Slc25a12 Disruption Alters Myelination and Neurofilaments: A Model for a Hypomyelination Syndrome and Childhood Neurodevelopmental Disorders

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Background: *SLC25A12*, a susceptibility gene for autism spectrum disorders that is mutated in a neurodevelopmental syndrome, encodes a mitochondrial aspartate-glutamate carrier (aspartate-glutamate carrier isoform 1 [AGC1]). AGC1 is an important component of the malate/aspartate shuttle, a crucial system supporting oxidative phosphorylation and adenosine triphosphate production.

Methods: We characterized mice with a disruption of the *Slc25a12* gene, followed by confirmatory in vitro studies.

Results: *Slc25a12*-knockout mice, which showed no AGC1 by immunoblotting, were born normally but displayed delayed development and died around 3 weeks after birth. In postnatal day 13 to 14 knockout brains, the brains were smaller with no obvious alteration in gross structure. However, we found a reduction in myelin basic protein (MBP)-positive fibers, consistent with a previous report. Furthermore, the neocortex of knockout mice contained abnormal neurofilamentous accumulations in neurons, suggesting defective axonal transport and/or neurodegeneration. Slice cultures prepared from knockout mice also showed a myelination defect, and reduction of *Slc25a12* in rat primary oligodendrocytes led to a cell-autonomous reduction in MBP expression. Myelin deficits in slice cultures from knockout mice could be reversed by administration of pyruvate, indicating that reduction in AGC1 activity leads to reduced production of aspartate/*N*-acetylaspartate and/or alterations in the dihydronicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide^{+} ratio, resulting in myelin defects.

Conclusions: Our data implicate AGC1 activity in myelination and in neuronal structure and indicate that while loss of AGC1 leads to hypomyelination and neuronal changes, subtle alterations in AGC1 expression could affect brain development, contributing to increased autism susceptibility.

Key Words: Malate/aspartate shuttle, mitochondria, *N*-acetylaspartate (NAA), neuron-oligodendrocyte interactions, pyruvate

Autism spectrum disorders (ASDs) are neurodevelopmental disorders with strong genetic components (1,2). *SLC25A12* (solute carrier family 25 member 12) is a gene on chromosome 2q31 that was identified as an autism susceptibility gene through both linkage and association studies (3). Recently, homozygous mutations in *SLC25A12* have been reported in a patient with seizures, severe hypotonia, and arrested psychomotor development with global hypomyelination (4). *SLC25A12* encodes the calcium ion (Ca^{2+})-dependent mitochondrial aspartate-glutamate carrier isoform 1 (AGC1), which is expressed in brain and skeletal muscle. A peripheral AGC isoform, called aspartate-glutamate carrier isoform 2 (AGC2) (encoded by *SLC25A13* on chromosome 7q21), is mainly expressed in liver, kidney, and heart. Aspartate-glutamate carrier isoform 1 and AGC2 function in the transport of aspartate from the mitochondrial matrix to the intermembrane space in exchange for glutamate and represent a component of the malate/aspartate shuttle (MAS), a crucial pathway that supports oxidative phosphorylation to produce adenosine triphosphate (ATP) by the transport of dihydronicotinamide adenine dinucleotide (NADH)-reducing equivalents into the mitochondrial matrix (5).

We and others have reported linkage of the 2q31 region to ASDs, and two single nucleotide polymorphisms (SNPs) of the *SLC25A12* gene—one immediately upstream of alternately spliced exon 4 (rs2056202) and one in the small intron between exons 16 and 17 (rs2292813)—have been shown to be associated with ASDs in two and three of six studies, respectively (3,6–11). In each of the positive associations, the effect was in the same direction, providing very strong evidence for replication. A follow-up study to the first association study indicated that one of the SNPs (rs2056206) was associated with the levels of routines and rituals in ASDs (12), supporting a functional role for these SNPs in AGC1 activity. The *SLC25A12* gene is expressed in developing human brain (13) and is expressed about 1.5 fold higher in individuals with ASDs in the dorsolateral frontal cortex in postmortem samples (13). An increase in AGC1 activity was also reported in postmortem samples, a finding that was attributed to altered calcium levels (10).

Based on these findings, we hypothesized that genetic alterations in *SLC25A12* and/or other mechanisms that alter AGC1 activity will affect neurodevelopment, resulting in phenotypes that can contribute to disorders such as ASDs. To gain insight into

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the possible mechanisms by which *SLC25A12* might affect neurodevelopment, we generated knockout mice with a disruption of *Slc25a12*. We analyzed *Slc25a12*-knockout mice from histological, molecular, and cell biological perspectives.

Methods and Materials

Development of *Slc25a12*-knockout mice, genotyping, and brain magnetic resonance imaging (MRI) analysis, as well as more detailed protocols for the methods summarized below, are found in Supplement 1.

Biochemical Analyses

Immunoblotting on brain extract was performed using standard methods using alkaline phosphatase (Sigma, St. Louis, Missouri) or horseradish peroxidise (HRP) (Jackson ImmunoResearch, West Grove, Pennsylvania) conjugated secondary antibodies.

The messenger RNA levels of the myelin genes, *Cldn11, Cnp1, Mag, Mobp, Olig2, Plp1, Qk5/6, Sox10, and Erbb4,* were measured by quantitative polymerase chain reaction using TaqMan MGB probes and primer sets (Applied Biosystems, Foster City, California) on RNA prepared from brains as described in Supplement 1.

Slice Cultures

Littermates (postnatal day [P]10) from heterozygote matings were used to prepare cerebellar slice cultures as described in Supplement 1. Following treatment, cultures were fixed with 4% paraformaldehyde at day 7 in vitro and processed for immunohistochemistry using rabbit anti-myelin basic protein (MBP) (Millipore, Billerica, Massachusetts) and mouse anti-calbindin (Sigma) and analyzed by the fluorescence microscopy.

Oligodendrocyte Precursor Cell Cultures

Oligodendrocyte precursor cells (OPCs) prepared from rat brain were nucleofected using the rat oligodendrocyte kit (VPG-1009, Amaxa, Cologne, Germany) following the manufacturer's protocol. After nucleofection, OPCs were plated in proliferation medium for 2 days and then switched to differentiation medium (day 2). Cultures were fixed with 4% paraformaldehyde and immunostained with anti-MBP antibody (Calbiochem, San Diego, California) followed by secondary antibody conjugated with Cy3 (Jackson ImmunoResearch). Cultures were analyzed by confocal microscopy in a blinded manner.

Histology

Sagittal and coronal sections (40 μm-thick) were prepared on a vibratome and immunostained with indicated antibodies. Secondary antibodies used were either fluorescently labeled or HRP-conjugated and visualized either by fluorescence microscopy or by bright field microscopy following 3,3'-Diaminobenzidine staining.

Statistical Analysis

All data represent mean and standard error of the mean (SEM) for three or more experiments. When applicable, Student *t* tests were used for statistical analysis comparing two groups.

Results

Developmental Abnormalities in *Slc25a12*-Knockout Mice

To clarify the function of AGC1 in brain, we generated mice with a disruption of the *Slc25a12* gene. The *Slc25a12* gene in mice consists of 18 exons, spread over ~100 kilobase (kb). We modified a mouse genomic DNA fragment by replacing exon one with a neomycin-resistance/green fluorescent protein cassette (Figure S1A in Supplement 1). The targeting construct was introduced into C57BL/6 embryonic stem cells and targeted clones were used to establish a mouse line carrying the disrupted allele on the C57BL/6Tac genetic background. Matings between heterozygous animals produced wild-type, heterozygous, and knockout progeny with apparently Mendelian frequencies (27%, 52%, and 21%, respectively, in 66 pups from 10 litters). AGC1 levels were below the level of detection in homozygous knockout mice, while in heterozygous mice, AGC1 levels were about half of that found in wild-type animals (Figure S1B, C in Supplement 1). Measurement of MAS activity using mitochondrial fractions prepared from P10 knockout brains showed very low MAS activity (wild-type, 14 ± .1 absorbance

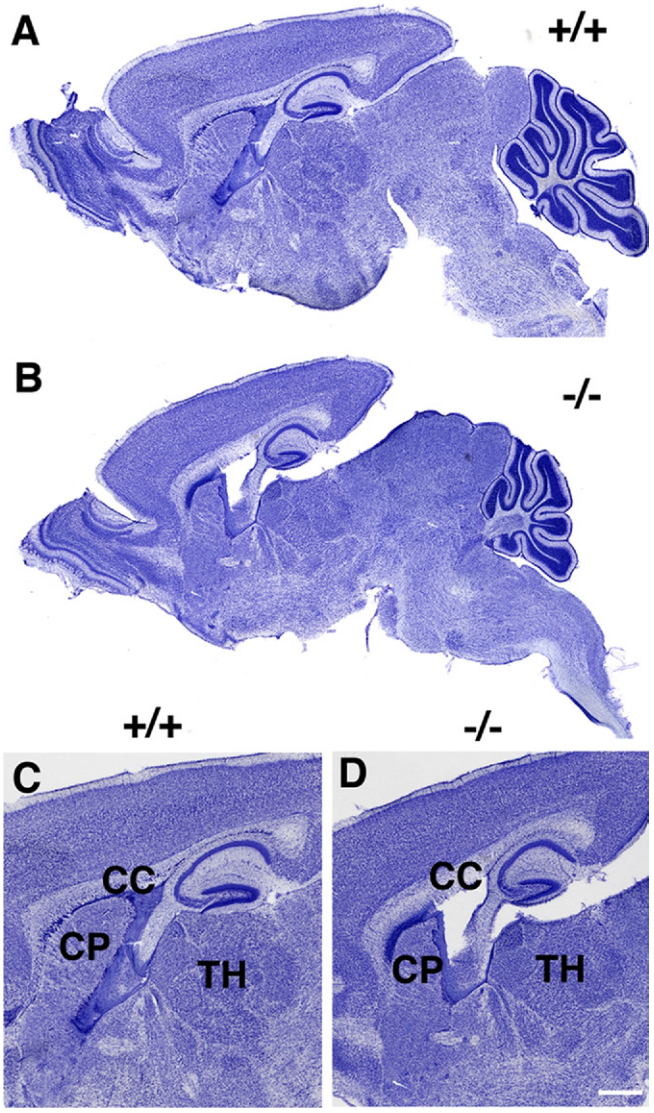

Figure 1. *Slc25a12*-knockout mice have smaller brains. Sagittal sections of postnatal day 13 brain from wild-type (+/+) **(A, C)** or knockout (−/−) **(B, D)** mice are shown stained with cresyl violet. Note the generally smaller size of all brain regions in the knockout animal. Panels **(C)** and **(D)** show higher power views of the images in **(A)** and **(B)**, with the corpus callosum (CC), caudate putamen (CP), and thalamus (TH) indicated. Scale bar: **(A)** and **(B)**, 750 μm; **(C)** and **(D)**, 500 μm.

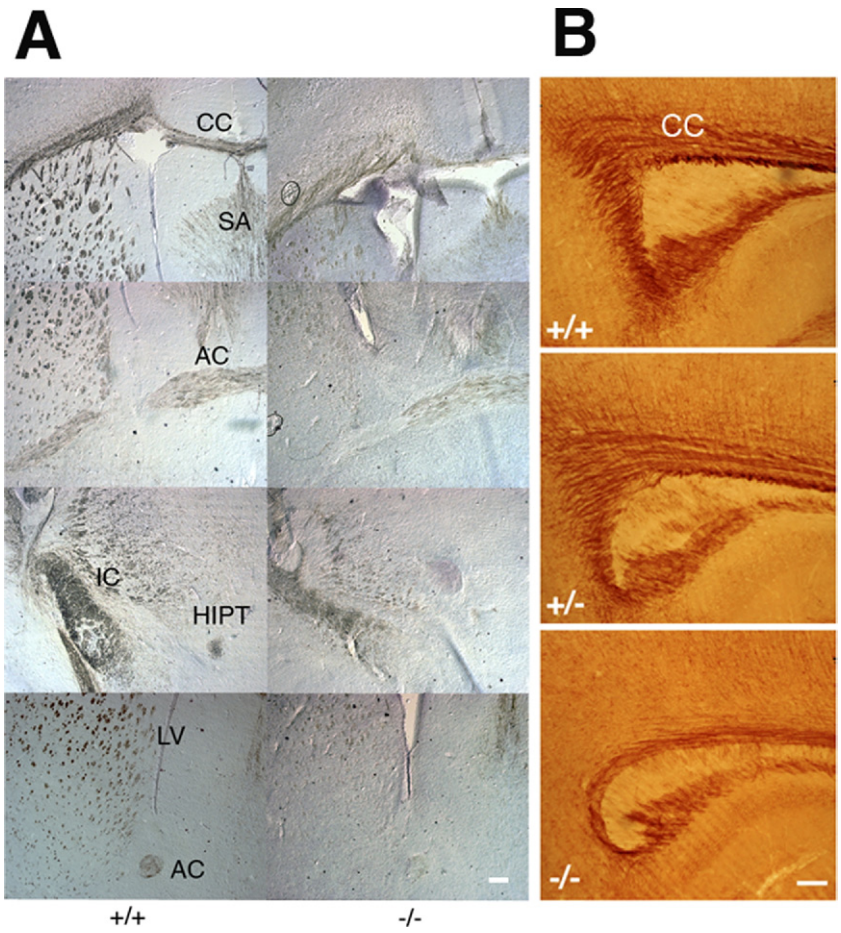

Figure 2. *Slc25a12*-knockout mice show myelination deficits. **(A)** Coronal sections through frontal cortex from postnatal day 14 wild-type (+/+) and knockout (−/−) brains were stained with anti-MBP antibody followed by HRP-conjugated secondary antibody visualized by the DAB reaction. **(B)** Sagittal sections from postnatal day 13 wild-type (+/+), heterozygous (+/−), and knockout (−/−) brains were stained with an anti-PLP antibody. Scale bars, 200 μm. AC, anterior commissure; CC, corpus callosum; DAB, 3,3′-Diaminobenzidine; HIPT, habenulo-interpeduncular tract; HRP, horseradish peroxidise; IC, internal capsule; LV, lateral ventricles; MBP, myelin basic protein; PLP, proteolipid protein; SA, septal area.

change/min/g; knockout, 1.4 ± .1 absorbance change/min/g; n = 3 per group), indicating that this pathway was functionally disrupted in the knockouts.

The knockouts were indistinguishable through the first few days of life from wild-type or heterozygous animals, but around P7, knockouts could be identified by their smaller size with continuing growth retardation. At P13 to P14, the average body weight of wild-type animals was 7.60 g ± .73 g (mean ± SEM; n = 8) and 8.24 g ± 1.06 g for heterozygotes (n = 17) (p = .068 compared with wild-type), whereas that of the knockouts was 3.79 g ± .74 g (n = 12) ($p = 1.35 \times 10^{-9}$ compared with wild-type and $p = 9.9 \times 10^{-15}$ compared with heterozygotes). Knockouts showed a wide-based, ataxic gait, as well as tremor, and died around 3 weeks postnatally. No knockouts survived by 4 weeks after birth.

Myelin and Neuronal Abnormalities in *Slc25a12*-Knockout Mice

We analyzed P13 to P14 knockout and control brains by histology and immunohistochemistry and found four major alterations in *Slc25a12*-knockout mice. Cresyl violet and hematoxylin-eosin staining revealed reduction of brain size, consistent with a previous report (14) (Figure 1). This reduction in size was observed in almost all brain structures, including hippocampus, striatum, and thalamus (Figure 1C, D), suggesting global neurodevelopmental delay. However, other than brain size, we did not find any obvious gross histological differences between knockout and control mice in cortical and subcortical structures, including brainstem (Figure 1A, B).

In P13 to P14 knockout brains, we identified a reduction in MBP-positive fibers (Figure 2A). Areas affected included the corpus callosum, anterior commissure, internal capsule, and the habenulo-interpeduncular tract. The alterations in myelination were also observed by proteolipid protein staining (Figure 2B). This myelin alteration was confirmed by quantitative immunoblotting of whole brain extracts prepared from P10 littermates. Myelin basic protein levels were significantly reduced (to 75% of wild-type) in knockouts at P10, whereas glial fibrillary acidic protein (an astrocyte marker), synaptophysin, and neurofilament (NF) (synaptic and neuronal markers) showed no differences in expression levels (Figure S1C in Supplement 1). We obtained similar results using whole cortex instead of whole brains (data not shown).

As a general assessment of neuronal integrity, we also performed a variety of immunostains on P13 to P14 brains, including staining for the NF triplet proteins (neurofilament-L [NF-L], neurofilament-M [NF-M], and neurofilament-H [NF-H]). Neurofilament staining was found widely in all brain regions in both knockout and control brains and in large fiber tracts such as the corpus callosum or fimbria; the staining was comparable in knockouts and control mice. However, throughout the neocortex there were fewer fine processes that exhibited NF staining in knockout animals. These changes were best seen with the anti-NF antibody SMI-31, which recognizes phosphorylated forms of the mouse NF-M and NF-H proteins that are found mainly in axons (Figure 3). Many more fine neuronal processes were present in layers IV to VI in the control animals (arrows in Figure 3A, C) than in the homozygous knockouts (Figure 3B, D), whereas in the knockouts, there were additional punctate accu-

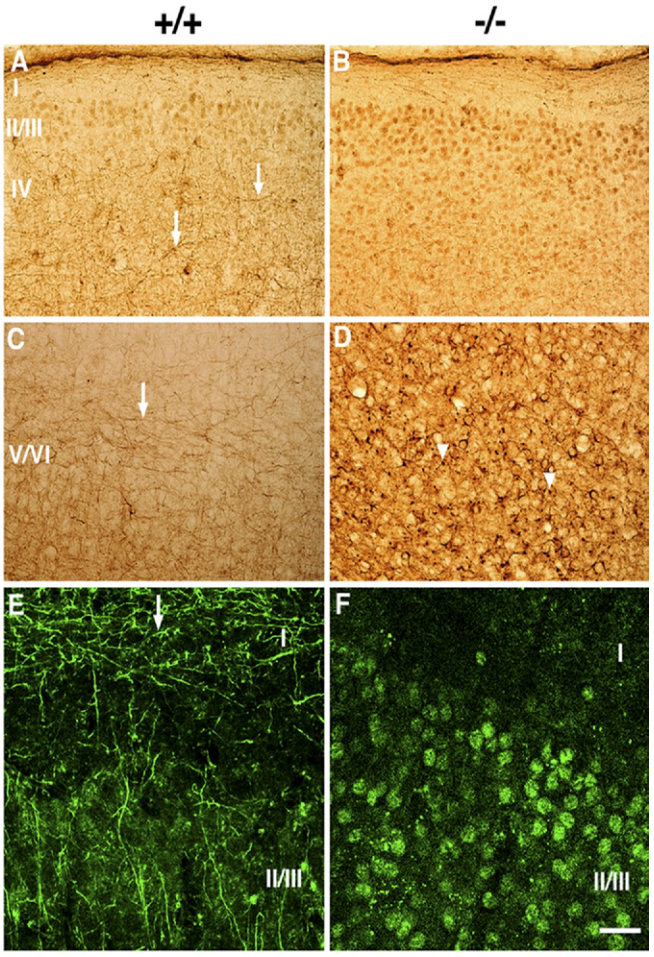

Figure 3. Altered neurofilament distribution in *Slc25a12*-knockout mice. Sagittal sections from postnatal day 13 cortex of wild-type (+/+) **(A, C, E)** and knockout (−/−) **(B, D, F)** mice were stained with antibody SMI-31, which recognizes phosphorylated forms of the mouse midsized and heavy NF proteins that are normally found primarily in axons. Sections are shown from the somatosensory cortex **(A–D)** and auditory cortex **(E, F)**, the latter with confocal imaging in superficial cortical layers. Cortical layers I to VI are indicated. Arrows in **(A)**, **(C)**, and **(E)** point to many fine axonal processes that are labeled in wild-type animals but absent in knockout animals. Arrowheads in **(D)** indicate neurofilamentous accumulations that are present in the knockout mice. Scale bar: **(A)** and **(B)**, 100 μm; **(C)** and **(D)**, 50 μm; **(E)** and **(F)**, 25 μm. NF, neurofilament.

mulations of NF proteins in the deeper cortical layers that were not seen in the wild-type animals (arrowheads in Figure 3D). In contrast, there was increased NF perikaryal staining in many neurons of the knockouts, especially in layers II to III (Figure 3B). By confocal imaging, there was decreased staining of the fine axons normally found in layer I (arrow in Figure 3E) in the knockouts (Figure 3F) with increased cell body staining in layers II/III (Figure 3F). A very similar staining pattern was seen with antibody RMO108 that recognizes phosphorylated forms of the mouse NF-M (data not shown). While the neurofilament accumulations and loss of fine processes were most prominent in layers II/III, they were also seen in deeper cortical layers as well and thus do not appear to be specific to a neuronal subset or lamina but rather an indication of a generalized alteration in neurofilament processing or transport. These findings could reflect a defect in the development of fine axonal processes or defective NF transport. Alternatively, they could also be a sign of neurodegeneration in *Slc25a12*-knockouts.

As an additional indicator of neuronal integrity, we also performed calbindin immunostaining. Calbindin is a calcium binding protein that labels a subset of gamma aminobutyric acid ergic neurons, as well as other neuronal types, including cerebellar Purkinje cells. In the cerebellum, a disrupted alignment of the Purkinje cell layer was observed in *Slc25a12*-knockouts (Figure 4). In contrast to wild-type mice, calbindin-immunoreactive Purkinje cells in the knockout mice frequently formed a layer that was several cells thick (Figure 4A–D). The Purkinje cell dendrites were also less elaborate in the knockouts and the molecular layer appeared generally thinner (compare Figure 4E and F). No differences in calbindin immunostaining were observed in the hippocampus and neocortex (data not shown).

Altered Mag Expression in Male Heterozygous Mice

We also looked at adult heterozygous mice. In these mice, we did not detect any structural changes by immunohistochemistry (data not shown). Furthermore, we did not identify any structural

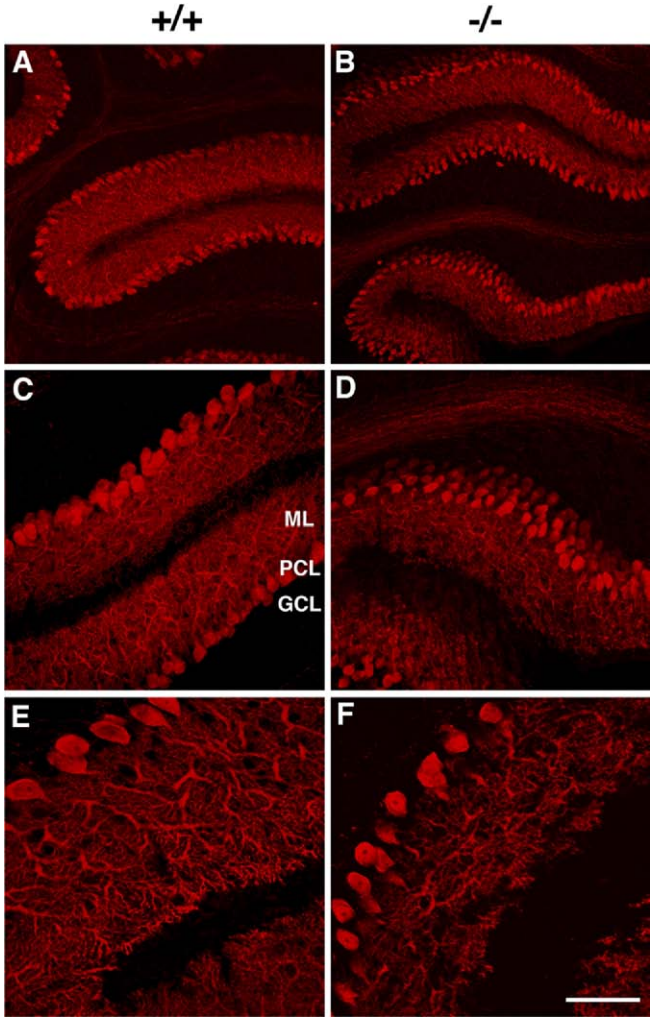

Figure 4. Altered Purkinje cell alignment in the cerebellum of *Slc25a12*-knockout mice. Confocal imaging of sagittal sections from postnatal day 13 wild-type (+/+) **(A, C, E)** and knockout (−/−) **(B, D, F)** cerebella stained with an anticalbindin antibody are shown. The location of the granule cell layer (GCL), Purkinje cell layer (PCL), and molecular layer (ML) are indicated. Note that the Purkinje cells in the mutant frequently form a layer several cells thick **(D)** and that Purkinje cell dendrites appear attenuated in the knock out **(F)**. Scale bar: **(A)** and **(B)**, 100 μm; **(C)** and **(D)**, 50 μm; **(E)** and **(F)**, 25 μm.

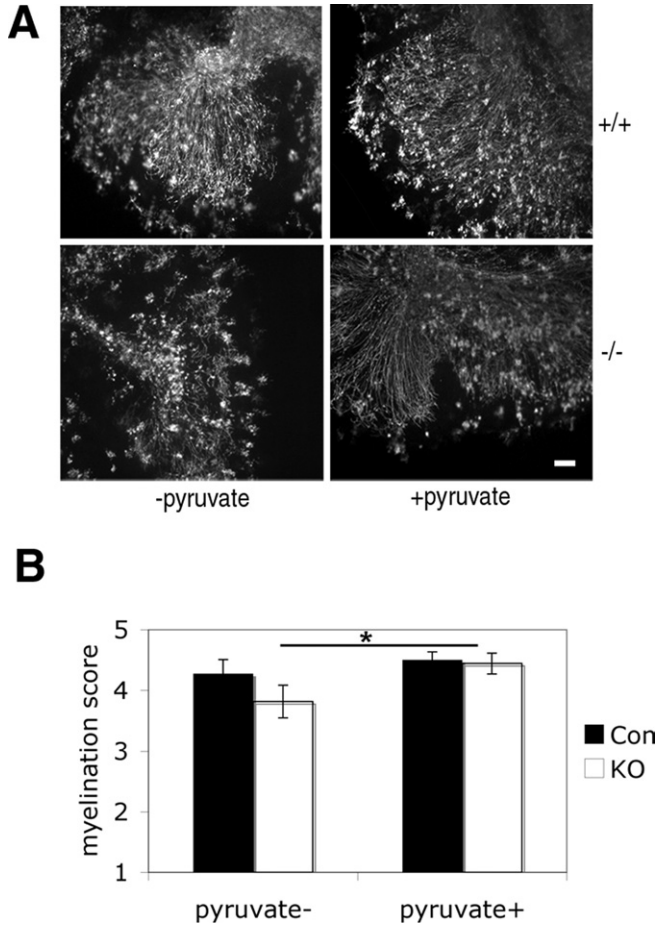

Figure 5. Pyruvate administration rescues myelination deficits of *Slc25a12*-knockout cultures in vitro. **(A)** Cerebellar slice cultures, prepared from postnatal day 10 wild-type (+/+) and knockout (−/−) mice, were treated with or without pyruvate for 7 days and stained for MBP. Scale bar: 100 μm. **(B)** Slice cultures were quantified by an observer blind to genotype and treatment condition, using a scale from 1 to 5 (see Methods and Materials). *p = .038. Con, control (wild-type and heterozygous) animals; KO, knockouts; MBP, myelin basic protein.

changes when we used MRI to measure ventricular size (male wild-type, 2442 ± 101 arbitrary units (AU); male heterozygotes, 2458 ± 198 AU; p = .47; female wild-type, 2447 ± 135 AU; female heterozygotes, 2300 ± 169 AU; p = .26). However, when we analyzed gene expression in adult brains focusing on several oligodendrocyte/myelin related genes (i.e., *Cldn11, Cnp1, Qk5/6, Mobp, Mbp, Olig2, Plp1, Sox10, Erbb4*, and *Mag*), we did observe a reduction in *Mag* expression (coding for myelin-associated glycoprotein [MAG]) in male heterozygotes compared with wild-type male animals (61% of control animals, p = .017 for the first set, 9 wild-type and 8 heterozygous male animals, and 62% of control animals, p = .032 for the second set, 10 wild-type and 13 heterozygous male animals), indicating the presence of subtle alterations in myelination-associated gene expression in the heterozygous animals. Interestingly, we did not observe change in female animals (data not shown). Other oligodendrocyte/myelin genes did not show significant changes.

Myelination Deficits in Cerebellar Slices from *Slc25a12*-Knockout Mice

To further study the role of AGC1 in neuronal development and myelination, we used in vitro systems. We prepared cere-

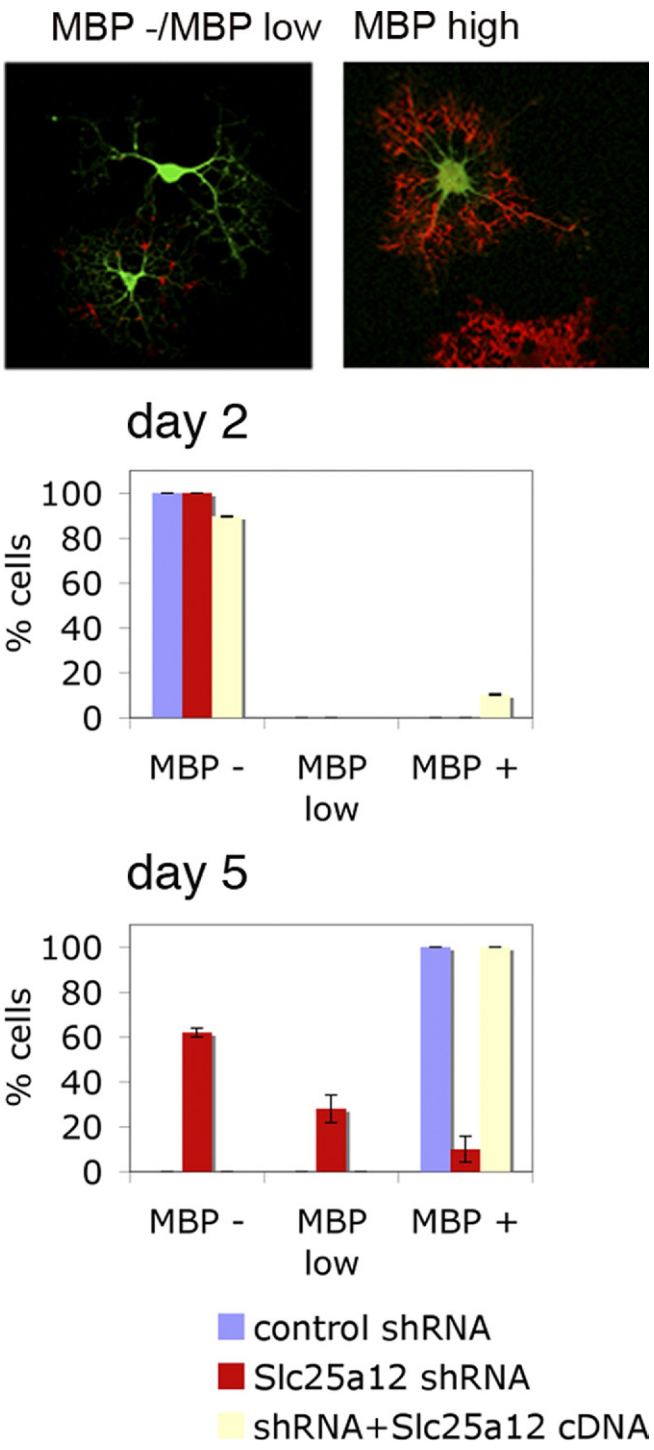

Figure 6. Inhibition of *Slc25a12* in OPCs inhibits MBP expression in vitro. OPC cultures prepared from P0 rat brains were nucleofected with a GFP construct, together with either control (scrambled sequence) shRNA (blue), shRNA against *Slc25a12* (red), or shRNA against *Slc25a12* with SLC25A12 cDNA (white). Transfected (GFP-positive) cells were classified as having no MBP expression (MBP −, see example in top left panel), low MBP expression (MBP low, see example in top left panel), or high MBP expression (MBP +, see example in top right panel). Cultures were assessed in a blinded fashion at day 2 (middle) and day 5 (bottom) and the proportion of cells in each stage shown. cDNA, complementary DNA; GFP, green fluorescent protein; MBP, myelin basic protein; OPC, oligodendrocyte precursor cells; shRNA, short hairpin RNA.

bellar slice cultures from P10 wild-type, heterozygous, and knockout mice and cultured them in vitro for 1 week. In wild-type and heterozygous cultures, normal development of Purkinje cell dendrites and axons was confirmed by calbindin immunostaining. Furthermore, normal myelination in vitro was confirmed by MBP staining (Figure 5A, left panel, and not shown). In contrast, in cultures from knockout animals, the axons of Purkinje cells were less myelinated than control animals (Figure 5A, left panel).

Rescue of Myelination Deficits In Vitro by Pyruvate Administration

Mutations in *SLC25A13*, which codes for AGC2, cause adult-onset type II citrullinemia in humans. It has been shown that pyruvate administration in *Slc25a13*-knockout mouse liver ameliorates the deficient phenotype ex vivo through modulation of cellular metabolism involving mitochondria (15). We hypothesized that pyruvate may be able to change cellular metabolism in the absence of AGC1, ultimately leading to recovery of myelination. To test if pyruvate administration would ameliorate the myelination deficits in *Slc25a12*-knockouts, we added pyruvate to cerebellar slice cultures. After 7 days of culture in the presence of pyruvate, more MBP-positive axons were observed in knockout cultures (Figure 5), indicating that myelination was significantly enhanced by pyruvate administration.

Reduced MBP Expression in *Slc25a12*-Deficient Oligodendrocyte Progenitor Cells

While AGC1 is highly expressed in neurons (16), we also observed expression of AGC1 in OPCs at their earliest stage of differentiation in vitro (data not shown), and hence, it was of interest to determine whether perturbation of AGC1 in oligodendrocytes can alter myelination. When we nucleofected short hairpin RNA (shRNA) against AGC1 into purified OPCs, we observed that fewer cells expressed MBP at day 5 compared with control cultures (Figure 6). In contrast, with the introduction of control (scrambled) shRNAs, most cells expressed high levels of MBP, comparable with the control cultures. Quantitative PCR showed that shRNA treatment resulted in an ~60% reduction of *Agc1* gene expression and ~60% reduction in *Mbp* gene expression at day 5 (note that transfection efficiency was ~60%). To exclude the possibility of off-target effects of shRNA, we also nucleofected shRNA together with AGC1 complementary DNA in a rescue experiment and found that most cells were high MBP expressers, similar to control cultures (Figure 6). These results indicate that perturbation of AGC1 expression in oligodendrocytes is sufficient to alter their development, i.e., that there is an oligodendrocyte-autonomous effect of AGC1 expression on myelination. This, however, does not exclude the possibility that reduced expression of AGC1 in neurons also contributes to the observed myelin deficits.

Discussion

SLC25A12 is a putative ASD susceptibility gene and a gene involved in a severe neurodevelopmental disorder. The gene encodes AGC1, a mitochondrial protein. To clarify its involvement in disease, understanding AGC1 function in brain development and function is important. In this article, we characterized AGC1 knockout mice morphologically. Four major alterations in AGC1 knockout mice were identified: 1) smaller brain size, 2) myelination defects in the brain, 3) altered neurofilament distribution in the cortex, and 4) Purkinje cell abnormalities in the cerebellum. Some of these deficits are similar to what was recently described in a newly identified syndrome, including brain size and myelin deficits, as well as some behavioral changes (4). Below we discuss these abnormalities and their relevance to neurodevelopment.

AGC1 Affects Brain Development

AGC1 is a Ca^{2+}-dependent mitochondrial aspartate/glutamate carrier involved in the MAS system, crucial for effective oxidative phosphorylation to produce ATP (5). Cells deficient in AGC1 may not be able to produce ATP in an efficient manner. Since AGC1 is mainly expressed in brain and skeletal muscle, when energy demand in those tissues is high, particularly during development, AGC1 deficiency may affect development of those tissues. Consistent with this idea, AGC1 knockout mice appear normal at birth, but as development proceeds, they became lethargic, showing severe delays in growth and development. We found that increase of body weight stops and that brain size is smaller compared with the wild-type or heterozygous littermates at P13 to P14 (Figure 1). This is consistent with the previous report by Jalil *et al.* (14) who also developed *Slc25a12*-knockout mice but in a different genetic background.

We also found Purkinje cell alterations in the cerebellum. Purkinje cells were frequently misaligned and had a less developed dendritic structure, producing a thinner molecular layer (Figure 4). This may be a consequence of developmental delay, as Purkinje cell development can be readily affected by developmental insults. Interestingly, AGC1 knockouts exhibited an ataxic gait and tremor, the underlying basis of which may be these cerebellar alterations, although we cannot exclude the possibility that general developmental delay of the animals as well as specific effects on skeletal muscle development might contribute to the phenotype (AGC1 is also highly expressed in skeletal muscle). Given the role of AGC1 in mitochondrial function, these alterations may also be related to insufficient ATP generation during the high-energy demand periods of development.

AGC1 Is Important for Myelin Formation

AGC1 is involved in the MAS system, which is important for ATP production, and therefore, it was at first surprising that the knockout mice showed major defects in myelination (14) (Figure 2). It appears, however, that AGC1 is also crucial for providing proper levels of aspartate to the cytoplasm, where it can be metabolized to *N*-acetylaspartate (NAA), as suggested by Jalil *et al.* (14). Jalil *et al.* (14) demonstrated that in *Slc25a12*-knockout mice, NAA levels in the brain are reduced. Myelination requires the coordinated interactions between neurons and oligodendrocytes, and considering the presence of neuronal alterations, the deficits in myelin in the knockout mice might thus be attributed to alterations in neuron-oligodendrocyte interactions. As AGC1 is highly expressed in neurons, it was proposed that NAA produced in neurons is transported to oligodendrocytes to provide a source for acetyl residues, necessary for myelin lipid formation (14,16). However, when we looked at purified OPCs, we observed that when AGC1 expression was perturbed, OPC differentiation and MBP expression were altered (Figure 6), demonstrating that OPCs themselves are directly affected by the absence of AGC1 and this, in turn, can affect myelination.

Administration of pyruvate restored myelination in our studies, as shown by MBP expression in slice cultures (Figure 5). Based on the possibility that myelin deficits are caused by the reduction of NAA levels in *Slc25a12*-knockout mice, we can

hypothesize that pyruvate administration provides an alternate source for generating aspartate, which can be converted to NAA, and in turn provide lipids for myelin formation (Figure S2 in Supplement 1). Furthermore, pyruvate administration may also restore the NADH/NAD^+ ratio, so that mitochondria can produce ATP more efficiently (Figure S2 in Supplement 1). Pyruvate has been shown to be effective in treating some of the clinical features related to mitochondrial dysfunction (17) (M. Tanaka, M.D., Ph.D., oral communication). It would be interesting to determine whether pyruvate administration to neonatal pups would rescue some of the *Slc25a12*-knockout phenotype and restore myelin formation. In any event, our pyruvate administration results further support the involvement of AGC1 in myelinating processes in the brain. Recently, a case of an AGC1 deficiency has been reported (4). The patient is homozygous for a missense mutation in the *SLC25A12* gene that abolishes AGC1 activity, and the child shows arrested psychomotor development, hypotonia, and seizures. Magnetic resonance imaging scans indicate that there is global hypomyelination in the cereberal hemispheres. Experimental interventions in the knockout animals (including those providing alternate sources of pyruvate and aspartate) might someday lead to potential interventions in such conditions.

In AGC1 heterozygotes, we have not seen any obvious defects in myelination other than reduction of *Mag* expression (to the ~60% of control animals). Interestingly, although reproducible, we only detect this change in male animals. The basis for this difference is still not clear. Myelin-associated glycoprotein is the first myelin membrane protein that appears at the contact points of axons with the processes of oligodendrocytes, supporting axon-oligodendrocyte interactions that are important for myelination. Since myelin is dynamic, even in adult brains where there is constitutive turnover related to remodeling and changes associated with neuronal plasticity, a slight reduction of MAG might mean that myelin cannot be as dynamically regulated in AGC1 heterozygous mice.

Slc25a12-Knockout Mice Show Neuronal Defects

In addition to myelination defects, we found that *Slc25a12*-knockout mice showed neuronal deficits, namely accumulation of NFs in axons of cortical neurons across multiple cortical layers (Figure 3). We do not know whether this is a direct effect of absence of AGC1 in neurons or an indirect effect of the myelination deficits. The accumulation of phosphorylated NFs detected by antibody SMI-31 within cell bodies is suggestive of an intrinsic defect in axonal transport or could be reflective of a neurodegenerative effect. Defects in myelination could cause axonal damage, leading to impaired NF transport and their accumulation within neuronal perikarya. Alternatively, AGC1 in neurons may be important for axonal transport, perhaps by facilitating the production of ATP. In either case, neuronal deficits could contribute to phenotype with altered AGC1 expression. However, neuronal deficits in heterozygotes may be subtle, as we have not observed morphological changes in these mice.

Autism Susceptibility and AGC1

SLC25A12/AGC1 has been shown to be associated with autism susceptibility (3,6,9,10), although there are studies showing no association (7,8). It is not surprising that genetic association studies of susceptibility genes do not replicate previous findings using different cohorts, as the effect sizes are generally quite weak and the sample sizes likely too small to have adequate power in every case. Interestingly, a recent large-scale genome-wide association study has demonstrated that the *SLC25A12* region is nominally associated with autism, although it did not reach genome-wide significance (J.D. Buxbaum, unpublished data), further supporting possible involvement of AGC1 in autism susceptibility. However, the replicated SNPs are all intronic and their functional relevance to AGC1 expression is not yet clear. In postmortem brain samples, AGC1 is upregulated in dorsolateral frontal cortex of patients with autism (10,13). This does not indicate that either overexpression or underexpression of AGC1 is associated with autism, as changes in expression may be a consequence of other aspects of the disorder and/or may reflect compensatory effects.

Since *SLC25A12* is a putative autism susceptibility gene that at most increases genetic risk, AGC1 animal models that have either elevated or reduced AGC1 expression would not be expected to show clear ASD phenotypes. In this study, we rather intended to clarify the role of AGC1 in brain development and function, as well as to clarify the processes that may be affected when AGC1 expression is changed, which may be involved in increasing risk for ASDs.

We found that the absence of AGC1 affects several processes in brain development, namely general brain growth, myelination, and neuronal development, the latter associated with NFs. Thus, when AGC1 activity is modulated genetically, global effects mediated by altered mitochondrial function could affect a wide range of processes related to brain development. Such global effects on brain development may be the basis for involvement of AGC1 in ASD susceptibility.

Our results support the idea that alterations in AGC1 activity could affect NAA levels as well as ATP production in brain. There are several reports on NAA levels in brains of individuals with ASDs using magnetic resonance spectroscopy, with most studies pointing to decreased NAA levels, although some studies have found no differences compared with control subjects and one study found elevated levels (18–29). Additional studies suggest that there may be alterations in white matter and myelination in ASDs (reviewed in [30]).

Synapse formation and plasticity, as well as myelination, demand a high-energy supply, and therefore, these processes may also be altered when AGC1 function is modulated. In fact, AGC1 overexpression in primary neuronal cultures in vitro induces rapid movement of mitochondria into dendrites (13), which is thought to be associated with synaptic plasticity (31).

Aspartate-glutamate carrier isoform 1 is also a Ca^{2+} sensor modulating mitochondrial activity in cells (5). Recently, it has been shown that Ca^{2+} levels are elevated in postmortem brains from patients with autism compared with control subjects, and AGC1 activity itself is upregulated (10). Because AGC1 is involved in Ca^{2+} signals that affect global cell metabolism, sustained elevations of cellular Ca^{2+} may result in oxidative stress and cellular dysfunction through excessive activation of AGC1 (10).

From our studies, we cannot pinpoint specific pathways that are affected in *Slc25a12*-knockout mice but rather found global effects in several aspects of brain development. Although we have not observed any obvious morphological changes in AGC1 heterozygous brains, either at the synaptic level or in neural circuitry, it is possible that subtle effects on several processes during brain development may lead to a behavioral phenotype. If so, then even subtle modulation of AGC1 expression may contribute to a behavioral phenotype, which may lead to an increased susceptibility to ASDs. In contrast, loss of functional AGC1 will lead to myelin abnormalities and severe neurodevelopmental deficits.

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JDB and NR hold a patent on the SLC25A12 *gene in autism and receive shared royalties on this gene. JDB and TS have submitted a provisional patent application on treatment of dysmyelination. All other authors reported no financial interests or potential conflicts of interest.*

Supplementary material cited in this article is available online.

1. Folstein SE, Rosen-Sheidley B (2001): Genetics of autism: Complex aetiology for a heterogeneous disorder. *Nat Rev Genet* 2:943–955.
2. Abrahams BS, Geschwind DH (2008): Advances in autism genetics: On the threshold of a new neurobiology. *Nat Rev Genet* 9:341–355.
3. Ramoz N, Reichert JG, Smith CJ, Silverman JM, Bespalova IN, Davis KL, Buxbaum JD (2004): Linkage and association of the mitochondrial aspartate/glutamate carrier *SLC25A12* gene with autism. *Am J Psychiatry* 161:662–669.
4. Wibom R, Lasorsa FM, Töhönen V, Barbaro M, Sterky FH, Kucinski T, *et al.* (2009): AGC1 deficiency associated with global cerebral hypomyelination. *N Engl J Med* 361:489–495.
5. Satrústegui J, Contreras L, Ramos M, Marmol P, del Arco A, Saheki T, Pardo B (2007): Role of aralar, the mitochondrial transporter of aspartate-glutamate, in brain *N*-acetylaspartate formation and Ca(2+) signaling in neuronal mitochondria. *J Neurosci Res* 85:3359–3366.
6. Segurado R, Conroy J, Meally E, Fitzgerald M, Gill M, Gallagher L (2005): Confirmation of association between autism and the mitochondrial aspartate/glutamate carrier *SLC25A12* gene on chromosome 2q31. *Am J Psychiatry* 162:2182–2184.
7. Rabionet R, McCauley JL, Jaworski JM, Ashley-Koch AE, Martin ER, Sutcliffe JS, *et al.* (2006): Lack of association between autism and *SLC25A12*. *Am J Psychiatry* 163:929–931.
8. Blasi F, Bacchelli E, Carone S, Toma C, Monaco AP, Bailey AJ, *et al.* (2006): *SLC25A12* and CMYA3 gene variants are not associated with autism in the IMGSAC multiplex family sample. *Eur J Hum Genet* 14:123–126.
9. Turunen JA, Rehnström K, Kilpinen H, Kuokkanen M, Kempas E, Ylisaukko-Oja T (2008): Mitochondrial aspartate/glutamate carrier *SLC25A12* gene is associated with autism. *Autism Res* 1:189–192.
10. Palmieri L, Papaleo V, Porcelli V, Scarcia P, Gaita L, Sacco R, *et al.* (2008): Altered calcium homeostasis in autism-spectrum disorders: Evidence from biochemical and genetic studies of the mitochondrial aspartate/glutamate carrier AGC1 [published online ahead of print July 8]. *Mol Psychiatry*.
11. Correia C, Coutinho CC, Diogo L, Grazina M, Marques C, Miguel T, *et al.* (2006): High frequency of biochemical markers for mitochondrial dysfunction in autism: No association with the mitochondrial asparate/glutamate carrier SLC25A12 gene. *J Autism Dev Disord* 36:1137–1140.
12. Silverman JM, Buxbaum JD, Ramoz N, Schmeidler J, Reichenberg A, Hollander E, *et al.* (2008): Autism-related routines and rituals associated with a mitochondrial aspartate/glutamate carrier *SLC25A12* polymorphism. *Am J Med Genet B Neuropsychiatr Genet* 147:408–410.
13. Lepagnol-Bestel AM, Maussion G, Boda B, Cardona A, Iwayama Y, Delezoide AL, *et al.* (2008): *SLC25A12* expression is associated with neurite outgrowth and is upregulated in the prefrontal cortex of autistic subjects. *Mol Psychiatry* 13:385–397.
14. Jalil MA, Begum L, Contreras L, Pardo B, Iijima M, Li MX, *et al.* (2005): Reduced *N*-acetylaspartate levels in mice lacking aralar, a brain- and muscle-type mitochondrial aspartate-glutamate carrier. *J Biol Chem* 280:31333–31339.
15. Moriyama M, Li MX, Kobayashi K, Sinasac DS, Kannan Y, Iijima M, *et al.* (2006): Pyruvate ameliorates the defect in ureogenesis from ammonia in citrin-deficient mice. *J Hepatol* 44:930–938.
16. Ramos M, del Arco A, Pardo B, Martínez-Serrano A, Martínez-Morales JR, Kobayashi K, *et al.* (2003): Developmental changes in the Ca2+-regulated mitochondrial aspartate-glutamate carrier aralar1 in brain and prominent expression in the spinal cord. *Brain Res Dev Brain Res* 143:33–46.
17. Tanaka M, Nishigaki Y, Fuku N, Ibi T, Sahashi K, Koga Y (2007): Therapeutic potential of pyruvate therapy for mitochondrial diseases. *Mitochondrion* 7:399–401.
18. Hardan AY, Minshew NJ, Melhem NM, Srihari S, Jo B, Bansal R, *et al.* (2008): An MRI and proton spectroscopy study of the thalamus in children with autism. *Psychiatry Res* 163:97–105.
19. Gabis L, Wei Huang, Azizian A, DeVincent C, Tudorica A, Kesner-Baruch Y, *et al.* (2008): 1H-magnetic resonance spectroscopy markers of cognitive and language ability in clinical subtypes of autism spectrum disorders. *J Child Neurol* 23:766–774.
20. Endo T, Shioiri T, Kitamura H, Kimura T, Endo S, Masuzawa N, Someya T (2007): Altered chemical metabolites in the amygdala-hippocampus region contribute to autistic symptoms of autism spectrum disorders. *Biol Psychiatry* 62:1030–1037.
21. Kleinhans NM, Schweinsburg BC, Cohen DN, Müller RA, Courchesne E (2007): *N*-acetyl aspartate in autism spectrum disorders: Regional effects and relationship to fMRI activation. *Brain Res* 1162:85–97.
22. DeVito TJ, Drost DJ, Neufeld RW, Rajakumar N, Pavlosky W, Williamson P, Nicolson R (2007): Evidence for cortical dysfunction in autism: A proton magnetic resonance spectroscopic imaging study. *Biol Psychiatry* 61: 465–473.
23. Friedman SD, Shaw DW, Artru AA, Dawson G, Petropoulos H, Dager SR (2006): Gray and white matter brain chemistry in young children with autism. *Arch Gen Psychiatry* 63:786–794.
24. Murphy DG, Critchley HD, Schmitz N, McAlonan G, Van Amelsvoort T, Robertson D, *et al.* (2002): Asperger syndrome: A proton magnetic resonance spectroscopy study of brain. *Arch Gen Psychiatry* 59:885–891.
25. Hisaoka S, Harada M, Nishitani H, Mori K (2001): Regional magnetic resonance spectroscopy of the brain in autistic individuals. *Neuroradiology* 43:496–498.
26. Otsuka H, Harada M, Mori K, Hisaoka S, Nishitani H (1999): Brain metabolites in the hippocampus-amygdala region and cerebellum in autism: An 1H-MR spectroscopy study. *Neuroradiology* 41:517–519.
27. Hashimoto T, Tayama M, Miyazaki M, Yoneda Y, Yoshimoto T, Harada M, *et al.* (1997): Differences in brain metabolites between patients with autism and mental retardation as detected by in vivo localized proton magnetic resonance spectroscopy. *J Child Neurol* 12:91–96.
28. Chugani DC, Sundram BS, Behen M, Lee ML, Moore GJ (1999): Evidence of altered energy metabolism in autistic children. *Prog Neuropsychopharmacol Biol Psychiatry* 23:635–641.
29. Fayed N, Modrego PJ (2005): Comparative study of cerebral white matter in autism and attention-deficit/hyperactivity disorder by means of magnetic resonance spectroscopy. *Acad Radiol* 12:566–569.
30. Hughes JR (2007): Autism: The first firm finding = underconnectivity? *Epilepsy Behav* 11:20–24.
31. Li Z, Okamoto K, Hayashi Y, Sheng M (2004): The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. *Cell* 119:873–887.