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Report

Impaired Mitochondrial Glutamate Transport in Autosomal Recessive Neonatal Myoclonic Epilepsy

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Severe neonatal epilepsies with suppression-burst pattern are epileptic syndromes with either neonatal onset or onset during the first months of life. These disorders are characterized by a typical electroencephalogram pattern—namely, suppression burst, in which higher-voltage bursts of slow waves mixed with multifocal spikes alternate with isoelectric suppression phases. Here, we report the genetic mapping of an autosomal recessive form of this condition to chromosome 11p15.5 and the identification of a missense mutation (p.Pro206Leu) in the gene encoding one of the two mitochondrial glutamate/H^+ symporters (*SLC25A22*, also known as "*GC1*"). The mutation cosegregated with the disease and altered a highly conserved amino acid. Functional analyses showed that glutamate oxidation in cultured skin fibroblasts from patients was strongly defective. Further studies in reconstituted proteoliposomes showed defective [^{14}C]glutamate uniport and [^{14}C]glutamate/glutamate exchange by mutant protein. Moreover, expression studies showed that, during human development, *SLC25A22* is specifically expressed in the brain, within territories proposed to contribute to the genesis and control of myoclonic seizures. These findings provide the first direct molecular link between glutamate mitochondrial metabolism and myoclonic epilepsy and suggest potential insights into the pathophysiological bases of severe neonatal epilepsies with suppression-burst pattern.

Severe neonatal epilepsies with suppression-burst pattern are early-onset epileptic syndromes characterized by a typical electroencephalogram (EEG) pattern—namely, suppression burst, in which higher-voltage bursts of slow waves mixed with multifocal spikes alternate with isoelectric suppression phases (Ohtsuka et al. 1993; Vigevano and Bartuli 2002). Early myoclonic encephalopathy (EME), first described by Aicardi and Goutières in 1978, is characterized by a very early onset (during the 1st month of life); erratic, fragmentary myoclonus; massive myoclonus; partial motor seizures; and late tonic spasms (Aicardi and Goutières 1978; Aicardi 1992). The prognosis of EME is poor, with no effective treatment, and children with the condition either die within 1–2 years after birth or survive in a persistent vegetative state. The lack of consistent neuropathological features suggests that etiology may vary from case to case. EME has been reported in nonketotic hyperglycinemia (MIM 605899), propionic acidemia (MIM 606054), and some malformative disorders (Guerrini et al. 2003). In most cases, however, the underlying mechanism of these disorders remains unknown.

Early infantile epileptic encephalopathy, which includes West syndrome and Lennox-Gastaut syndrome, is characterized by a very early onset (during the first few months of life), frequent tonic spasms, and a suppression-burst EEG pattern. Partial motor seizures may also occur. The course is severe, with early death or marked psychomotor retardation and intractable seizures with frequent evolution to West syndrome. Brain imaging usually discloses gross structural abnormalities, in the majority of cases. Metabolic disorders are also present in a few cases, but the proportion of cryptogenic cases is high.

We have investigated a sibship of four affected chil-

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dren (two girls and two boys) and four healthy children born to Arab Muslim parents from Jerusalem who are first cousins (fig. 1*A*). The proband (patient II.7 in fig. 1*A*) was first examined at age 2 d because of intractable seizures and neonatal hypotonia. At age 3 years, computerized tomography of the head revealed brain atrophy, and EEG showed myoclonic seizures and burst suppression. Nonketotic hyperglycinemia was ruled out. His eldest sister (patient II.1 in fig. 1*A*) also had hypotonia and seizures a few hours after birth. Computerized tomography of the head at age 3 years showed brain atrophy. Electroretinogram (ERG) was normal, whereas visual-evoked potential (VEP) showed a low amplitude signal and a slow response suggestive of abnormal visual nerve-conduction velocity. Electron-microscopy examination of a skin biopsy ruled out storage disease, and high-resolution karyotype was normal. The eldest brother (patient II.2 in fig. 1*A*) also had hypotonia and neonatal myoclonic seizures. Plasma amino acid, copper, biotinidase lactate, carnitine, and urinary organic acids were normal. He died at age 8 years. Finally, the fourth affected child (patient II.4 in fig. 1*A*) was hypotonic and presented with neonatal seizures. At age 7 wk, ERG-VEP examination showed abnormal conduction and retinal function. At 3 years of age, brain magnetic resonance imaging (MRI) showed subarachnoid enlargement, especially in the frontal region. EEG showed myoclonic seizures with burst suppression. Extensive metabolic work-up was consistently normal. Electron-microscopy examination of skin fibroblasts and optic examination of muscle biopsy were normal as well. In conclusion, very-early-onset intractable myoclonus epilepsy with progressive microcephaly, periodic EEG with suppression burst, and abnormal VEPs evolving into a severe encephalopathy and spasticity were consistent features in the four affected siblings.

A genomewide screening, performed using the Perkin Elmer Biosystems linkage mapping set (version 1), identified a single homozygous marker, *D11S4046*, on chromosome 11p15.5 in the four affected sibs. Further marker typing and haplotype analyses reduced the genetic interval to a 2.5-Mb interval between marker *D11S4088* and the telomere (fig. 1*A*). Linkage analysis using LINKAGE software (version M-LINK) gave a Z_{max} of 3.06 at $\theta = 0$ at the *D11S4177* locus. Concordant data from the National Center for Biotechnology Information (NCBI) and the University of California–Santa Cruz (UCSC) database genome browsers indicate that 54 known genes and ~25 EST clusters map to this interval. On the basis of the hypothesis that this condition is of metabolic origin, nine genes were considered as candidates (fig. 1*B*). Among them, *SLC25A22* (also known as "*GC1*") was regarded as a strong candidate gene because of position, expression, and function. The

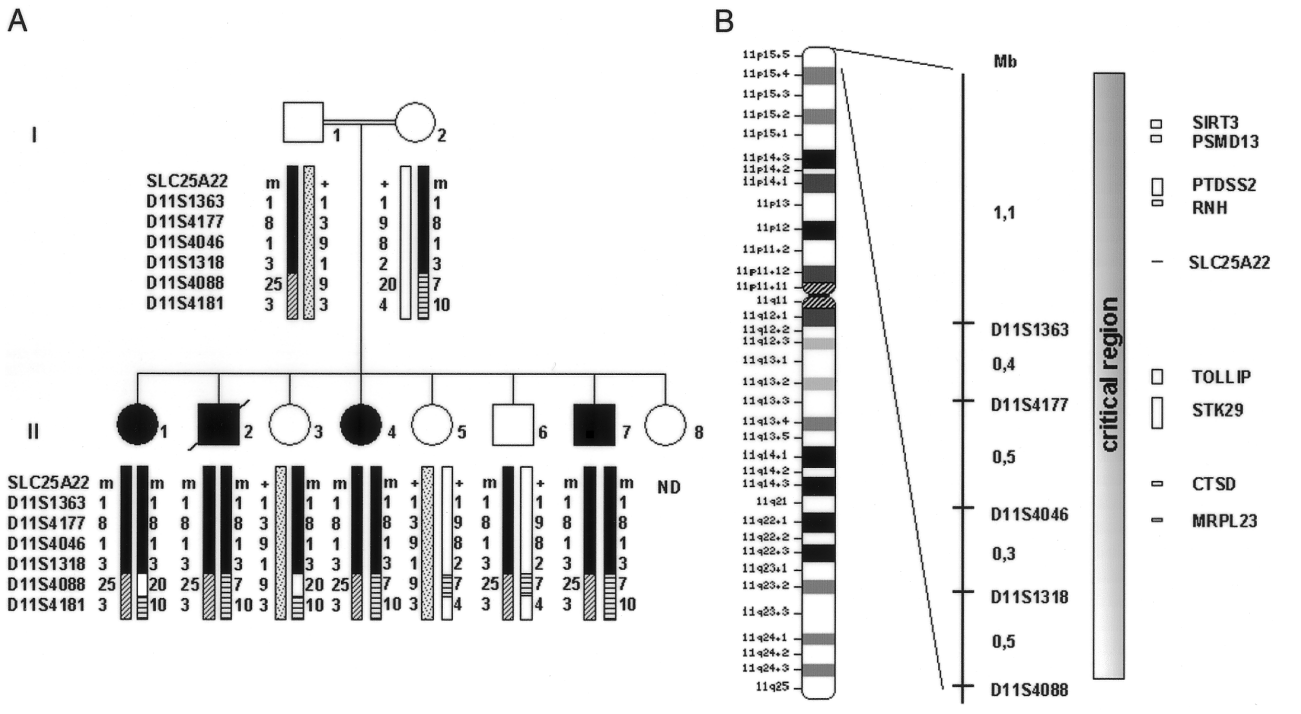

Figure 1 *A*, Pedigree and haplotype of the family. The blackened symbols indicate affected individuals, and the haplotype segregation with EME is shown. A plus sign (+) = wild-type sequence; m = mutation. *B*, Physical map of the critical interval, indicating the candidate genes in this region of chromosome 11p15.

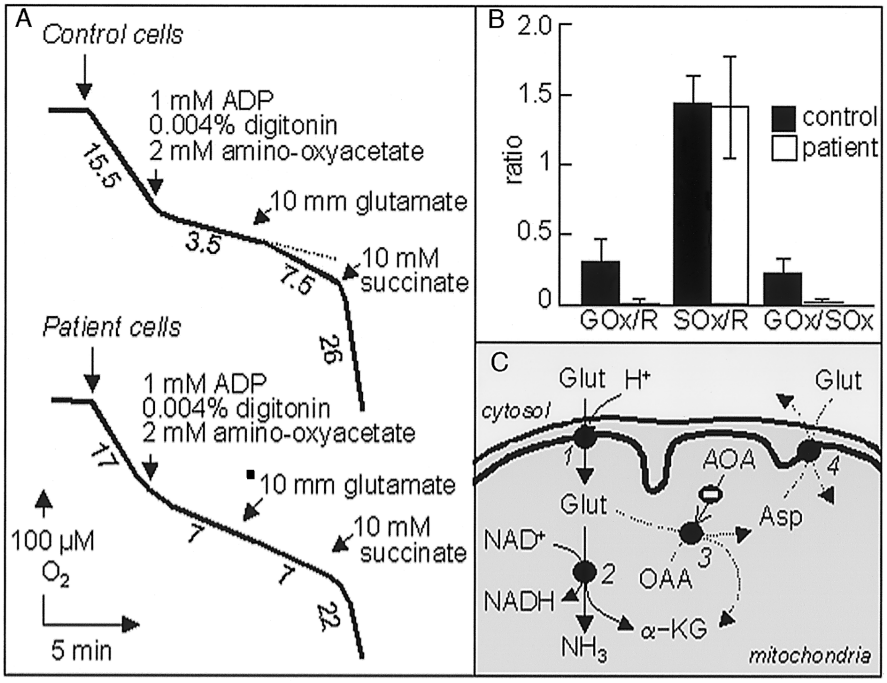

Figure 2 Respiration and mitochondrial substrate oxidation in cultured skin fibroblasts from patients and controls. *A*, After polarographic measurement of intact cell respiration, digitonin-permeabilized cells were loaded with adenosine diphosphate (ADP) and amino oxyacetate. This compound inhibits the aspartate-amino transferase enzyme activity (see *panel C*). A subsequent addition of glutamate allowed estimation of mitochondrial glutamate oxidation under phosphorylation conditions (i.e., presence of ADP). Note the lack of glutamate-triggered oxygen uptake in patient cells. A similar succinate oxidation rate was measured in control and patient cells. Numbers along the traces are nmol O_2 consumed per minute per microgram of protein. *B*, Various ratios showing the profound defect of glutamate oxidation (GOx) in patient cells (*unblackened boxes*), as compared with cell respiration (R) or succinate oxidation (SOx). Values are the means (±1 SD) of six experiments. *C*, Features of mitochondrial glutamate metabolism, with inner membrane–associated glutamate-H^+ symporter (*1*) and aspartate/glutamate antiporter (*4*), and the matrix-located enzymes, glutamate dehydrogenase (*2*) and amino-oxyacetate–sensitive aspartate-amino transferase (*3*). AOA = amino oxyacetate; Asp = aspartate; Glut = glutamate; α-KG = α-ketoglutarate; and OAA = oxaloacetate. Experimental conditions were as described in the "Methods" section of appendix A (online only).

SLC25 gene family encodes mitochondrial carriers that transport a variety of metabolites across the inner mitochondrial membrane (Palmieri 2004). The SLC25A22 protein catalyzes either the cotransport of L-glutamate with H^+ or its exchange with OH^- (Fiermonte et al. 2002). The genomic structure of the human *SLC25A22* gene was identified by alignment of the cDNA sequence (GenBank accession number NM_024698) with the sequence of PAC clone RP13-569C6 (GenBank accession number AC132936). Nine coding exons were identified, and primers were designed for direct sequencing of all exons and exon-intron junctions (see table A1 [online only]). Homozygosity for a missense mutation in exon 8 of the *SLC25A22* gene was detected in patient II.1, with the change of a proline into a leucine in the protein (p.Pro206Leu). Sequence analyses showed cosegregation of the mutation with the disease in all affected individuals, whereas both parents were heterozygous (fig. 1*A*). This mutation was not found in 200 unrelated control individuals (100 of Arab origin and 100 of various ethnic origins). Real-time PCR analysis of fibroblast RNAs from patient II.1 detected normal amounts of the mutant gene transcripts, compared with those of various controls (data not shown). To estimate the prevalence of *SLC25A22* mutations in EME, we collected DNA from 25 unrelated children with EME and progressive microcephaly and sequenced the coding region of the *SLC25A22* gene, but no mutation was identified.

The changed proline residue is highly conserved across species in the glutamate and aspartate/glutamate carriers (fig. A1 [online only]), suggesting that this base change might impair glutamate transport. To test this hypothesis, we investigated the oxidation of glutamate in cultured skin fibroblasts from patients (fig. 2*A* and the "Methods" section of appendix A [online only]). Because a balanced proportion of oxidizing activities is consistently observed in cultured fibroblasts and is required for optimum function of the respiratory chain and for continuous metabolic fluxes, activity ratios rather than absolute values were used to evaluate glutamate oxidation efficiency. Figure 2*B* presents the mean (± 1 SD) of six independent experiments. Under stan-

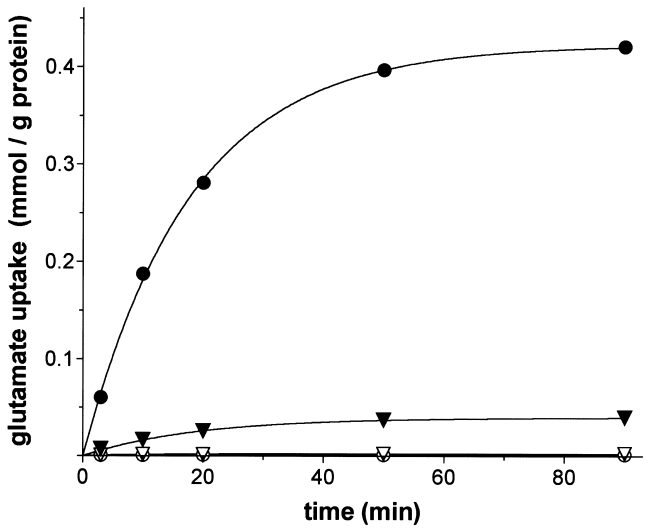

Figure 3 Kinetics of [^{14}C]glutamate uniport and [^{14}C]glutamate/glutamate exchange catalyzed by the wild-type and mutated GC1 proteins. Proteoliposomes were reconstituted with the recombinant wild-type (*blackened symbols*) or the mutated GC1 protein (*unblackened symbols*). At time zero, 1 μM [^{14}C]glutamate was added to the proteoliposomes containing 10 μM glutamate (exchange [*circles*]) or 10 μM NaCl and no substrates (uniport [*triangles*]). At the indicated time intervals, the uptake of labeled substrate was stopped by addition of 30 μM pyridoxal 5′-phosphate and 10 μM bathophenantroline. Data are means ($\pm$ SD) of four independent experiments. No transport activity was detected when the mutant protein was used.

dard conditions, polarographic studies showed normal cell respiration by intact cells, indicating that glutamate does not represent a major respiratory substrate, at least in the cell type tested. However, after permeabilization of cell membranes by digitonin in the presence of amino-oxyacetate, a specific inhibitor of amino-aspartate transferase (fig. 2*C*), patient cells failed to normally oxidize glutamate, as compared with controls, whereas oxidation of another substrate, succinate, was normal in patient cells (fig. 2*B*). Simultaneous spectrophotometric studies detected similar glutamate dehydrogenase activities in patient and control cells (data not shown). These results provide evidence that patient cells show a clear defect in mitochondrial glutamate metabolism.

To get further insight into the functional consequences of this base change, wild-type and mutant SLC25A22 proteins were overexpressed in *Escherichia coli* and were purified (see the "Methods" section of appendix A [online only]). Equivalent amounts of neosynthesized proteins were then used for in vitro reconstitution of the transporter into liposomes (Palmieri et al. 1995). Observation of the time course of [^{14}C]glutamate uniport and [^{14}C]glutamate/glutamate exchange in reconstituted proteoliposomes showed that the wild-type protein efficiently catalyzed glutamate transport and exchange, whereas, despite normal insertion of the mutant protein in the liposomal membrane, neither transport nor exchange activities were detected with the mutant construct (fig. 3).

Northern blot analysis of human fetal tissues revealed a strong hybridization signal with brain mRNA, whereas a very faint signal was detected in the lung, kidney, and liver mRNAs (data not shown). Further expression analyses, by use of in situ hybridization experiments, detected no signal in 32-, 44-, and 47-d-old human embryos (data not shown). By contrast, at 15 wk of development, prominent expression was detected in the cortex (fig. 4*A*–4*D*), the hippocampal formation (fig. 4*E* and 4*F*), and the pontine nuclei (data not shown). In the cortex, signal was detected in all the laminae of the cortical plate, from laminae II through VI. In the hippocampal formation, *SLC25A22* was uniformely expressed in the CA1, CA2, and CA3 pyramidal cell fields as well as in the granular layer of the dentate gyrus. No expression was detected in the spinal cord. At a later stage (20 wk of development), the highest gene expression was detected in the cerebral cortex, the brain stem, and the cerebellum (fig. 4*G*–4*L*). In the brainstem, a moderate-to-high level of *SLC25A22* mRNA expression was observed in the red nuclei, the substantia nigra, and the cranial nerves nuclei III, as well as in the olivary complexes. Finally, *SLC25A22* expression was also detected in the cerebellum, within the dentate nucleus.

Mutations in mitochondrial carriers in humans have been reported elsewhere (Palmieri 2004), but none of these mutations have been shown to cause early myoclonic epilepsy. *SLC25A13* (encoding aspartate/glutamate carrier 2) mutations cause type II citrullinemia (MIM 603471), *SLC25A15* (encoding ornithine carrier 1) mutations are associated with hyperornithinaemia-hyperammonaemia-homocitrulliniria syndrome (triple H syndrome [MIM 238970]), *SLC20A19* (encoding deoxynucleotide carrier) mutations cause Amish microcephaly (MIM 607196), *SLC25A4* mutations cause autosomal dominant progressive external ophthalmoplegia (adPEO [MIM 157640]), and *SLC25A20* (encoding carnithine/acylcarnithine carrier) mutations are responsible for Stanley syndrome (MIM 212138). These genes encode proteins involved in the urea cycle, mtDNA metabolism, or translocation of fatty acids into the mitochondria. An increasing number of studies have also shown that mitochondrial dysfunction can result in seizures and drug-resistant forms of severe epilepsy (Wallace et al. 1988; Goto et al. 1990; Degoul et al. 1995). Presumably, all these mutations act by impairing mitochondrial respiratory chain function and ATP synthesis. By contrast, the present study provides the first evidence that, despite normal oxidative phosphorylation, impaired mitochondrial glutamate import/metabolism leads to an alteration of neuronal excitability, possibly

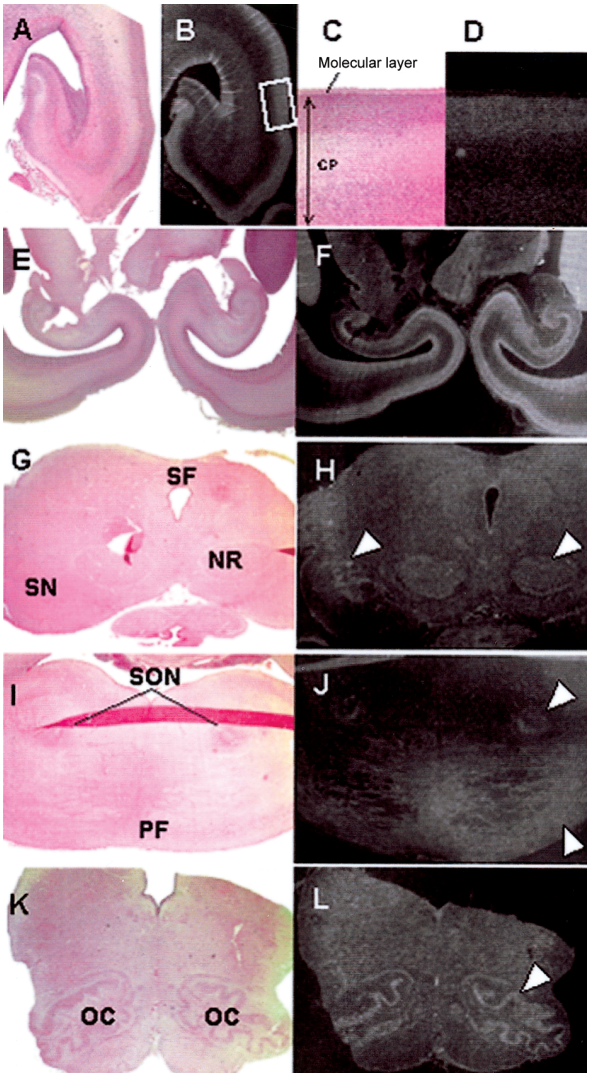

Figure 4 *SLC25A22* gene expression in the 15-wk-old (*A–F*) and 20-wk-old (*G–L*) fetal human brain. Sections counterstained with hematoxylin and eosin are show under bright-field (*A, C, E, G, I,* and *K*) and dark-field illumination to reveal the localization of the in situ hybridization signal (*B, D, F, H, J,* and *L*). CP = cortical plate; SN = substantia nigra; NR = nucleus ruber; SON = superior olive nucleus; SF = sylvian fissure; OC = olivary complex; and PF = pontocerebellar fibers.

linking glutamate transport to the pathogenesis of myoclonic seizures.

Previously published data regarding *SLC25A22* expression showed that it is ubiquitously expressed in adult tissues and is particularly abundant in liver and pancreas (Fiermonte et al. 2002). To understand why the *SLC25A22* mutation results in a pure neurological phenotype, we studied the *SLC25A22* gene expression during human development. Northern blot analyses and in situ hybridization experiments confirmed that *SLC25A22* expression is restricted to the brain. Interestingly, *SLC25A22* expression was found within territories proposed to contribute to the genesis and control of myoclonic seizures. It is worth remembering that the substantia nigra is the central part of the nigral control of the epilepsy system that, when activated, inhibits seizure generation and propagation (Depaulis et al. 1994; Deransart et al. 2001). More interesting is that several cases of neonatal epilepsy with suppression-burst EEG pattern and olivary-dentate dysplasia have been reported (Harding and Boyd 1991; Robain and Dulac 1992), and the olivocerebellar pathway was proposed to contribute to the genesis of myoclonic seizures. Several lines of evidence, based on immunohistochemical, pharmacological, and electrophysiological experiments, suggest that glutamate plays an important role in olivocerebellar transmission (Clements et al. 1987; Zhang et al. 1990; Trinka et al. 2001). The mitochondrial component of the glutamate system may thus be a key player in this pathway.

How mutations in *SLC25A22* cause epilepsy remains an open question. Neuronal dysfunction may result from an abnormal glutamate pool in the cytoplasm, from a secondary alteration of electron flow through the respiratory chain, or from impaired oxygen handling in a discrete subset of neurons. The mitochondrion is a major site of ammonia synthesis, especially through the glutamate dehydrogenase reaction. Hence, impaired ammonia synthesis (due to altered glutamate import) could also play a role in the pathogenesis of the disease.

Epilepsy might also result from abnormal glutamate turnover. Indeed, it is worth remembering that glutamate is the major excitatory neurotransmitter in the mammalian brain. Efficient neurotransmission requires (1) low intrasynaptic glutamate concentration, so as to maximize the signal-to-noise ratio, and (2) rapid replacement of the transmitter that is released from presynaptic ends. This is achieved by the rapid uptake of glutamate into astrocytes, which converts glutamate into glutamine, thus ensuring the replenishment of the internal neuronal glutamate pool. In addition, high extracellular glutamate concentrations have been identified as a likely cause of epileptic seizures in mesial temporal lobe epilepsy (During and Spencer 1993). Thus, glial cells may play an important role in the pathogenesis of the disorder. This hypothesis is also favored by the observation that, at variance with neurons, adult glial cells do not significantly express the aspartate/glutamate carriers (Ramos et al. 2003), so that glial mitochondrial glutamate import may be dependent on glutamate carrier activity only. We therefore speculate that impaired GC1 function could result in a severe alteration of glutamate metabolism in glial cells.

Intact glutamate metabolism is crucial for amino acid synthesis and oxidation, for ureogenesis, and for normal function of the CNS. The identification of mutant GC1

as an etiology of early myoclonic epilepsy emphasizes the unexpected importance of the mitochondrial component of glutamate metabolism in normal brain function, thus opening a novel field in the pathophysiology of EME.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *SLC25A22* cDNA [accession number NM_024698] and PAC clone RP13-569C6 [accession number AC132936])
NCBI, http://www.ncbi.nlm.nih.gov/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for nonketotic hyperglycinemia, propionic acidemia, type II citrullinemia, triple H syndrome, Amish microcephaly, adPEO, and Stanley syndrome)
UCSC Genome Bioinformatics, http://genome.ucsc.edu/ (for the human genome working draft)

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