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Ablating N-Acetylaspartate Prevents Leukodystrophy in a Canavan Disease Model

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Abstract

Canavan disease is caused by inactivating *ASPA* (aspartoacylase) mutations that prevent cleavage of N-acetyl-L-aspartate (NAA), resulting in marked elevations in central nervous system (CNS) NAA and progressively worsening leukodystrophy. We now report that ablating NAA synthesis by constitutive genetic disruption of *Nat8l* (N-acetyltransferase-8 like) permits normal CNS myelination and prevents leukodystrophy in a murine Canavan disease model.

Canavan disease is a recessively inherited spongiform leukodystrophy caused by deficiency of functional aspartoacylase (ASPA).[1,2] N-acetyl-L-aspartate (NAA) is synthesized in neurons from acetyl-CoA and aspartate by *NAT8L* (N-acetyltransferase-8 like; EC 2.3.1.17),[3,4] and then cleaved to acetate and aspartate by oligodendroglial *ASPA* (EC 3.5.1.15).[5] Mammalian central nervous system (CNS) NAA is normally maintained at approximately 10mM, but is substantially higher in the ASPA-deficient brain.[6] Radiotracer studies have shown that oligodendroglia use NAA-derived acetate as a precursor for lipogenic acetyl-CoA,[7] and brain acetate and acetyl-CoA levels have been reported to be diminished in mice lacking functional *Aspa* alleles.[8,9] These observations suggested the hypothesis that impaired myelination in Canavan disease is attributable to oligodendroglial deficiency of acetate and acetyl-CoA required for myelin lipid synthesis.[7] An alternative hypothesis, supported by the prominence of astroglial swelling and vacuolation in ASPA-deficient brains,[1] and the expression by rodent astroglia of *Nadc3* (*Slc13a3*), a Na^+-coupled plasma membrane dicarboxylate transporter with high NAA affinity,[10] is that leukodystrophy in Canavan disease is a consequence of a deleterious effect of elevated brain NAA, perhaps due to osmotic disequilibrium.[11]

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Authorship

F.G. and P.B. are co–first authors.

Potential Conflicts of Interest

Nothing to report.

We reasoned that preventing NAA synthesis by disrupting both alleles of *Nat8l* in mice would be an efficient means for evaluating both the "oligodendroglial acetate/acetyl-CoA starvation" and "elevated NAA osmotic toxicity" hypotheses. If oligodendroglial acetate/acetyl-CoA starvation attributable to oligodendroglial inability to derive acetate from NAA is responsible for leukodystrophy in a CNS that is deficient in ASPA enzymatic activity, it would be predicted that the absence of NAA would inhibit CNS myelination in mice with normal *Aspa* alleles, but would neither exacerbate nor prevent leukodystrophy in mice in which both *Aspa* alleles have been mutated to block translation of functional ASPA. If, instead, leukodystrophy in ASPA-deficient mice is attributable to a toxic effect of elevated CNS NAA, it would be predicted that absence of NAA would prevent leukodystrophy in these ASPA-deficient mice.

Materials and Methods

Constitutive *Nat8l* knockout ($Nat8l^{KO}$) mice were from the University of California, Davis (UC Davis) KOMP Repository (Project ID VG11213), and $Aspa^{nur7}$ mice[6] were from the Jackson Laboratory (Bar Harbor, ME; stock number 008607). The mice were maintained on a C57BL/6J background, and genotyped by polymerase chain reaction. $Nat8l^{KO/KO}$, $Aspa^{nur7/nur7}$, and double homozygous $Nat8l^{KO/KO}/Aspa^{nur7/nur7}$ mice were bred by heterozygous mating, and offspring were obtained in expected Mendelian ratios. Both male and female mice were used in all studies. Motor function was tested by measuring accelerating rotarod retention times (starting speed = 4.0 rotations per minute [rpm], speed step = 1.3rpm every 10 seconds). For this purpose, 5-minute training sessions were repeated daily for 10 days, at which point plateau performances had been achieved and were recorded. At the time of sacrifice, mice were deeply anesthetized with ketamine/xylazine prior to euthanasia by decapitation and brain flash freezing (for biochemical studies) or by cardiac perfusion (for histological studies). These procedures were conducted in accord with a UC Davis institutional animal care and use committee–approved protocol. NAA was extracted from whole brain (including cerebellum) and assayed by ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS).[12] A spectrophotometric method was used to assay acetate in whole brain (including cerebellum).[13] Immunohistology was performed with first antibodies against myelin basic protein (mouse anti-MBP IgG2b, Covance [Princeton, NJ] SMI-99P, 1:1,000) and glial fibrillary acidic protein (mouse anti-GFAP IgG1, Millipore [Billerica, MA] MAB360, 1:400), and imaged by laser scanning confocal microscopy. Transmission electron microscopy (TEM) was performed on EPON-embedded tissues.

Results

$Nat8l^{KO/KO}$ Mice

Both male and female adult homozygous constitutive *Nat8l* knockout ($Nat8l^{KO/KO}$) mice were, on average, 20% heavier than littermate wild-type ($Nat8l^{WT/WT}$) mice of the same sex, but were otherwise clinically undistinguishable from adult $Nat8l^{WT/WT}$ mice. Brain NAA was not detectable in the $Nat8l^{WT/WT}$ mice by ^{1}H-MRS (Fig 1A). TEM demonstrated intact CNS myelination in the $Nat8l^{KO/KO}$ mice; linear regression analysis of myelin G-ratios of 3 p60 $Nat8l^{WT/WT}$ control versus 3 p60 $Nat8l^{KO/KO}$ mice showed a slightly lower G ratio

(ie, thicker myelin) in *Nat8l*$^{KO/KO}$ corpus callosum (G-ratio intercept = 0.6392, standard error of the mean [SEM] = 0.0145) than in *Nat8l*$^{WT/WT}$ corpus callosum (G-ratio intercept = 0.6601, SEM = 0.0081, $p < 0.01$, Student 2-tailed *t* test). However, linear regression analysis of dorsal corticospinal tract G-ratios in the same mice showed no significant differences between the 2 groups (data not shown). Higher magnification TEMs showed myelin lamellar thickness did not differ significantly between the *Nat8l*$^{KO/KO}$ and *Nat8l*$^{WT/WT}$ mice (data not shown).

Aspa$^{nur7/nur7}$ Mice

As previously reported,[6] ataxia appeared a few weeks postweaning in *Aspa*$^{nur7/nur7}$ mice, and worsened progressively thereafter. In addition, short generalized seizures were often seen in these homozygous *Aspa* mutant mice as they aged. Brain NAA concentrations in *Aspa*$^{nur7/nur7}$/*Nat8l*$^{WT/WT}$ mice were approximately 2-fold higher than in littermate *Aspa*$^{WT/WT}$/*Nat8l*$^{WT/WT}$ mice (see Fig 1A). Accelerating rotarod performance of the *Aspa*$^{nur7/nur7}$–Nat8l$^{WT/WT}$ mice was markedly impaired (see Fig 1B). Astrogliosis, vacuolation, and remyelinating axons were prominent in Aspa$^{nur7/nur7}$/Nat8l$^{WT/WT}$ cerebellum (Figs 2 and 3) and forebrain (not shown). In wild-type (*Aspa*$^{WT/WT}$/*Nat8l*$^{WT/WT}$) control mice, the brain acetate concentration was 0.1136 ± 0.0061mg/g (mean ± SEM, n = 8), but was diminished by 38% (0.0702 ± 0.0086mg/g, n = 6, $p < 0.01$) in *Aspa*$^{WT/WT}$/*Nat8l*$^{KO/KO}$ mice, and by 52% (0.0548 ± 0.0110mg/g, n = 4, $p < 0.001$) in *Aspa*$^{nur7/nur7}$/*Nat8l*$^{WT/WT}$ mice (1-way analysis of variance with Bonferroni multiple comparison test).

Aspa$^{nur7/nur7}$/Nat8l$^{KO/KO}$ Mice

Brain NAA was not detectable in *Aspa*$^{nur7/nur7}$/*Nat8l*$^{KO/KO}$ mice (see Fig 1A). In contrast to *Aspa*$^{nur7/nur7}$/*Nat8l*$^{WT/WT}$ mice, *Aspa*$^{nur7/nur7}$/*Nat8l*$^{KO/KO}$ mice did not develop ataxia, and their accelerating rotarod performance was normal (see Fig 1B). Also in contrast to *Aspa*$^{nur7/nur7}$/*Nat8l*$^{WT/WT}$ mice, immunohistology and transmission electron microscopy showed no evidence of astroglial vacuolation, astrogliosis, or demyelination in *Aspa*$^{nur7/nur7}$/*Nat8l*$^{KO/KO}$ cerebellum (see Figs 2 and 3) or forebrain (not shown).

Discussion

The substantial diminutions in brain acetate levels in *Aspa*$^{nur7/nur7}$/*Nat8l*$^{WT/WT}$ mice, in which NAA cannot be cleaved to acetate and aspartate because of the absence of functional *Aspa*, and also in *Aspa*$^{WT/WT}$/*Nat8l*$^{KO/KO}$ mice, in which NAA cannot be synthesized because of the absence of functional *Nat8l*, support a component of the oligodendroglial acetate/acetyl-CoA starvation in Canavan disease hypothesis, by indicating that acetate derived from NAA is important in maintaining a normal brain acetate pool. Additional support for “acetate starvation” in Canavan disease was provided by previous reports that dietary supplements to enhance brain acetyl-CoA supplies diminished the severity of leukodystrophy in ASPA-deficient rodents.[9] But our demonstration that CNS myelination is intact in both *Aspa*$^{WT/WT}$*Nat8l*$^{KO/KO}$ mice, in which NAA cannot be synthesized, and in *Aspa*$^{nur7/nur7}$/*Nat8l*$^{KO/KO}$ mice, in which NAA cannot be cleaved, has substantially weakened this hypothesis. Importantly, *Aspa*$^{nur7/nur7}$ mice were entirely protected against

development of leukodystrophy when their capacity to synthesize NAA was ablated by constitutive deletion of *Nat8l*, thus supporting the "elevated NAA osmotic toxicity" hypothesis,[11] a conclusion also compatible with the recent report of redistribution of the water channel protein aquaporin 4 in astroglia in *Aspa*$^{nur7/nur7}$ mice.[14]

Although homozygous constitutive deletion of *Nat8l* did not impede accelerating rotarod performance or CNS myelination, abnormal social interactions have been documented in *Nat8l*$^{KO/KO}$ mice,[15] and the single homozygous *NAT8L* mutant human reported thus far was developmentally delayed.[3,16] Those observations suggest that NAA, although not essential to support myelination, is required for other aspects of normal CNS development.

Since neither *Aspa*$^{nur7/nur7}$ nor *Nat8l*$^{KO/KO}$ mice can employ NAA-derived acetate as a precursor for brain acetyl-CoA, what substrates do they use instead for myelin lipid synthesis? Studies of cultured brown adipocytes, which like neurons express *Nat8l*, showed that the lipogenic acetyl-CoA pool can be maintained at a normal level in the absence of NAA synthesis by induction of the ATP-citrate lyase pathway, which generates acetyl-CoA from citrate exported from mitochondria,[17] a pathway known to be active in the CNS.[18]

Provision of functional ASPA to brain by viral *Aspa* transduction has been reported to be effective in suppressing leukodystrophy in *Aspa* mutant mice, and to a lesser extent by viral *ASPA* transduction in children with Canavan disease.[19,20] An important implication of the "elevated NAA toxicity" hypothesis is that delivery of functional ASPA to any CNS cells capable of NAA uptake would likely benefit children with Canavan disease by diminishing brain NAA overload. Development of CNS-permeant NAT8L inhibitors, and of methods to accelerate clearance of NAA from the CNS, may also prove useful as adjunctive therapies for Canavan disease.

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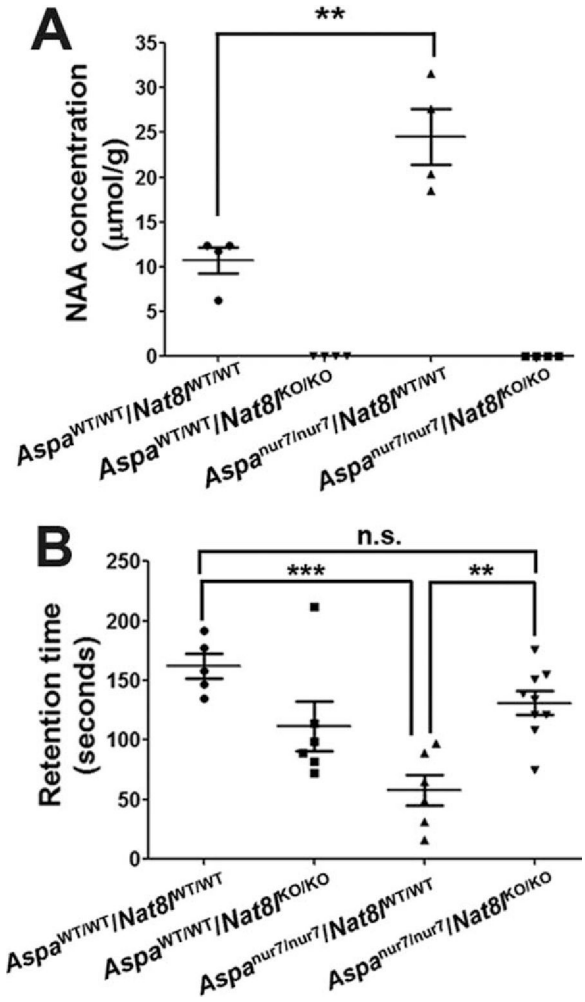

FIGURE 1:

(A) Brain N-acetyl-L-aspartate (NAA) concentrations were >2-fold higher in p60 Aspa$^{nur7/nur7}$/*Nat8l*$^{WT/WT}$ mice than in p60 littermate control *Aspa*$^{WT/WT}$/*Nat8l*$^{WT/WT}$ mice, but NAA was not detectable in the brains of either *Aspa*$^{WT/WT}$/*Nat8l*$^{KO/KO}$ or *Aspa*$^{nur7/nur7}$/*Nat8l*$^{KO/KO}$ mice. (B) In comparison to p60 *Aspa*$^{WT/WT}$/*Nat8l*$^{WT/WT}$ normal control mice (2 females, 3 males), accelerating rotarod performance was substantially diminished in p60 *Aspa*$^{nur7/nur7}$/*Nat8l*$^{WT/WT}$ mice (4 females, 2 males). Accelerating rotarod performances of p60 *Aspa*$^{WT/WT}$/*Nat8l*$^{KO/KO}$ mice (2 females, 4 males) and of p60 *Aspa*$^{nur7/nur7}$/*Nat8l*$^{KO/KO}$ mice (5 females, 5 males) did not differ significantly from those of normal control mice. Vertical bars denote standard error of the mean; statistical analysis in both A and B was by 1-way analysis of variance with Bonferroni multiple comparison post-test. n.s5not significantly different. $^{**}p < 0.01$, $^{***}p < 0.001$.

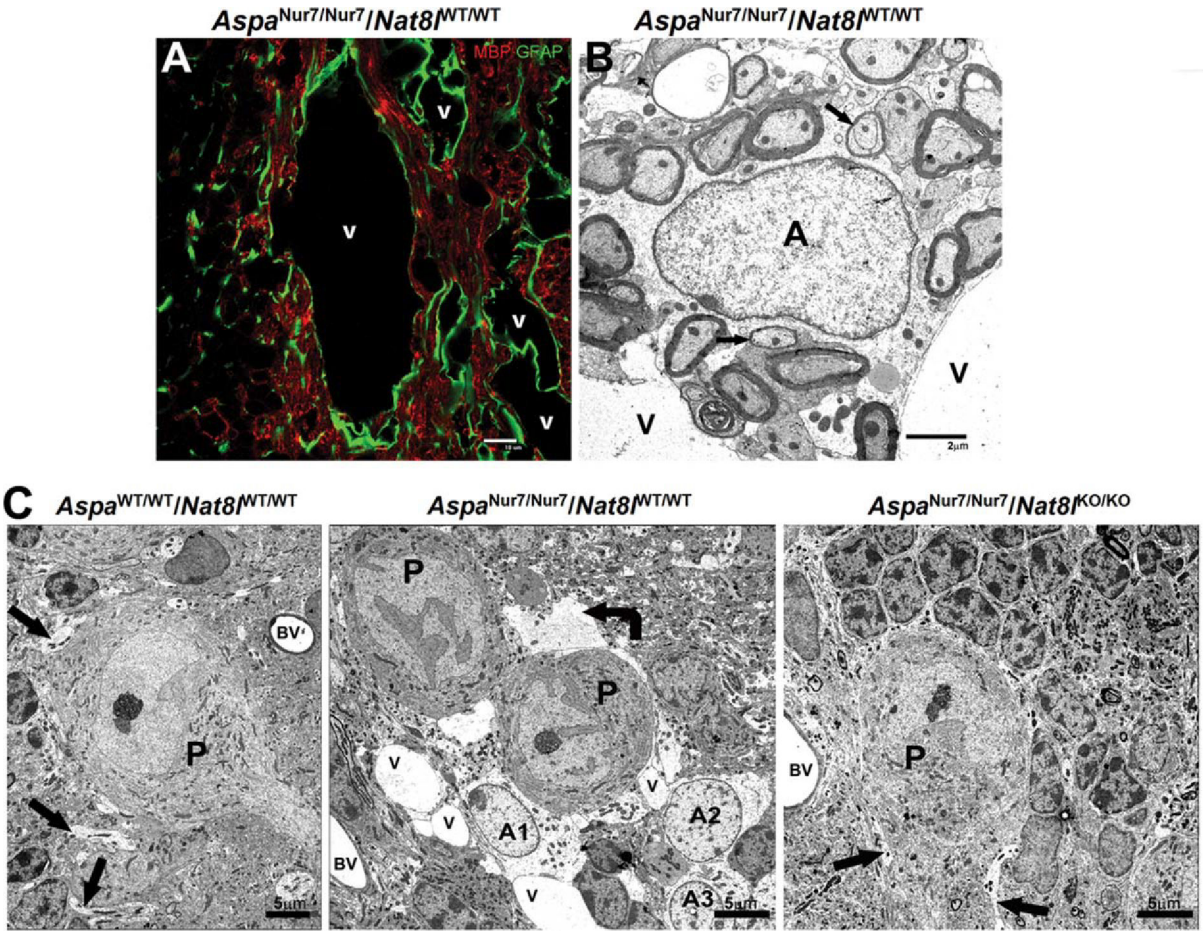

FIGURE 2:

(A) A 0.5μm confocal optical slice of cerebellar white matter from a p60 *Aspa*$^{nur7/nur7}$/*Nat8l*$^{WT/WT}$ mouse, immunostained for glial fibrillary acidic protein (GFAP; green) and myelin basic protein (MBP; red), showing small and large vacuoles (v) apparently lined by GFAP. Size bar = 10μm. (B) Transmission electron microscopy (TEM) of cerebellar white matter from a p60 *Aspa*$^{nur7/nur7}$/*Nat8l*$^{WT/WT}$ mouse. A = astrocyte nucleus. Remyelinating axon is indicated by an arrow. Size bar = 2μm. (C) TEM showing cerebellar gray matter vacuolation in a p60 *Aspa*$^{nur7/nur7}$/*Nat8l*$^{WT/WT}$ mouse (center panel) that was not present in a p60 *Aspa*$^{WT/WT}$/*Nat8l*$^{WT/WT}$ control mouse (left panel) or a p60 *Aspa*$^{nur7/nur7}$/*Nat8l*$^{KO/KO}$ mouse (right panel). P = Purkinje cell; BV = blood vessel; V = vacuole. Straight arrows point to astroglial processes. The curved arrow in the center panel points to a pale astroglial process containing glial filaments. Size bars = 5μm. Results shown are representative of those obtained with 3 mice in each of these 3 genetic groups.

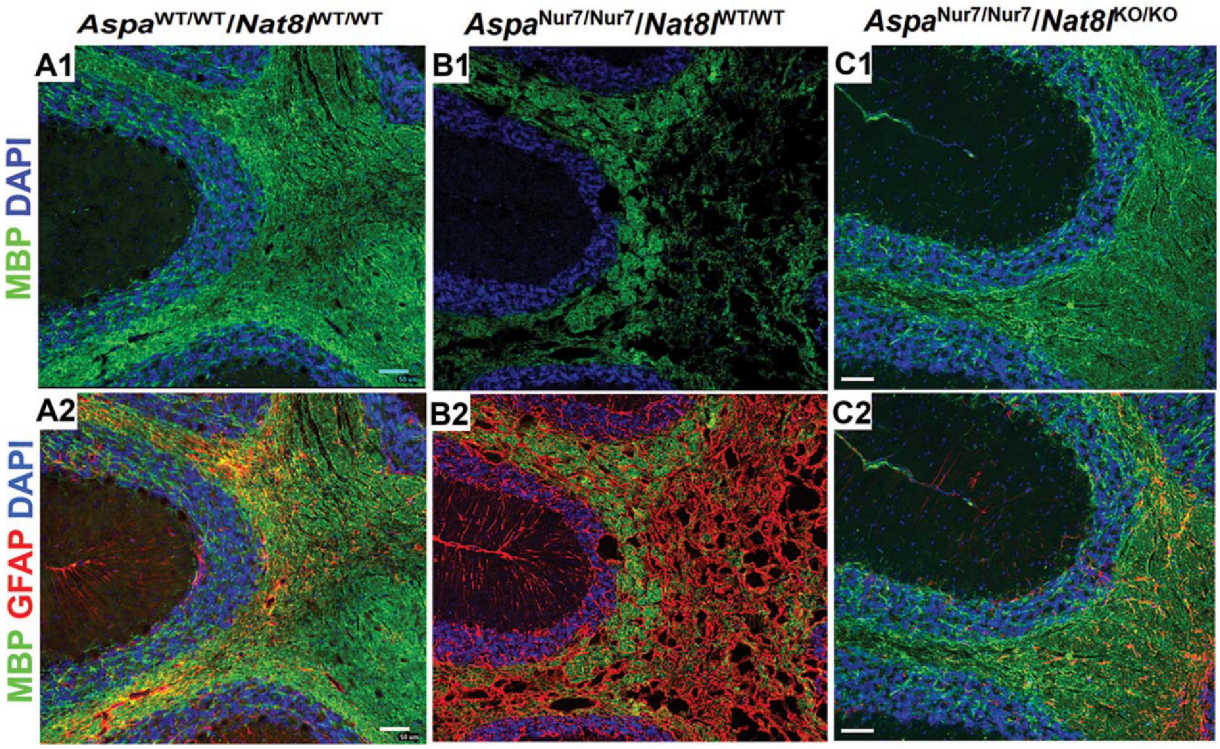

FIGURE 3:

Laser-scanning confocal stack cerebellar images immunostained for myelin basic protein (MBP; green; A1, B1, C1) or both MBP (green) and glial fibrillary acidic protein (GFAP; red; A2, B2, C2) showing demyelination, astrogliosis, and vacuolation in a p60 *Aspa*$^{nur7/nur7}$/*Nat8l*$^{WT/WT}$ mouse (B1, C1); these abnormalities were not present in a p60 wild-type (*Aspa*$^{WT/WT}$/*Nat8l*$^{WT/WT}$) control mouse (A1, A2) or a p60 *Aspa*$^{nur7/nur7}$/*Nat8l*$^{KO/KO}$ mouse (C1, C2). All panels were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Size bars = 5μm.