Glutaric Acid Neurotoxicity: Mechanisms and Actions

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Abstract

Glutaric acid (GA) is found in high concentrations in glutaric acidemia type 1 (GA 1), an inherited neurometabolic disorder of intoxication characterized by acute striatal degeneration, progressive extrastriatal abnormalities, white matter changes, and cerebral atrophy. Untreated GA 1 patients present increased mortality and manifest with a severe complex movement disorder due to striatum injury. Mounting evidence indicates that GA has a central role in the brain damage of GA 1, by disturbing essential processes necessary for cerebral development and functioning. This chapter will update the current knowledge on GA neurotoxic effects, which were mainly obtained from animal studies. GA disrupts neurotransmission, redox homeostasis, bioenergetics, blood-brain barrier, brain vasculature, and myelination, besides inducing reactive astrogliosis and neuronal death. In addition, GA in vivo administration to rats provokes neurodevelopmental motor delay, cognitive impairment, and convulsions. Studies performed in humans support most of the animal data relative to GA-induced neurotoxic effects. Potential novel therapies targeting the deleterious effects of GA to improve the outcome of GA 1 patients will also be discussed.

Keywords

Glutaric acid · Glutaric acidemia type 1 · Glutaric acidemia type 2 · Glutaric acidemia type 3 · Neurotoxicity · Excitotoxicity · Bioenergetics · Redox homeostasis · Myelination · Reactive astrogliosis · Neuroinflammation · Blood-brain barrier · Vascular alterations

Abbreviations

3HGA	3-Hydroxyglutaric acid
AMPA	α-Amino-3-hydroxy-5-methylisoxazole propionic acid
BBB	Blood-brain barrier

C5DC	Glutarylcarnitine
CA	Citric acid cycle
CK	Creatine kinase
CNS	Central nervous system
CSF	Cerebrospinal fluid
ER	Endoplasmic reticulum
ETF	Electron transfer flavoprotein
ETFDH	Mitochondrial electron transfer flavoprotein dehydrogenase
FAD	Flavin adenine dinucleotide
GA 1	Glutaric acidemia type 1
GA 2	Glutaric acidemia type 2
GA 3	Glutaric acidemia type 3
GA	Glutaric acid
GABA	γ-Aminobutyric acid
GCDH	Glutaryl-CoA dehydrogenase
Gcdh-/-	Glutaryl-CoA dehydrogenase deficient mice
GDH	Glutamate dehydrogenase
GFAP	Glial fibrillary acidic protein
GPx	Glutathione peroxidase
GSH	Reduced glutathione
HGMD	Human Gene Mutation Database
IL	Interleukin
Lys	L-Lysine
MDA	Malondialdehyde
Mit-CK	Mitochondrial creatine kinase
NaC3	Sodium dicarboxylate cotransporter 3
NMDA	N-Methyl-D-aspartic acid
OAT	Organic anion transporter
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SUGCT	Succinate-hydroxymethylglutarate CoA-transferase
TNF-α	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor

1 Key Points

- Glutaric acid is the major accumulating metabolite in patients affected by glutaric acidemia type 1, which is caused by glutaryl-CoA dehydrogenase deficiency and characterized by striatum degeneration and progressive extrastriatal abnormalities.
- Glutaric acid is also found in high concentrations in glutaric acidemia type 2 and glutaric acidemia type 3, which are due to deficient activity of mitochondrial electron transfer flavoprotein dehydrogenase and succinate-hydroxymethyl-glutarate CoA-transferase, respectively.

- Glutaric acid is generated within the brain from mitochondrial catabolism of lysine, hydroxylysine, and tryptophan and accumulates in the central nervous system due to its poor efflux through the blood-brain barrier.
- Excitotoxicity, oxidative stress, reactive astrogliosis, astrocyte dysfunction, neuroinflammation, bioenergetics disruption, blood-brain barrier breakdown, vascular alterations, and myelination disturbance are major neurotoxic mechanisms induced by glutaric acid.
- Glutaric acid neurotoxicity is mainly involved in the neuropathological alterations of patients with glutaric acidemia type 1.

2 Glutaric Acid in Inherited Metabolic Disorders

Glutaric acid (GA), also known as 1,5-pentanedioic acid, is a C5 linear chain dicarboxylic acid produced in mammals predominantly from spontaneous hydrolysis of glutaryl-CoA in the catabolic pathway of lysine (Lys), tryptophan, and hydroxylysine. This organic acid accumulates in biological fluids and tissues predominantly when glutaryl-CoA oxidation is blocked. GA tissue accumulation is observed in three different inherited metabolic disorders, namely, glutaric acidemia type 1 (GA 1) (OMIM 231670), glutaric acidemia type 2 (GA 2) (OMIM 231680), and glutaric acidemia type 3 (GA 3) (OMIM 231690).

2.1 Glutaric Acidemia Type 1: Glutaryl-CoA Dehydrogenase Expression, Biochemical and Clinical Phenotypes

GA 1, also known as glutaric aciduria type 1 or glutaryl-CoA dehydrogenase (GCDH) deficiency, is an autosomal recessive neurometabolic disorder caused by absent or deficient activity of GCDH (EC 1.3.8.6). GCDH is a flavoprotein enzyme with ~43.3 kDa, which belongs to the acyl-CoA dehydrogenase family and participates in the catabolic pathway of Lys, tryptophan, and hydroxylysine. It is located in the mitochondria, catalyzing a two-step oxidative decarboxylation of glutaryl-CoA that generates crotonyl-CoA.

2.1.1 Glutaryl-CoA Dehydrogenase Expression in Rodents and Humans

Variable expression of GCDH protein occurs in tissues of embryonic and adult rats (Braissant et al., 2017), as well as in mice (Olivera-Bravo et al., 2015; Woontner et al., 2000; Zinnanti et al., 2007). GCDH is well expressed in the peripheral tissues of rodents, mainly in the liver, and in various brain areas, but at lower levels (Table 1). Human GCDH is also highly expressed in peripheral tissues of humans, such as the liver, kidney, heart, and skeletal muscle, with lower expression in the cerebral structures (Table 2) (Uhlén et al., 2015) (Human Protein Atlas, available from http://www.proteinatlas.org on April 15th, 2021).

Table 1 Tissue protein expression of glutaryl-CoA dehydrogenase in rodents

Rats				
	Tissue	Protein expression (WB or IM)		References
Adult	**Liver**	+++		Braissant et al., 2017
	Intestine	+++		
	Kidney	+++		
	Heart	+++		
	Skeletal muscle	++		
	Skin	+		
	Spleen	++		
	Brain	Cerebral cortex Cerebellum Striatum Hippocampus Olfactory bulbs Thalamus Pons Medulla oblongata	++ ++ ++ ++ + + ++ ++	
Embryonic	**Liver**	++		
	Heart	+		
	Kidney	++		
	Skeletal muscle	+		
	Lung	++		
	Bone	++		
	Skin	+++		
	Intestine	+++		
	Brain	Cerebral cortex Cerebellum Striatum	++ + ++ + ++ +	
Mice				
Adult	**Liver**	+++		Woontner et al., 2000
	Kidney	+++		
	Heart	++		
	Skeletal muscle	+		
	Brown fat	++		
	Brain	Cerebral cortex Cerebellum Striatum Hippocampus Thalamus	+ ++ + ++ ++	
	Cortical neurons	+++		Zinnanti et al., 2007
	Hippocampal neurons	+++		

(continued)

Table 1 (continued)

Rats			
	Tissue	Protein expression (WB or IM)	References
	Hippocampal astrocytes	ND	
	Hippocampal endothelial cells	ND	
Embryonic	**Striatal neurons**	+++	Olivera-Bravo et al., 2015
Neonatal	**Cortical astrocytes**	+	

ND, not detected; +, low; ++, medium; +++, high; IM, immunoreactivity; WB, Western blot

Table 2 Tissue expression of enzymes causing glutaric acidemia type 1, 2, and 3 in humans

Protein expression					
Tissue	GA 1	GA 2			GA 3
	GCDH	ETF-A	ETF-B	ETFDH	SUGCT
Brain					
Cerebral cortex	++	+	++	+	++
Hippocampus	++	++	++	ND	++
Caudate	+	+	++	ND	++
Cerebellum	+++	++	+++	+	ND
Liver	+++	++	+++	++	ND
Kidney	+++	+++	+++	+++	+++
Heart	+++	+++	+++	+++	++
Skeletal muscle	+	++	++	+	ND
mRNA expression					
Tissue	GA 1	GA 2			GA 3
	GCDH	ETF-A	ETF-B	ETFDH	SUGCT
Brain					
Cerebral cortex	+	+	++	+	+
Hippocampus	+	+	++	+	+
Basal ganglia	+	+	++	+	+
Cerebellum	+	+	+	+	+
Liver	+++	+++	+++	+++	++
Kidney	++	++	++	++	+++
Heart	++	+++	+++	+++	+
Skeletal muscle	++	+++	+++	++	+

ND, not detected; +, low; ++, medium; +++, high (Uhlén et al., 2015; Human Protein Atlas, available from https://www.proteinatlas.org/ on April 15th, 2021); ETF, electron transfer flavoprotein; ETFDH, mitochondrial ETF dehydrogenase; GA 1, glutaric acidemia type 1; GA 2, glutaric acidemia type 2; GA 3, glutaric acidemia type 3; GCDH, glutaryl-CoA dehydrogenase; SUGCT, succinate-hydroxymethylglutarate CoA-transferase

The human *GCDH* gene encodes a 438-amino-acid precursor peptide with a mitochondrial localization sequence at the amino terminus, which is cleaved, yielding a 394-residue mature subunit (Goodman et al., 1995). Oligomerization of four

GCDH monomers, each containing a non-covalently bound FAD, builds up to the enzymatically active GCDH homotetramer. The *GCDH* gene is located on chromosome 19p13.2 (Greenberg et al., 1994), spanning about ~7 kb region and containing 12 exons. To date, approximately 200–250 pathogenic mutations have been reported in the Human Gene Mutation Database (HGMD), being missense mutations the most common type (Human Gene Mutation Database, available from http://www.hgmd.cf.ac.uk/ac/index.php on April 15th, 2021).

2.1.2 Biochemical Findings

Deficient or absent activity of GCDH due to pathogenic mutations in the *GCDH* gene results in deficient glutaryl-CoA degradation and accumulation and spontaneous hydrolysis to GA (Fig. 1). Thus, blockage at the GCDH step leads to tissue high concentrations of GA, predominantly in the brain, as well as in cerebrospinal fluid (CSF), urine, blood, and other tissues of GA 1 patients. Additional biochemical hallmarks of GA 1 include elevated concentrations of 3-hydroxyglutaric acid (3HGA) and glutarylcarnitine (C5DC). 3HGA is formed by the nonspecific oxidation of glutaryl-CoA by medium-chain acyl-CoA dehydrogenase, generating glutaconyl-CoA, which originates 3-hydroxyglutaryl-CoA by a reaction catalyzed by 3-methylglutaconyl-CoA hydratase and 3HGA by spontaneous hydrolysis (Goodman & Woontner, 2019; Peters et al., 2019). C5DC is synthesized by the conjugation of glutaryl-CoA with carnitine by an acyltransferase. GA and 3HGA are mainly detected in urine, whereas C5DC is measured in blood.

GA concentrations in biological fluids and postmortem brain of GA 1 patients vary considerably (Tables 3 and 4). Based on the urinary GA levels, GA 1 patients are classified into two different biochemical phenotypes, the low excretors (below 100 mmol/mol creatinine) and the high excretors (usually between 850 and 1700 mmol/mol creatinine), although 3HGA urinary concentrations are high in both variants (Baric et al., 1999). The activity of GCDH is generally nondetectable in the high excretors, whereas a residual activity of up to 30% is found in the low excretors (Boy et al., 2017). Interestingly, the disease course and risk for neurological dysfunction are similar in both biochemical phenotypes (Boy et al., 2017). Blood GA and C5CD levels are usually decreased in low excretors, making newborn screening and postnatal diagnosis of GA 1 more problematic in these patients, therefore increasing the number of false negatives. Variable blood and brain levels of C5DC have been reported, and plasma C5DC levels seem to correlate with the urinary excretion of GA, but not with the clinical manifestations. Importantly, blood C5DC levels substantially decrease with treatment, therefore potentially being important to monitor therapy efficacy in this disorder (Guerreiro et al., 2020; Kölker et al., 2003; Kulkens et al., 2005; Tortorelli et al., 2005).

2.1.3 Clinical Manifestations

GA 1 untreated patients commonly present a complex movement disorder with predominant dystonia, choreiform movements, dyskinesia, and truncal hypotonia following acute degeneration of the basal ganglia associated with encephalopathic

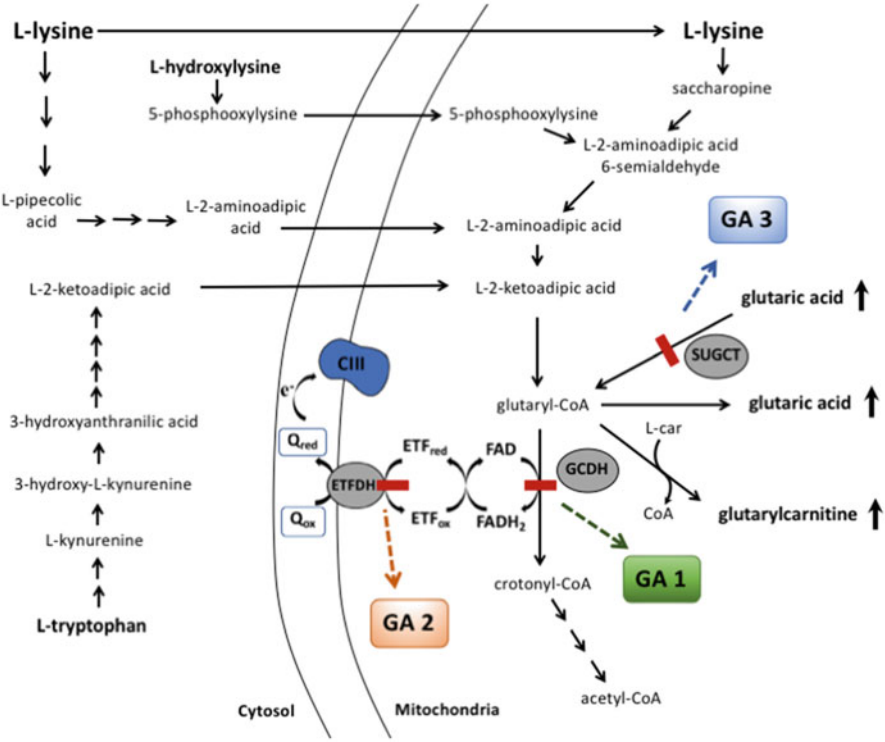

Fig. 1 *Glutaric acid accumulation and enzymatic defects in glutaric acidemia type 1 (GA 1), glutaric acidemia type 2 (GA 2), and glutaric acidemia type 3 (GA 3)*. GA 1 is caused by deficiency in the activity of glutaryl-CoA dehydrogenase (GCDH) of the catabolism of L-lysine, L-hydroxylysine, and L-tryptophan, leading to gutaryl-CoA accumulation, which is subsequently transformed in glutaric acid. GA 2 is due to pathogenic mutations in the *ETFA*, *ETFB*, or *ETFDH* genes that encode the two subunits of the electron transfer flavoprotein (ETF) and the mitochondrial ETF dehydrogenase (ETFDH), respectively, impairing the mitochondrial downstream electron transport of GCDH through the respiratory chain, subsequently leading to cellular glutaryl-CoA increase. On the other hand, glutaric acid accumulates in GA 3 due to a deficiency in succinate-hydroxymethylglutarate CoA-transferase (SUGCT) activity that forms glutaryl-CoA from free glutaric acid

crises. Other nonspecific neurologic symptoms, accompanied by progressive leukodystrophy, and cerebral atrophy occur (Boy et al., 2017, 2019; Harting et al., 2015).

2.2 Glutaric Acidemia Type 2: Expression of Mitochondrial Electron Transfer Flavoprotein Dehydrogenase and Electron Transfer Flavoproteins – Biochemical and Clinical Phenotypes

Brain accumulation and increased plasma and urinary excretion of GA are observed in multiple acyl-CoA dehydrogenase deficiency, also known as GA 2. GA 2 is an

Table 3 Glutaric acid concentrations in urine and plasma of patients with glutaric acidemia types 1, 2, and 3

		Urine (mmol/mol creatinine)	Plasma (μmol/L)	References
GA 1	**High excretors**	500–5000	255	Bennett et al., 1986
		5470	–	Goodman et al., 1977
		5138–6457	4390	Leibel et al., 1980
		1998–3608	–	Kölker et al., 2003
		1600	–	Kulkens et al., 2005
	Low excretors	33–450	–	Funk et al., 2005
		58	–	
		99–118	–	
		6–97	–	
GA 2		9683	2270	Przyrembel et al., 1976
		22,350	–	Goodman et al., 1980
		8563	–	Gregersen et al., 1980
		5526	620	Sweetman et al., 1980
		407	<10	
		8040	–	Baric et al., 1999
		7.4	–	
		7509	–	Tortorelli et al., 2005
GA 3		500	–	Knerr et al., 2002
		1460	–	
		290	–	
		375	–	Tortorelli et al., 2005
		78.5	–	Sherman et al., 2008
		45	–	Waters et al., 2018
		153–940	–	
		38–393	–	

GA 1, glutaric acidemia type 1; GA 2, glutaric acidemia type 2; GA 3, glutaric acidemia type 3. The data correspond to a single or range of measurements in individual patients

autosomal recessive disorder caused by pathogenic mutations in the *ETFA*, *ETFB*, or *ETFDH* genes that encode the two subunits of the electron transfer flavoprotein (ETF) and the mitochondrial ETF dehydrogenase (ETFDH, also known as ETF-ubiquinone oxidoreductase, EC 1.5.5.1), respectively. ETFs are heterodimers containing a single equivalent of flavin adenine dinucleotide (FAD). Human ETF structure comprehends three distinct domains: two domains are found in the α-subunit and the third domain is made up entirely by the β-subunit. On the other hand, ETFDH is an intrinsic membrane protein located in the inner mitochondrial membrane that contains single equivalents of FAD and a $[4Fe4S]^{2+,1+}$ cluster (Watmough & Frerman, 2010). Together with ETF, ETFDH forms a short pathway in the mitochondrial respiratory chain that transfers electrons from flavoprotein dehydrogenases to ubiquinone (Watmough & Frerman, 2010). Deficiency of ETF

Table 4 Clinical, biochemical, and postmortem neuroradiological features in patients with glutaric acidemia type 1

Clinical	Biochemical (brain glutaric acid levels)	Neuroradiological	References
Mental retardation, dystonia, and athetosis Episodes of high fever, vomiting, and diarrhea Death after an acute metabolic crisis	Frontal cortex: 0.83μmol/g wet weight (approximately 1.04 mM)	Marked neuronal shrinkage and degeneration associated with edema in the cerebral cortex Massive degeneration with severe neuronal loss and fibrous gliosis in putamen and caudate	Goodman et al., 1977
Recurrent episodes of metabolic decompensation with vomiting, diarrhea, and seizures Lethargy, developed choreoathetoid movements Death following a crisis with high fever	Frontal cortex: 0.67μmol/g (approximately 0.84 mM) Basal ganglia: 1.25μmol/g (approximately 1.56 mM)	Shrinkage of the caudate and putamen associated with severe loss of nerve cells and fibers with proliferation of astrocytes	Leibel et al., 1980
Headache, tremor (hand), seizures, ataxia, and orofacial dyskinesia Psychiatric disease and dementia	Brain = 5.28 mM	Generalized atrophy in the cerebral cortex Confluent signal changes of supratentorial white matter Basal ganglia were normal	Kulkens et al., 2005
Developmental delay, hypotonia, vomiting, diarrhea, fever, and severe dehydration Death after an acute metabolic crisis	Cerebellum: 21240 nmol/g protein = (approximately 26.55 mM)	Hemorrhagic infarction of the right parietal and temporal cerebrum Atrophy with severe loss of medium-sized neurons in the caudate and putamen Astrocyte hypertrophy and microglial activation in the striatum	Funk et al., 2005
Developmental delay and relatively sudden onset of dystonia and seizures Patient died suddenly after developing fever	Cerebellum: 8020 nmol/g protein = (approximately 10.02 mM) Thalamus: 10960 nmol/g protein = (approximately 13.7 mM) Caudate: 5960 nmol/g protein = (approximately 7.45 mM) Frontal cortex:	Atrophy of caudate and putamen, whereas lateral ventricles were mildly enlarged Striatum loss of medium-sized neurons, reactive astrocytes, and reactive microglia in caudate Scattered pyknotic	

(continued)

Table 4 (continued)

Clinical	Biochemical (brain glutaric acid levels)	Neuroradiological	References
	7760 nmol/g protein = (approximately 9.7 mM)	neurons in cerebral cortex	
Fever associated with dystonia and athetoid limb movements, as well as seizures Patient died during a febrile illness	Frontal cortex: 3770 nmol/g protein = (approximately 4.71 mM)	Pronounced widening of the Sylvian fissures Symmetric lateral ventricle enlargement The caudate and the putamen exhibited severe neuronal loss with pronounced reactive astrocytes	
Generalized hypotonia in infancy Patient developed seizures and choreoathetoid movements after a metabolic crisis, followed by severe spastic quadriparesis Patient died after acute pneumonia	Frontal cortex: 5990 nmol/g protein = (approximately 7.49 mM) Caudate/internal capsule: 8310 nmol/g protein = (approximately 10.39 mM)	Mild gyral flattening, slightly enlarged ventricles, and severe striatal atrophy The caudate and putamen presented a severe loss of medium-sized neurons and some reactive astrocytes Small foci of spongiform change in the frontal and insular cortex	

or ETFDH impairs the dehydrogenation catalyzed by at least 12 flavoprotein dehydrogenase enzymes involved in the oxidation of different molecules, including GCDH, therefore resulting in glutaryl-CoA accumulation and its subsequent conversion to GA (Prasun, 2020) (Fig. 1).

The ETFDH transcript can be found throughout brain structures and in many peripheral tissues. In the cerebral cortex, ETFDH is found only in neurons, whereas in the cerebellum it is expressed in Purkinje cells and cells of the granular layer. Transcript levels and ETF alpha and beta proteins are also expressed in the brain and peripheral tissues. The protein content of both ETF alpha and beta is found in neurons and glial cells of the cerebral cortex, hippocampus, and basal ganglia, as well as in cells of granular and molecular layers and Purkinje cells of the cerebellum (Table 2) (Uhlén et al., 2015) (Human Protein Atlas, available from http://www.proteinatlas.org on April 15th, 2021).

Patients affected by GA 2 usually present a severe neonatal-onset form characterized by hypotonia, hepatomegaly, hyperammonemia, nonketotic hypoglycemia, and metabolic acidosis or a milder late-onset form manifesting with myopathy, hepatomegaly, encephalopathy, episodic lethargy, vomiting, and hypoglycemia (Boyer et al., 2015). High urinary excretion and elevated plasma and CSF levels of GA are commonly found in these patients (Baric et al., 1999; Coude et al., 1981; Gregersen et al., 1980; Przyrembel et al., 1976; Sweetman et al., 1980;

Tortorelli et al., 2005) (Table 3). Accumulation of GA was also demonstrated in the postmortem frontal cortex and associated with brain damage in GA 2 (Goodman et al., 1982). Blood acylcarnitine profile characteristically shows increased concentrations of several short-, medium-, and long-chain acylcarnitines (Boyer et al., 2015), including C5DC (Tortorelli et al., 2005). Although high GA levels are found in GA 2 patients, there is no correlation between GA concentrations and the clinical phenotype of the disease, both in the severe neonatal-onset and in the milder late-onset form. It is important to emphasize that these patients also accumulate and excrete large amounts of ethylmalonic, adipic, 3-hydroxyisovaleric, 2-hydroxyglutaric, 5-hydroxyhexanoic, suberic, sebacic, and dodecanedioic acids, as well as acylglycine conjugates, especially of C4 and C5 carboxylic acids (Prasun, 2020).

2.3 Glutaric Acidemia Type 3: Expression of Succinate-Hydroxymethylglutarate CoA-Transferase – Biochemical and Clinical Phenotypes

Tissue accumulation and high urinary excretion of GA are also observed in individuals affected by deficiency of succinate-hydroxymethylglutarate CoA-transferase (SUGCT) (EC 2.8.3.13) activity, a very rare disorder also called GA 3. SUGCT deficiency is caused by mutations in the *SUGCT* gene (*C7orf10*), preventing the formation of glutaryl-CoA from free GA, thereby resulting in the accumulation of this organic acid (Sherman et al., 2008; Waters et al., 2018) (Fig. 1).

SUGCT transcripts are found in various tissues, with the highest levels in the kidney, liver, and muscle. In the brain, SUGCT is expressed in all regions, with higher levels in the hippocampus, basal ganglia, pons, and medulla. SUGCT is mainly expressed in neurons of the cerebral cortex, hippocampus, and basal ganglia. In contrast, low content of SUGCT is observed in glial cells of the hippocampus and basal ganglia (Table 2) (Uhlén et al., 2015) (Human Protein Atlas, available from http://www.proteinatlas.org on April 15th, 2021).

Only a few patients have been described with GA 3. GA urinary levels were shown to be increased, varying from 45 to 1460 mmol/mol creatinine (normal levels $<$10 mmol/mol creatinine) (Knerr et al., 2002; Sherman et al., 2008; Tortorelli et al., 2005; Waters et al., 2018) (Table 3). Clinical presentation in the few reported patients is variable and inconsistent (Waters et al., 2018).

3 Glutaric Acid Transport Across Membranes. Blood-Brain Barrier – Glutaric Acid Accumulation in the Central Nervous System (Trapping Theory)

3.1 Glutaric Acid Transport Across Membranes

The sodium dicarboxylate cotransporter 3 (NaC3) and the organic anion transporters (OAT) have been advocated as candidates to mediate intra- and intercellular transport of GA across biological membranes in several tissues (Sauer et al., 2006).

NaC3 is found in renal proximal tubules, placenta, and hepatocytes, as well as in synaptosomes and astrocytes (Yodoya et al., 2006). Furthermore, GA is a substrate for the NaC3 expressed in astrocytes (but not in the blood-brain barrier (BBB)), competing with other citric acid cycle (CAC) intermediates for the same carrier and therefore compromising the astrocytic supply of essential substrates to neurons (Lamp et al., 2011). On the other hand, the isoforms 1 (OAT1) and 3 (OAT3) of OAT are located in renal proximal tubular cells (Robertson & Rankin, 2006), whereas isoform 1 is also expressed in cortical and hippocampal neurons, as well as in the choroid plexus (Alebouyeh et al., 2003). OAT1 and OAT3 are expressed in the BBB but have a limited capacity to transport dicarboxylates, including GA (Sauer et al., 2006).

3.2 Blood-Brain Barrier

The BBB is a term used to describe the restrictive characteristics and properties of the microvasculature of the central nervous system (CNS). It is formed by continuous nonfenestrated vessels associated with other components tightly regulating the movement of molecules, ions, and cells between blood and the CNS (Daneman, 2012). This restricting barrier allows BBB endothelial cells to protect the brain against toxins, pathogens, inflammation, and injury. The restrictive nature of the BBB also provides an obstacle for molecules to enter the CNS, including dicarboxylates like GA and similar products, which at physiological pH behave as hydrophilic anions, rendering these molecules impermeable to cellular membranes, thereby requiring specific transport systems (Sauer et al., 2006).

3.3 Glutaric Acid Accumulation in the Central Nervous System (Trapping Theory)

The brain levels of GA and 3HGA may be up to 1000-fold higher relatively to serum values, so that the huge accumulation of particularly GA (in the millimolar range) and also 3HGA (in the micromolar range) in the brain of GA 1 patients is mostly attributed to their intracerebral de novo synthesis mainly from Lys oxidation and their very limited efflux from the brain (trapping theory) (Table 4, Fig. 2) (Sauer et al., 2006). Noteworthy, Lys is transported into the brain by the BBB SLC7A1 transporter and enters into the mitochondria through the SLC25A29 transporter, being thereafter degraded to generate GA and 3HGA to a lesser extent (Fig. 1) (Sauer et al., 2011). Experiments using the hepatic GCDH knockout mice showed that the levels of GA and 3HGA were only slightly increased in the brain, whereas the complete GCDH knockout mice presented much higher cerebral concentrations of these organic acids (Sauer et al., 2006; Zinnanti et al., 2006), corroborating with the trapping theory.

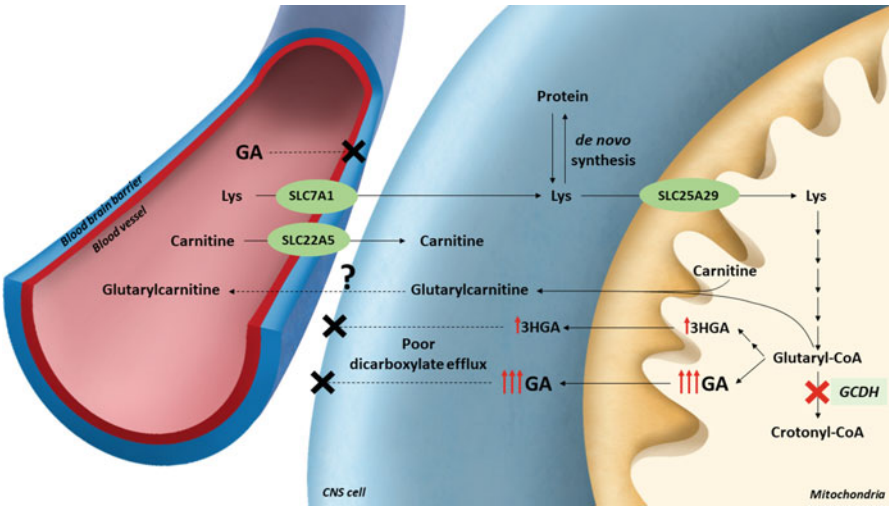

Fig. 2 *Glutaric acid (GA) accumulation in the brain of glutaric acidemia type 1 (GA 1) patients: trapping theory.* L-lysine (Lys) is transported from the circulation into the brain through the blood-brain barrier by SLC7A1 protein transporter and then is uptaken by mitochondria through the SLC25A29 transporter. Intramitochondrial Lys catabolism generates high amounts of GA, and 3-hydroxyglutaric acid (3HGA) to a lesser extent, in the brain of GA 1 patients due to glutaryl-CoA dehydrogenase (GCDH) deficiency. These organic acids are then trapped into the central nervous system because of the very limited dicarboxylate efflux through the blood-brain barrier, thus resulting in marked intracerebral accumulation of GA and 3HGA

4 Human Evidence of Glutaric Acid Neurotoxicity in Glutaric Acidemia Type 1

Mounting data indicate that GA is a central neurotoxin in GA 1 neuropathology. Thus, very high concentrations of GA in the cerebral cortex of late-onset high excretor GA 1 patients were associated with extensive white matter changes (Harting et al., 2015). In addition, high millimolar GA concentrations were found in different postmortem cerebral structures of GA 1 patients that suffered encephalopathic crises that led them to death and were correlated with brain damage, supporting the presumption that cerebral GA may play a crucial role in the neurological dysfunction and brain abnormalities found in this disease (Funk et al., 2005; Goodman et al., 1977; Leibel et al., 1980) (Table 4). This is supported by the acute basal ganglia degeneration occurring in GA 1 patients during crises of metabolic decompensation, which are characterized by accelerated proteolysis and rapid buildup of Lys that is further metabolized to GA after its entrance into the brain, giving rise to high intracerebral concentrations of this neurotoxin (Boy et al., 2019).

Another indication of the toxic role of brain GA accumulation on the neurological dysfunction in GA 1 patients is that the recommended therapy based on low dietary Lys and carnitine supplementation aiming to decrease the levels of the accumulating

metabolites dramatically improves the neurological manifestations and outcome of the affected patients (Kölker et al., 2011).

5 In Vitro and In Vivo Animal Evidence of Glutaric Acid Neurotoxicity

Animal evidence revealing neurotoxic properties of GA was obtained mostly by in vitro, but also by in vivo, experimental approaches utilizing distinct brain cellular preparations. Thus, in vitro experiments achieved by supplementing GA to neural cells or to subcellular preparations from the brain of rodents have shown to (1) compromise glutamatergic and GABAergic neurotransmission by decreasing glutamate uptake and binding to glutamate transporters and receptors presumably due to competition with glutamate, as well as by inhibiting the activities of Na^+/K^+-ATPase and glutamate decarboxylase, that potentially increases glutamate concentrations in the synaptic cleft and reduces brain γ-aminobutyric acid (GABA) content, respectively; (2) induce oxidative stress by causing lipid and protein oxidative damage and by increasing reactive oxygen species (ROS) generation and decreasing the antioxidant defenses; and (3) impair mitochondrial bioenergetics, by decreasing CO_2 production from acetate, mitochondrial respiration, and MTT reduction, besides inhibiting respiratory chain complexes and creatine kinase (CK) activities, as well as ATP production (see reviews Wajner et al., 2004, 2020). These pathomechanisms were presumed to reduce the viability of neural cells exposed to GA (Gerstner et al., 2005; Isasi et al., 2019; Kölker et al., 2000; Komatsuzaki et al., 2018).

5.1 Chemically Induced and Genetic Models of Glutaric Acidemia Type 1

In vivo experimental models of GA 1 also evidenced neurotoxic properties of GA corroborating with the in vitro findings. In this context, chronic and acute systemic administration of GA, as well as acute intrastriatal or intracerebroventricular injection of GA to developing rats, was demonstrated to induce excitotoxicity, oxidative stress, and mitochondrial dysfunction in the brain of developing rats. Astrogliosis, BBB disruption, increase of inflammatory cytokines, decrease of neurotrophic factors, and neuronal, astrocytic, and oligodendrocyte injury have also been observed in the cerebral cortex and striatum of neonatal rats injected with GA, as well as in cultured neural cells from these cerebral structures submitted to these in vivo models (Isasi et al., 2014; see review Olivera-Bravo & Barbeito, 2015; Rodrigues et al., 2019; Wajner et al., 2020).

The development of the genetic mice model of GA 1 (*Gcdh-/-*) by Koeller and collaborators (2002), later improved (Zinnanti et al., 2006), provided an in vivo experimental model of GA 1, presenting similar biochemical and neuropathological aspects of the human disease. Zinnanti et al. (2006) showed that *Gcdh-/-* mice fed a Lys-enriched chow (4.7%) increased the production and intracerebral accumulation

of GA, concomitantly with severe striatum damage, which was not observed when these mice received a normal chow (0.9% Lys) and presented lower intracerebral GA concentrations and no striatum lesions, indicating therefore a neurotoxic role for GA. A high percentage of adolescent *Gcdh-/-* animals receiving a high Lys chow became hypoactive, developed paralysis and seizures after 24 h, and died a few days later. These symptoms occurred in parallel with a 20-fold increase (2.5 mM) of GA serum levels, as compared to asymptomatic *Gcdh-/-* mice that presented 0.27 mM serum concentrations and also to *Gcdh-/-* mice treated with normal chow, whose GA levels were much lower. More importantly, *Gcdh-/-* animals with severe neurological outcomes presented brain GA levels of the order of 3.5 mM in fresh tissue, relative to 0.67 mM of *Gcdh-/-* asymptomatic animals. There was also an accumulation of 3HGA in serum and brain samples from *Gcdh-/-* mice exposed to the high Lys diet, but at a much lower degree than that of GA, implying a major role for GA (Zinnanti et al., 2006).

Furthermore, *Gcdh-/-* mice fed a high Lys chow revealed disturbance of glutamatergic and GABAergic neurotransmission, as well as of redox and energy homeostasis (see review Wajner et al., 2019). Importantly, these alterations preceded the brain histopathological and immunochemical abnormalities in the cerebral cortex and striatum of *Gcdh-/-* animals submitted to dietary Lys overload, including neuronal loss, reactive astrogliosis, and severe vacuolation of striatal patches, suggesting, therefore, a causal relationship between biochemical alterations and brain damage. Biochemical and neuropathological alterations, such as marked vacuolation/edema in the striatum accompanied by oxidative stress induction, neuronal loss, and astrocyte reactivity, were also detected in *Gcdh-/-* mice submitted to a single intrastriatal injection of Lys, mimicking an acute decompensation (Amaral et al., 2019).

Therefore, in vivo experimental models of GA 1 support the hypothesis that increased brain concentrations of GA are related to the brain degeneration observed in GA 1 patients, particularly during episodes of metabolic decompensation in which the cerebral GA concentrations are highly increased.

5.2 Neurobehavioral Disturbances Caused by In Vivo Administration of Glutaric Acid

Studies performed in rodents at various developmental stages (from neonatal to adults) demonstrated that acute and chronic administration of GA causes cognitive deficits and convulsions, further supporting neurotoxic properties for GA. Thus, chronic GA administration to infant rats from postnatal day 5 to 28 caused a long-lasting spatial behavioral deficit in rats (da Costa Ferreira et al., 2008). Furthermore, chronic administration of GA to rats from postnatal day 5 to 28 (Rodrigues et al., 2019) also caused deficits in the procedural and working memories, which were attributed to activated glial cells (reactive astrogliosis) and decrease of nerve growth factor, Bcl-2 and NeuN, as well as increase of oxidative stress, cytokine generation, and caspases levels in the striatum and cerebral cortex of the animals. Noteworthy,

the biochemical and behavioral changes induced by GA in vivo were prevented by the antioxidant N-acetylcysteine, reinforcing the role of oxidative stress in the cognitive impairment induced by GA in vivo (Rodrigues et al., 2019). Furthermore, acute administration of GA in the striatum (a cerebral structure highly vulnerable in GA 1) of adult rats provoked convulsions and other behavioral alterations with the involvement of the GABAergic and glutamatergic systems (Wajner et al., 2004).

Motor and neurodevelopment impairment, deficit of short- and long-term memories, and climbing behavior observed in the inhibitory avoidance task have been also found in *Gcdh-/-* mice (Busanello et al., 2013; Koeller et al., 2002), reflecting brain damage in these animals.

In conclusion, a great deal of experimental evidence indicates that neurodevelopment delay, cognitive deficit, and convulsions are elicited by GA in rodents and exacerbated by inflammatory processes. Some of these effects were accompanied by derangement of redox homeostasis, reactive astrogliosis, glutamatergic and GABAergic neurotransmission, and other biochemical alterations that were probably involved in the behavior disturbances observed. Furthermore, neuroinflammation may also play an important role in the infection-related seizures that are associated with encephalopathic episodes and striatum degeneration observed in GA 1 patients, especially after crises of metabolic decompensation commonly triggered by infections (Boy et al., 2019; Harting et al., 2015).

6 Pathomechanisms of Glutaric Acid Neurotoxicity

A myriad of mechanisms has been proposed to contribute to GA 1 neuropathology, including disruption of neurotransmitter systems, redox homeostasis and bioenergetics, as well as reactive astrogliosis, neuroinflammation, vascular alterations, BBB breakage, and disturbed myelination. These pathomechanisms will be discussed separately, as follows:

6.1 Disruption of Glutamatergic and GABAergic Neurotransmission

Glutamate is the main excitatory neurotransmitter in the CNS and plays key roles in brain development and functioning at physiological concentrations. It is also the immediate precursor of GABA, the principal inhibitory neurotransmitter in the brain, which is formed by glutamate decarboxylation catalyzed by glutamate decarboxylase. Glutamate also generates a-ketoglutarate that supports brain energy metabolism through the activities of glutamate dehydrogenase (GDH) and transaminases (Schousboe et al., 2014). However, at pathological concentrations, glutamate is excitotoxic. Excitotoxicity is a complex process resulting from overactivation of the glutamate receptors N-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methylisoxazole propionic acid (AMPA), and voltage-gated Ca^{2+} channels that lead to excessive neuronal influx of Na^{+} and Ca^{2+} through the neuronal plasma

membrane, resulting in degeneration of dendrites and neuronal death. Glutamate also activates GTP-binding protein-coupled metabotropic glutamate receptors, stimulating inositol trisphosphate production and Ca^{2+} release from the endoplasmic reticulum (ER). Excessive intracellular Ca^{2+} promotes ROS and reactive nitrogen species (RNS) generation, mitochondrial dysfunction associated with low ATP synthesis, and induction of necrotic and apoptotic pathways. Energy deprivation leads to decreased activity of membrane synaptic Na^+/K^+-ATPase, resulting in increased K^+ extracellular concentrations and glutamate release to the synaptic cleft (secondary excitotoxicity) (Greene & Greenamyre, 1996). Susceptibility of neurons to excitotoxicity depends on their ability to remove and buffer Ca^{2+} that is carried out by Ca^{2+}-ATPases located in the plasma membrane and ER, Na^+/Ca^{2+} exchangers, the mitochondrial Ca^{2+} uniporter, and various Ca^{2+}-binding proteins. Also important to excitotoxic-induced neuronal death is the increase in duration and extent of intracellular Ca^{2+} concentrations after glutamate receptor activation. Thus, excessive Ca^{2+} leads to the activation of calpains that can degrade cytoskeletal proteins, membrane receptors, and enzymes. Glutamate-induced Ca^{2+} influx also triggers cyclooxygenases and lipoxygenases, increasing ROS production and membrane lipid peroxidation that subsequently disrupts mitochondrial metabolism. Membrane lipid peroxidation may also render neurons more susceptible to excitotoxicity by impairing the function of membrane ion-motive ATPases, as well as of glucose and glutamate transporters. In this particular, neuronal cells are particularly vulnerable to excitotoxicity under conditions of energy deprivation such as hypoglycemia mainly because of the high amounts of energy (ATP) neurons required to maintain and restore ion gradients (Greene & Greenamyre, 1996).

Postsynaptic vacuolation characteristic of glutamate-mediated brain damage and massive death of striatal medium spiny neurons, which are highly vulnerable to excitotoxicity, were observed in postmortem basal ganglia and cerebral cortex of GA 1 patients accumulating GA at millimolar concentrations (Funk et al., 2005; Goodman et al., 1977). Characteristic excitotoxic-like cerebral lesions were also observed in the brain of *Gcdh-/-* mice (Zinnanti et al., 2007). These histopathological alterations support the view that GA-induced excitotoxicity possibly represents an important pathomechanism of brain dysfunction in GA 1.

Noteworthy, GA was shown to alter the glutamatergic and GABAergic neurotransmitter systems through distinct mechanisms. Some of these effects could be ascribed to the chemical structure similarities between GA and glutamate. Thus, in vitro studies showed that GA decreases glutamate uptake into rat synaptosomes probably by interaction with glutamate transporters. Other observations indicate an interaction between GA and glutamate transporters, particularly the GLAST transporter, in cerebral cortex of infant but not adult rats, and that oxidation of these transporters seems to play a role in the decreased GA-induced glutamate uptake, which was rescued by the antioxidant N-acetylcysteine. It is emphasized that GA elicits oxidative stress in the rat cerebral cortex and that glutamate transporter activity can be inhibited by oxidation (Wajner et al., 2004).

Age-dependent inhibition of $[Na^+]$-independent glutamate binding to synaptic plasma membranes (receptors) from cerebral cortex and striatum by GA was demonstrated only in infant rats (aged 7 and 15 days), but not in adult rats (60-day-old).

The ontogenetic and organ-specific differences of glutamate receptor and transporter expression may explain the lack of effect of GA on glutamate binding in the brain of older rats. Other data revealed that GA binds to non-NMDA receptors, especially to kainate receptors. GA also inhibited glutamate binding to glutamate receptors in synaptic membranes and glutamate uptake into synaptic vesicles in the forebrain of young rats (Wajner et al., 2004).

Studies using the genetic mouse model of GA 1 (*Gcdh-/-* mice) also revealed that GA inhibits Na^+-dependent glutamate binding to synaptic membranes (binding to transporters) and glutamate uptake into the striatum of *Gcdh-/-* mice by inhibition of astrocyte glutamate transporters. High mRNA and protein expression of various NMDA glutamate receptors, and more particularly the subtypes NR2A and NR2B, was also observed in the striatum and in the cerebral cortex of developing *Gcdh-/-* mice. Furthermore, elevated expression of the glutamate transporter subunits GLAST and GLT1 was found in the cerebral cortex and striatum of adult *Gcdh-/-* mice, whereas in infant mice only GLAST mRNA levels were augmented in the striatum. Noteworthy, when exposing these *Gcdh-/-* animals to high Lys chow, a further increase of the expression of these glutamate receptors and transporters occurred, implying the participation of GA and 3HGA in these effects (Wajner et al., 2019). Overall, these data demonstrated that *Gcdh-/-* mice overexpress glutamate receptors and transporters, potentially facilitating excitotoxicity, and that these alterations are maximized by the accumulation of GA and 3HGA.

Other observations showed that GA markedly inhibits in vitro and in vivo Na^+/K^+-ATPase activity in rat striatum, as well as in cultured chick embryo neurons. Since this activity is necessary for glutamate uptake by astrocytes, the release of glutamate to the synaptic cleft may further aggravate excitotoxicity (secondary excitotoxicity). Creatine administration was neuroprotective by preventing the inhibition of Na^+/K^+-ATPase activity and of glutamate uptake into synaptosomes, as well as protein oxidation and convulsions elicited by intrastriatal injection of GA (Wajner et al., 2004, 2020).

On the other hand, decreased intracerebral amounts of GABA found in postmortem brain tissue from patients with GA 1 and from *Gcdh−/−* mice (Funk et al., 2005; Leibel et al., 1980; Zinnanti et al., 2007), which were attributed to inhibition of glutamate decarboxylase by GA and 3HGA, allied to altered electrophysiological measures observed in *Gcdh−/−* mice indicate impairment of the GABAergic neurotransmission system in GA 1 (Wajner et al., 2019).

Therefore, it is conceivable that disturbances of glutamatergic and GABAergic systems may predispose the striatum and cerebral cortex of GA 1 patients to excitotoxicity, contributing, therefore, to the pathophysiology of this disorder.

6.2 Oxidative Stress

Oxidative stress is a deleterious condition that results from an imbalance between cellular prooxidants and antioxidants in favor of the former, leading to disruption of redox signaling and oxidative damage. This condition is considered a relevant

pathomechanism of tissue damage in a variety of diseases, including common neurodegenerative disorders and some inborn errors of metabolism (Niedzielska et al., 2016; Wajner et al., 2020). Regarding GA 1, a growing body of evidence indicates the metabolites that accumulate in tissues and biological fluids of individuals with GA 1.

Mounting experimental evidence has shown that GA induces oxidative stress in vitro and in vivo, inducing ROS generation; oxidative attack toward lipids, proteins, and DNA; and decreasing cellular antioxidant defenses in the brain. Thus, GA causes lipoperoxidation (increased chemiluminescence signal) and decreases the antioxidant defenses (total radical-antioxidant potential and glutathione peroxidase – GPx – activity) in vitro in the rat brain. Accordingly, in vivo chronic subcutaneous administration of GA to developing rats provokes marked lipid peroxidation (increased malondialdehyde – MDA – levels) and decreased enzymatic (GPx activity) and nonenzymatic (total antioxidant reactivity – TAR) antioxidant defenses in the midbrain. Noteworthy, peripheral tissues including the liver, erythrocytes, and heart were mildly affected by the same treatment compared to the CNS. Acute subcutaneous administration of GA to adolescent rats also caused a marked decrease of antioxidant defenses in the midbrain and cerebellum and a mild to moderate oxidative stress in peripheral erythrocytes. On the other hand, acute injection of GA into the striatum of adult rats caused lipid peroxidation (increased MDA levels) and protein oxidation (carbonylation) in this cerebral structure, which were prevented by the neuroprotective agent GM1 ganglioside. Creatine supplementation by intragastric gavage also prevented the increased protein carbonylation in the striatum of rats injected with GA. Furthermore, increased ROS production in rat striatal synaptosomes exposed to GA was prevented by trolox (a hydrosoluble analog of the antioxidant a-tocopherol) and by CNQX (a non-NMDA glutamate receptor antagonist), suggesting the involvement of glutamatergic neurotransmission in GA-induced increased generation of ROS. GA was also able to elicit oxidative stress in rat cortical astrocytes and C6 glioma cells, by increasing lipid peroxidation (MDA values) and decreasing antioxidant defenses (reduced glutathione – GSH – levels) (Wajner et al., 2020).

Induction of oxidative stress was also shown in the striatum and cerebral cortex of the genetic mouse model of GA 1 (*Gcdh-/-* mice) submitted to Lys overload. Chronic treatment of *Gcdh-/-* mice with a high Lys chow showed marked disruption of redox homeostasis, as verified by increases of MDA concentrations (lipid oxidative damage) and of 2,7-dihydrodichlorofluorescein oxidation (free radical production), as well as by a decrease of GSH levels and changes of various antioxidant enzyme activities (antioxidant defenses), associated with histopathological injury only in the striatum and cerebral cortex, with no alterations in the hippocampus, liver, and heart. Furthermore, oxidative stress was more accentuated in symptomatic as compared to asymptomatic *Gcdh-/-* mice, establishing therefore a correlation between symptomatology and redox homeostasis disruption (Wajner et al., 2019). Additional data evidenced that L-carnitine supplemented to *Gcdh-/-* mice submitted to high Lys diet significantly attenuated or normalized lipid and protein damage, as well as the increased ROS production and the changes in antioxidant enzyme activities

observed in the striatum of these animals (Guerreiro et al., 2019). Alterations of oxidative stress parameters (ROS production, lipid peroxidation, protein oxidative damage, overexpression of superoxide dismutase, and GPx) in *Gcdh-/-* mice fed a Lys-enriched chow were positively correlated to C5DC plasma levels (reflecting GA concentrations), reinforcing the toxic prooxidant effects of GA, which accumulates in the brain of *Gcdh-/-* mice under Lys overload (Guerreiro et al., 2019; Zinnanti et al., 2006). Moreover, intrastriatal administration of Lys to adolescent *Gcdh-/-* mice was also shown to increase Nrf2 and NF-κB expression in the nuclear fraction and to decrease heme oxygenase-1 content, implying disturbances of oxidative stress signaling pathways (Amaral et al., 2019).

Finally, *Gcdh-/-* mice receiving a normal chow and injected with lipopolysaccharide, which induces an inflammatory response, revealed increased lipid oxidative damage (MDA levels) and decreased antioxidant defenses (GSH concentrations) in the striatum and cerebral cortex, but not in the hippocampus, liver, and heart (Seminotti et al., 2020), suggesting that an inflammatory insult that usually occurs during infections can disturb redox status in the most vulnerable cerebral structures. The increased S100 calcium-binding protein B and NF-κB protein levels found in the brain of *Gcdh-/-* mice injected with lipopolysaccharide and receiving a high Lys indicate activation of oxidative stress signaling pathways. The mounting evidence implies that GA disrupts redox homeostasis, particularly in the brain (striatum and cerebral cortex), and that this process is accentuated by inflammation in animal models of GA 1. It is emphasized that the brain is highly susceptible to oxidative stress because of their high rate of oxidative metabolism and oxygen consumption that generates more ROS, as well as due to the low antioxidant defenses compared to other tissues, and the elevated content of polyunsaturated fatty acids leading to increased peroxidation potential (Salim, 2017).

Noteworthy, GA-induced oxidative stress in chemically induced models of GA 1 and the genetic knockout animal with GA 1 (*Gcdh-/-* mice) is supported by the findings obtained in GA 1 patients. Induction of lipid peroxidation (increased isoprostanes), protein oxidative damage (increased Di-tyrosine), DNA damage (oxidized guanine species), and increased RNS generation are markedly increased in the urine of untreated patients affected by this disease, and, importantly, these alterations were attenuated or abolished by L-carnitine treatment (Guerreiro et al., 2018), implying a protective effect of this compound against oxidative damage. It is concluded that oxidative stress may represent an important pathomechanism in the neuropathology of GA 1.

6.3 Reactive Astrogliosis and Astrocyte Dysfunction

Astrocytes consist of a large proportion of neural cells, which are involved in many brain processes. They provide structural and metabolic support to neurons, synapsis, and other glial cells, besides controlling glutamate reuptake and participating in the maintenance of the BBB by interacting with endothelial cells, release of substrates to neuronal intermediary metabolism, redox balance, and neurotransmitter metabolism

(Tan et al., 2021). Astrocyte expression of the enzyme glutamine synthetase enables de novo formation of glutamine in these cells, which is important in the maintenance of intracellular pools of glutamate and GABA in the brain (Schousboe et al., 2014).

Reactive astrogliosis is a condition usually triggered by CNS injury and characterized by significant cellular and functional alterations of glial cells, particularly astrocytes, including increased cell number and/or hypertrophic cell body, as well as overexpression of glial fibrillary acidic protein (GFAP), inflammatory markers, and other products (Pekny et al., 2016).

Gliosis has been observed in postmortem brain of GA 1 patients (Funk et al., 2005; Goodman et al., 1977; Leibel et al., 1980). Of note, astrocytes can uptake GA possibly by their capacity to transport dicarboxylic acids through the NaC3 transporter (Lamp et al., 2011; Yodoya et al., 2006). In this particular, rat cortical astrocytes exposed to GA display morphological changes, increased cell proliferation, and mitochondrial dysfunction (Olivera-Bravo & Barbeito, 2015). On the other hand, when GA was incubated with astrocytes under starvation culture conditions (low glucose, without glutamine and fetal calf serum) or hypoxia, it induced astrocytic cell death, which was prevented by glutamine supplementation to the culture media (Komatsuzaki et al., 2018). Striatal astrocytes activated by GA exposition were also demonstrated to secrete toxic soluble factors that cause the death of cultured striatal and cortical neurons, signaling for a deleterious effect of these activated neural cells. In this particular, intracerebral administration of GA to newborn rats provoked sustained increase of astrocyte proliferation and elevated nitrotyrosine levels in the striatum that preceded neuronal loss, implying that astrocytic reactivity primarily induced by GA in vivo leads to striatal neuronal degeneration in this GA 1 animal model. These data strongly indicate that GA induces astrocyte dysfunction and glial reactivity, followed by deleterious effects toward neurons (Olivera-Bravo & Barbeito, 2015).

Similar findings were observed in the genetic mice model of GA 1 (*Gcdh-/-*). Marked astrogliosis accompanied by increased number of reactive astrocytes adjacent to blood vessels was found in the cerebral cortex, being less obvious in the striatum of *Gcdh-/-* mice fed a high Lys chow (Zinnanti et al., 2006). Furthermore, intrastriatal injection of Lys to these mutant mice caused astrocyte reactivity, neuronal loss, and marked vacuolation and edema in the striatum (Amaral et al., 2019). Astrocyte proliferation and oxidative stress were also verified after exposition of cultured astrocytes of the striatum of *Gcdh-/-* mice to Lys or GA (Olivera-Bravo et al., 2015). *Gcdh-/-* astrocytes exposed to Lys synthesize and release GA and 3HGA in the culture medium, besides becoming hyperactive and provoking striatal and cortical neuronal death. Since these deleterious effects were prevented by the antioxidants melatonin and alpha-tocopherol, it is presumed that hyperactive astrocytes become neurotoxic through oxidative stress-dependent mechanisms that can kill striatal and cortical neurons (Olivera-Bravo et al., 2015).

Changes in astrocyte morphology, such as shorter, thicker, and wavy astrocytic fibers, were also found in *Gcdh-/-* 3D organotypic brain cell cultures, and these changes were aggravated by Lys exposure (Cudré-Cung et al., 2019). Altogether, these animal and cell experimental data corroborate with the histopathological and

immunohistochemical findings observed in the autopsied brain of GA 1 patients, showing reactive and hypertrophic astrocytes, as well as microglial activation especially in the basal ganglia, which is characteristic of astrogliosis (Funk et al., 2005; Goodman et al., 1977; Leibel et al., 1980), therefore supporting the participation of astrocyte and glial reactivity in the pathogenesis of the brain damage in this disease.

6.4 Neuroinflammation

Inflammatory responses within the CNS are associated with high production of pro-inflammatory agents by microglia and astrocytes, such as interleukin (IL)-1β, IL-6, IL-18, and tumor necrosis factor-alpha (TNF-α), and with reactive species, which are key events in neuroinflammation (Schain & Kreisl, 2017). Infiltrating peripheral macrophages, leukocytes, and systemic pro-inflammatory mediators also contribute to this process, particularly when the BBB is damaged (Serrats et al., 2010). Neuroinflammation is usually caused by infection, trauma, stroke, and epilepsy and is also observed in common neurodegenerative disorders, such as multiple sclerosis, Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis. Elevated concentrations of neuroinflammatory biomarkers have been found in the plasma and brain of patients affected by some of these disorders (Schain & Kreisl, 2017). Pro-inflammatory insults to the brain may damage healthy neurons, leading to synaptic dysfunction, neuronal death, and inhibition of neurogenesis, mainly due to reduced production of brain plasticity-related molecules, such as brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor (Lyman et al., 2014).

Human and animal evidence suggests that neuroinflammation may contribute to brain injury in GA 1. In this particular, it is emphasized that acute striatum degeneration in GA 1 patients is characteristically observed during episodes of metabolic decompensation that are commonly triggered by infections, which are accompanied by inflammatory processes (Boy et al., 2019). Furthermore, severe neuronal loss and gliosis that are characteristically seen in neuroinflammation were shown in the putamen and caudate of these patients (Chow et al., 1988; Funk et al., 2005; Goodman et al., 1977; Soffer et al., 1992). Thus, it is reasonable to postulate that acute neuroinflammation may contribute to striatum degeneration during these acute episodes. This presumption is supported by the observations that the levels of the inflammatory and neurodegeneration biomarkers cathepsin-D and the brain-derived neurotrophic factor, respectively, are significantly elevated in GA 1 patients (Guerreiro et al., 2020).

On the other hand, activation of the kynurenine pathway, which is triggered by pro-inflammatory cytokines, generating high amounts of kynurenic acid, kynurenine, and particularly the neurotoxin quinolinic acid, was proposed as a pathomechanism of acute striatum damage in GA 1 (Varadkar & Surtees, 2004). Quinolinic acid is an NMDA glutamate receptor agonist that has been implicated in

the cerebral damage of a variety of neurological conditions, such as hypoxia, Huntington's disease, and stroke (Stone et al., 2012).

Experimental studies showed that GA induces inflammation in the brain. Chronic subcutaneous injections of GA to rats from the 5th to the 28th day of life resulted in increased levels of the pro-inflammatory cytokines TNF-α and IL-1b in the striatum (Rodrigues et al., 2019). Increased GFAP and Iba-1 immunoreactivity were also observed in the striatum of these animals, indicating astrocyte activation and microglia reactivity, respectively, that are also related to neuroinflammation (Rodrigues et al., 2019). This treatment also caused decreased NeuN immunoreactivity and increased proapoptotic proteins caspases 1, 3, and 8 in the striatum, signaling for neuronal loss and apoptotic induction. Therefore, it can be presumed that GA per se induces neuroinflammation in vivo, resulting in neuronal death and apoptosis induction. Importantly, most of these effects were attenuated or prevented by the antioxidant N-acetylcysteine, indicating the involvement of oxidative stress (Rodrigues et al., 2019). Other studies showed that neuroinflammation induced by intrastriatal quinolinic acid injection or systemic lipopolysaccharide administration further aggravated the neurochemical alterations observed in the brain of *Gcdh-/-* mice (Seminotti et al., 2020; Wajner et al., 2019).

These results indicate that GA toxicity toward the brain is potentialized during neuroinflammation and possibly related to the poor outcome of GA 1 patients during intercurrent infections (Boy et al., 2019), implying a synergistic action between neuroinflammatory mediators and brain GA accumulation.

6.5 Bioenergetics Disruption

The brain relies on a remarkably high metabolic activity, which is maintained by a continuous supply of oxygen and glucose from the blood. Brain weight accounts for only about 2% of total body weight and for approximately 20% of total oxygen consumption at rest (Mergenthaler et al., 2013). Such an extraordinary metabolic rate is necessary to fulfill the energy needed to hold the multiple brain functions. Even though the brain is able to oxidize various carbon sources during development, glucose is the main fuel in the mature brain and ATP is mainly obtained from glucose aerobic oxidation through the CAC and the oxidative phosphorylation. Metabolic compartmentation between neurons and glial cells, particularly astrocytes, is crucial for normal brain energy homeostasis. Nevertheless, there is no consensus yet regarding the precise metabolic contribution of each neural type to the brain energy expenditure (Sonnay et al., 2017). Energy transfer by the coordinated activities of the mitochondrial and cytosolic CK isoforms grants ATP availability in the cytosol during periods of high-energy demand (Wallimann et al., 1992). On the other hand, there is evidence that transient multienzyme complexes with a very dynamic associative/dissociative behavior can greatly influence metabolism. These complexes can direct

substrates toward specific pathways responding to local alterations in cellular milieu (McKenna & Ferreira, 2016). Physical associations were reported for GCDH with other important enzymes such as the ETF subunit beta, as well as to dihydrolipoamide S-succinyl-transferase, GDH 1, and ATP synthase alpha and beta subunits (Schmiesing et al., 2014).

GA-induced bioenergetics dysfunction through distinct mechanisms has been reported, indicating that energy deprivation may play a role in GCDH deficiency. In this scenario, much evidence supports the deleterious role of GA in disrupting brain bioenergetics by interfering with important systems related to energy production, transfer, and utilization. GA was demonstrated to inhibit in vitro the activities of the respiratory chain complexes I-III and II-III in the cerebral cortex, as well as the activities of succinate dehydrogenase and mitochondrial CK in subcortical structures of the rat brain. A decrease of complex I-III activity in subcortical structures was also found in vivo following subcutaneous GA injections. The data indicate a compromised electron flow through the respiratory chain, therefore disturbing oxidative phosphorylation. GA also decreases MTT reduction in rat brain synaptosomes and cultured astrocytes, reflecting impairment of mitochondrial dehydrogenase activities (Wajner et al., 2020). GA-induced inhibition of succinate uptake into cultured astrocytic and neuronal cells through the Na^+-coupled dicarboxylate transporter, potentially impairing the CAC activity, was also demonstrated (Lamp et al., 2011). On the other hand, GA inhibits GDH activity in astrocytes (Komatsuzaki et al., 2018), compromising the oxidation of glutamate taken up by these cells from the synaptic cleft to form a-ketoglutarate, an important anaplerotic intermediate of CAC and mitochondrial oxidative metabolism (McKenna et al., 2016). Thus, GDH activity inhibition by GA may also indirectly trigger excitotoxicity by increasing glutamate concentrations in astrocytes, leading to higher extravasation of this excitatory neurotransmitter to the synaptic cleft.

Disturbance of mitochondrial homeostasis was also observed in *Gcdh-/-* mice fed a high Lys diet, as shown by swollen and disintegrating brain mitochondria, associated with depletion of ATP, phosphocreatine, coenzyme A, α-ketoglutarate, glutamate, glutamine, and GABA. Reduction in the activities of CK, aconitase, and Na^+/K^+-ATPase was also found in brain structures of *Gcdh-/-* mice submitted to Lys dietary overload (Wajner et al., 2019). These results are presumably secondary to increased GA production and accumulation in the brain of these animals when exposed to a high Lys diet (Sauer et al., 2006; Zinnanti et al., 2006).

Glutarylation of mitochondrial enzymes, including glutamate dehydrogenase and various CAC enzymes, was also observed in glial cells from *Gcdh-/-* mice and presumed to be due to glutaryl-CoA accumulation (Schmiesing et al., 2018). It is stressed that native proteins can undergo posttranslational covalent modifications, such as glutarylation, that can modulate their biological activity and impact important biochemical pathways. Indeed, since abnormal protein glutarylation may alter enzyme activities (Tan et al., 2014), it is conceivable that this posttranslational modification may be also implicated in the pathophysiology of GA 1.

6.6 Blood-Brain Barrier Breakdown and Vascular Alterations

Vascular alterations and hemorrhages associated with abnormal cerebral blood flow and BBB damage have been observed in the brain of the knockout animal model and of affected patients with GA 1 (Strauss et al., 2010; Woelfle et al., 1996; Zinnanti et al., 2006). Some of these changes were also obtained by in vitro and in vivo studies evaluating the impact of GA on BBB and brain vasculature. Thus, high permeability of striatal microvessels was observed after exposition to GA for a short period, implying endothelial cell injury and disrupted BBB (Zinnanti et al., 2006). Increased pericyte contractility and decreased pericyte migration caused by soluble factors produced by dysfunctional astrocytes exposed to GA were also demonstrated (Isasi et al., 2019) and may explain the reduction of cerebral blood flow observed in GA 1 patients (Strauss et al., 2010). It is stressed that pericytes are cells that wrap around endothelial cells, regulating the blood-brain flow, being essential for normal BBB functioning and brain vasculature (Hall et al., 2014). Striatal vascular alterations and BBB breakage were also caused by in vivo intracerebral administration of GA to rat pups (Isasi et al., 2014), supporting the in vitro findings.

BBB breakdown, engorged blood vessels, and subarachnoid hemorrhage can be also observed in the striatum of *Gcdh-/-* mice fed a high Lys chow. Upregulation of the vascular endothelial growth factor (VEGF, isoforms A and C), which plays a central role in vascular permeability, as well as of genes involved in regulating angiogenesis and vascular permeability, allied to a displacement of the BBB tight-junction protein occludin was also reported in cerebral cortex and striatum of *Gcdh-/-* mice. In addition, *Gcdh-/-* mice fed a high protein chow presented cortical and striatal capillary occlusion associated with ischemia, neuronal swelling, and vacuole formation (Wajner et al., 2019).

Summarizing, vascular alterations caused by GA and upregulation of angiogenic genes and the VEGF factor may explain, at least in part, the cerebral vasculature changes and the acute and chronic subdural and subarachnoid hematomas observed in about 20% of GA 1 patients (Strauss et al., 2010; Woelfle et al., 1996). BBB breakage may also contribute to the neuropathology of GA 1, but further studies in humans are necessary to confirm these findings observed in *Gcdh-/-* mice and induced by GA.

6.7 Myelination Disturbance

CNS myelination consists of the formation of myelin sheaths by oligodendrocytes, which surround neuronal axons (Saab & Nave, 2017). It takes place from the fifth month of fetal life up to childhood in humans, although it continues at a much slower

pace during adulthood. Myelination of the CNS plays a crucial role in brain development and functioning, notably increasing the speed of information conduction and assisting axonal metabolism (Saab & Nave, 2017). Defective myelination is associated with the pathogenesis of common neurodegenerative diseases (Wolf et al., 2021) and also inherited metabolic disorders, including GA 1 (van der Knaap & Bugiani, 2017).

GA 1-affected patients generally develop progressive leukoencephalopathy associated with delayed myelination and periventricular white matter changes whose pathogenesis is still poorly known (Chow et al., 1988; Funk et al., 2005; Soffer et al., 1992). These alterations occur despite the characteristic acute encephalopathic crises, suggesting that white matter disease is a chronically developing process not primarily dependent on metabolic decompensation (Harting et al., 2009). Postmortem analyses from the brain of GA 1 patients show spongiform myelinopathy characterized by marked white matter vacuolation in many brain regions, including the fiber tracts in the putamen (Chow et al., 1988; Soffer et al., 1992). White matter changes are already present in MRI imaging of GA 1 newborns, implying that chronic neurotoxicity affecting brain maturation starts prenatally (Harting et al., 2009). Neuropathological findings of progressive white matter abnormalities have also been described in the genetic mouse model of GA 1 (Koeller et al., 2002; Zinnanti et al., 2006).

Elevated GA concentrations were detected in vivo in the white matter of high excretor GA 1 patients (Harting et al., 2015) so that a role for GA accumulation in the white matter abnormalities and myelinopathy in this disease is likely. This is consistent with previous reports showing high concentrations of GA in postmortem brain (Funk et al., 2005; Kölker et al., 2003; Leibel et al., 1980) and brain biopsies (Kulkens et al., 2005) of affected patients, as well as in the brain of the genetic mouse model of GA 1 (Koeller et al., 2002; Sauer et al., 2006; Zinnanti et al., 2006). Interestingly, higher concentrations of GA occurred in parallel with the extensive white matter changes verified in older high excretor patients (Harting et al., 2015), supporting a role for GA in myelinopathy in these patients. This presumption is further supported by the findings that a single GA injection into the cisterna magna of newborn rats provokes long-lasting striatal myelination delay, associated with abnormal myelin sheaths and thinner axons, besides decreased expression of the myelin-associated glycoprotein and the myelin binding protein in the striatal bundles (Olivera-Bravo & Barbeito, 2015).

Oligodendrocytes are the principal neural cells responsible for myelin formation, although astrocytes and microglia also contribute to myelination (Saab & Nave, 2017). Thus, it was shown that a single injection of GA in the cisterna magna of rat pups causes oligodendrocyte damage associated with medium- and long-term white matter abnormalities (Olivera-Bravo & Barbeito, 2015). In this particular, apoptosis of immature oligodendrocytes caused by GA was previously proposed as a novel mechanism of white matter degeneration in GCDH deficiency (Gerstner et al., 2005).

Other studies revealed oligodendrocyte ultrastructural alterations related to astrocyte dysfunction and disruption of the astrocyte-oligodendrocyte communication in the brain of GA-injected neonatal rats (Olivera-Bravo & Barbeito, 2015) and in the genetic animal GA 1 model (Olivera-Bravo et al., 2015). Notably, oligodendrocyte maturation depends on trophic factors produced by astrocytes (Nash et al., 2011). Destruction of the highly myelinated striatal patches associated with intense edema and vacuolation has been also found in *Gcdh-/-* mice submitted to chronic or acute Lys overload that leads to brain GA accumulation (Amaral et al., 2019; Zinnanti et al., 2006).

Therefore, mounting evidence indicates that GA is involved the progressive disruption of myelination associated with white matter changes and probably in the leukodystrophy observed in GA 1 patients (Chow et al., 1988; Soffer et al., 1992) and in the genetic mouse model of GA 1 (Koeller et al., 2002; Zinnanti et al., 2006).

7 Conclusion

Soaring evidence obtained from humans and especially from animal studies supports the hypothesis that GA is primarily involved in the neuropathology of GA 1 through multiple mechanisms. This organic acid, which mostly accumulates in the brain of patients with this intoxicating neurometabolic disorder, has been shown to markedly disrupt glutamatergic and GABAergic neurotransmission, redox homeostasis, and bioenergetics, besides inducing reactive astrogliosis, neuroinflammation, BBB breakage, cerebral vascular dysfunction, and white matter changes in the striatum and cerebral cortex of rats and in the genetic mouse model of GA 1 (Fig. 3). Some of these pathomechanisms presumably contribute to neurodevelopment delay, convulsions, cognitive impairment, and neuropathological changes occurring in GA 1 patients since brain homeostasis relies on an intricate network of interactions involving different CNS systems. It is conceivable that some of these deleterious mechanisms act synergistically, therefore potentiating one another. Thus, it is reasonable to postulate that brain accumulation of GA may significantly contribute to the progressive and acute neurological symptoms and brain abnormalities observed in GA 1 patients. However, the available data in humans and experimental models revealing impairment of these CNS crucial systems by GA must be taken with caution to better evaluate the relative relevance of each of these pathomechanisms, relative to the others. Nevertheless, unraveling these mechanisms is probably crucial to understand the pathophysiology of this unique disease and to the development of novel neuroprotective drugs for the affected patients to be first tested in the genetic animal model of GA 1 and thereafter as potential therapeutic targets for humans affected by this disease. On the other hand, the role of GA on the pathogenesis of GA 2 and GA 3 is practically unknown and should be a matter of future investigation.

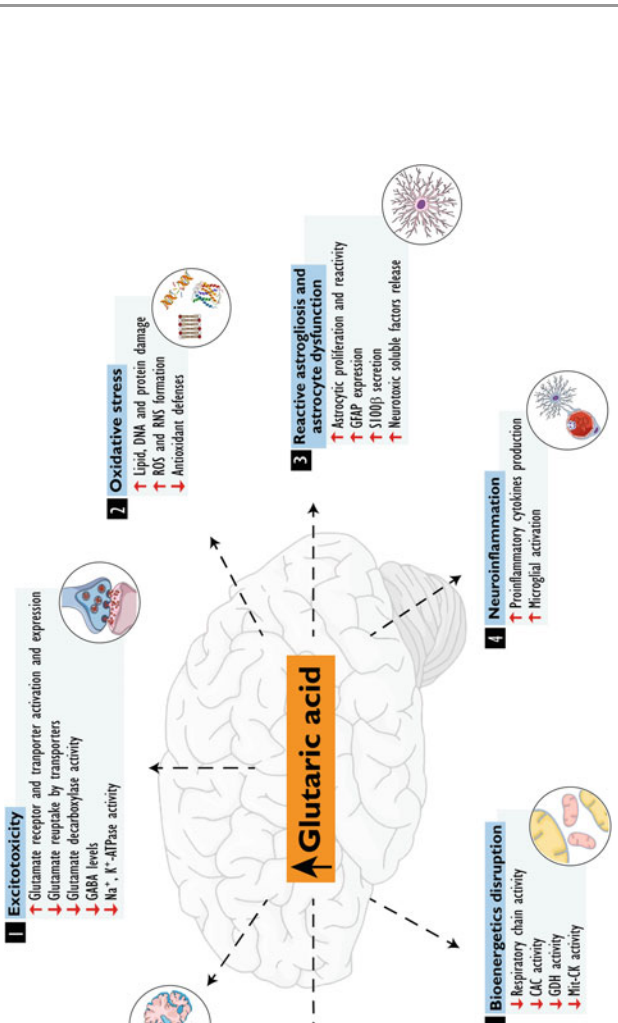

Fig. 3 *Potential pathomechanisms of glutaric acid neurotoxicity.* (1) Excitotoxicity caused by activation of glutamate receptors and transporters, decreased glutamate reuptake by transporters, decrease of glutamate decarboxylase with reduced GABA levels and Na^+/K^+-ATPase activities; (2) oxidative stress by increased reactive species generation and decreased antioxidant defenses, inducing lipid, protein, and DNA damage; (3) reactive astrogliosis and astrocyte dysfunction as determined by increased astrocytic proliferation and reactivity associated with release of neurotoxic factors; (4) neuroinflammation due to enhanced pro-inflammatory cytokine production and microglial activation; (5) bioenergetics disruption provoked by impairment of respiratory chain and citric acid cycle (CAC) functioning, as well as decreased glutamate dehydrogenase (GDH) and mitochondrial creatine kinase (Mit-CK) activities; (6) blood-brain barrier breakdown and vascular alterations associated with increased striatal microvessel permeability, pericyte dysfunction, and occludin displacement, besides upregulation of vascular endothelial growth factor (VEGF); and (7) myelination disturbance triggered by oligodendrocyte dysfunction related to reduced myelin basic protein and myelin-associated glycoprotein expressions

8 Cross-References

- ▶ 3-Hydroxyglutaric Acid as a Neurotoxin
- ▶ Glutamate as a Neurotoxin
- ▶ Glutamate Neurotoxicity Related to Energy Failure
- ▶ Glutamate Neurotoxicity, Transport and Alternate Splicing of Transporters
- ▶ Glutaric Acidemia Type 1: An Inherited Neurometabolic Disorder of Intoxication
- ▶ Neurotoxicity: A Complex Multistage Process Involving Different Mechanisms
- ▶ Quinolinic Acid and Related Excitotoxins: Mechanisms of Neurotoxicity and Disease Relevance
- ▶ The NMDA Receptor System and Developmental Neurotoxicity

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