Reduced N-Acetylaspartate Levels in Mice Lacking Aralar, a Brain- and Muscle-type Mitochondrial Aspartate-glutamate Carrier*

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Aralar is a mitochondrial calcium-regulated aspartate-glutamate carrier mainly distributed in brain and skeletal muscle, involved in the transport of aspartate from mitochondria to cytosol, and in the transfer of cytosolic reducing equivalents into mitochondria as a member of the malate-aspartate shuttle. In the present study, we describe the characteristics of aralar-deficient (Aralar\(-\)/-) mice, generated by a gene-trap method, showing no aralar mRNA and protein, and no detectable malate-aspartate shuttle activity in skeletal muscle and brain mitochondria. Aralar\(-\)-/- mice were growth-retarded, exhibited generalized tremoring, and had pronounced motor coordination defects along with an impaired myelination in the central nervous system. Analysis of lipid components showed a marked decrease in the myelin lipid galactosyl cerebroside. The content of the myelin lipid precursor, N-acetylaspartate, and that of aspartate are drastically decreased in the brain of Aralar\(-\)-/- mice. The defect in N-acetylaspartate production was also observed in cell extracts from primary neuronal cultures derived from Aralar\(-\)-/- mouse embryos. These results show that aralar plays an important role in myelin formation by providing aspartate for the synthesis of N-acetylaspartate in neuronal cells.

Aralar (1), also known as aralar1 (2), encoded by SLC25A12 located on chromosome 2q31 (3), is a mitochondrial aspartate-glutamate carrier (AGC)\(^1\) (4) with high expression in the brain, skeletal muscle, and heart. AGC functions in the transport of aspartate from mitochondria to cytosol in exchange of glutamate and plays a role in the transport of NADH reducing equivalents from cytosol to mitochondria as a member of malate-aspartate shuttle (MAS). The structure of aralar and another AGC isoform, citrin (5), 77.8% identical to aralar at the amino acid sequence level, is different from those of the other mitochondrial carriers in that they have a long N-terminal extension harboring EF-hand Ca\(^{2+}\) binding motifs.

Citrin, encoded by SLC25A13 on chromosome 7q21.3 and expressed mainly in the liver, kidney, and heart, is deficient in adult-onset type II citrullinemia (5, 6), which is characterized biochemically by a liver-specific deficiency of argininosuccinate synthetase and clinically by neuropsychotic symptoms (7). It was proposed that the argininosuccinate synthetase deficiency in adult-onset type II citrullinemia was because of the lack of aspartate, one of the two substrates of argininosuccinate synthetase, which is produced in mitochondria and transported to cytosol via citrin (4).

During embryogenesis, aralar and citrin have a widely overlapping expression pattern, and the full expression of aralar is only attained postnatally (8, 9). Aralar, expressed in almost every tissue except the adult liver (1, 8, 10), is the only AGC isoform present in the adult central nervous system. Aralar expression in the central nervous system is restricted to neurons (9). It has no expression in white matter and low levels in cultured glial cells (10). Neuronal maturation is associated with an increase in aralar expression and a prominent rise in MAS function (9).

Here, we describe a new function of aralar discovered after the generation of aralar-deficient (Aralar\(-\/-\)) mice. In addition to a total lack of muscle and brain MAS activity, and a drastic fall in respiration on glutamate plus malate, Aralar\(-\)-/- mice had severe growth defects, a restricted lifespan, and developed motor coordination deficits along with an impaired myelination. Dysmyelination in Aralar\(-\)-/- mice is associated with a deficient synthesis of myelin lipids and a striking reduction in the levels of aspartate and N-acetylaspartate (NAA), a metabolite produced in neuronal mitochondria and microsomes (11–13) believed to be a precursor of myelin lipids (14–16). Our results reveal that the neuronal AGC is required for the syn-

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thesis of aspartate and NAA in the adult mammalian brain, suggesting that aralar deficiency in humans could have impact in the brain levels of these metabolites and their derivatives.

**EXPERIMENTAL PROCEDURES**

*Generation of Aralar−/− Mice—*Gene trapping was performed at Lexicon Genetics (The Woodlands, TX) in SVJ129 ES cells using the insertion vector that was based on the gene trap technology of Lexicon (17). Slc25a12−/− ES cells were injected into C57BL blastocysts, and chimera mice were bred to C57BL (albinos) wild-type mice. Subsequent breeding was with SVJ129/C57BL albino hybrids to maintain background. The resulting slc25a12−/− (Aralar−/−) offspring were interbred to produce slc25a12−/− (Aralar−/−) mice. Mice were kept in climate-controlled quarters at 23 °C with free access to water and food, and a 12-h/day light cycle. The experimental protocols fulfilled the requirements of the Committee for Experimental, Kagoshima University, and European guidelines.

*Determination of Insertion Site of the Trap Vector—*One targeted allele of the slc25a12 gene (OmniBank OST clone 123999) was obtained from a library of randomly targeted embryonic stem cell lines at Lexicon Genetics. Because the insertion was in intron 13, which is larger than 20 kb, we searched for mouse genomic clones containing sequences downstream of intron 13 or upstream fragments of slc25a12. As a result, the AAZ41829 clone with the 5′-sequence of exon 14 (Fig. 1A). PCR of genomic DNA from the slc25a12−/− mice as template with a forward primer derived from BTK in the gene trap vector and a reverse primer derived from 5′-end sequence of exon 14 of slc25a12 yielded a 3.5-kb band. Comparison of the sequences of the 3.5-kb fragment and AAZ41829 revealed the precise insertion site of the trap vector.

*Generation of Slc25a12 Knockouts—*After determining the insertion site of the trap vector (see Fig. 1A), the slc25a12 genotype was determined by PCR using genomic DNA from mouse ear with the following primers: sense primer-a (mAra /H9262 3′-GTTCTCTAGAAACTGCTGAGG-3′) and antisense primer-b (mAra int-13F1: 5′-GTTCTCTAGAAACTGCTGAGG-3′) for wild-type alleles, sense primer-b (mAra int-13F1: 5′-GATTGTGAACTCTCAGCTGAGT-3′) for wild-type alleles, and antisense primer-c (mAra int-13B: 5′-ACCACACACCGTGTCAGC-3′) for both mutant and wild-type alleles. Mutant (406 bp) fragments and wild-type (271 bp) were separated by electrophoresis on a 2% agarose gel (Fig. 1B). Both PCR products were verified by sequencing.

*Northern and Western Blot Analyses—*Total RNA was extracted from mouse tissues by the method of Chomczynski and Sacchi (18), and products were verified by sequencing. Separated by electrophoresis on a 2% agarose gel (Fig. 1B) for both mutant and wild-type alleles. Mutant (406 bp) fragments and wild-type (271 bp) were separated by electrophoresis on a 2% agarose gel (Fig. 1B). Both PCR products were verified by sequencing.

*Histology and Immunocytochemistry—*Mice at 18–20 days were deeply anesthetized with sodium pentobarbital and perfused transcardially with 50 ml of heparinized physiological saline followed by 200 ml of fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, containing 0.2% picric acid at room temperature. The brains were removed and immersed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 3 days at 4 °C. The brain was cryoprotected in 20% phosphate-buffered sucrose solution. Serial 40-μm thick coronal sections were cut, dehydrated with ethanol, cleared in xylene, and coverslipped. Number of animals histologically examined was 6 for each genotype. Immunocytochemical controls for specificity in which antigen-absorbed antibody was used showed no immunostaining.

*Neuronal Cell Culture—*Cortical neuronal cultures were prepared from 16-day-old mouse embryos as described previously (9), and grown on a serum-free defined medium, as described by Ruiz et al. (28). In these culture conditions, neurons represented more than 90% of the cell population.

*Determination of Metabolites in Brain and Cultured Neurons—*Mice were sacrificed by cervical dislocation, and the brain was immediately removed from the skull on dry ice and homogenized with 4 volumes of 3% sulfosalicylic acid or 5% perchloric acid. The sulfosalicylic acid supernatant was used for amino acid analysis with a JEOI JLC-500 amino acid analyzer (JEOI Ltd., Tokyo, Japan). NAA and N-acetylaspartylglutamate were extracted from brain by homogenization with methanol and purification with a Bio-Rad AG-50Wx8 column, as described by Bjartmarz et al. (27). The eluate was analyzed by HPLC with UV detection at 206 nm using a SAX anion exchange column (250 × 4.6 mm, 5 μm, Waters) at room temperature (28). Cultured neurons were
mechanically detached and extracted with 3% perchloric acid. The extracts were dissolved in 0.1 M potassium dihydrogen phosphate and 0.025 M potassium chloride, pH 4.5, filtered (0.45 μm), and applied to HPLC for NAA analysis. Total lipid fractions were isolated and dissolved in chloroform/methanol (2:1, v/v) as 1 ml/100 mg wet weight of brain and 2 ml/100 mg wet weight of sciatic nerve. 20 μl of total RNA per lane were used. Aralar, fusion gene (5′-Ara + Neo, open arrowhead), neo, citrin, and glyceraldehyde dehydrogenase (GAPDH) mRNAs were detected. Br, brain; H, heart; SkM, skeletal muscle. D, Western blot analysis of aralar and citrin. Four μl of 10% homogenate per lane were resolved on a 10% SDS-polyacrylamide gel and immunoblotted with anti-aralar and anti-citrin antibodies. E, respiratory rates in SkM (ii–iii) and Br (iv) mitochondria derived from Aralar+/− (open bars), Aralar+++ (gray bars), and Aralar−− (solid bars) mice, in the presence of 5 mM glutamate plus 5 mM malate (i), 5 mM pyruvate plus 5 mM malate (ii), or 5 mM succinate (iii and iv), without (state 4: St 4) or with (St 3) of 0.5 mM ADP. AO, atomic oxygen. F, MAS activity in SkM and Br mitochondria. Data are mean ± S.E. of at least three independent experiments performed in triplicate. Differences among the different genetic backgrounds were analyzed with one-way analysis of variance followed by Bonferroni tests. Mean values not sharing a common superscript letter denote significant differences (p < 0.05).

RESULTS

Generation of Aralar−− Mice—The original Aralar+/− founder mice were created by using a gene-trapping method (17). Wild-type (Aralar+/+) and Aralar−− mice were derived by breeding the Aralar+/− founder mice, which are in a hybrid genetic background (50% C57BL/6 albino and 50% 129SvEvBrd strains). Of the total 92 mouse embryos (at E15-E17) derived from inter-crosses of heterozygote mice, the proportions of Aralar+/+, Aralar−−, and Aralar−− genotypes were 26, 49, and 25%, respectively, indicating that the lack of aralar does not affect fetal development up to embryonic day 17. On the other hand, at 10 to 15 days of age out of 94 Aralar−− mice expected, only 54 were found (p < 0.001). Many Aralar−− mice died between E18 and 15 days postnatal at the time when the pattern of aralar-specific expression is established (8–10).

Aralar mRNA and Protein Levels in Aralar−− Mice—The two major aralar transcripts of 2.8 and 3.3 kb (1, 8, 9) and a third minor band of about 6.6 kb were detected in the brain, heart, and skeletal muscle of Aralar+/+ and Aralar+++ mice, and not in Aralar−− mice (Fig. 1C). A faint 2.6-kb band found in Aralar+/+ and Aralar−− tissues was identified as fusion mRNA composed of the 5′-portion of aralar and the gene for neomycin resistance. Citrin mRNA levels were not different among the three genotypes. Western blot analysis revealed a total loss of aralar protein in the brain, heart, and skeletal muscle of Aralar−− mice and about half of the wild-type levels in Aralar−− mice (Fig. 1D), confirming the functional disruption of the slc25a12 gene in the null mice. The same amount of citrin peptide was detected in the heart of all genotypes.

Respiration, MAS Activity, and Aspartate Formation in Skeletal Muscle and Brain Mitochondria—Skeletal muscle and brain mitochondria from Aralar−− mice did not show any difference in the respiratory rate or respiratory control ratio with succinate as substrate (Fig. 1E) indicating that aralar deficiency did not have any effect on overall mitochondrial function. Respiration rate with glutamate plus malate, studied in skeletal muscle, revealed a dramatic decrease in Aralar+/+ and Aralar−− mice, specially in state 3, to about 50 and 12% of that in Aralar+/+ mice, respectively (Fig. 1E, i), but, no significant difference was found when using pyruvate plus malate. MAS activity in skeletal muscle and brain mitochondria was halved in Aralar−− mice and was extremely low in Aralar−− mice (Fig. 1F). Aspartate formation from glutamate and malate by isolated brain mitochondria was reduced accordingly: 100 ± 4.2, 69.2 ± 5.1, and 16.0 ± 2.0 nmol per min/mg of protein in Aralar+/+, Aralar−−, and Aralar−− mice, respectively.

General Phenotype of Aralar−− Mice—As shown in Fig. 2A, Aralar−− mice were smaller than Aralar+/+ and Aralar−− mice.
mice from 6 days of age and growth-retarded thereafter. Their life expectancy was greatly reduced: almost none survived beyond 22 days, and they had a 59% weight reduction at death (Fig. 2A). At 10 days, all the tissue weights of Aralar-/- mice were significantly lower than those of Aralar +/- and Aralar++ mice, which were the same (Fig. 2B).

Behavioral Analysis—In the wire hanging test that evaluates neuromuscular strength (23), Aralar++ mice gradually increased the time to fall between 10 and 16 days, with a clear overshoot in performance from then onwards. Aralar-/- mice behaved as Aralar++ mice until 14 days, but showed a much smaller increase in performance thereafter (Fig. 2C). In the bar test that examines balance coordination and neuromuscular strength (24), Aralar++ mice increased the time on the bar in a linear way from 13 days onwards, a time at which motor coordination is established (23), whereas Aralar-/- mice progressed only till 14 days and stopped or even decreased thereafter (Fig. 2D). Disability of muscle coordination and tremor were apparent beyond 16–17 days in Aralar-/- mice. It became more and more severe with age so that by 19–21 days mice showed haunched posture (Fig. 2E), and convulsions.

Histological and Immunocytochemical Observation—Histological analysis of the central nervous system in Aralar++ and Aralar-/- mice at 18–20 days showed a prominent hypomyelination throughout the myelinated areas of the central nervous system, such as the corpus callosum, white matter of the cerebral cortex, anterior commissure, internal capsule, and pyramidal tract in Aralar-/- mouse (Fig. 3, A and B). However, no change in the neuronal numbers, morphology, or cytoarchitecture was observed either in the brain (Fig. 3, C and D) or the spinal cord (results not shown), except that Aralar-/- mice had enlarged lateral ventricles (Fig. 3B). Immunocytochemical study using the antibody to MBP confirmed the results of the myelin staining, showing a decrease in the intensity and numbers of the immunostained fibers throughout the myelinated regions of the central nervous system (Fig. 3, E and F).

Analysis of Myelin Proteins and Lipid Components—Western blot analysis and quantification of the brain proteins during development are shown in Fig. 4. The results revealed significantly lower levels of the myelin-specific proteins, MBP and MOBP, in the brain of Aralar-/- mice at 20 days of age (about 40% of Aralar++ mice), but no significant differences in the Coomassie Brilliant Blue staining pattern and in the neuron- or glia-specific proteins, GFAP and NF-M, respectively.

Brain lipids were analyzed by high-performance thin-layer chromatography (Fig. 5A). There was a clear decrease in the major myelin-specific lipid, galactocerebroside, in the brain from Aralar-/- mice (about 40% decrease, as compared with Aralar++ levels), and differences in other non-myelin-specific lipids, such as cholesterol, phosphatidylethanolamine, and phosphatidylcholine, were very small, if any. On the other hand, there was no such difference in the lipids from sciatic nerve (Fig. 5A), indicating that the defect is restricted to the central nervous system. The results of high-performance thin-layer chromatography were confirmed by further quantitative analysis of the brain lipids by determination of galactose, phosphorous, and cholesterol contents (Fig. 5B).

NAA in the Brain and Cultured Neurons—NAA content increased markedly in the brain of Aralar++ mice from 10 to 20 days, whereas it remained much lower in Aralar-/- mice, at 36 and 29% of Aralar++ values at 10 and 20 days, respectively (Fig. 6A). Changes in the related metabolite, N-acetylaspartylglutamate, were much smaller. N-Acetylaspartylglutamate levels decreased in the brain from Aralar++ mice between 10 and 20 days (610 ± 60 and 330 ± 10 nmol/g brain, respectively), and in Aralar-/- mice, remained at about 60 and 80% of...
Aralar−/− mice at 19 days. A and B, myelin stained sections of the brain of the mice according to the method of Heidenhain-Woelcke, showing a hypomyelination in the corpus callosum (CC), white matter of the cerebral cortex, and caudate-putamen area in the Aralar−/− mouse (A). Aralar−/− mouse has enlarged lateral ventricles (LV). AC denotes anterior commissure. Scale = 0.5 mm. C and D, Nissl-stained sections of the motor cortex of the mice, suggesting no gross changes in the neuronal numbers, morphology, or cytoarchitecture between the Aralar+/+ (C) and Aralar−/− (D) mouse. I and WM denote layer I and white matter, respectively. Scale = 50 μm. E and F, myelin basic protein-like immunostained sections, showing a decrease in the intensity and numbers of the immunopositive fibers distributed through the cingulate bundle (bottom) to the motor cortex (upper) in the Aralar−/− (F) as compared with the Aralar+/+ mouse (E). Scale = 50 μm.

Aralar+/+ values, respectively. On the other hand, the concentration of aspartate was very low in the brain of Aralar−/− mice; about 11 and 6% of those of Aralar+/+ mice at 10 and 20 days, respectively (Fig. 6B).

We tested whether NAA synthesis was impaired in cultured cortical neurons derived from Aralar−/− mice. As shown in Fig. 6C, NAA levels were markedly reduced in cultured neurons from Aralar−/− mice. Therefore, the results indicate that the decrease in brain NAA levels is because of a reduced NAA synthesis in aralar-deficient neurons.

DISCUSSION

As expected from the essential role of AGC in the malate-aspartate NADH shuttle, we have found that the Aralar−/− mice first established showed a total lack of MAS activity in mitochondria from brain and muscle, two tissues that have only aralar as AGC isoform, a very drastic fall of respiration with glutamate plus malate, and a great reduction in aspartate efflux from mitochondria. The lack of any detectable neuronal loss in Aralar−/− mice indicates that MAS is not essential for survival of neurons.

Based on the overlap of aralar and citrin expression in fetal tissues (8, 10), we anticipated that Aralar−/− mice would be viable throughout embryogenesis. Indeed, Aralar−/− mouse embryos are produced in normal numbers; however, soon after birth many Aralar−/− mice develop severe growth defects and neuromuscular deficiencies. Along with that, Aralar−/− mice have a reduced myelination in the central nervous system, but not in peripheral nerves. The dysmyelination is associated with a drastic and central nervous system-restricted reduction of myelin lipids, particularly galactocerebroside, accompanied by similar reductions in the levels of the major proteins in myelin, MBP, and MOBP. This combined reduction in galactocerebroside and myelin protein suggests decreased function of oligodendrocytes.

Myelin deficiencies, such as that in the shiverer mouse,
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caused by mutations in the MBP gene, are accompanied by reductions in myelin proteins and lipids, particularly galactolipids (33). The only mouse mutants with a defect in a specific myelin lipid component known so far are mice with targeted disruption of the enzymes responsible for galactolipid synthesis, UDP-galactose:ceramide galactosyltransferase (34, 35), mice deficient in cerebroside sulfotransferase (36), and mice with targeted disruption in squalene synthase, the enzyme of cholesterol biosynthesis, in oligodendrocytes (37). The marked phenotype of the ceramide galactosyltransferase knock-out mouse, with small body weights, tremor, and locomotion defects, and early death, between 18 and 30 days, is comparable with that of the shiverer or jimpy mouse mutants (34, 35, 38), whereas that of cerebroside sulfotransferase mice is milder (36). However, in ceramide galactosyltransferase knock-out mice, myelination is normal, but the properties of this mutant myelin are altered, and nerve conduction velocity is drastically decreased (34, 35), possibly because galactolipids are necessary for proper axo-glial interactions (39). On the other hand, the lack of sphingomyelin in oligodendrocytes causes a very drastic hypomyelination in white matter of the central nervous system with ataxia and tremor in mutant mice at 20 days, showing that oligodendrocyte-formed cholesterol is essential in myelin formation, as these defects were only slowly reversed along many months of postnatal life (37).

Aralar–/– mice have a neurological phenotype comparable with that of the ceramide galactosyltransferase knock-out or shiverer mouse, and more severe than that of the cerebroside sulfotransferase or squalene synthase mice. It is important to note that this Aralar–/– phenotype is associated with impaired synthesis of myelin proteins and lipids, particularly galactolipids. Whether myelin structure and/or composition are defective, or whether other defects in addition to reduced myelin formation are involved in the phenotype remains to be established.

There is long standing evidence that NAA plays a role in myelin lipid formation through the supply of acetyl groups (14–16, 40, 41). NAA produced in neurons (42, 43) undergoes transaxonal transfer to oligodendrocytes where it supplies acetyl groups for the synthesis of myelin lipids. The NAA cleavage enzyme, aspartoacylase, is restricted primarily to oligodendrocytes (44, 45). A number of lipid components of myelin including ceramide and cerebrosides, but not cholesterol, are preferentially labeled by [14C]NAA rather than from [14C]acetate in the optic nerve (15), cerebrosides showing a particularly high preference toward [14C]NAA in the brain (14). Mutations in aspartoacylase cause Canavans disease, characterized by a spongy degeneration of the central nervous system, increased levels of NAA in the brain and body fluids, and an extensive loss of myelin (46). Aspartoacylase-deficient mice, generated as model for Canavans disease (47), show decreased myelin lipid synthesis, 35–38% reductions in the levels of myelin-associated polar lipids, and a very drastic (about 80%) reduction in the brain acetate concentrations (48), showing that the lack of NAA results in a lack of acetyl groups for myelin lipid synthesis. A reduction in the levels of galactocerebroside and sulfatide was also observed in the brain of a Canavans disease patient (48).

We have found that aralar deficiency results in a drastic drop of NAA and aspartate in the brain and neurons from Aralar–/– mice, together with a drop in myelin lipids, particularly galactolipids, similar to that found in Canavans disease (48). The fall in brain aspartate levels and the lack of aspartate production in brain mitochondria from Aralar–/– mice demonstrate that the major route of aspartate production in the central nervous system is mitochondrial and it depends on aralar for aspartate efflux to the cytosol. Neurons are the brain cells with highest aralar expression (10), and neuronal aralar emerges as responsible for brain aspartate synthesis. NAA is derived from neuronal aspartate, and its synthesis is catalyzed by aspartate-N-
acetyltransferase in brain mitochondria (11, 12) and microsomes (13). Thus, the fall in NAA in the brain and cultured neurons from Aralar−/− mice is clearly associated with the defect in aspartate production. The high activity of the aspartate-N-acetyltransferase microsomal system, about 4–5 higher than the mitochondrial one (13), explains why aspartate efflux from mitochondria is necessary for neuronal NAA synthesis. Taken together, our results suggest that impaired NAA formation in neurons from Aralar−/− mice is responsible for the defects in central nervous system myelination observed in these animals. On the other hand, the absence of defects in myelin lipids in the peripheral nervous system of Aralar−/− mice is explained by the fact that NAA is probably not a myelin lipid precursor in the peripheral nervous system, because aspartoacylase is restricted primarily to myelin-synthesizing cells in the central nervous system, but not present in Schwann cells (45).

Our results provide the first indications of the role of the brain AGC, aralar, in NAA synthesis and myelin formation. This is important, because NAA contributes the most prominent signal in proton magnetic resonance spectroscopy of human brain but its role is still controversial (16, 49, 50). On the other hand, aralar deficiency may contribute to reduced myelin formation in neurons from SK/CAM mice (45).

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10. Very recently, Ramoz et al. (52) reported a strong linkage and association of the SLC25A12 gene with autism, although the functional relevance of the polymorphisms associated with the disease is yet unknown. NAA content appears to be reduced in certain brain regions of autistic patients (53–55). Studies with viable aralar−/− mice may be very important to address this issue.

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