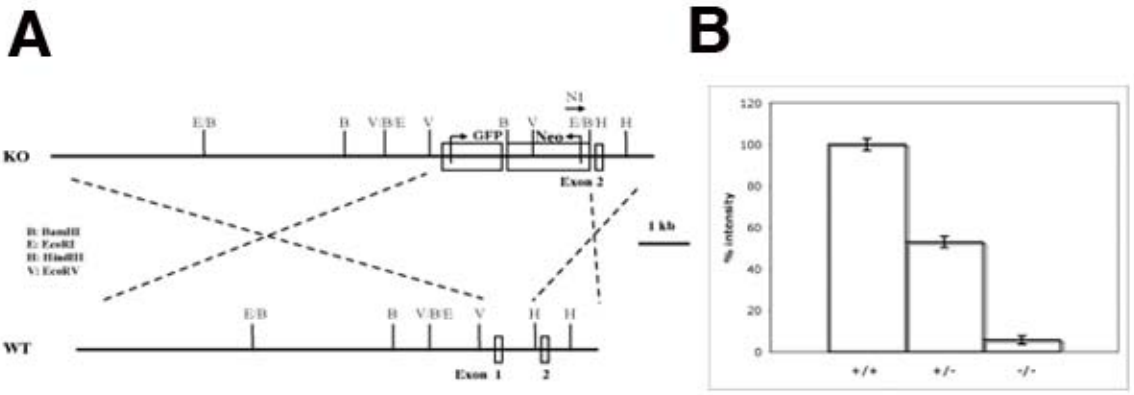
A
B
KO
WT
GFP
Neo
Exon 2
Exon 1
1 kb
% intensity
+/+
+/-
-/-

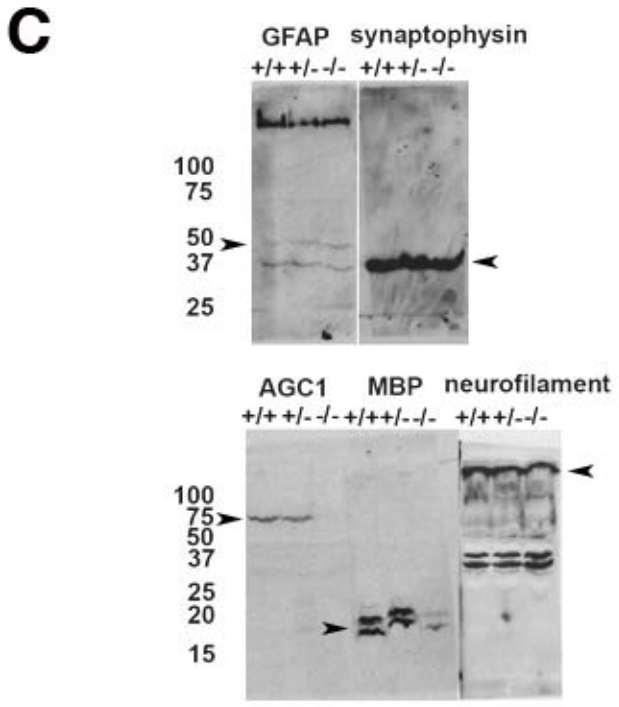
C
GFAP
synaptophysin
AGC1
MBP
neurofilament
100
75
50
37
25
20
15

Figure 1

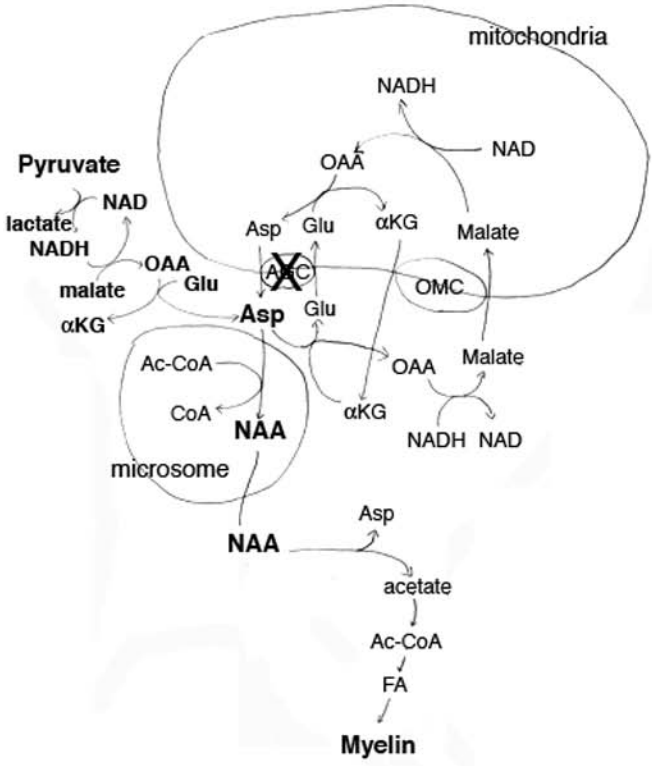
mitochondria
NADH
NAD
OAA
Pyruvate
NAD
lactate
NADH
Asp
Glu
αKG
Malate
OAA
AGC
OMC
malate
Glu
αKG
Asp
Glu
Ac-CoA
OAA
Malate
CoA
αKG
NAA
NADH
NAD
microsome
Asp
NAA
acetate
Ac-CoA
FA
Myelin

Figure 2

Supplemental Materials

Materials and Methods

Development of C57BL Slc25a12-knockout mice

All animal studies were approved by the relevant Institutional Animal Care and Use Committees (IACUC), at the Mount Sinai School of Medicine, the James J. Peters Veterans Affairs Medical Center, and/or the Memorial Sloan Kettering Cancer Center (MSKCC). A ~9.8kb fragment used to construct the targeting vector was first subcloned, using the Red/ET recombineering technique (Gene Bridges), from a C57BL/6-derived BAC clone that includes genomic DNA encompassing the murine *Slc25a12* gene (Fig. 1). The region was chosen such that a short homology arm would extend 2 kb downstream of exon 1 and a long homology arm (7.2 kb) would start at the 5' end of exon 1, before the ATG. A *GFP/neo* cassette was inserted before the ATG of exon 1 and replaces ~560 bp of the gene including part of exon 1. The targeting construct was sequenced to confirm correct genomic organization. The linearized targeting vector was electroporated into C57BL/6-derived ES cells and correctly targeted ES cell clones were identified and used to develop chimeric mice, which were then mated with C57BL/6Tac females. F1 mice were screened to identify heterozygotes that were used to develop the *Slc25a12*-deficient line. Mice for experimentation were obtained from either heterozygote x heterozygote matings or heterozygote x wild type matings, always using littermates as controls.

Genotyping

For the wild-type allele, PCR primers that recognize the region in exon 1 deleted in the *Slc25a12*-deficient mice were used (5'-agcatggcggtcaaggtgact and 5'-catcttggcgacccttctcc, with a 65^{o}C annealing temperature). For the disrupted allele, primers that identify the presence of the *GFP* gene were used (5'-aagttcatctgcaccaccg and 5'-tccttgaagaagatggtgcc, with a 60^{o}C annealing temperature).

Biochemical analyses

For immunoblotting, brains were homogenized in extraction buffer (62.5 mM Tris.Cl, pH 6.8, 5% β-mercaptoethanol, 100 mM DTT, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 50 μg/ml TLCK, 8 ml extraction buffer per 1 g tissue) at 0.5^{o}C. Subsequently, 10% SDS was added to a final concentration of 2% and homogenates were boiled for 10 min. After centrifugation, protein concentration was measured by the BCA assay (Pierce) and equal amounts of protein were subjected to SDS-PAGE (Invitrogen). Immunoblotting was performed using primary antibodies, followed by secondary antibodies conjugated with alkaline phosphatase (Sigma). Bands were visualized by BCIP/NBT and quantified by densitometry. For some blots, chemiluminescence detection system was used (Pierce). Primary antibodies used were: rabbit anti-AGC1 (a generous gift of Dr. Araceli del Arco), mouse anti-myelin basic protein (MBP) (Covance), mouse anti-neurofilament (NF) (Covance), rabbit anti-synaptophysin (Chemicon), and rabbit anti-glial fibrillary acidic protein (GFAP) (Dako).

MAS activity was assayed as previously reported (1). Briefly, mitochondrial fractions were obtained from brains and MAS was reconstituted in the presence of aspartate aminotransferase, malate dehydrogenase, NADH, aspartate, malate, ADP, ruthenium red, and $CaCl_2$. The reaction was started by the addition of glutamate and was followed by the decay in NADH fluorescence using a Fusion Universal Microplate Analyzer (PerkinElmer).

Gene expression

Brains were cut midsagittally and stored at -80°C until use. Total RNA was isolated from the right half of the brain and cDNA was prepared using High-Capacity cDNA Archive Kit (Applied Biosystems). The mRNA levels of the myelin genes, *Cldn11*, *Cnp1, Mag, Mobp, Olig2, Plp1, Qk5/6*, *Sox10, and Erbb4* were measured by qPCR using TaqMan MGB probes and primer sets (Applied Biosystems). Four housekeeping genes (*Ppia, Actb, Gusb*, and *B2m*) were also analyzed as a method of normalization using qBase (2); of these, the geometric mean expression of three housekeeping genes (*Actb*, *Gusb* and *B2m*) were selected by qBase as optimal for control reference genes.

Slice cultures

Littermates (P10) from heterozygote x heterozygote matings were used to prepare cerebellar slice cultures. 400 μm-thick sagittal cerebellar slices were cut with a tissue chopper (McIlwain) and the largest slices, prepared from the mid portion of the cerebellum, were placed on Millicell filter inserts (0.4 μm, Millipore; 4-5 slices per filter) in 6-well plates (Falcon) (3). 1.2 ml of medium (BME with 10% horse serum, Invitrogen) was added to the cultures and the medium changed every other day. Where indicated, pyruvate (Invitrogen) was added to a final concentration of 5 mM (note that BME does not contain pyruvate nor aspartate). Cultures were fixed with 4% paraformaldehyde at day 7 *in vitro* and processed for immunohistochemistry. Cultures were stained with rabbit anti-MBP (Chemicon) and mouse anti-calbindin (Sigma), followed by secondary antibodies (Jackson Immuno Laboratories), mounted on slides in fluoromount-G (Southern Biotech) and analyzed by the fluorescence microscopy.

We chose the same lobes for quantification to avoid potential regional differences in myelin formation. Purkinje cell axons were identified by calbindin staining, and the degree of myelination of those axons quantified with MBP staining (from Purkinje cell bodies to white matter in the middle of the lobe, but excluding the white matter) in a blinded manner. By switching the channels back and forth, axons were identified and if any MBP positive segments were observed, those axons were scored as myelin positive. Scoring was done on a scale of 1 through 5, where a 1 indicated that 0-20% of axons in the field were myelin positive, and 5 indicated that 80-100% of axons were myelin positive. We averaged the score for each well, and pooled these data from 3 experiments for statistical analyses.

OPC cultures

Rat oligodendrocyte progenitor cell (OPC) cultures were prepared by shaking mixed cortical cell cultures prepared from rat neonatal pups as described previously (4). OPCs (2×10^6 cells) were nucleofected with shRNA constructs

(Mission shRNA plasmid DNA, selected from Sigma Mission TRC shRNA libraries, shdnac-trcn000006991) with pmaxGFP (Amaxa), shRNA scrambled DNA with pmaxGFP, or an shRNA construct with the *Slc25a12* gene fused with EGFP at the N-terminus (5) (using a total 3 μg of DNA at a 1:1 ratio) using the rat oligodendrocyte kit (VPG-1009, Amaxa) following the manufacturer's protocol (program number A-033 in the Amaxa nucleofector). After nucleofection, OPCs were plated onto poly-D-lysine-coated MatTek glass bottom 35-mm plastic dishes (1×10^6 cells) in proliferation medium for 2 days, and then switched to differentiation medium (day 2). Note that the culture media requires DMEM with high glucose that contains 1 mM pyruvate (although DMEM does not contain aspartate, the medium can supply pyruvate that can be converted to aspartate, a material for myelin lipid synthesis, see Fig. 2). Cultures were fixed with 4% paraformaldehyde and immunostained with anti-MBP antibody (Calbiochem) followed by secondary antibody conjugated with Cy3 (Jackson). Cultures were analyzed by confocal microscopy in a blinded manner. GFP-positive cells were identified and analyzed for morphology and then, classified into no, low, or high MBP expressers. Cell numbers were counted in 3 randomly chosen fields per dish. In parallel, 1×10^6 cells were plated into 1 well of a 6-well plate (coated with poly-D-lysine as above) and used for gene expression analysis. Cells in one well were washed with PBS and lysed in SideStep lysis buffer (Stratagene). After DNAse treatment, 2.5 μl of lysate (~10^4 cells/μl) were used for qRT-PCR reactions (Stratagene). The probe used was a rat *Slc25a12* TaqMan probe (Applied Biosystems) and data were analyzed by qBASE as above. Three housekeeping genes (*Pp1a, B2m,* and *Gapdh*) were used as the endogenous reference genes and the experiments were performed in triplicates.

Histology

Littermates (P13-14) from heterozygote x heterozygote matings were perfused with 4% paraformaldehyde and brains were dissected out and further fixed in 4% paraformaldehyde. Sagittal and coronal sections (40 μm-thick) were prepared on a Vibratome and immunostained for MBP (SMI-94, Covance),

proteolipid protein (PLP, gift of Dr. Robert A. Lazzarini), calbindin (Chemicon), the light (L), mid-sized (M) and heavy (H) NF proteins (all from Chemicon), RMO108 (gift of Dr. Virginia Lee), and SMI-31 (Covance). Secondary antibodies used were either fluorescently labeled or HRP-conjugated and visualized either by fluorescence microscopy or by bright field microscopy following DAB staining. Some of the immunofluorescent slides were analyzed and imaged using a Zeiss LSM410 inverted laser scanning confocal microscope with Zeiss-Plan-Neofluar objectives and an ArKr laser.

Brain MRI analysis

Heterozygotes and wild type controls (male heterozygotes, 6, wild type, 6; female heterozygotes 7, wild type 6) were analyzed by brain MRI. All MR imaging was performed on a Bruker Biospec 47/40 scanner at 4.7T using ParaVision 3.0.2 console software (Bruker Biospin MRI Inc., Billerica, MA) at MSKCC. A custom-made 32 mm (ID) quadrature birdcage volume coil (Stark contrast MRI coils research, Erlangen, Germany) was used for both excitation and detection. The mouse was laid prone inside the coil under anesthesia with 1.5% isofluorane. Fast spin-echo RARE (Rapid Acquisition with Relaxation Enhancement) scout images, with RARE factor 8, TR 3.6 s, and TE 56 ms, in 3 orthogonal orientations were first acquired to locate the region of interest. T2-weighted mouse brain coronal images were then scanned using a T2 RARE method with a slice thickness of 0.7 mm, FOV of 3 x 2 cm, 256x128 matrix, TR/TE 3.5 s/50 ms, 40 averages and a total scanning time of 38 minutes. Image processing was done off-line using XTIP image display software suite (Bruker Biospin MRI Inc., Billerica, MA). The brain regions of interest were manually traced and the areas were measured. The total volume for each region was expressed as the sum of the ROI area for each slice multiplied by the slice separation.

Statistical analysis

All data represent mean and standard error of the mean (SEM) for 3 or more experiments. When applicable, Student's *t* tests were used for statistical analysis comparing two groups.

Supplemental figure legends

Figure 1. *Slc25a12*-knockout mice do not express AGC1.

A. Genomic organization of the murine *Slc25a12* gene and the targeted allele. The ATG start codon is located in exon 1, which is replaced, in the targeted allele, by a *GFP/Neo* fusion cassette. White boxes with numbers represent exons and restriction enzyme sites (BamHI, EcoRI, HindIII, EcoRV) are indicated. The arrow indicates the N1 sequencing primer at the 5' of the neo cassette that was used for screening of the mutated allele. KO; targeted allele, WT; wild type allele. B. Brain extracts from wild type (+/+), heterozygous (+/-), and homozygous (-/-) mice (P10) were subjected to quantitative immunoblotting using an anti-AGC1 antibody. The Y-axis indicates percent expression relative to wild type. Values in the knockout are at background level. C. Brain extracts from wild type (+/+), heterozygous (+/-), and homozygous (-/-) mice were subjected to immunoblotting using anti-GFAP, anti-synaptophysin, anti-AGC1, anti-MBP, and anti-neurofilament antibodies. Amounts of GFAP (~50kDa), synaptophysin (~34kDa), and neurofilament H (~200kDa) are similar among all genotypes, whereas AGC1 (~70kDa) and MBP (~18-20kDa) are not detectable or very low in the homozygotes, as compared to wild type mice or heterozygotes. There is also reduced expression of AGC1 in heterozygotes, as compared to homozygotes.

Figure 2. Metabolic map of potential effects of pyruvate.

In *Slc25a12*-knockouts, no aspartate (Asp) is available in the cytoplasm for production of NAA,and there is a higher $NADH/NAD^+$ ratio in the cytoplasm, and lower $NADH/NAD^+$ ratio in mitochondria. Administration of pyruvate reduces the $NADH/NAD^+$ ratio, while also providing a substrate for the production of Asp that is metabolized into NAA to supply sources for myelin lipid formation.

References

1. Jalil, M.A., et al., *Reduced N-acetylaspartate levels in mice lacking aralar, a brain- and muscle-type mitochondrial aspartate-glutamate carrier.* J Biol Chem, 2005. **280**(35): p. 31333-9.
2. Hellemans, J., et al., *qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data.* Genome Biol, 2007. **8**(2): p. R19.
3. Dunaevsky, A., et al., *Developmental regulation of spine motility in the mammalian central nervous system.* Proc Natl Acad Sci U S A, 1999. **96**(23): p. 13438-43.
4. McCarthy, K.D. and J. de Vellis, *Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue.* J Cell Biol, 1980. **85**(3): p. 890-902.
5. Lepagnol-Bestel, A.M., et al., *SLC25A12 expression is associated with neurite outgrowth and is upregulated in the prefrontal cortex of autistic subjects.* Mol Psychiatry, 2008. **13**(4): p. 385-97.