3-Hydroxyglutaric Acid as a Neurotoxin

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Abstract

3-Hydroxyglutaric acid (3HGA) accumulates predominantly in the brain and biological fluids of individuals affected by glutaric acidemia type 1 (GA 1), being considered the most reliable biochemical marker for the diagnosis of this disease. GA 1 is a hereditary neurometabolic disease clinically characterized by acute episodes of encephalopathy resembling intoxication, which are associated with extensive striatal damage and followed by a complex movement disorder. Progressive striatal and extrastriatal abnormalities associated with white matter changes attributed to defective myelination are also common in this disease. Although brain concentrations of 3HGA in GA 1 are still unknown, an important characteristic of this organic acid is that, once produced mainly from lysine, it cannot leave the central nervous system because of very limited efflux, therefore, accumulating in this tissue. The pathogenesis of the brain damage of GA 1 is still poorly established, although neurotoxic effects have been attributed to 3HGA. In this particular, experimental data indicate that 3HGA *(i)* induces excitotoxicity, possibly due to its similar chemical structure to glutamate, the main excitatory neurotransmitter; *(ii)* disrupts redox homeostasis, increasing production of mitochondrial reactive species, decreasing cellular antioxidant defenses, and inducing oxidative damage to biomolecules; *(iii)* impairs bioenergetics, by inhibition of mitochondrial respiration and compromising the citric acid cycle activity; *(iv)* promotes reactive astrogliosis; and *(v)* causes blood-brain barrier breakage and cerebral vascular alterations. However, the pathophysiological relevance of the aforementioned deleterious effects on the neuropathology of GA 1 should be taken cautiously since some of these data were obtained with supraphysiological concentrations of 3HGA.

Keywords

Lysine catabolic pathway · Glutaric acidemia type 1 · 3-Hydroxyglutaric acid · Neurotoxicity · Excitotoxicity · Oxidative stress · Bioenergetics · Reactive astrogliosis · Blood-brain barrier breakage · Vascular alterations

Abbreviations

3HGA	3-Hydroxyglutaric acid
3-MGH	3-Methylglutaconyl-CoA hydratase
AMPA	α-Amino-3-hydroxy-5-methylisoxazole propionic acid
BBB	Blood-brain barrier
C5DC	Glutarylcarnitine
CNS	Central nervous system
CPT 1	Carnitine palmitoyltransferase 1
CSF	Cerebrospinal fluid
EC	Enzyme Commission

GA	Glutaric acid
GA 1	Glutaric acidemia type I
GABA	γ-Aminobutyric acid
GCDH	Glutaryl-CoA dehydrogenase
$Gcdh^{-/-}$	Homozygous glutaryl-CoA dehydrogenase deficient mice
GFAP	Glial fibrillary acidic protein
GluRs	Glutamatergic receptors
GPx	Glutathione peroxidase
GSH	Reduced glutathione
HADH	3-Hydroxy-acyl-CoA dehydrogenase
LCAD	Long-chain acyl-CoA dehydrogenase
L-NAME	Nω-Nitro-L-arginine
Lys	Lysine
MCAD	Medium-chain acyl-CoA dehydrogenase
MDA	Malondialdehyde
MK-801	(5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5-10-imine
NaC3	Sodium dicarboxylate cotransporter 3
NBQX	2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline
NMDA	N-Methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
OAT	Organic anion transporter
OMIM	Online Mendelian Inheritance in Man
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RS	Reactive species
S100B	S100 calcium-binding protein B
VEGF	Vascular endothelial growth factor

1 3-Hydroxyglutaric Acid Synthesis

3-Hydroxyglutaric acid (3HGA) is an organic acid endogenously produced at high amounts in patients affected by glutaric acidemia type 1 (GA 1; OMIM # 231670), also known as glutaric aciduria type 1 or glutaryl-CoA dehydrogenase deficiency. GA 1 is a neurometabolic error of metabolism caused by inherited deficient activity of the mitochondrial complex glutaryl-CoA dehydrogenase (GCDH; E.C. 1.3.8.6). GCDH catalyzes the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA and CO_2 during the catabolism of L-lysine (Lys), L-hydroxylysine, and L-tryptophan (Fig. 1). The deficient activity of this enzyme provokes the accumulation of glutaryl-CoA, which is further converted to glutarylcarnitine (C5DC) and glutaric acid (GA) (Fig. 1) (Larson and Goodman 2019). The production of 3HGA and glutaconic acid occurs due to non-specific dehydrogenation of glutaryl-CoA to glutaconyl-CoA mainly by mitochondrial medium-chain acyl-CoA dehydrogenase,

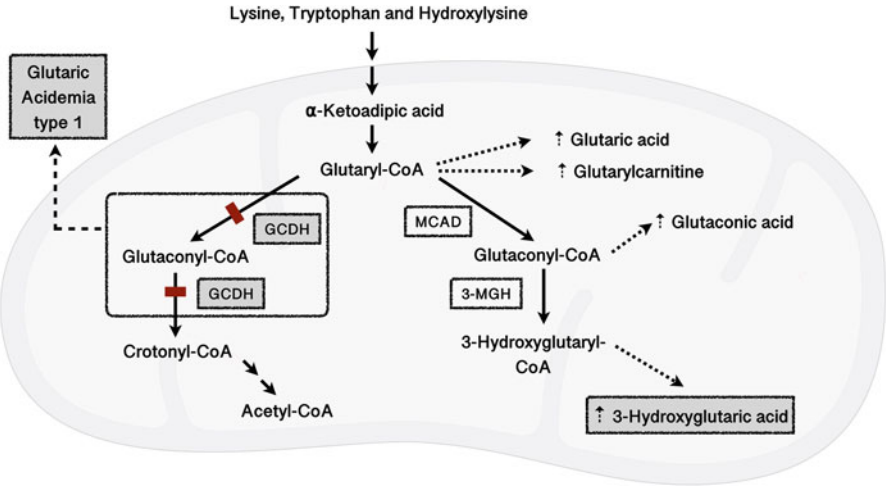

Fig. 1 Catabolic pathway of lysine, hydroxylysine, and tryptophan: 3-hydroxyglutaric acid synthesis. Glutaric acidemia type 1 is caused by deficiency of glutaryl-CoA dehydrogenase (GCDH) activity, resulting in high levels of glutaryl-CoA, which is spontaneously hydrolyzed to glutaric acid or combined to L-carnitine to form glutarylcanitine with CoA release. Glutaryl-CoA is mainly metabolized by the medium-chain acyl-CoA dehydrogenase (MCAD) to glutaconyl-CoA, which is spontaneously hydrolyzed to glutaconic acid or converted to 3-hydroxyglutaryl-CoA by 3-methylglutaconyl-CoA hydratase (3-MGH). 3-Hydroxyglutaric acid arises from 3-hydroxyglutaryl-CoA spontaneous hydrolysis

although short-chain 3-hydroxy-acyl-CoA dehydrogenase (HADH; EC:1.3.8.1) and long-chain acyl-CoA dehydrogenase (LCAD; EC:1.3.8.8) are also able to catalyze the conversion of glutaryl-CoA to glutaconyl-CoA (Peters et al. 2019). Glutaconyl-CoA is then hydrated to form 3-hydroxyglutaryl-CoA by 3-methylglutaconyl-CoA hydratase (3-MGH; EC 4.2.1.18) in a reversible reaction. After spontaneous hydrolysis of the CoA precursors, GA, 3HGA, and glutaconic acid are formed, whereas L-carnitine binds to glutaryl-CoA with CoA loss to generate C5DC in a reaction catalyzed by L-carnitine acyltransferase (Peters et al. 2019) (Fig. 1).

2 3-Hydroxyglutaric Acid in Glutaric Acidemia Type 1 (GA 1), Carnitine Palmitoyltransferase 1 Deficiency, and Ketosis

Elevations of 3HGA concentrations are found mainly in biological fluids of patients with GA 1, carnitine palmitoyltransferase 1 (CPT 1) deficiency, and in ketotic patients (Table 1). Patients with these pathological conditions present high

Table 1 3-Hydroxyglutaric acid levels (3HGA) in urine and cerebrospinal fluid of healthy individuals and in patients affected by glutaric acidemia type 1 (GA 1), carnitine palmitoyltransferase 1 (CPT 1) deficiency, and ketosis

3-Hydroxglutaric acid levels in urine and cerebrospinal fluid of healthy individuals			
Biological fluid		**Age**	**Reference**
Urine (mmol/mol creatinine)	**CSF** (μmol/L)		
1.4–8.0 (before hydrolysis) 2.6–11.7 (after hydrolysis)	<0.2	Children <5 years old	(Barić et al. 1999)
0.88–4.5	0.022–0.067	Children <10 years old	(Schor et al. 2002)
1.3 ± 0.7	0.07 ± 0.03	Adults >18 years old	(Shigematsu et al. 2005)
3-Hydroxglutaric acid levels in urine and cerebrospinal fluid of patients affected by metabolic disorders			
GA 1			
44–395 [a] 14–122[b] (before hydrolysis) 37–480 [a] 13–175[b] (after hydrolysis)	4.45	Children <5 years old	(Barić et al. 1999)
55–360	–	6.5 months old	(Bennett et al. 1986)
618[a] 153[b]	–	–	(Christensen et al. 2004)
110	–	8.5 years old	(Harting et al. 2009)
109	–	66 years old	(Külkens et al. 2005)
590	2.2	10 weeks old	(Pöge et al. 1997)
262 (patient 1) – –	0.52 (patient 1) 0.36 (patient 2) 5.4 (patient 3)	– – –	(Schor et al. 2002)
CPT 1 deficiency			
9.8 (patient 1) 24.7 (patient 2) 14.7 (patient 3)	– – –	14 months old (patient 1) 3 years old (patient 2) 23 months old (patient 3)	(Korman et al. 2005)

(continued)

Table 1 (continued)

Ketosis			
37.9 (patient 1) 23.5 (patient 2) 23.0 (patient 3)	– – –	2.5 years old (patient 1) – (patient 2) 10 months old (patient 3)	(Pitt et al. 2002)

[a]High excretors
[b]Low excretors
CSF cerebrospinal fluid
– not reported
Results represent 3HGA levels (single or range of values) from each patient

glutaryl-CoA levels and a variable degree of neurological manifestations. Table 1 shows the concentrations of 3HGA in urine and cerebrospinal fluid (CSF) of patients with GA 1, CPT 1 deficiency, and during ketosis.

GA 1 is an autosomal recessive disease caused by mutations in the gene *GCDH* that encodes the enzyme complex GCDH. Individuals affected by GA 1 present brain abnormalities, including macrocephaly and frontotemporal hypoplasia that may be present at birth. Encephalopathic crises usually triggered by infections, or other metabolic stress associated with prolonged fasting, are commonly observed and characterized by acute striatum degeneration. Dystonia, dyskinesia, muscle stiffness, and general developmental deterioration follow these crises. Chronically progressive white matter changes with leukodystrophy are also found in practically all patients (Harting et al. 2009; Külkens et al. 2005; Larson and Goodman 2019; Wajner 2019). Biochemical diagnosis of GA 1 is based on the identification of increased levels of 3HGA (main biochemical marker) and GA in high excretor patients in the urine, as well as C5DC in the blood. Elevation of GA levels may be quite subtle or even undetectable in a group of GA 1 patients called low excretors, although increased levels of 3HGA are still found in the urine of these patients (Barić et al. 1999). The disease is confirmed by the detection of pathogenic biallelic mutations and by deficient GCDH activity in fibroblasts or leukocytes (Larson and Goodman 2019).

Deficiency of CPT 1 (EC 2.3.1.21, OMIM # 255120) is a rare autosomal recessively inherited defect of mitochondrial fatty acid oxidation (Bennett and Santani 2016). CPT 1 is located in the outer mitochondrial membrane and catalyzes the transesterification of long-chain acyl-CoA into long-chain acylcarnitine, which is the rate-limiting step of mitochondrial fatty acid oxidation, and the first step in the carnitine shuttle for the import of acyl-CoA from the cytosol to the mitochondrial matrix (Bennett and Santani 2016). CPT 1 deficiency severely impairs energy production in the liver because long-chain fatty acids are major energy substrates in this tissue. CPT 1A is the only isoform identified in human deficiency. The encoding gene is predominantly expressed in the liver and kidney. Patients with

CPT 1A deficiency usually manifest during infancy with acute Reye-like hepatic encephalopathy, which is precipitated by fasting or metabolic stress usually triggered by a febrile illness. Typical features include altered consciousness, hepatomegaly, hypoketotic hypoglycemia, and liver dysfunction. The diagnosis is based on the increased ratio of free carnitine to the sum of palmitoylcarnitine plus C18 acylcarnitines in the blood (Bennett and Santani 2016). Patients with CPT 1A deficiency excrete large amounts of dicarboxylic acids in the urine, including 3HGA, with normal levels of GA in urine and C5DC in blood. The origin of 3HGA in this disorder is not clear, but it may be due to increased Lys degradation, resulting in augmented levels of glutaryl-CoA, favoring 3HGA formation (Korman et al. 2005).

Ketotic patients have been also reported with increased excretion of 3HGA, which normalizes when the patients are nonketotic (Pitt et al. 2002). The levels of 3HGA observed in ketotic patients are of the same order of magnitude as those found in patients with GA 1 (Table 1), and this may represent a potential confounder in the diagnosis of GA 1. 3HGA formation during ketosis is thought to result from increased protein catabolism and increased flux through the Lys catabolic pathway, glutaryl-CoA, ultimately leading to 3HGA synthesis.

3 3-Hydroxyglutaric Acid in Glutaryl-CoA Dehydrogenase Deficient Mice

A genetic mouse model of GA 1 (*Gcdh*−/− mice) was developed by Koeller and coworkers (Koeller et al. 2002) and further improved by feeding the mutant animals with a high Lys (4.7%) chow (Zinnanti et al. 2006). This model has been largely used to study the pathogenesis of the brain damage in GA 1 because it mimics the clinical and biochemical phenotypes of the human GA 1. Thus, *Gcdh*−/− mice present high levels of GA and 3HGA in their biological fluids, similar to those found in GA 1 patients, and manifest with neuroradiological and histopathological findings, including spongiform myelinopathy and striatum degeneration with neuronal loss and reactive astrogliosis when under Lys overload (Zinnanti et al. 2006, 2007). Vasogenic edema, blood-brain barrier (BBB) breakdown, hemorrhage within the striatum, mild motor deficits, paralysis, seizures, and death are also observed in 4-week-old *Gcdh*−/− mice exposed to Lys or protein overload (Zinnanti et al. 2006).

The elevated brain GA and 3HGA levels demonstrated in 8-week-old *Gcdh*−/− mice fed a high Lys chow indicates central nervous system (CNS) de novo synthesis of this toxic organic acid from Lys, which easily penetrates into the brain and enters mitochondria through specific carriers. Brain 3HGA concentrations increase proportionally to GA levels after Lys overload, although they are 20-fold lower than GA levels (Zinnanti et al. 2007). Young adult $Gcdh^{-/-}$ mice exposed to a high protein diet also show a significant increase of 3HGA in plasma and urine (Table 2) (Keyser et al. 2008).

Table 2 Levels of 3-hydroxyglutaric acid in biological fluids of Gcdh$^{-/-}$ mice fed a normal or high protein chow

Condition	Specimen	
	Blood (μmol/L)	Urine (mmol/mol creatinine)
Gcdh$^{-/-}$ (normal chow)	7.2 ± 2.1	898 ± 126
Gcdh$^{-/-}$ (high protein chow)	10.5 ± 1.8	1414 ± 168

Gcdh$^{-/-}$ = glutaryl-CoA dehydrogenase deficient mice; *normal chow* = 18% protein content = 0.9% Lys; *high protein chow* = 62% protein content = 4.7% Lys (Keyser et al. 2008; Zinnanti et al. 2006)

4 3-Hydroxyglutaric Acid Transport Through Cellular Membranes

Due to its hydrophilic activity, 3HGA requires carrier proteins to cross biological membranes (Mühlhausen et al. 2008). In this particular, renal transport membrane proteins involved in the uptake and secretion of dicarboxylates, such as the organic anion transporters 1 (OAT1) and 4 (OAT4) and the sodium dicarboxylate cotransporter 3 (NaC3), have been suggested to mediate 3HGA transport (Hagos et al. 2008; Mühlhausen et al. 2008). These carrier proteins are responsible for the translocation of a wide variety of endogenous and exogenous organic anions, especially dicarboxylates (Burckhardt and Burckhardt 2011).

OAT1 is mainly expressed in the proximal tubular renal cells, as well as in the choroid plexus and in cortical and hippocampal neurons, mediating mainly the translocation of neurotransmitters (Rizwan and Burckhardt 2007). As for OAT4, it is predominantly found in the liver and kidney proximal tubule cells, exhibiting specificity for dicarboxylates and sulfated conjugates of xenobiotics and steroid hormones. On the other hand, NaC3 is expressed in the kidney, liver, placenta, and brain and regulates the cellular concentrations of Krebs cycle intermediates, such as α-ketoglutarate, succinate, and citrate, being, therefore, important for the maintenance of Krebs cycle activity (Hagos et al. 2008; Mühlhausen et al. 2008; Pajor 2014). NaC3 translocates GA with high affinity in the kidney, similarly to the dicarboxylates α-ketoglutarate and succinate, whereas 3HGA is transported through this carrier with much lower affinity (Stellmer et al. 2007). On the other hand, OAT4 acts in concert with NaC3 and OAT1 for the renal clearance of GA and 3HGA. During this process, GA and 3HGA are taken up from the plasma across the basolateral membrane by OAT1 and NaC3 and are secreted into the urine via OAT4 (Hagos et al. 2008).

Since OATs and NaC3 are also expressed in the brain, although at lower levels than in the kidney (*source: proteinatlas.org*), it is conceivable that these carriers may mediate 3HGA membrane transport also in the CNS. In this regard, it was shown that 3HGA is transported by OAT1 in a sodium-dependent and ATP-independent mechanism in an experimental model of blood-brain barrier (BBB) (Sauer et al. 2010). On

the other hand, the NaC3 transporter that is highly expressed in astrocyte cell membranes, where it mediates succinate transport, is compromised by 3HGA (Lamp et al. 2011; Mühlhausen et al. 2008). Furthermore, the uptake of Krebs cycle intermediates by neuronal cells, likely to be facilitated by the protein carriers NaC2 and/or NaC3 (Lamp et al. 2011; Mühlhausen et al. 2008), is also inhibited by 3HGA (Lamp et al. 2011).

Overall, these observations indicate that 3HGA accumulation in the brain may impair the transport of anaplerotic dicarboxylates, including succinate, potentially disrupting brain bioenergetics. It is also conceivable that the intracellular neuronal pool of the neurotransmitters glutamate and γ-aminobutyric acid (GABA), whose synthesis depends on extracellular dicarboxylate intermediates (Lamp et al. 2011), is also impaired by 3HGA.

5 3-Hydroxyglutaric Acid Neurotoxicity: Implications for GA 1 Neuropathology

Neurotoxicity occurs when endogenous or exogenous substances called neurotoxins or neurotoxicants alter the normal activity of the CNS. Accumulating endogenous metabolites behave as neurotoxins in several inherited metabolic disorders, by acting as excitotoxins, compromising bioenergetics, inducing oxidative stress, promoting a sustained inflammatory environment, and causing other deleterious toxic effects. It is thought that the combined and synergistic interplay among these deleterious actions may contribute to the neuropathological findings characteristically seen in these disorders (Wajner 2019).

An increasing body of evidence obtained from in vitro and in vivo experimental studies performed in rodents and cultured cells from rats and chicks exposed to 3HGA, as well as in the animal genetic model of GA 1, has shown a wide spectrum of neurotoxic mechanisms elicited by 3HGA.

5.1 3-Hydroxyglutaric Acid Behaves as an Excitotoxin

Post-mortem examination of the brain of GA 1 patients revealed postsynaptic vacuolation in the striatum and cerebral cortex similar to the lesions observed in glutamate-mediated damage (Funk et al. 2005), suggesting that excitotoxicity could be a pathomechanism of neurodegeneration in these patients. In this context, some data indicates that 3HGA could potentially act as a false neurotransmitter, occupying the same sites of glutamate on specific glutamate receptors and transporters, because of its similar chemical structure of that of L-glutamate (Fig. 2).

L-glutamate plays fundamental roles in neural cell function and brain plasticity mainly through activation of the glutamatergic receptors (GluRs; Fig. 2) (Traynelis et al. 2010). However, acute or sustained overstimulation of these receptors can elicit a deleterious condition called excitotoxicity (Dong et al. 2009). Overactivation or sustained activation of GluRs leads to increased Ca^{2+} influx, triggering a deadly cell

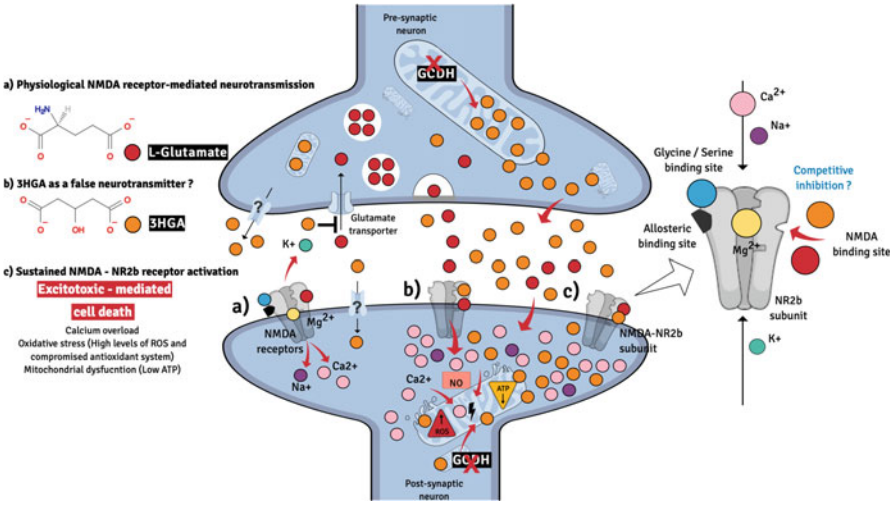

Fig. 2 Presumed excitotoxic effects of 3-hydroxyglutaric acid. (**a**). Physiological neurotransmission mediated by L-glutamate binding to NMDA receptors, with consequent Ca^{2+} and Na^{+} influx. (**b**). 3-Hydroxyglutaric acid (3HGA) may act as a false neurotransmitter, leading to NMDA receptor activation, subsequently increasing Ca^{2+} and Na^{+} influx in the post-synaptic neuron. (**c**). Sustained activation of NMDA receptors containing NR2b subunit leads to intracellular Ca^{2+} overload, ultimately causing excitotoxic mediated neuronal death. GCDH, Glutaryl-CoA dehydrogenase; ROS, reactive oxygen species; NMDA, N-methyl-D-aspartate; NO, nitric oxide

cascade of events, such as mitochondrial membrane depolarization, caspase, and nitric oxide synthase stimulation, as well as generation of toxic reactive oxygen and nitrogen species, which may ultimately lead to cell death by apoptosis or necrosis (Dong et al. 2009).

Mounting experimental evidence shows that 3HGA may induce excitotoxicity through overstimulation of ionotropic GluRs. 3HGA induces massive cell death in rat hippocampal organotypic cultures and cultured mouse neocortical neurons with the involvement of α-amino-3-hydroxy-5-methylisoxazole propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) glutamate receptors (Kölker et al. 2004; Lund et al. 2004). Furthermore, a great deal of data have demonstrated that the subtype NR2b NMDA receptor is mainly involved in the neuronal death provoked by 3HGA in chick neuronal cultures from embryo telencephalons and mixed neuronal and glial cell cultures from rat hippocampus (Kölker et al. 1999, 2004). In addition, 3HGA neuronal cytotoxicity was shown to increase in parallel with the expression of the NR2b subunit in primary neuronal cultures from chick embryo telencephalons in a maturation-dependent manner (Kölker et al. 2004). Whole-cell patch-clamp recordings also evidenced that 3HGA activates recombinant NMDA but not recombinant AMPA receptors in HEK 293 cells transfected with different ionotropic glutamate receptor subtypes (Kölker et al. 2004). 3HGA was also shown to increase neuronal Ca^{2+} influx, subsequently provoking a deadly cell cascade activation that was prevented by NMDA receptor antagonists in cultured chick embryo neurons

(Kölker et al. 2004). Altogether, these data provide solid evidence of the relevance of NMDA and particularly NR2b receptors on the excitotoxicity provoked by 3HGA.

As regards the mechanisms underlying 3HGA excitotoxicity, 3HGA markedly decreases Na^+-dependent (binding to glutamate transporters) and Na^+-independent glutamate binding (binding to GluRs), presumably due to a competition between glutamate and 3HGA for the same places on glutamate transporters and receptors (Dalcin et al. 2007; Rosa et al. 2004). It was also shown that 3HGA significantly increases (5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5-10-imine (dizocilpine) (MK-801) binding, which is a common effect of NMDA agonists (Rosa et al. 2004), indicating that this organic acid acts as an agonist of NMDA receptors, potentially activating these receptors. This agonistic effect is further supported by the observations that 3HGA increases extracellular Ca^{2+} uptake by cerebral cortex slices (Rosa et al. 2004). Further experiments using the competitive glutamate transporter inhibitor L-trans-pyrrolidine-2,4-dicarboxylate suggest a competition between 3HGA and glutamate for its transporters in striatum of adolescent rats (Dalcin et al. 2007).

Nonetheless, other study verified that the toxic effects provoked by 3HGA in neuronal cultures from embryonic rat striatum were not prevented by the use of the glutamate antagonists MK-801 and 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F) quinoxaline (NBQX), suggesting that 3HGA toxicity in this embryonic system does not seem to be mediated by NMDA and non-NMDA GluRs (Bjugstad et al. 2001). Furthermore, the viability of primary neuronal cells of neonatal rats exposed to 3HGA was not changed relative to untreated control cultures, implying that 3HGA is not toxic in this neonatal model despite the presence of functional NMDA receptors (Freudenberg et al. 2004). Distinct methodology and doses of 3HGA, and of glutamate antagonists used in the distinct assays, may possibly explain these apparently conflicting results.

As regard the GABAergic system, 3HGA significantly inhibits glutamate decarboxylase, decreasing the generation of the inhibitory neurotransmitter GABA in rat and rabbit brain, which may explain the decreased GABA concentrations found in *post-mortem* analysis of striatum of GA 1 patients (Leibel et al. 1980; Wajner 2019).

In vivo experiments also support excitotoxicity as an underlying pathomechanism of 3HGA. Intrastriatal administration of 3HGA to Wistar rats provokes marked and dose-dependent increase in the number and duration of seizures, accompanied by striatum histopathological alterations with the involvement of NMDA and GABA receptors since the NMDA antagonist blocker MK-801 and the GABA receptor agonist muscimol prevented seizures and striatal damage (De Mello et al. 2001). The protective effect of muscimol could be tentatively attributed to a higher stimulation of GABA receptors despite 3HGA-elicited inhibition of glutamate decarboxylase, decreasing GABA formation (see review Wajner 2019). These in vivo results support the hypothesis of NMDA and GABA receptors mediate 3HGA-induced excitotoxic cell damage.

Summarizing, the mounting in vitro and in vivo experimental data supports the hypothesis that 3HGA induces excitotoxicity by activating specific glutamate receptors and transporters and reducing GABA concentrations (Fig. 2). It is presumed,

therefore, that this pathomechanism may mediate, at least in part, the neuropathological abnormalities observed in GA 1, such as postsynaptic vacuolation in the striatum and cerebral cortex (Funk et al. 2005).

5.2 3-Hydroxyglutaric Acid Induces Oxidative Stress in the Brain

Oxidative stress is defined as an imbalance between cellular pro-oxidant and antioxidant defenses in favor of the former. This condition usually results from increased reactive species (RS) generation, potentially leading to oxidative damage to biomolecules (lipids, carbohydrates, proteins, and polynucleotides), and eventually cell death (Halliwell and Gutteridge 2015).

Increasing evidence has shown that 3HGA disrupts cellular redox homeostasis in the CNS. 3HGA induces nitric oxide production and lipid oxidation and reduces the antioxidant defenses in the cerebral cortex and striatum of rats (Latini et al. 2002, 2005a). Significantly augmented reactive oxygen species (ROS) production and moderate increase of nitric oxide associated with neuronal death was also observed after exposing neuronal cultures from chick embryo telencephalons to 3HGA (Kölker et al. 2004). Noteworthy, these deleterious effects were prevented by the antagonist of NMDA receptors MK-801, as well as by the free radical scavengers α-tocopherol and melatonin, indicating a central role of NMDA stimulation in 3HGA toxicity and the importance of free radical scavenging as a potential therapy for GA 1 (Kölker et al. 2004). Furthermore, 3HGA-induced moderate increase of nitric oxide production was normalized by the nitric oxide synthase (NOS) inhibitor Nω-nitro-L-arginine (L-NAME), although it did not prevent neuronal death, indicating that the augmented reactive nitrogen species (RNS) was not cytotoxic in this model (Kölker et al. 2004). 3HGA also provokes oxidative stress and mitochondrial dysfunction associated with astrocyte proliferation in rat cortical astrocytes, which were prevented by antioxidants (Olivera et al. 2008). Lipid peroxidation secondary to excessive RS was also observed in C6 rat astroglial cells exposed to 3HGA (Quincozes-Santos et al. 2010). Altogether, these data indicate that oxidative stress may contribute to 3HGA-induced neurototoxicity affecting both neurons and astrocytes.

5.3 3-Hydroxyglutaric Acid Disrupts Bioenergetics Homeostasis in the Brain

Disturbances of bioenergetics have been proposed to contribute to GA 1 neuropathology (Strauss and Morton 2003). 3HGA was shown to moderately compromise oxidative phosphorylation in rat cerebral cortex and C6 glioma cells, by inhibiting mitochondrial respiratory chain complex II activity (Latini et al. 2005b). 3HGA also reduces the respiratory control ratio and increases state IV respiration (resting respiration) in rat brain mitochondria, indicating low efficiency to synthesize ATP and mitochondrial uncoupling, respectively (Latini et al. 2005b). This is in agreement with the mitochondrial depolarization of primary cultured astrocytes from

neonatal rodents exposed to 3HGA (Olivera et al. 2008). Further studies using organotypic mixed cerebral cortex cultures from neonatal rats revealed that high concentrations of 3HGA significantly reduce the activity of the respiratory chain complex II with a mild decrease of complex V activity and phosphocreatine levels (Kölker et al. 2004). Succinate uptake by Na^+-dicarboxylate transporters is also inhibited by 3HGA in both astrocyte and neuronal cell cultures from *Gcdh−/−* mice, as well as succinate efflux in astrocytes prepared from wild type and *Gcdh−/−* mice (Lamp et al. 2011), potentially decreasing astrocyte anaplerotic supply of Krebs cycle intermediates to neurons. Summarizing, the available data suggest that 3HGA disturbs brain bioenergetics through multiple mechanisms, by acting as a metabolic inhibitor and an uncoupler of oxidative phosphorylation, and also compromising the anaplerotic pathway.

5.4 3-Hydroxyglutaric Acid Induces Reactive Astrogliosis

Astrocytes constitute a large proportion of neural cells in humans and are actively involved in the synapsis, also providing structural and metabolic support to neurons and other glial cells at physiological conditions. Astrocytes are also involved in the formation of gap junction-coupled functional networks in the BBB, besides participating in the antioxidant balance, neurotransmitter synthesis, and myelination (Traiffort et al. 2020).

In response to CNS injury, infection, or other cerebral pathological condition, astrocytes become reactive and undergo a number of morphological changes, including swelling and proliferation, which are accompanied by biochemical, metabolic, and physiological remodeling, a condition known as reactive astrogliosis (Escartin et al. 2021). It was initially thought that astrocyte reactivity exclusively aimed to protect neurons from damage, but recent evidence shows that reactive astrocytes may improve, not change or even worse the progression and outcome of neurologic diseases. Thus, accumulating evidence indicates that reactive astrogliosis associated with overexpression and release of glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100B), as well as pro-inflammatory and neurotoxic factors, may be involved in neuronal damage (Escartin et al. 2021; Olivera et al. 2008).

Since gliosis has been observed in *post-mortem* brain of GA 1 patients and in the genetic murine model of GA 1 ($Gcdh^{-/-}$ mice) (Funk et al. 2005; Zinnanti et al. 2006), emphasis has been given on the investigation of the underlying mechanisms of reactive astrogliosis and its participation in the pathophysiology of this disease. In this scenario, it was shown that 3HGA induces astrocyte proliferation and causes mitochondrial dysfunction in rat cortical astrocytes (Olivera et al. 2008). MAPK inhibitors and antioxidant iron porphyrins prevented the increased astrocyte proliferation caused by 3HGA, implying that MAPK signaling pathways and oxidative stress are involved in these effects (Olivera et al. 2008). Further studies revealed increased S100B secretion and lipid peroxidation in C6 astroglial cells exposed to 3HGA, signaling astrocyte oxidative injury (Quincozes-Santos et al. 2010). Changes of cell morphology from a round flat to a spindle-differentiated shape associated with

disturbances of mitochondrial respiration were also observed after exposition of C6 glioma cells to 3HGA (Latini et al. 2005b). Therefore, it is conceivable that mitochondrial dysfunction and oxidative stress promoted by 3HGA may lead astrocytes to adopt morphological and functional changes as an adaptive response characteristic of reactive astrogliosis. However, further studies are still needed to establish whether this response is beneficial or harmful to GA 1 neurodegeneration.

5.5 3-Hydroxyglutaric Acid Causes Blood-Brain Barrier Breakdown and Cerebral Vascular Alterations

Vascular abnormalities such as acute and chronic subdural hemorrhagic effusions have been found in the brain of GA 1 patients (Strauss et al. 2003), whereas BBB breakdown and vascular dysfunction were demonstrated in the striatum of the genetic murine model of GA 1 (*Gcdh*$^{-/-}$ mice) on dietary lysine overload and in rat striatum exposed to GA and 3HGA (Zinnanti et al. 2006). In this regard, it was demonstrated that developing *Gcdh*$^{-/-}$ mice fed a high Lys diet for 3 days have extravasation of Evans blue dye in their striatum, which is indicative of BBB breakdown. While the dye was found to escape from smaller vessels of the striatum of *Gcdh*$^{-/-}$ mice, some larger vessels concentrated blue staining and vasodilatation consistent with congestion. In contrast, BBB permeability was not altered in striatum of wild-type mice fed a high Lys diet and of *Gcdh*$^{-/-}$ mice receiving a normal chow, suggesting that BBB breakdown was caused by high concentrations of GA and 3HGA derived from Lys (Zinnanti et al. 2006). This presumption is supported by the observations that 3HGA alters in vitro vascular functions and disturbs BBB integrity in isolated microvessels prepared from rat striatum (Zinnanti et al. 2006).

Moreover, 3HGA inhibits basal and vascular endothelial growth factor (VEGF)-induced endothelial cell migration in a cell line of human dermal microvascular endothelial cells (Mühlhausen et al. 2004, 2006). This inhibitory effect is presumed to cause endothelial disorganization during capillary-like tube formation, actin cytoskeleton disruption, and adhesion molecule content reduction, indicating that angiogenesis is disturbed by 3HGA (Mühlhausen et al. 2006). Further experiments showed that 3HGA causes abnormal blood vessel dilatation and hemorrhage in chorioallantoic membranes isolated from chicken (Mühlhausen et al. 2004, 2006). Altogether, these experimental findings denote a significant role of 3HGA inducing vascular alterations and disrupting BBB permeability in striatum, and may clarify the vascular abnormalities described in patients affected by GA 1 and in the genetic murine model of this disease.

6 Conclusion

Patients with GA 1 commonly suffer extensive striatum degeneration during acute metabolic decompensation that is presumably caused by acute toxicity due to high concentrations of the major accumulating metabolites, namely, GA and 3HGA, whose

synthesis is markedly increased during these crises. Progressive striatal and extrastriatal abnormalities including white matter changes, leukodystrophy, and brain atrophy, possibly as a consequence of chronic intoxication, are also observed in GA 1. Mounting evidence indicates that multiple mechanisms contribute to GA 1 neuropathology and that the toxic effects of the accumulating organic acids play a central role in the pathogenesis of this disease. This chapter updates the present knowledge mostly obtained from animal experimental studies on the toxicity of 3HGA on critical cerebral systems necessary for normal brain development and performance. The available data demonstrate that 3HGA induces excitotoxicity, probably because of its similar chemical structure to glutamate, besides disturbing cellular redox state and altering bioenergetics homeostasis (Fig. 3). Reactive astrogliosis, BBB breakdown, and vascular alterations are also caused by 3HGA, mainly in the striatum, which corresponds to the most damaged cerebral structure in GA 1. However, the available experimental data revealing alterations of these processes must be taken with caution to better evaluate the relative relevance of each of these pathomechanisms relative to the others. Furthermore, it is emphasized that many of these effects were observed with supraphysiological concentrations of 3HGA, so that their pathophysiological relevance should be also taken prudently. Unraveling these harmful mechanisms and the precise role of 3HGA on these deleterious alterations at the brain concentrations found in GA 1 patients will be important for the development of new therapeutic strategies to prevent the long-term neurological complications that still compromise a considerable number of affected patients.

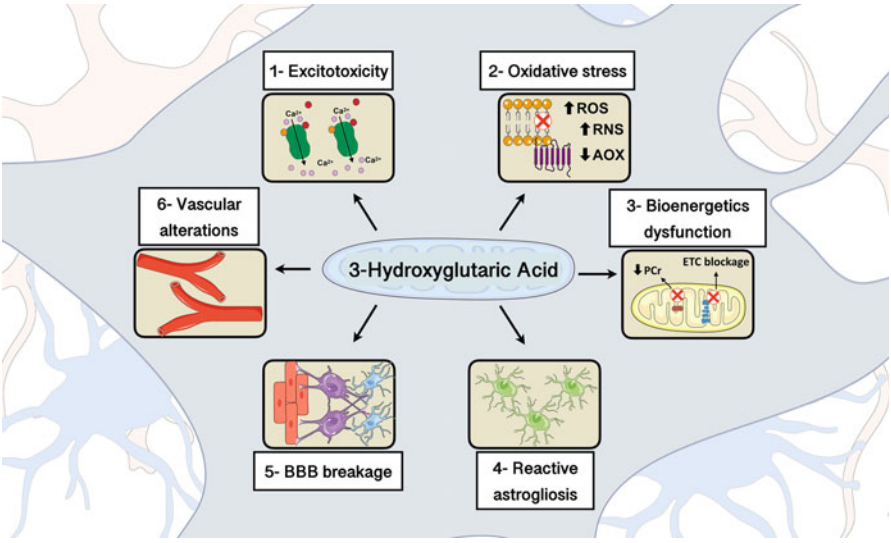

Fig. 3 Presumed mechanisms of 3-hydroxyglutaric acid neurotoxicity. High levels of 3-hydroxyglutaric acid in the brain may cause (1) excitotoxicity, (2) oxidative stress, (3) bioenergetics dysfunction, (4) reactive astrogliosis, (5) BBB breakage, and (6) vascular alterations. ROS, reactive oxygen species; RNS, reactive nitrogen species; AOX, antioxidants; ETC, electron transfer chain; PCr, phosphocreatine; BBB, blood-brain barrier

7 Cross-References

- ▶ Glutamate as a Neurotoxin
- ▶ Glutamate Neurotoxicity Related to Energy Failure
- ▶ Glutaric Acid Neurotoxicity: Mechanisms and Actions
- ▶ Glutaric Acidemia Type 1: An Inherited Neurometabolic Disorder of Intoxication
- ▶ Ionotropic Receptors in the Central Nervous System and Neurodegenerative Disease
- ▶ Neurotoxicity: A Complex Multistage Process Involving Different Mechanisms
- ▶ Role of Ionotropic Glutamate Receptors in Neurodegenerative and Other Disorders
- ▶ The NMDA Receptor System and Developmental Neurotoxicity

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