Glutaric acidemia type I (GA-I) is an inherited disorder of lysine and tryptophan metabolism presenting with striatal lesions anatomically and symptomatically similar to Huntington disease. Affected children commonly suffer acute brain injury in the context of a catabolic state associated with nonspecific illness. The mechanisms underlying injury and age-dependent susceptibility have been unknown, and lack of a diagnostic marker heralding brain injury has impeded intervention efforts. Using a mouse model of GA-I, we show that pathologic events began in the neuronal compartment while enhanced lysine accumulation in the immature brain allowed increased glutaric acid production resulting in age-dependent injury. Glutamate and GABA depletion correlated with brain glutaric acid accumulation and could be monitored in vivo by proton nuclear magnetic resonance (1H NMR) spectroscopy as a diagnostic marker. Blocking brain lysine uptake reduced glutaric acid levels and brain injury. These findings provide what we believe are new monitoring and treatment strategies that may translate for use in human GA-I.

Introduction

Glutaryl-CoA dehydrogenase (EC 1.3.99.7) (GCDH) activity is required for complete oxidation of lysine and tryptophan. Autosomal recessive inheritance of GCDH deficiency, known as glutaric acidemia type I (GA-I), is one of the more common inherited metabolic disorders affecting 1 out of 30,000–100,000 children worldwide (1,2). GA-I results in accumulation of glutaric acid and glutaryl carnitine with carnitine deficiency (3–5). Affected individuals accumulate substantial glutaric acid in the brain and 3-hydroxyglutaric acid (3-OHGA) to a lesser extent (6–8). Following a period of normal development, affected children may experience irreversible striatal injury during an encephalopathic crisis, commonly precipitated by a nonspecific illness between 6 and 18 months of age (2). The neuropathology associated with GA-I is similar to that of Huntington disease, in which a systemic genetic defect results in specific striatal degeneration involving loss of medium spiny neurons and astroglisisis (6–10).

Current therapy for GA-I includes dietary lysine restriction, carnitine supplementation, and emergency treatment of intercurrent illness including intravenous glucose (3–5). Unfortunately, one-third of affected children, prospectively identified by newborn screening, do not respond to therapy and experience striatal degeneration despite careful clinical management (11). Children with GA-I who escape encephalopathy in their first 5 years typically remain asymptomatic (2). We recently developed an animal model of GA-I encephalopathy in GCDH-deficient (Gcdh−/−) mice by introducing added dietary protein or lysine (12). This model shows age-dependent susceptibility to acute brain injury similar to human GA-I. Weaning (4-week-old) Gcdh−/− mice accumulate substantial glutaric acid and suffer seizures, paralysis, hemorrhages, and death within 3–6 days of lysine diet exposure. In contrast, adult (8-week-old) Gcdh−/− mice survive lysine exposure and accumulate less glutaric acid, though all develop bilateral striatal lesions after 6 weeks of lysine diet exposure.

In the current study, we first localized the origin of brain injury by identifying the cell type or types in the brain that express Gcdh and the earliest pathological events after protein and lysine supplementation in weanling Gcdh−/− mice. We then compared the biochemical responses in weanling and adult Gcdh−/− mice after lysine diet challenge to identify differences associated with age-dependent susceptibility. Our current findings identified developmental differences that control glutaric acid accumulation and age-dependent brain injury. Brain injury was preceded by biochemical changes that may be used as a diagnostic marker. These findings led to what we believe are novel monitoring and treatment strategies.
used double-label immunohistochemistry to determine whether Gcdh expression is limited to neurons, astrocytes, or endothelial cells. We then used electron and confocal microscopy to detect the earliest pathologic events in the brain after special diet exposure. Sections of motor cortex and CA1 hippocampus from Gcdh−/− mice revealed that Gcdh expression was limited to neurons (indicated by β-gal labeling; see Methods) (Figure 1, A–F).

Pathologic changes were limited to Gcdh−/− mice and were evident within 24–48 hours of protein or lysine diet exposure in weanlings (Figure 2, A–H). Moderately vacuolated neurons were found in the motor cortex with pathological changes previously associated with necrosis (13) (Figure 2E). Dendritic processes in the motor cortex showed moderately enlarged mitochondria and disorganization of neurofilaments (Figure 2F). Swollen and disintegrating mitochondria were found in postsynaptic regions with edema of dendritic spines (Figure 2G), resembling excitotoxic lesions (9). Similar changes, though less severe, were found in adult Gcdh−/− mice after 60 hours (Figures 2H and 3B). Although adult Gcdh−/− mice do not show obvious behavioral differences (12), further testing may reveal deficits associated with these pathologic changes after long-term diet exposure.

To confirm the cell types associated with vacuoles in a larger field, we compared confocal images of neurofilament- and astrocyte-labeled sections with semithin sections prepared for electron microscopy (Figure 3, A–D). The vacuoles that formed in and around the pyramidal cell layer of CA3 hippocampus (Figure 3B) were surrounded by neurofilaments (shown by NF2 labeling), indicating a neuronal origin of the vacuoles (Figure 3D). Vacuolation of astrocytes (glial fibrillary acidic protein [GFAP]) was not apparent. Additionally, labeling of cholinergic neurons showed vacuole formation in the cell bodies and processes of these large neurons of the striatum within 48 hours of lysine exposure.
diet exposure in weanling Gcdh<sup>−/−</sup> mice (Figure 3G). These changes were less noticeable in adults after 60 hours (Figure 3H).

**Brain lysine accumulation and age-dependent susceptibility.** To determine the mechanism of age-dependent susceptibility to encephalopathy in Gcdh deficiency, we considered that brain glutaric acid levels were previously shown to correlate with severity of disease presentation (12). Therefore, we considered the possibilities that (a) brain lysine catabolism, which produces glutaric acid, may be reduced with age; (b) brain lysine uptake may be reduced with age; or (c) clearance of glutaric acid from the brain may be enhanced with age. Based on recent findings that showed limited blood-brain barrier (BBB) permeability for glutaric acid (14), we pursued the first 2 possibilities.

We first tested for differences in brain lysine and glutaric acid accumulation between susceptible weanling Gcdh<sup>−/−</sup> mice and encephalopathy-resistant adults. Since there were no significant differences between WT and heterozygotes with and without lysine diet exposure, we report these changes compared with heterozygous controls. All mice accumulated similar serum lysine levels with lysine diet exposure (P < 0.01) regardless of age or genotype (Figure 4A). Brain lysine levels were 46% higher in weanling Gcdh<sup>−/−</sup> mice compared with heterozygous controls, both of which were on a normal diet (P < 0.01) consistent with disrupted lysine catabolism in Gcdh<sup>−/−</sup> mice (Figure 4A). Lysine diet exposure raised brain lysine levels by 66% in weanling heterozygotes (P < 0.01) without any increase in glutaric acid levels (Figure 4A). Weanling but not adult Gcdh<sup>−/−</sup> mice showed a 3-fold increase in brain lysine accumulation (P < 0.001) accompanied by a 2-fold increase in glutaric acid (P < 0.01) after 48 hours of lysine diet exposure (Figure 4A). Between 48 and 60 hours, a decrease in brain lysine was accompanied by an equimolar increase in glutaric acid, suggesting production of glutaric acid within the brain from available lysine (Figure 4A). At 8 weeks of age, adult Gcdh<sup>−/−</sup> mice showed no increase in brain lysine or glutaric acid levels on the lysine diet, consistent with a substantial reduction in brain lysine uptake with maturity. Changes in brain 3-OHGA paralleled those of glutaric acid although 3-OHGA concentrations were 20-fold lower than glutaric acid levels (not shown). Although symptoms of encephalopathy were less evident at the earlier time points examined in the current work, brain glutaric acid levels correlated with acute injury of symptomatic mice as previously shown (12).

To determine whether lysine diet exposure had any effect on other basic or neutral amino acids, we compared serum and brain alanine and arginine levels. Both serum arginine and alanine levels were 2-fold higher only in weanling Gcdh<sup>−/−</sup> mice on the lysine diet (P < 0.01) (Figure 4B). On a normal diet, brain arginine levels were 2-fold higher in weanling Gcdh<sup>−/−</sup> mice compared with heterozygotes (P < 0.01) (Figure 4B). Lysine diet exposure raised brain arginine 60% in both heterozygotes and weanling Gcdh<sup>−/−</sup> mice but not adults (P < 0.01 and P < 0.001 respectively, compared with heterozygote normal diet controls) (Figure 4B). Brain alanine levels were unaffected by lysine diet exposure in all groups (Figure 4B). These data are consistent with increased flux of the basic amino acid transporter in the immature brain that carries both lysine and arginine (15).

**Serum amino acid accumulation, other than lysine.** Since there were no significant differences between WT and heterozygotes with and without lysine diet exposure, we report these changes compared with heterozygous controls. All mice accumulated similar serum lysine levels with lysine diet exposure (P < 0.01) regardless of age or genotype (Figure 4A). Brain lysine levels were 46% higher in weanling Gcdh<sup>−/−</sup> mice compared with heterozygous controls, both of which were on a normal diet (P < 0.01) consistent with disrupted lysine catabolism in Gcdh<sup>−/−</sup> mice (Figure 4A). Lysine diet exposure raised brain lysine levels by 66% in weanling heterozygotes (P < 0.01) without any increase in glutaric acid levels (Figure 4A). Weanling but not adult Gcdh<sup>−/−</sup> mice showed a 3-fold increase in brain lysine accumulation (P < 0.001) accompanied by a 2-fold increase in glutaric acid (P < 0.01) after 48 hours of lysine diet exposure (Figure 4A). Between 48 and 60 hours, a decrease in brain lysine was accompanied by an equimolar increase in glutaric acid, suggesting production of glutaric acid within the brain from available lysine (Figure 4A). At 8 weeks of age, adult Gcdh<sup>−/−</sup> mice showed no increase in brain lysine or glutaric acid levels on the lysine diet, consistent with a substantial reduction in brain lysine uptake with maturity. Changes in brain 3-OHGA paralleled those of glutaric acid although 3-OHGA concentrations were 20-fold lower than glutaric acid levels (not shown). Although symptoms of encephalopathy were less evident at the earlier time points examined in the current work, brain glutaric acid levels correlated with acute injury of symptomatic mice as previously shown (12).

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These changes are consistent with induction of catabolic stress after lysine diet exposure. Additionally, the reduced serum glucose with increased serum alanine levels may represent an impairment of gluconeogenesis (16).

Increased brain glutaric acid accumulation in weanling Gcdh−/− mice was associated with 20% 12-day survival compared with 100% survival of adults on the lysine diet (Figure 4D). Weanlings showed brain swelling by MRI at 48 hours and development of bilateral striatal lesions at 6 days on the lysine diet (Figure 4E). Together, these data indicate that enhanced lysine uptake in the immature brain provides increased glutaric acid accumulation and susceptibility to brain injury.

Brain biochemical changes consistent with disrupted mitochondrial function. Mitochondrial swelling and disruption in the brain of Gcdh−/− mice indicate disrupted mitochondrial function with lysine diet exposure. Gcdh deficiency may lead to the intramitochondrial
accumulation of glutaryl-CoA, which may result in sequestration of intramitochondrial free CoA. Alternatively, increased ketone body utilization leads to acetyl-CoA accumulation, which can also reduce free CoA levels (17, 18). Therefore, we measured acetyl-CoA, glutaryl-CoA, free CoA, ATP, phosphocreatine, and α-ketoglutarate in the brain of weanling and adult Gcdh−/− mice or heterozygous controls with normal and lysine diet exposure (Figure 5, A–D). Heterozygote controls on normal or lysine diets. Mean ± SEM. *P < 0.01; **P < 0.001. n = 6 each group. (C) ATP, phosphocreatine (PCr), and α-ketoglutarate changes in cortex of weanling and adult Gcdh−/− mice and heterozygous controls on the lysine diet compared with normal diet. Mean ± SEM, *P < 0.04; †P < 0.02. n = 4 each group. (D) Glutamate, glutamine, and GABA levels in weanling and adult Gcdh−/− mice and heterozygous controls on the lysine diet compared with normal diet. Mean ± SEM. n = 6 each group.

Increased ketone body utilization was previously shown to sequester free CoA and cause accumulation of α-ketoglutarate, since free CoA is a limiting substrate for α-ketoglutarate dehydrogenase (17). Depletion of α-ketoglutarate with acetyl-CoA accumulation in Gcdh−/− mice suggests a different mechanism, involving glutaric acid accumulation and resulting in CoA sequestration. We propose that loss of Krebs cycle intermediates through α-ketoglutarate may be a primary cause of mitochondrial disruption as diagrammed in Figure 6. Accumulation of saccharopine, previously reported in human GA-I (8), may sequester α-ketoglutarate directly. Alternatively, glutaric acid accumulation may deplete Krebs cycle intermediates through exchange with α-ketoglutarate (Figure 6). Loss of Krebs cycle intermediates would prevent the regeneration of oxaloacetate, which is needed to combine with acetyl-CoA to form citrate for continued Krebs cycle function. The resulting accumulation of acetyl-CoA depletes available free CoA and depresses the buildup of glutaryl-CoA due to the limited mitochondrial CoA pool.

Since α-ketoglutarate is a precursor of glutamate synthesis (19) and the product of glutamate dehydrogenation (20), depletion of α-ketoglutarate suggests that glutamate may be depleted as well. Additionally, GABA is produced from glutamate decarboxylation and was previously shown to be depleted in human GA-I (21). Therefore we compared glutamate, glutamine, and GABA levels in the brain of weanling Gcdh−/− mice with and without lysine diet exposure (Figure 5D). Glutamate and GABA levels were depleted...
to half the normal concentrations ($P < 0.001$) after lysine diet exposure while glutamine levels were reduced by only 24% ($P < 0.01$) (Figure 5D). Since glutamine is exclusively produced in glia (22), depletion of more glutamate than glutamine in this model suggests a neuronal origin of metabolic impairment. GABA depletion may be responsible for the seizures observed in children and mice with Gcdh deficiency (7, 12).

**Limiting brain lysine uptake and catabolism provides successful treatment.** The increase in brain lysine and glutaric acid associated with susceptibility in weanling Gcdh–/– mice suggested the possibility that (a) blocking brain lysine uptake or (b) reducing brain lysine catabolism may be protective. We tested the first possibility by administration of homoarginine, previously shown to effectively compete with brain lysine uptake (23). We tested the second possibility by administration of glucose to reduce lysine catabolism. We administered these treatments separately or combined to weanling Gcdh–/– mice on the lysine diet and monitored these animals for survival, biochemical changes, and brain injury by MRI (Figures 7 and 8). Each treatment substantially reduced brain glutaric acid accumulation, improved survival, and prevented brain injury in surviving animals at 6 days (Figure 7) although mildly increased transverse relaxation time constant ($T_2$) values, representing vasogenic edema (24), were found in the striatum of some glucose-only treated mice (Figure 7F). Glucose treatment reduced accumulation of brain lysine by one-third ($P < 0.03$) and glutaric acid by 45% ($P < 0.001$), resulting in a 3-fold increase in 12-day survival (Figure 7, A and D). Homoaarginine treatment reduced brain lysine accumulation by 50% ($P < 0.01$), resulting in 59% less glutaric acid accumulation ($P < 0.001$) and a 4-fold increase in 12-day survival (Figure 7, B and D). Combined homoaarginine/glucose treatment provided similar control of lysine and glutaric acid compared with homoaarginine alone but improved 12-day survival to 100% (Figure 7, C and D).

Treatment success correlated directly with control of brain glutaric acid levels and maintenance of glutamate and GABA. Glutamate and GABA levels were higher with glucose treatment compared with lysine diet controls (Figure 8A). However, homoaarginine alone and combined treatment maintained glutamate and GABA levels closest to normal (Figure 8A). Glutamate levels were further reduced with each treatment ($P < 0.01$) (Figure 8A). Glucose and homoaarginine treatment each protected free CoA, glutaryl-CoA, and α-ketoglutarate levels ($P < 0.01$) although only combined treatment decreased acetyl-CoA accumulation ($P < 0.01$), which resulted in substantially higher levels of free CoA compared with either treatment alone (Figure 8B). These effects are consistent with protection of Krebs cycle function.

The protection of free CoA with combined treatment suggested that carnitine supplementation, routinely used to treat human GA-I (2), may be protective in this mouse model. However, carnitine supplementation at up to 4 times the level given to children during encephalopathic crisis (100 mg/kg every 6 hours) (5) did not provide any improvement in survival. The lack of benefit from carnitine in this model may be related to limited access of dietary supplementation (25, 26) or a limited ability to conjugate glutarate in the brain as previously suggested (14).

**Glutamate, glutamine, and GABA levels measured by 1H NMR spectroscopy provide a diagnostic marker.** Serum glutaric acid levels do not correlate with risk or progression of brain injury in human GA-I (11). Our current and previous data with this mouse model indicate that brain glutaric acid levels correlate with injury (12). However, brain glutaric acid levels currently cannot be monitored noninvasively and a clinical marker is needed to help guide treatment efforts (27). Glutamate and GABA depletion are shown here to correlate with elevated brain glutaric acid levels and progression of injury. The magnitude of glutamate and GABA depletion detected in brain extracts suggested that this biochemical change may be detectable by proton nuclear magnetic resonance (1H NMR) spectroscopy and used as a diagnostic marker. Therefore, we performed 1H NMR spectroscopy and MRI to correlate progression of encephalopathy with glutamate and GABA levels (Figure 9). Weaning Gcdh–/– mice showed worsening symptoms associated with progression of encephalopathy between 48 and 60 hours of lysine diet exposure. A substantial reduction in the glutamate and
glutamine/creatine (Glx/Cr) ratio ($P < 0.03$) and the GABA/Cr ratio ($P < 0.05$) but not the N-acetyl aspartate (NAA)/Cr ratio was detectable by $^1$H NMR spectroscopy after 48 hours of lysine diet exposure (Figure 9, A and B). These changes are consistent with our data from brain extracts (Figure 5D) and further show that glutamate and GABA depletion precede substantial neuronal damage, detected by a decrease in the NAA/Cr ratio as previously shown in human GA-I (28). Additionally, Glx/Cr and GABA/Cr ratios were higher with homoarginine ($P < 0.01$) and combined treatment ($P < 0.01$) but not glucose alone (Figure 9B). These data are consistent with glutamate and GABA levels from brain extracts in treated compared with untreated controls (Figure 8A) and suggest that $^1$H NMR spectroscopy may be used to indicate impending brain injury.

Discussion

In this work we present several lines of evidence to suggest that the brain injury of GA-I involves mitochondrial disruption precipitated by glutaric acid production in the neuronal compartment from available lysine. Consistent with initial neuronal injury, expression of Gcdh and the first enzyme in lysine degradation, lysine-oxoglutarate reductase (29), are both limited to neurons. These data implicate the neuronal compartment as the predominant location of lysine catabolism and glutaric acid production in the brain. Intracellular glutaric acid accumulation may cause direct mitochondrial toxicity within neurons. Previous studies with isolated mitochondria showed swelling induced with 500–1,000 μM glutaric acid (30). Higher concentrations of glutaric acid are consistently found in the brains of Gcdh−/− mice (12) and GA-I patients associated

Figure 7
Glucose and homoarginine treatments increased survival and reduced brain injury in weanling Gcdh−/− mice on the lysine diet. Twelve-day survival of weanling Gcdh−/− mice on the lysine diet alone (black diamonds, $n = 30$) or mice treated (black squares) with glucose (A, $n = 30$), homoarginine (HA) (B, $n = 30$), or combined glucose and homoarginine (C, $n = 30$). (D) Serum lysine, brain lysine, and brain glutaric acid levels and (E) serum glucose and β-hydroxybutyrate levels in weanling Gcdh−/− mice on the lysine diet with glucose, homoarginine, or both for 48 hours compared with lysine diet alone. Mean ± SEM, *$P < 0.01$; **$P < 0.001$. $n = 6$ each group. (F) $T_2$ maps (top) and $T_2$-weighted images (bottom) of weanling Gcdh−/− mice with indicated treatments for 6 days show mild brain injury development in those treated with glucose alone (red arrow). Color bar indicates different $T_2$ values (right side).
with injury (6). In the current study, mitochondrial swelling and biochemical changes consistent with Krebs cycle dis-ruption were observed when brain glutaric acid levels reached more than 1,000 μM. Glutaric acid is a preferred substrate for the mitochondrial oxodicarboxylate carrier that normally exchanges α-ketoadipate for α-ketoglutarate in a strict counter-exchange mechanism (31). The level of glutaric acid accumulation, depletion of α-ketoglutarate, and accumulation of acetyl-CoA are all consistent with disruption of Krebs cycle function through loss of cycle intermediates as diagrammed in Figure 6.

Previous studies have shown a developmental change in BBB transport kinetics for basic and neutral amino acids that switch from low affinity/high capacity in the immature brain to high affinity/low capacity with maturity (15). Consistent with our current findings, this developmental difference was previously shown to provide a 3- to 4-fold decrease in brain lysine influx between suckling and adult rats (32). High-capacity amino acid uptake in the immature brain supports the increased metabolic demand and protein turnover during rapid growth and myelination of the brain. However, this enhanced influx of amino acids also provides age-dependent susceptibility to GA-I and other metabolic disorders. The immature brain readily uses alternate energy substrates such as ketone bodies, which may be supplied to the brain as ketogenic amino acids (i.e., lysine and branched-chain amino acids). Ketogenic amino acids have been shown to provide a substantial proportion of ketone bodies used for myelin synthesis (33). As brain growth and myelination are completed, metabolic demands are reduced and the brain uses glucose more exclusively (15). Accordingly, transport and catabolic enzyme activity for lysine are reduced in the brain with maturity (32, 34). Therefore the immature brain is especially susceptible to metabolic disorders of amino acid metabolism such as GA-I and maple syrup urine disease (35). This susceptibility is typically realized in the context of catabolic stress associated with fasting during a nonspecific illness. Catabolic stress leads to breakdown of muscle protein to free amino acids and enhanced amino acid uptake and turnover in the liver for gluconeogenesis (16). Hypoglycemia in children with GA-I during metabolic crisis (8) and in Gcdh−/− mice on a lysine diet suggests that the process of gluconeogenesis from available amino acids is impaired. Decreased glucose levels place greater dependence on ketone bodies and ketogenic amino acids in the brain. The combination of catabolic stress and enhanced amino acid access to the immature brain provides the opportunity for large accumulations of aberrant metabolites in GA-I and other disorders of amino acid metabolism. These age-dependent susceptibilities, related to GA-I, and the proposed effect of treatments are diagrammed in Figure 10.

Recent evidence shows that glutaric acid has limited BBB permeability, suggesting de novo synthesis rather than diffusion or transport of glutaric acid into the brain (14). To confirm that glutaric acid is produced in the brain de novo from available lysine, we demonstrated that blocking brain lysine uptake with homoarginine decreased glutaric acid levels, resulting in reduced brain injury and increased survival. These findings are likely to have clinical relevance, since elevated brain glutaric acid levels and brain injury in GA-I patients occur despite the use of low lysine diets (4, 7). In the present study, controlling brain glutaric acid levels with homoarginine and glucose prevented encephalopathy, underscoring the importance of this strategy for treatment.

Currently, there is no reliable marker that can be used noninvasively for predicting the risk of brain injury in human GA-I.
Brain glutaric acid levels were previously shown to correlate with injury in this mouse model (12) but currently cannot be measured noninvasively. Glutamate and GABA depletion monitored by $^1$H NMR spectroscopy correlates with increased brain glutaric acid levels and may translate for use in human GA-I to detect risk of brain injury. Glutamate depletion in this mouse model is consistent with reduced brain glucose utilization, as previously shown with immature rats (36). Reduced brain glucose utilization was previously shown in human GA-I using $^{18}$fluoro-2-deoxyglucose uptake studies (37), indicating that glutamate levels may also be compromised in human GA-I with encephalopathy. Glutamate is the precursor for GABA (38), and reduced GABA levels in this mouse model and human GA-I (7) correlate with glutamate depletion and further indicate that glutamate levels may be compromised in human GA-I. GABA levels may be initially reduced along with glutamate as brain glucose utilization is compromised during encephalopathy (37). Glutaric acid accumulation suppresses GABA production (21), and the return of glutamate levels with restored brain glucose utilization may set up unopposed excitatory neurotransmission resulting in seizures and excitotoxic lesions found in this model and in human GA-I (9). This possibility may explain the differences between glucose and homoarginine treatments and emphasizes the importance of controlling brain glutaric acid accumulation for neuroprotection.

Establishment of this mouse model provides the opportunity to test the effects of current and novel treatment strategies. Intravenous glucose administration is standard treatment for GA-I encephalopathy, but there are currently no data on the effect of this treatment in the brain. The lysine diet induces a catabolic state in Gcdh$^{-/-}$ mice similar to that seen in children with GA-I during crisis, including hypoglycemia and ketosis (Figure 4C). Catabolic stress leads to increased amino acid accumulation and breakdown in the liver (16). For lysine, increased catabolism was shown to be regulated by mitochondrial influx (39), and glucose availability reduces this effect (39, 40). We propose that a similar mechanism regulates lysine utilization in the immature brain. In the context of catabolic stress with reduced serum glucose levels, the immature brain depends on alternate energy substrates such as ketone bodies and ketogenic amino acids. Glucose supplementation provides adequate preferred substrate and reduces the demand for alternate substrates. This reduced demand correlates with reduced brain lysine accumulation in adult brains that

Figure 9

Glx and GABA depletion detected by $^1$H NMR spectroscopy correlates with treatment success. (A) Comparison of $^1$H NMR spectra from weanling Gcdh$^{-/-}$ mice on a normal (left) or lysine diet (middle) or with combined glucose/homoarginine treatment (right) indicates a large decrease in the Glx peak (red arrow, center) and a smaller decrease in NAA after 48 hours of lysine diet exposure that was prevented by combined treatment (right). Multiple resonance peaks for GABA are not indicated. (B) Glx/Cr ratio, GABA/Cr ratio, and NAA/Cr ratio detected by $^1$H NMR spectroscopy in weanling Gcdh$^{-/-}$ mice on the lysine diet or with indicated treatments. Data are reported as percentage of normal diet control. Mean ± SEM, *P < 0.03. n = 4 per group. Lower left panel indicates voxel location used to acquire NMR spectroscopy data.
utilize glucose more exclusively (15). Here we demonstrate that dietary glucose therapy reduced lysine catabolism (glutaric acid formation) more than lysine accumulation in the brain. Serum glutaric acid levels were also lower with glucose treatment (data not shown), consistent with the influence of glucose to reduce amino acid turnover in the liver (41).

Combined homoarginine and glucose treatment may provide the best protection by reducing the substrate and the drive for glutaric acid production. However, the lack of cumulative effect on glutaric acid levels suggests that each treatment affects a different part of the same pathway. Glutaric acid is produced from lysine breakdown in the context of GCDH deficiency. The rate-limiting step of lysine breakdown at the mitochondrial level was shown to be lysine influx, which was increased by a high-protein diet and reduced by glucose availability (39). Our current data support a model in which lysine access is limited by homoarginine at the BBB and by glucose at the mitochondrial level. Both glucose and homoarginine reduced lysine breakdown (glutaric acid formation) similarly, but brain lysine accumulation remained higher with glucose treatment compared with homoarginine. Therefore, both treatments reduce brain glutaric acid production by limiting lysine access, although to different compartments.

Similar to Huntington disease, the expression of the affected gene in GA-I is not specific to the striatum but results in selective striatal degeneration. Medium spiny neurons are primarily affected in both disorders (4,10). Although the clinical presentation of GA-I is commonly associated with acute onset, the resulting neuropathology in this mouse model is strikingly similar to that of Huntington disease, including the involvement of cortical pyramidal neurons (12, 42). Corticostriatal circuitry may play a role in the pathophysiology of both disorders (43). Further study of this mouse model may reveal factors underlying striatal-specific susceptibility that can be used to develop protective strategies.

The current findings provide insight into the age-dependent mechanism, treatment, and monitoring of GA-I and offer new strategies for the prediction and prevention of brain injury. Enhanced amino acid accumulation in the immature brain may contribute to the age-dependent susceptibility in other neurometabolic disorders (44). Competitive transport inhibition, shown in this study using homoarginine, provides an attractive approach to developing potential treatments for these disorders.

Methods

Materials. All chemicals were purchased from Sigma-Aldrich unless otherwise specified.

Animals. All animal experiments were approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee. Gdb−/− mice and age-matched WT controls, both of mixed C57Bl/6J x 129SvEv background (45), were generated from homozygotes maintained at Penn State College of Medicine (Department of Comparative Medicine) in accordance with Institutional Animal Care and Use Committee research guidelines set forth by Pennsylvania State University and the Society for Neuroscience policy on the use of animals in neuroscience research. Heterozygotes (Gdb+/−) were generated by mating Gdb−/− and WT mice. Gdb−/− mice were previously produced by replacement of the first 7 exons of Gdb with an in-frame ß-gal cassette including a nuclear localization sequence. Therefore, Gdb−/− and Gdb+/− mice express ß-gal under the control of the Gdb regulatory elements (45). To detect expression of ß-gal, we used heterozygotes to avoid loss of cells that require GCDH during development.

Special diets. Diets were purchased from Harland Teklad. The normal diet was the Harland Teklad 2018 diet, which is 18% protein. The high-protein diet (TD.03637) (70% casein) contained 62% protein, which is 4.7% lysine by weight. The lysine diet (TD.04412) was prepared by adding free lysine to a standard diet to achieve 4.7% total lysine. This level of lysine is not toxic in normal animals (46,47). The lysine-homoarginine diet was prepared by adding 5% homoarginine to the lysine diet. Glucose was administered by adding 5% glucose monohydrate by weight to water and provided ad libitum.

For intervention studies, male and female Gdb−/− mice were placed on special diets at 4 weeks of age, housed 2 per cage, and observed every 12 hours; mice and diets were weighed daily (n = 30 for each study group). All special diet–treated animals and corresponding controls were evaluated on special diets for 4 weeks of age. Mice and diets were weighed daily (n = 30 for each study group). All special diet–treated animals and corresponding controls were evaluated on special diets for 4 weeks of age.

Neuropathology. To follow the earliest pathologic events, 12 Gdb−/−, 5 Gdb+/−, and 5 WT mice were placed on a high-protein diet at 4 weeks of age daily for symptoms as previously described (12). Special diets–treated animals and corresponding controls were evaluated on special diets for 4 weeks of age daily for symptoms as previously described (12).
MRI was performed on a 7.0 T Bruker system. Serum samples and brain extracts were analyzed by gas chromatography–mass spectroscopy using stable-isotope dilution as previously described (51). Deuterium-labeled internal standards for GA (d4-GA) (0.05 mg/ml) and 3-OHGA (d5-HGA) (0.05 mg/ml) were added at 0.05 ml each to 0.5 ml serum samples or 1.5 ml brain homogenates. Sulfosalicylic acid at 0.15 ml of 9.33% or 70 mg was added to serum or brain samples, respectively. Samples were extracted twice with 3 ml diethyl ether and 1.5 ml ethyl acetate. Combined organic phases were dried at 30°C under nitrogen and derivatized with 0.05 ml BTSA/1% TMCs [N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane] for 20 minutes at 80°C. Injected volume of 0.001 ml was analyzed using a Hewlett-Packard 6890 gas chromatograph with a 5973 mass spectrometer. The mass spectrometer monitored ions at 265/261 (with check of ratio at 237/233) for glutaric and 188/185 (check ratio at 262/259) for 3-OHGA in separate runs.

ATP and phosphocreatine. Gcdh–/– and WT mice at 4 and 8 weeks of age were placed on the normal or lysine diet in groups of 4 (8 different groups by genotype, diet, and age). All mice were sacrificed by focused beam microwave irradiation to preserve phosphorylation status as previously described (52). The cortex, hippocampus, and striatum were dissected from both sides of the brain from each mouse and frozen. Samples from each brain area were weighed and extracted with 8% PCA as described above and neutralized. Neutralized PCA extracts were assayed for ATP and phosphocreatine enzymatically (49). ATP was measured by following the formation of NDPDP spectrophotometrically using hexokinase coupled with glucose-6-phosphate dehydrogenase in the presence of glucose and NADP+. Phosphocreatine was measured by adding Cr kinase to the above reaction and following the additional formation of NADPH. Protein concentrations were measured by the method of Lowry (53).

Coenzyme A, glutaryl-CoA and α-ketoglutarate. PCA extracts of brain samples from each group described above were used to analyze free reduced CoA and glutaryl-CoA by HPLC as previously described (54). For α-ketoglutarate, brain extracts were extracted with ethyl acetate. Dried residues were reconstituted in 25 mM potassium phosphate buffer at pH 2.5. CoA, acetyl-CoA, glutaryl-CoA, and α-ketoglutarate standards were used to develop a curve for quantification.

MRI and H1 spectroscopy. MRI was performed on a 7.0 T Bruker system using a 2-mm birdcage coil at 48 hours, 72 hours, and 6 days following the start of special diets (n = 4 mice per diet group). Mice were anesthetized with isoflurane (1–1.5%) and imaged with a T1-weighted multiecho, spin echo sequence (5 0.5 mm thick slices, TR/TE = 3,000/10.6–14.84 ms, 14 echoes, 117 × 117 μm2 resolution, 2 averages; TR, repetition time; TE, echo time). T2 values were calculated on a pixel-by-pixel basis from the corresponding exponential fits using CCHIPS software (55). 1H NMR spectroscopy data were acquired between 48 and 72 hours, using PRESS sequence (TR/TE = 2656/7 ms, 512 averages, 4.2 ml voxel covering the cortex and the striatum; Figure 6A). NMR spectroscopy data was processed using LCModel software (56). Data quality was assessed by visual inspection of the peaks in addition to quantitative measurement of the line width (full width as half maximum), automatically provided by LCModel software (56). Mean SD was greater than 20% for estimated concentration of reported metabolites. Mann-Whitney U test was used for statistical analysis. Data are presented as a ratio to Cr, with the assumption that its concentration was unaffected.

Statistics. Normally distributed data sets were analyzed by ANOVA with Fisher least-significant-difference post hoc test. Kruskal-Wallis 1-way ANOVA on ranks was performed with Student-Neuman-Keuls post hoc test on samples that were not normally distributed. SigmaStat software (Jandel Scientific) was used for analysis. All P values of less than 0.05 were considered statistically significant.
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