulting product was gene-cleaned and used for restriction analysis. Two enzymes were used in restriction analysis: *Hin*dIII and *Pst*I. The expected sizes of the restriction fragments from the RT-PCR product by digestion with these two enzymes are 734 and 215 bp for *Hin*dIII and 660 and 289 bp for *Pst*I.

2.5. In situ hybridization

Enucleated eyes from albino mice were frozen in Tissue-Tek OCT (Miles Laboratories, Elkhart, IN, USA), sectioned at 10-μm thickness, and fixed in 4% paraformaldehyde. Treatment of the sections for in situ hybridization was done according to the method as described previously [19,20]. Hybridization signals were detected by using digoxigenin-labeled probes and the anti-DIG-AP (the alkaline phosphatase-coupled anti-digoxigenin antibody) (DIG nucleic acid detection kit, Boehringer-Mannheim, Indianapolis, IN, USA). The color reaction was developed in NBT/BCIP (provided in the detection kit).

For the preparation of probes specific for NaDC3, a *Kpn*I/*Hin*dIII digestion fragment (~ 0.85 kbp) of the mouse NaDC3 cDNA was subcloned into pSPORT vector. For the preparation of the sense riboprobe, used as a negative control, the subcloned plasmid was linearized with *Kpn*I and the cDNA insert was transcribed using SP6 RNA

polymerase. For the preparation of the antisense riboprobe, the subcloned plasmid was linearized with *Hin*dIII and the cDNA insert was transcribed using T7 RNA polymerase. The riboprobes were labeled with digoxigenin using the digoxigenin-lebeling kit (Boehringer-Mannheim). For the preparation of probes specific for aspartoacylase II, a RT-PCR product specific for mouse aspartoacylase II was generated using a primer pair designed on the basis of the reported sequence of the mouse aspartoacylase II [21] and poly(A)$^+$mRNA isolated from mouse eyes. The primers were 5′-CATGGAAATGAACTGACCGGAGTGT-3′ (sense) and 5′-AACAGGATACTTGGCAATGGAACGA-3′ (antisense). The expected size of the RT-PCR product was 465 bp. The product was subcloned into pGEM-T vector and sequenced to establish its identity and orientation. For the preparation of the sense riboprobe, used as a negative control, the subcloned plasmid was linearized with *Apa*I and the cDNA insert was transcribed using SP6 RNA polymerase. For the preparation of the antisense riboprobe, the subcloned plasmid was linearized with *Sal*I and the cDNA insert was transcribed using T7 RNA polymerase. The riboprobes were labeled with digoxigenin using the digoxigenin-labeling kit (Boehringer-Mannheim).

3. Results

3.1. Succinate transport via the cloned mouse NaDC3

The cloned mouse NaDC3 cDNA is 3227-bp long with a 1803-bp-long open reading frame (including the termination codon). The cDNA codes for a protein of 600 amino acids and the sequence is identical to that of the recently reported NaDC3 cloned from mouse brain [22]. The mouse NaDC3 cDNA was functionally expressed in a heterologous expression system using HRPE cells. The uptake of succinate, a substrate for NaDC3, was compared in control cells transfected with vector alone and in cells transfected with mouse NaDC3 cDNA. When measured in the presence of NaCl at pH 7.5, the uptake of succinate (20 nM) in vector-transfected cells was 0.06 ± 0.01 pmol/10^6 cells/2 min. The uptake increased 85-fold to 5.08 ± 0.62 pmol/10^6 cells/2 min in cDNA-transfected cells. The cDNA-specific uptake was obligatorily dependent on Na^+ because the uptake decreased to 3% of control when measured in the absence of Na^+.

3.2. Substrate specificity of mouse NaDC3

The substrate specificity of mouse NaDC3 was investigated in this heterologous expression system by assessing the ability of various dicarboxylates and monocarboxylates to compete with succinate for uptake via mouse NaDC3 (Fig. 1). Unlabeled succinate (a dicarboxylate containing four carbon atoms), malate (also a dicarboxylate containing four carbon atoms), and α-ketoglutarate (a dicarboxylate

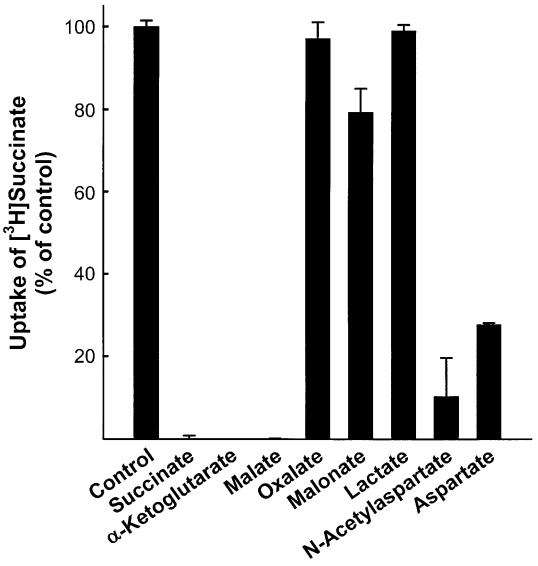

Fig. 1. Substrate specificity of mouse NaDC3. Uptake of [^{3}H]-succinate (20 nM) was measured in cells transfected with vector alone and in cells transfected with mouse NaDC3 cDNA in the absence or in the presence of various unlabeled dicarboxylates and monocarboxylates (2 mM). The cDNA-specific uptake was calculated by adjusting for the endogenous uptake activity. The cDNA-specific uptake measured in the absence of inhibitors was taken as control (100%) and the uptake in the presence of inhibitors is given as percent of this control value.

containing five carbon atoms), at a concentration of 2 mM, inhibited completely the uptake of [^{3}H]-succinate (20 nM) via mouse NaDC3. In contrast, the oxalate (a dicarboxylate with two carbon atoms) and malonate (a dicarboxylate with three carbon atoms) showed little or no inhibitory activity. Similarly, the monocarboxylate lactate did not compete with succinate for uptake via mouse NaDC3. These data show that NaDC3 prefers dicarboxylates with a backbone containing four or five carbon atoms as substrates. The carbon-chain length of the backbone seems to be very critical for recognition as a substrate because dicarboxylates with backbones containing less number of carbon atoms do not interact with the transporter. Aspartate, an amino acid with two carboxylate groups and an amino group and a backbone with four carbon atoms, caused 75% inhibition of NaDC3-mediated succinate uptake. The inhibitory potency of aspartate increased significantly with covalent modification of the amino group by acetylation. The resulting compound, *N*-acetylaspartate (NAA), is a dicarboxylate with no positive charge associated with the amino group. Consequently, this derivative of aspartate is recognized as a substrate by NaDC3 much better than aspartate. We compared the potency of aspartate and NAA to inhibit NaDC3-mediated succinate uptake (Fig. 2). Aspartate inhibited the uptake with an IC_{50} value (i.e., concentration of the inhibitor necessary to cause 50% inhibition) of 870 ± 70 μM. In contrast, NAA inhibited the uptake with an IC_{50} value of 66 ± 10 μM. These data demonstrate that NAA interacts with mouse NaDC3 with high affinity. We have shown recently that rat NaDC3 is able to transport NAA with high

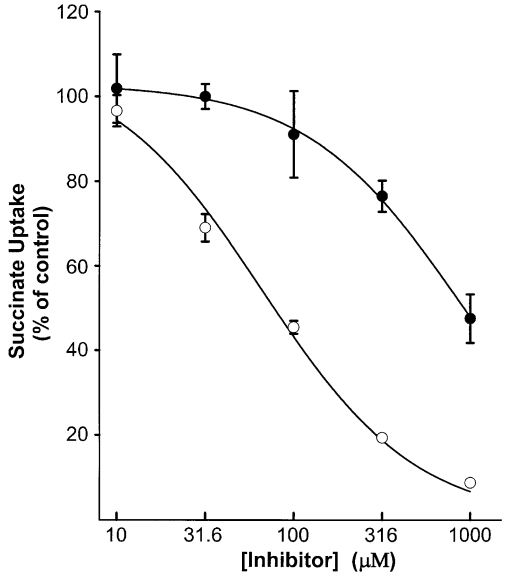

Fig. 2. Inhibition of NaDC3-mediated succinate uptake by aspartate and *N*-acetylaspartate. Uptake of [^{3}H]-succinate (20 nM) was measured in cells transfected with vector alone and in cells transfected with mouse NaDC3 cDNA in the absence or in the presence of increasing concentrations of aspartate (●) and *N*-acetylaspartate (○). Results represent cDNA-specific uptake activity after adjusting for endogenous activity. Data are given as percent of control uptake (100%) measured in the absence of inhibitors.

affinity (Michaelis–Menten constant, ~60 μM) in a Na^+-coupled manner [15]. The results with mouse NaDC3 obtained in the present study are comparable to those obtained with rat NaDC3.

We also performed similar dose-response studies for succinate, α-ketoglutarate, and malate to determine the IC_{50} values for these compounds from their abilities to inhibit the uptake of [^{3}H] succinate (20 nM) (data not shown). The values were 17 ± 4, 17 ± 3, and 27 ± 3 μM for succinate, α-ketoglutarate, and malate, respectively.

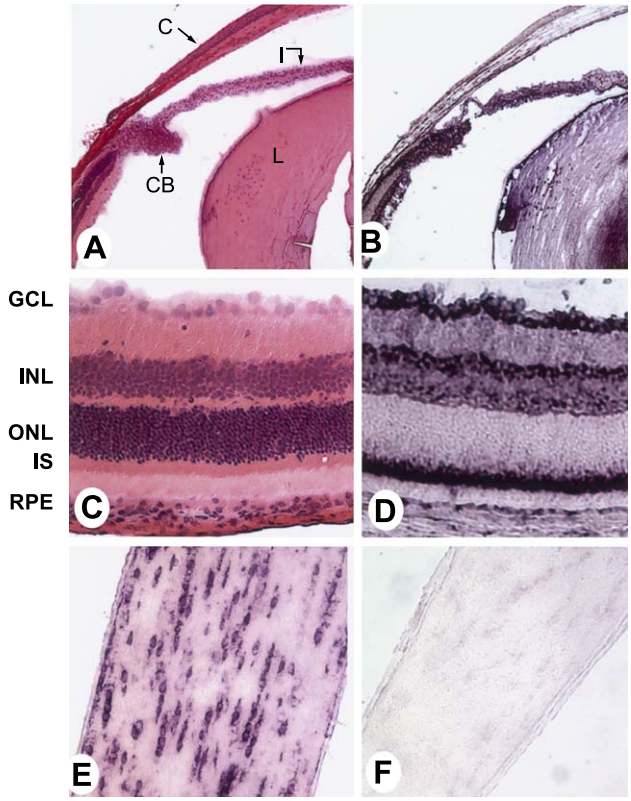

Fig. 4. Expression pattern of NaDC3 mRNA in mouse ocular tissues as assessed by in situ hybridization. (A) Hematoxylin and eosin-stained cryosection of the anterior portion of the eye showing the cornea (C), ciliary body (CB), iris (I), and lens (L). (B) Anterior portion of the eye probed with antisense digoxigenin-labeled mouse NaDC3 riboprobe. (C) Hematoxylin and eosin-stained retinal section for comparison to adjacent unstained retinal sections. Several layers of the retina are identified: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS, inner segment; RPE, retinal pigment epithelium. (D) Retina probed with antisense digoxigenin-labeled mouse NaDC3 riboprobe. (E) Optic nerve probed with antisense digoxigenin-labeled mouse NaDC3 riboprobe. (F) Optic nerve probed with sense digoxigenin-labeled mouse NaDC3 riboprobe (negative control).

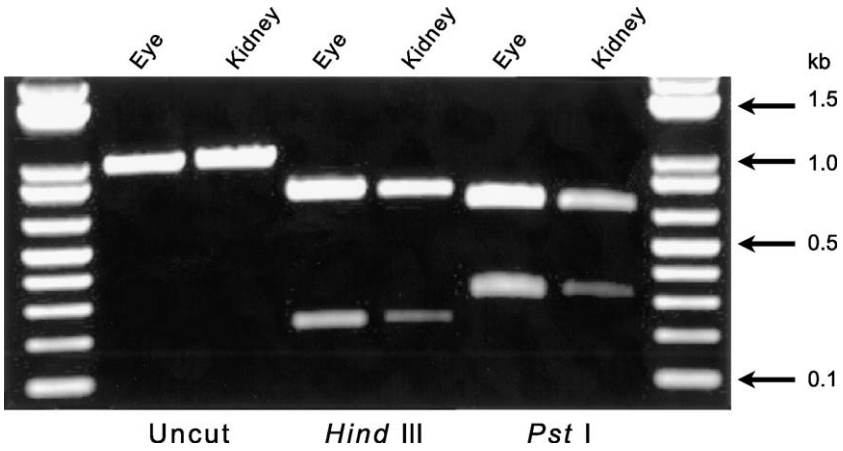

Fig. 3. Evidence for the expression of NaDC3 mRNA in mouse eye. Total RNA, isolated from mouse eyes, was used for RT-PCR with primers specific for mouse NaDC3. The RT-PCR product was gene-cleaned and used for digestion with *Hin*dIII and *Pst*I. Uncut and digested samples were size-fractionated in parallel along with DNA markers to estimate the sizes of the RT-PCR product and the restriction fragments. Total RNA, isolated from mouse kidneys, was used as a positive control.

These data show that the affinity of mouse NaDC3 for NAA is about two- to fourfold less than that for the dicarboxylates.

3.3. Evidence for the expression of NaDC3 in ocular tissues

The expression of NaDC3 in ocular tissues was determined in the mouse eye by RT-PCR and in situ hybridization. RT-PCR using mouse eye total RNA and mouse NaDC3-specific primers yielded a product of expected size (949 bp) based on the nucleotide positions of the primers in mouse NaDC3 cDNA (Fig. 3). Since the cDNA was cloned from mouse kidney, RT-PCR was done with the same pair of primers using mouse kidney total RNA as a positive control. The molecular identity of the RT-PCR products was established by restriction analysis. With two different enzymes (*Hin*dIII and *Pst*I), the restriction pattern of the RT-PCR products from the eye and kidney was similar and the sizes of the restriction fragments were as expected from the restriction map of the mouse NaDC3 cDNA.

3.4. Expression pattern of NaDC3 mRNA in mouse eye

In situ hybridization experiments were carried out using frozen sections of adult mouse eyes. The expression of NaDC3 mRNA as assessed by the labeling with a mouse NaDC3-specific antisense riboprobe was evident in the retina and in other parts of the eye (Fig. 4). A low magnification of a hematoxylin and eosin-stained section of the anterior part of the eye is shown in Fig. 4A. The results of in situ hybridization in this section are shown in Fig. 4B. The ciliary body and iris are intensely positive for NaDC3 mRNA as are the epithelial cells at the equator and periphery of the lens. A hematoxylin and eosin-stained section of the retina is shown in Fig. 4C. The results of in situ hybridization in this section, shown in Fig. 4D, demonstrate abundant expression of NaDC3 mRNA in the neurons of the ganglion cell layer, cells of the inner nuclear layer and the inner segments of the photoreceptor cells. The retinal pigment epithelial cells that constitute part of the blood-retinal barrier also express NaDC3 mRNA. The optic nerve also shows intense positive reaction, primarily in the glial columns, for the presence of NaDC3 mRNA as shown in Fig. 4E. The sense riboprobe did not yield a positive signal in the optic nerve (Fig. 4F) or anywhere else in the eye (not shown) under identical experimental conditions, demonstrating the specificity of the signals seen with the antisense riboprobe.

3.5. Expression pattern of aspartoacylase II mRNA in mouse eye

NaDC3 is the transporter that is responsible for the uptake of extracellular NAA into cells. Since aspartoacylase II, the enzyme responsible for the hydrolysis of NAA, is a cytosolic enzyme, NaDC3 and aspartoacyalse II must function in a coordinated manner in the breakdown of extracellular NAA. This would predict a similar expression pattern for these two proteins in cell types involved in NAA metabolism. Fig. 5 describes the expression pattern of aspartoacylase II mRNA in ocular tissues. The expression was evident, as indicated by the hybridization signals with a

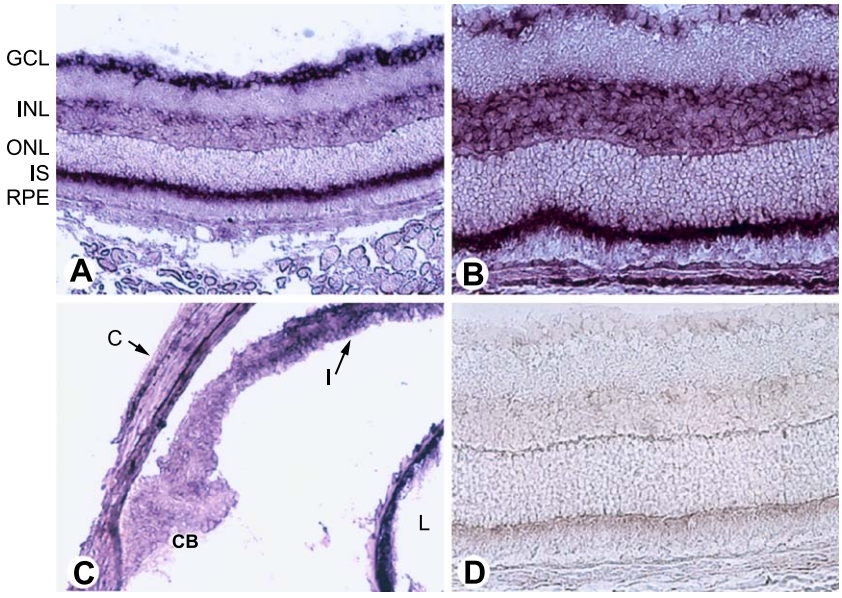

Fig. 5. Expression pattern of aspartoacylase II mRNA in mouse ocular tissues as assessed by in situ hybridization. (A) Retina probed with antisense digoxigenin-labeled mouse aspartoacylase II riboprobe. Different layers of the retina are identified: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS, inner segment; RPE, retinal pigment epithelium. (B) Higher magnification of (A). (C) Anterior portion of the eye probed with antisense digoxigenin-labeled mouse aspartoacylase II riboprobe (C, cornea; CB, ciliary body; I, iris; L, lens). (D) Retina probed with sense digoxigenin-labeled mouse aspartoacylase II riboprobe (negative control).

NaDC3-specific antisense riboprobe, in the neurons of the ganglion cell layer, cells of the inner nuclear layer and inner segments of the photoreceptor cells as well as in retinal pigment epithelial cells (Fig. 5A and B). The iris and, to a lesser extent, the ciliary body were also positive for expression of this enzyme (Fig. 5C). The epithelial cells at the periphery of the lens were also positive for the hybridization signals. There was no positive signal with the sense riboprobe under identical experimental conditions (Fig. 5D).

4. Discussion

We have demonstrated here that the murine NaDC3 is able to transport *N*-acetylaspartate (NAA) with high affinity. Acetylation of the amino group in aspartate increases the affinity for NaDC3 several-fold. These findings may have physiological significance. NAA is the second most abundant amino acid in the nervous system, only glutamate occurring at higher concentrations [1–3]. Glial cells express a Na^+-coupled transport system for NAA [14]. Interestingly, the characteristics of NAA transport in glial cells are similar to those of NAA transport via NaDC3. It is very likely that NaDC3 is responsible for the observed transport of NAA in glial cells. Neurons synthesize NAA and intraneuronal concentrations of this compound are usually in the range of 10–15 mM [1–3]. NAA is released from the neurons by a hitherto unknown mechanism and the extracellular concentration of NAA in the brain interstitial space is ~ 100 μM [1–3]. Glial cells possess aspartoacylase II, the enzyme responsible for breakdown of NAA [10,11]. Therefore, NAA has to first enter the glial cells from the extracellular space for subsequent hydrolysis by aspartoacylase II. Thus, NaDC3 and aspartoacylase II must function in a coordinated manner in the breakdown of NAA. The brain content of NAA is decreased in pathological conditions associated with neuronal loss because NAA, released from the deceased neurons, is efficiently broken down by glial cells. This is the basis of the current clinical use of measurements of NAA in the brain by proton magnetic resonance spectroscopy to assess neuronal loss in various pathological conditions associated with disorders of the central nervous system [1–3].

NAA functions as an important source of acetyl groups in the synthesis of brain lipids, a process closely related to the process of myelination [7]. Several cerebral pathologies such as multiple sclerosis, hypothyroidism, and mitochondrial encephalopathies with a dysfunction of the myelination process are associated with alterations in the content and metabolism of NAA in the brain [23,24]. The importance of NAA metabolism in myelination is underscored by the clinical consequences of a genetic defect in aspartoacylase II. Canavan disease is an autosomal recessive disorder with mutations in the gene coding for aspartoacylase II [12,13]. This is a neurodegenerative disease of infancy and early childhood characterized by demyelination, leukodystrophy, megaloencephalopathy, and spongiform degeneration. Recent studies have shown that targeted disruption of the gene coding for this enzyme in mice produces central nervous system pathologies similar to those observed in patients with Canavan disease [25]. Since NaDC3 functions in a concerted manner with aspartoacylase II in the hydrolysis of NAA, we hypothesize that dysfunction of the transporter may also lead to similar clinical consequences.

Since Canavan disease is also associated with dysfunction of the retinal and optic nerve [12,13], the metabolism of NAA apparently plays an important role in the eye. The presence of NAA has been demonstrated in the retina [1–3]. But, there is no information available in the literature on the expression of aspartoacylase II nor NaDC3. We speculated that aspartoacylase II and NaDC3, which are both obligatory for the metabolism of NAA, are expressed in the neural retina. In the present study, we provide evidence that supports this speculation. NaDC3 mRNA, as assessed by in situ hybridization, is expressed in several layers of the neural retina. The expression is evident in the ganglion cells, photoreceptor cells and in the optic nerve. A similar pattern of expression is also seen with aspartoacylase II. These findings suggest that the metabolism of NAA is disrupted in Canavan disease not only in the brain but also in the neural retina, thus providing the molecular basis for the retinal and optic nerve dysfunction in the disease. NaDC3 and aspartoacylase II are expressed in glial cells as expected from the published reports on the predominant metabolism of NAA associated with these cells. Interestingly, these two proteins are also expressed in neuronal cells such as the retinal ganglion cells and the photoreceptor cells. The nonneuronal cells such as the retinal pigment epithelial cells and the cells of the ciliary body, iris, and lens are also positive for the expression of NaDC3 and aspartoacylase II. The physiological relevance of the expression of NaDC3 and aspartoacylase II in these sites remains to be established.

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