Int. J. Devl Neuroscience 27 (2009) 65–72

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

journal homepage: www.elsevier.com/locate/ijdevneu

Kinetic characterization of L-[^{3}H]glutamate uptake inhibition and increase oxidative damage induced by glutaric acid in striatal synaptosomes of rats

Danieli Valnes Magni [a], Ana Flávia Furian [a,c], Mauro Schneider Oliveira [a,c], Mauren Assis Souza [a,e], Fabiane Lunardi [d], Juliano Ferreira [d], Carlos Fernando Mello [a], Luiz Fernando Freire Royes [a,d,e], Michele Rechia Fighera [a,b,f,*]

[a] Centro de Ciências da Saúde, Laboratório de Psicofarmacologia e Neurotoxicidade, Departamento de Fisiologia, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil
[b] Centro de Ciências da Saúde, Departamento de Pediatria, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil
[c] Programa de Pós-graduação em Ciências Biológicas: Bioquímica, Universidade Federal do Rio Grande do Sul, 90035-003 Porto Alegre, RS, Brazil
[d] Centro de Ciências Naturais e Exatas, Laboratório de Neurotoxicidade, Departamento de Química, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil
[e] Centro de Educação Física e Desportos, Departamento de Métodos e Técnicas Desportivas, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil
[f] Universidade Luterana do Brasil, Campus Santa Maria, Santa Maria, RS, Brazil

ARTICLE INFO

Article history:
Received 24 June 2008
Received in revised form 30 August 2008
Accepted 23 September 2008

Keywords:
Glutaric acid
Striatum
Excitotoxicity
Glutamate
Synaptosomes
Reactive oxygen species
Kinetic

ABSTRACT

Glutaric acidemia type I (GA-I) is an inherited metabolic disease characterized by accumulation of glutaric acid (GA) and striatal degeneration. Although growing evidence suggests that excitotoxicity and oxidative stress play central role in the neuropathogenesis of this disease, mechanism underlying striatal damage in this disorder is not well established. Thus, we decided to investigate the *in vitro* effects of GA 10 nM (a low concentration that can be present initial development this disorder) on L-[^{3}H]glutamate uptake and reactive oxygen species (ROS) generation in synaptosomes from striatum of rats. GA reduced L-[^{3}H]glutamate uptake in synaptosomes from 1 up to 30 min after its addition. Furthermore, we also provided some evidence that GA competes with the glutamate transporter inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC), suggesting a possible interaction of GA with glutamate transporters on synaptosomes. Moreover, GA produced a significant decrease in the V_{MAX} of L-[^{3}H]glutamate uptake, but did not affect the K_D value. Although the GA did not show oxidant activity *per se*, it increased the ROS generation in striatal synaptosomes. To evaluate the involvement of reactive species generation in the GA-induced L-[^{3}H]glutamate uptake inhibition, trolox (0.3, 0.6 and 6 μM) was added on the incubation medium. Statistical analysis showed that trolox did not decrease inhibition of GA-induced L-[^{3}H]glutamate uptake, but decreased GA-induced reactive species formation in striatal synaptosomes (1, 3, 5, 10, 15 and 30 min), suggesting that ROS generation appears to occur secondarily to glutamatergic overstimulation in this model of organic acidemia. Since GA induced DCFH oxidation increase, we evaluate the involvement of glutamate receptor antagonists in oxidative stress, showing that CNQX, but not MK-801, decreased the DCFH oxidation increase in striatal synaptosomes. Furthermore, the results presented in this report suggest that excitotoxicity elicited by low concentration of GA, could be in part by maintaining this excitatory neurotransmitter in the synaptic cleft by non-competitive inhibition of glutamate uptake. Thus the present data may explain, at least partly, initial striatal damage at birth, as evidenced by acute bilateral destruction of caudate and putamen observed in children with GA-I.

1. Introduction

Glutaric acidemia type I (GA-I) is an autosomal recessive inherited neurometabolic disease caused by deficiency of the activity of the mitochondrial enzyme glutaryl-CoA dehydrogenase (GCDH; EC 1.3.99.7), characterized biochemically by an accumulation of glutaric acid (GA), and 3-hydroxyglutaric acid (3-OH-GA) in

* Corresponding author at: Centro de Ciências da Saúde, Departamento de Pediatria, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil. Fax: +55 55 3220 9378.
E-mail address: mrfighera@yahoo.com.br (M.R. Fighera).

0736-5748/$34.00 © 2008 ISDN. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijdevneu.2008.09.004

the body fluids and brain tissue (GA, 500–5000 μM; 3-OH-GA, 40–200 μM) of affected patients (Goodman et al., 1977; Liesert et al., 1999; Goodman and Frerman, 2001; Strauss and Morton, 2003; Strauss et al., 2003). Clinical manifestations of GA-I are predominantly neurological, including generalized convulsions, progressive dystonia and dyskinesia, especially after encephalophatic crises, which are accompanied by bilateral and irreversible destruction of vulnerable brain regions, *i.e.* striatum and cortex (Morton et al., 1991; Hoffmann and Zschocke, 1999).

In this scenario, a considerable body of evidence have indicated that GA and 3-GA can induce brain damage by energy depletion (Ullrich et al., 1999; Silva et al., 2000; Das et al., 2003; Ferreira et al., 2005; Latini et al., 2005a), oxidative stress (Latini et al., 2002, 2005b; de Oliveira Marques et al., 2003; Fighera et al., 2006) and primary or secondary excitotoxicity (Kölker et al., 1999, 2000a, 2002a,b; de Mello et al., 2001; Porciúncula et al., 2004; Rosa et al., 2004). Furthermore, it has been suggested that these mechanisms might cooperate in a synergistic way to cause the neuropathological alterations found in GA-I patients (Kölker et al., 2004a; Wajner et al., 2004).

With regard to excitotoxicity, postmortem examination of the basal ganglia and cerebral cortex of patients with GA-I revealed post-synaptic vacuolization characteristic of glutamate-mediated brain damage indicating that this process may represent an important mechanism underlying the pathophysiology of this disorder (Goodman et al., 1977; Forstner et al., 1999; Hoffmann and Zschocke, 1999). Nevertheless, although there is convincing evidence of the participation of glutamate in the toxicity of GA, the primary cause of striatum degeneration in GA-I is still not well defined (Kölker et al., 2001, 2002a,b; Koeller et al., 2002; Latini et al., 2005a; de Oliveira Marques et al., 2003; Ferreira et al., 2005). While some experimental findings demonstrate that GA and 3-OHGA are excitotoxic to cultured neurons and may interact with glutamate receptors or transporters (Flott-Rahmel et al., 1997; Kölker et al., 1999, 2002a,b, 2004b; Rosa et al., 2004; Wajner et al., 2004), recent works did not show excitotoxic actions by 3-OHGA (Lund et al., 2004; Freudenberg et al., 2004). Thus, the role of excitotoxicity in GA-I neuropathophysiology is still under intense debate.

Glutamate is the main excitatory neurotransmitter in the brain, and its interaction with specific membrane receptors is responsible for many functions such as cognition, memory and movement (Ozawa et al., 1998). The role of glutamate in mammalian brain is mediated by activation of ionotropic receptors and metabotropic receptors (Nakanishi, 1992; Hollmann and Heinemann, 1994; Ozawa et al., 1998). Ionotropic receptors can be divided into *N*-methyl-D-aspartate (NMDA: NR1 and NR2A-D) and non-NMDA, the latter including the a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA: GluR1-4) and kainate (GluR5-9 and KA1-2) receptors. Metabotropic glutamate receptors (mGluRs) have been divided into groups I, II and III (Conn and Pin, 1997; Ozawa et al., 1998). Glutamate receptors are involved in a variety of physiological processes during brain development, including synaptogenesis and synaptic plasticity, and present a unique profile of susceptibility to toxicity mediated by differential activation of the receptor subtypes (McDonald and Johnston, 1990). Ionotropic receptor ontogeny is characterized by rapid maturational changes in various forebrain structures in the rat. NMDA receptor expression reaches the highest level in hippocampus and neocortex in the first postnatal week, whereas AMPA receptors density peaks occur in the second postnatal week (Insel et al., 1990; Petralia et al., 1999). This variable receptor expression profile generates a regional- and age-specific window of susceptibility to many neurotoxins and diseases (Kölker et al., 2000a; Haberny et al., 2002; Jensen, 2002).

The synaptic actions of glutamate are terminated by its removal from the synaptic cleft by a high-affinity sodium-dependent excitatory amino acid transporter (EAAT) system, mainly located in the astrocytic membranes (Danbolt, 2001; Amara and Fontana, 2002). The astroglial glutamate transporters GLAST (EAAT1) and GLT1 (EAAT2) are mainly responsible for the clearance of extracellular glutamate (Rothstein et al., 1996; Danbolt, 2001).

Besides its physiological effects, glutamate is also a potent neurotoxin and the presence of high amounts of this neurotransmitter in the synaptic cleft may lead to excitotoxicity by overstimulation of glutamate receptors, a process related to neuropathology of acute (brain hypoxia, ischemia and trauma) and chronic (Parkinson's disease, Alzheimer's disease and organic acidemias) brain disorders (Lipton and Rosenberg, 1994; Maragakis and Rothstein, 2001; Wajner et al., 2004; Kölker et al., 2008).

In models of glutaric acidemia, a considerable body of evidence suggest that neurotoxic actions elicited by GA and 3-OHGA are due the similar chemical structure of this organic acids with glutamate (Flott-Rahmel et al., 1997; Lima et al., 1998; Kölker et al., 1999, 2000a,b, 2002a,b; Ullrich et al., 1999; Porciúncula et al., 2000, 2004; de Mello et al., 2001; Rosa et al., 2004). In this context, it has been demonstrated that 3-OH-GA decreases cell viability by *N*-methyl-D-aspartate (NMDA) receptors stimulation (Kölker et al., 2000a,b) and ROS generation (Kölker et al., 2001). Furthermore, recent studies have suggested the GA-induced neurotoxicity can be due inhibition of glutamate uptake related with an interaction of GA with glutamate transporters, leading to an increase of the glutamate levels in synaptic cleft and overstimulation glutamate receptors (Porciúncula et al., 2000, 2004; Magni et al., 2007). On the other hand, the activity of glutamate transporters can also be reduced by indirect mechanisms, including reactive species formation (Volterra et al., 1994; Nanitsos et al., 2004).

Thus, considering that mechanism involved the primary striatal damage in the GA-I is not well established, we decided investigate whether low concentration of GA (that can be present in the initial development of this disorder −10 nM) alter L-[^{3}H]glutamate uptake. In this context, we evaluated the kinetic parameters dissociation constant (K_D) and maximum velocity (V_{MAX}) of glutamate transport to evaluate whether GA-induced reduction on L-[^{3}H]glutamate uptake could be due to competitive or non-competitive mechanism. Furthermore, we investigated the involvement of striatal ROS generation induced by GA on the L-[^{3}H]glutamate uptake. In addition, we also evaluated the effects of the glutamate receptor antagonists and transporters inhibitor on the oxidative stress and L-[^{3}H]glutamate uptake, respectively.

2. Experimental procedures

2.1. Animal and reagents

Adult male Wistar rats (270–300 g) maintained under controlled light and environment (12:12 h light-dark cycle, 22 ± 1 °C, 55% relative humidity) with free access to food (Guabi, Santa Maria, Brazil) and water were used. Animal utilization protocols followed the Official Government Ethics guidelines and were approved by the University Ethics Committee. L-[^{3}H]glutamate (49 Ci/mmol) was purchased from Amersham International, UK. All other reagents, including glutaric acid free acid (GA, 99% pure), were purchased from Sigma (St. Louis, MO, USA).

2.2. Synaptosomal preparation

The animals were sacrificed by decapitation and had their brain exposed by the removal of the parietal bone. A punch of the striatum was rapidly removed and synaptosomal preparation was obtained by isotonic Percoll/sucrose discontinuous gradients at 4 °C, as previously described (Dunkley et al., 1988). Briefly, homogenates (10%, w/v) from striatum were made in 5 mM HEPES and 320 mM sucrose (pH 7.4), and centrifuged twice at 800 × *g* for 10 min to produce a pellet (P1) and a supernatant (S1). P1 was discarded and S1 was subjected to 16, 10, 7.5% Percoll solution density gradient centrifugation at 24,000 × *g* for 10 min. The

synaptosomal fractions were isolated, suspended and homogenized in Krebs' buffer (pH 7.4), containing in mM: 145 NaCl, 5 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 20 HEPES, 10 glucose and 1.2 $CaCl_2$ and centrifuged at 15,000 × *g* for 10 min. The supernatant was removed and the pellet resuspended in Krebs' buffer. The synaptosomal fraction used contained approximately 1 mg of protein/ml. This fraction also contained approximately 5% contamination with fragments of the inner and outer mitochondrial membranes, microsomes, myelin, as well as neural and glial plasma membranes (Dunkley et al., 1988; Nagy et al., 1986; Migues et al., 1999).

2.3. Synaptosomal L-[^{3}H]glutamate uptake

Assays were performed in a final volume of 500 μl in a standard incubation medium composed of Krebs' buffer and 5 μM (3700 Bq) of L-[^{3}H]glutamate (1.81 GBq/mol, Amersham International, UK).

The synaptosomal L-[^{3}H]glutamate uptake was performed in the presence of GA (10 nM). This concentration of GA was previously reported to reduce L-[^{3}H]glutamate uptake in forebrain synaptosomes (Porciúncula et al., 2000). Controls did not contain glutaric acid. The uptake was carried out for 1, 3, 5, 10, 15 and 30 min at 37 °C after the addition of synaptosomes (100 μg of protein/tube) and stopped by centrifugation (16,000 × *g* for 1 min at 4 °C). Radioactivity in the synaptosomal pellet was measured in a Wallac 1409 liquid scintillation counter. The specific L-[^{3}H]glutamate uptake was calculated as the difference between total uptake at 37 °C and the uptake at 4 °C (non-specific uptake).

Kinetic analysis assay of L-[^{3}H]glutamate uptake was performed in an incubation medium composed of Krebs' buffer in a final volume of 500 μl containing labeled and unlabeled glutamate at final concentrations ranging from 0.625 to 320 μM. The synaptosomal L-[^{3}H]glutamate uptake was performed in the presence of GA (10 nM). Controls did not contain GA. The uptake was carried out for 10 min at 37 °C after the addition of synaptosomes from striatum (100 μg of protein/tube) and followed how above described. Kinetic parameters K_D and V_{MAX} for synaptosomal L-[^{3}H]glutamate uptake were determined by nonlinear regression analysis (GraphPad Software, San Diego, CA).

In order, to evaluate the involvement of glutamate transporters on the GA-induced L-[^{3}H]glutamate uptake reduction, the experiments were done in the presence of 50 μM L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC), which is a substrate inhibitor of glutamate transporters.

To evaluate whether oxidative stress could alter GA-induced L-[^{3}H]glutamate uptake reduction, other experiment was done in the presence of the synthetic antioxidant Trolox (0.3, 0.6 and 6 μM), a water-soluble derivative of vitamin E with potent antioxidant properties, for 10 min at 37 °C (Dreiem and Seegal, 2007).

2.4. Synaptosomal reactive species formation

Reactive oxygen species were assayed using 2′,7′-dichlorofluorescein diacetate (DCFH-DA), which is de-esterified within synaptosomes to the ionized free acid, dichlorofluorescein, DCFH. This is trapped within cells and thus accumulated. DCFH is capable of being oxidized to the fluorescent 2′,7′-dichlorofluorescein diacetate by reactive oxygen. The utility of this probe in isolated subcellular cerebral systems has been described (Bondy et al., 1998). Assays were performed in a final volume of 2000 μl in a standard incubation medium composed of Krebs' buffer, 100 μl synaptosomes (1 mg/ml). The synaptosomes were loaded with 5 μM DCFH-DA for 15 min at 37 °C. This incubation was stopped by centrifugation (16,000 × *g* for 5 min). The synaptosomal pellet was resuspended in Krebs' buffer in a final volume of 2000 μl. In this solution was added GA 10 nM and was read a long time for 1, 3, 5, 10, 15 and 30 min at 37 °C. Controls did not contain GA. The fluorescence was determined on a spectrofluorometer, with excitation wavelength at 488 nm, and emission wavelength 525 nm.

Other experiment was done in the presence of trolox (0.3, 0.6 and 6 μM) for 10 min at 37 °C (Dreiem and Seegal, 2007), to evaluate whether this antioxidant could decrease GA-induced synaptosomal reactive species formation.

The synaptosomal reactive species formation was also determinate in the presence of NMDA glutamate receptor antagonist, dizocilpine (MK-801; 5 μM) (Tavares et al., 2000) and non-NMDA glutamate receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 100 μM) (Barnes et al., 1994), to investigate the involvement of glutamate receptors on the GA-induced DCFH oxidation increase in striatal synaptosomes.

2.5. Lactate dehydrogenase (LDH) assay

Synaptosomes were incubated at 37 °C for 30 min in Krebs' buffer in the presence or absence of 10 nM GA. Viability was assessed by measuring the release of the cytosolic enzyme lactate dehydrogenase (LDH). LDH measurement was carried out in 25 μl aliquots using the LDH kit from Labtest reagents, Brazil. The LDH activity in the incubation medium was assayed spectrophotometrically at a wavelength of 340 nm. The results were expressed as percentage of total LDH release. Total LDH release (100% release) was achieved with 1% Triton X-100 in the incubation medium. Under the experimental conditions used, no changes in LDH were observed.

2.6. Chemiluminescence generated in cell-free systems

The assays were conducted in a standard medium composed of phosphate buffered saline (PBS, 10 mM KH_2PO_4 and 150 mM NaCl, pH 7.4) and luminol (50 mM, prepared daily in PBS) mixture. In this medium was added H_2O_2 (3.5 mM) or GA (10 nM). Controls contained only phosphate buffered saline and luminol mixture. Chemiluminescence generated was measured continuously for 3 min (Yildiz et al., 1998).

2.7. Protein determination

Protein content was measured colorimetrically by the method of Bradford (1976) using bovine serum albumin (1 mg/ml) as a standard.

2.8. Statistics

The synaptosomal L-[^{3}H]glutamate uptake and DCFH oxidation were analyzed by one- or two-way ANOVA, with time of measures treated as within subject factor, depending on the experimental design, followed by a Student–Newman–Keuls test. Data from kinetic analysis of Na^+-dependent transport of synaptosomal L-[^{3}H]glutamate uptake in the presence and absence the GA were analyzed by a student's *t*-test for paired and independent samples. Effect of trolox in the synaptosomal L-[^{3}H]glutamate uptake and DCFH oxidation were analyzed by a 2 (presence or absence of trolox) × 2 (presence or absence of GA) factorial ANOVA. Effect of PDC in the synaptosomal L-[^{3}H]glutamate uptake was analyzed by a 2 (presence or absence of PDC) × 2 (presence or absence of GA) factorial ANOVA. Effect of glutamate receptor antagonists, MK-801 and CNQX, in the DCFH oxidation was analyzed by a 2 (presence or absence of MK-801/CNQX) × 2 (presence or absence of GA) factorial ANOVA. *Post hoc* analyses were carried out by the *F* test for simple effect or the Student–Newman–Keuls test, when appropriate. All data are expressed as mean ± S.E.M., $P < 0.05$ was considered significant.

3. Results

We first evaluated the effect GA (10 nM) on L-[^{3}H]glutamate uptake by striatum synaptosomes of rats. Fig. 1 shows that GA significantly decreased L-[^{3}H]glutamate uptake into striatum synaptosomes as compared to controls [$F(5, 60) = 23.56$; $P < 0.001$]. In order to verify whether cellular death could be responsible for the GA-induced glutamate uptake reduction, we evaluated the viability of striatum synaptosomes measured by LDH release assays. Statistical analysis revealed that synaptosomes incubated for 30 min with GA (10 nM) showed no significant leakage of the cytosolic marker LDH as compared to controls. Percentages of the total LDH content achieved by synaptosomal disruption with Triton X-100 were: 25.71 ± 2.51 (control); 25.06 ± 2.04 (GA 10 nM, $n = 4$ experiments).

Next experiments, we evaluated the involvement of inhibitor of glutamate transporters PDC on the Na^+-dependent L-[^{3}H]gluta-

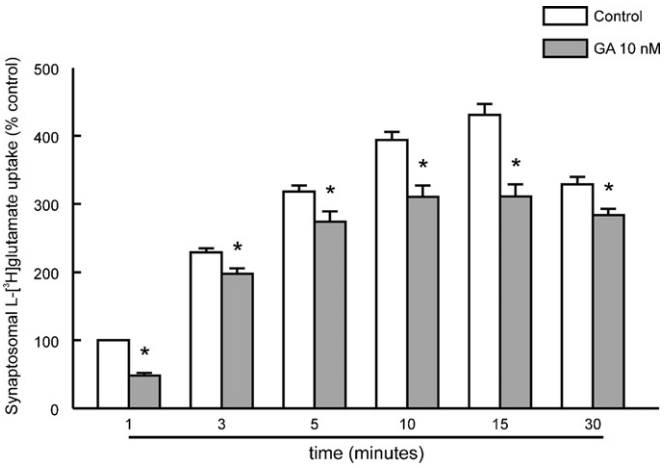

Fig. 1. Effect of glutaric acid (10 nM) on L-[^{3}H]glutamate uptake by synaptosome from striatum of rats. Data are mean ± S.E.M. for $n = 6$ in each group. *$P < 0.001$ as compared to control (Student–Newman–Keuls test).

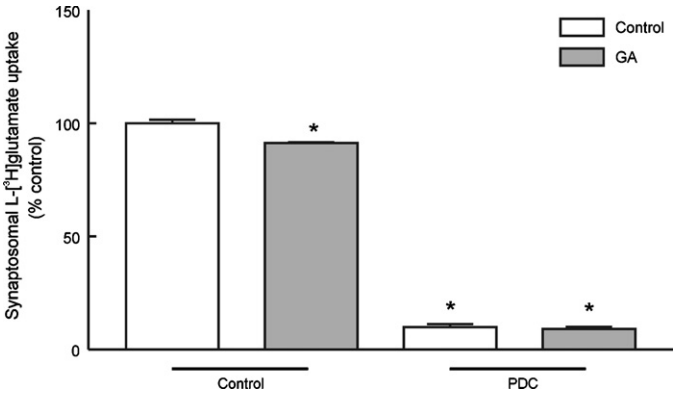

Fig. 2. Effect of PDC (in the absence and presence glutaric acid) on L-[^{3}H]glutamate uptake by synaptosome from striatum of rats. Data are mean ± S.E.M. for $n = 5$ in each group. $^*P < 0.005$ compared with control (Student–Newman–Keuls test).

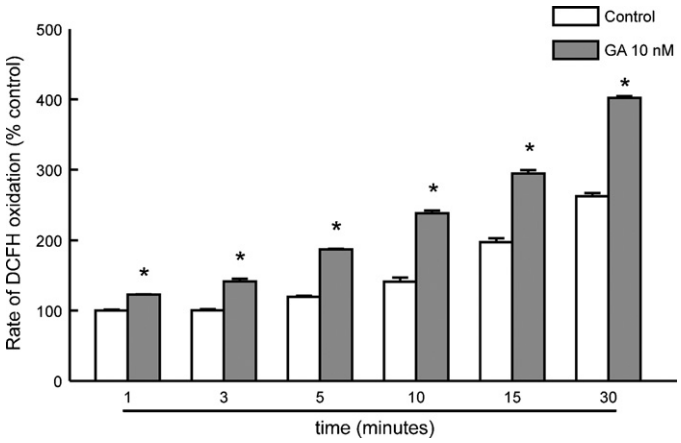

Fig. 4. Effect of glutaric acid on DCFH oxidation by synaptosome from striatum of rats. The DCFH oxidation increase initiated 1 min after GA administration and persisted up to the end of the incubation period (30 min). Data are the means ± S.E.M. $n = 4$ animals in each group. $^*P < 0.001$ compared with control (Student–Newman–Keuls test).

mate uptake in the presence or absence of GA in striatal synaptosomes. We observed that both GA and PDC inhibited Na^+-dependent L-[^{3}H]glutamate uptake. Furthermore, GA did not change the inhibitory effect of PDC [$F(1, 16) = 12.7$; $P < 0.005$] (Fig. 2).

Fig. 3 shows alterations on kinetic parameters of the glutamate uptake induced by the low concentration of GA. The hyperbolic glutamate concentration–velocity curve demonstrates typical substrate saturation kinetics expected of Na^+-dependent glutamate transport in synaptosomes, with K_D value of 29.8 ± 4.4 μM and V_{MAX} of 77.5 ± 3.5 pmoles/(mg protein min). Statistical analysis revealed that GA significantly decreased [$t = 2.35$; $P < 0.05$] V_{MAX} (to 66.6 ± 2.8 pmoles/(mg protein min)) and did not changed [$t = 0.91$; $P > 0.05$] K_D value (23.6 ± 3.4 μM) of glutamate uptake as compared to controls.

Considering that glutamate uptake transporters can also be reduced by indirect mechanisms, including reactive species formation, we decided to verify the effect of GA on the synaptosomal oxidative stress. Statistical analysis that GA did not show oxidant activity *per se* as compared to H_2O_2 (data not shown), but increased [$F(5, 30) = 80.06$; $P < 0.001$] DCFH oxidation in striatal synaptosomes at all times (1, 3, 5, 10, 15 and 30 min), as compared to control (Fig. 4). In addition, we observed that trolox (0.3, 0.6 and 6 μM) decreased [significant treatment (control or trolox) by treatment (control or GA) interaction: $F(3, 24) = 8.37$; $P < 0.001$] GA-induced oxidative stress production by synaptosomal preparations from striatum (Fig. 5). However, statistical analysis revealed that trolox (0.3, 0.6 and 6 μM) did not decrease inhibition of glutamate uptake [$F(3, 56) = 0.76$; $P > 0.05$] induced by GA (Fig. 6).

Since GA increased DCFH oxidation in striatal synaptosomes, we decided to investigate whether ionotropic glutamate receptors were involved in the currently described increase of oxidative stress by GA. Statistical analysis showed that the non-NMDA glutamate receptor antagonist CNQX decreased [$F(2, 24) = 6.49$; $P < 0.01$] GA-induced DCFH oxidation increase (Fig. 7). In contrast, the addition of the NMDA glutamate receptor antagonist MK-801 in medium of incubation did not protect against GA-induced oxidative stress. These results agree with previous studies that demonstrated the lack of effect of NMDA glutamate antagonists against GA-induced decrease of Na^+-independent glutamate binding to synaptic membranes and convulsions (Lima et al., 1998; Dalcin et al., 2007).

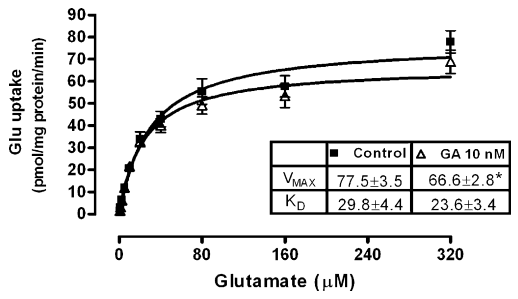

Fig. 3. Effect of glutaric acid (10 nM) on kinetic analysis of high affinity, Na^+-dependent transport L-[^{3}H]glutamate uptake by synaptosome from striatum of rats. Data are mean μmol/(kg protein min) ± S.E.M. for $n = 6$ in each group. Corresponding kinetic analysis (V_{MAX} = μmol/kg protein; K_D = μM) are provided and insert in table. $^*P < 0.05$ as compared to control (Student's *t*-test).

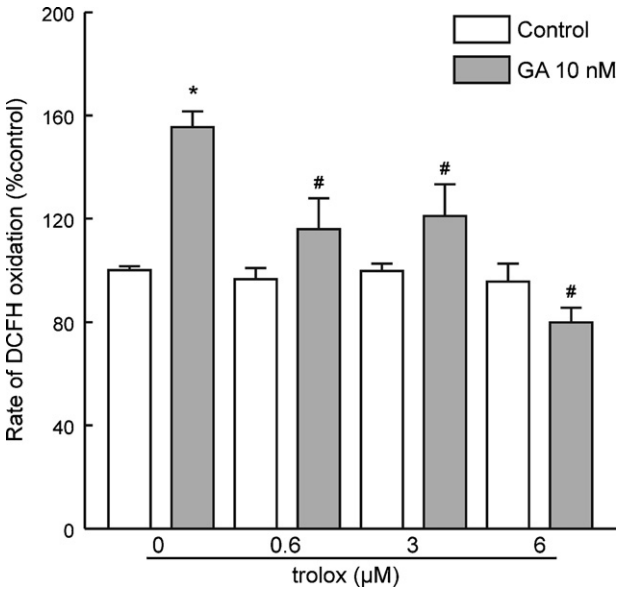

Fig. 5. Effect of trolox (in the absence and presence glutaric acid) on DCFH oxidation by synaptosome from striatum of rats. Data are mean ± S.E.M. for $n = 6$ in each group. $^*P < 0.001$ compared with control; $^\#P < 0.001$ as compared to GA group (Student–Newman–Keuls test).

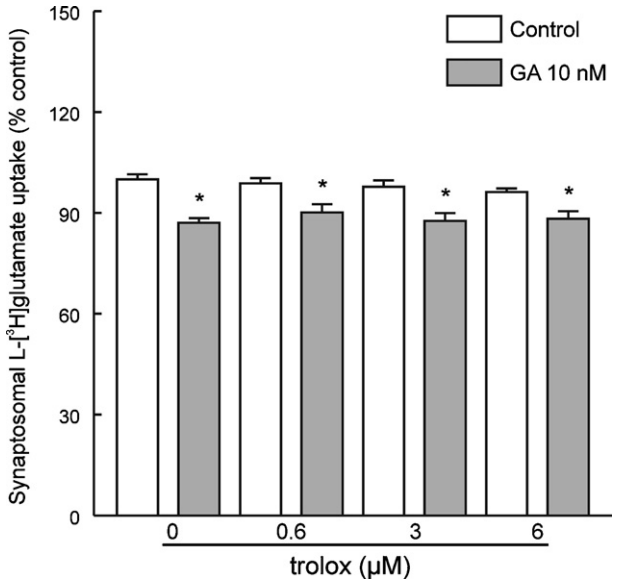

Fig. 6. Effect of trolox (in the absence and presence glutaric acid) on L-[^{3}H]glutamate uptake by synaptosome from striatum of rats. Data are mean ± S.E.M. for $n = 6$ in each group. $^*P < 0.05$ compared with control (Student–Newman–Keuls test).

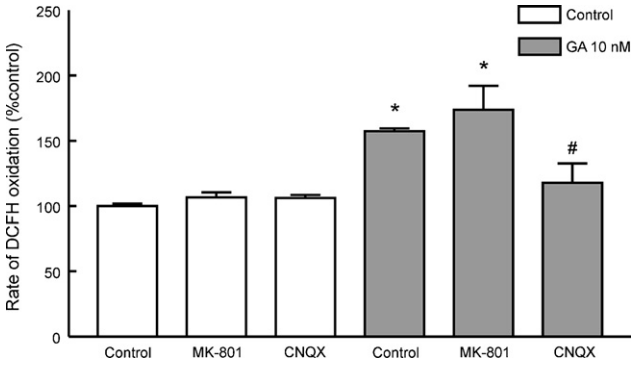

Fig. 7. Effect of NMDA (5 μM) and CNQX (100 μM) (in the absence and presence glutaric acid) on DCFH oxidation by synaptosome from striatum of rats. Data are mean ± S.E.M. for $n = 5$ in each group. $^*P < 0.01$ compared with control; $^\#P < 0.001$ as compared to GA group (Student–Newman–Keuls test).

4. Discussion

Although the pathophysiology of GA-I is not yet fully established, the excitotoxicity has been proposed as an important neurotoxic mechanism in GA-I, especially due to the structural similarity between glutamate, GA and 3-OH-GA (Flott-Rahmel et al., 1997; Lima et al., 1998; Kölker et al., 1999, 2004b; Wajner et al., 2004; Rosa et al., 2004). It is conceivable that some conflicting results about GA-induced excitotoxicity could be due to the ontogenetic and organ specific differences of glutamate receptor and transporter expression (McDonald and Johnston, 1990; Ullensvang et al., 1997; Furuta et al., 1997; Ozawa et al., 1998). Therefore, the present investigation was undertaken to evaluate the effect of GA, at the lower concentration present during initial development of GA-I, on L-[^{3}H]glutamate uptake and reactive species formation from striatum synaptosomal of rats. Furthermore, we evaluated, for first time, what kinetic parameters of glutamane uptake could be altered by GA.

In the present study, we have initially shown a time curve where the GA decreased L-[^{3}H]glutamate uptake from striatal synaptosomes in all times tested. However, this effect was not due to cellular death, as evidenced by LDH viability test, suggesting that specific glutamate transporters localized in striatum could be responsible for that effect. Considering that the glial GLAST and GLT1 glutamate transporters and the neuronal glutamate transporter EAAC1 can be present in synaptosomes preparations (Tanaka et al., 1997; Danbolt, 2001), it is plausible to propose that one or more of these transporters could serve as a target for GA inhibitory effect. In agreement with this view, previous studies have demonstrated that glutamate uptake in synaptosomal preparations is mediated by GLT1 transporters (Robinson et al., 1993; Bridges et al., 1999). Moreover, it has been demonstrated that synaptosomes prepared from GLT1 deficient mutant mice have very low uptake activities (Tanaka et al., 1997; Danbolt, 2001). Furthermore, Western blotting analysis revealed that EAAC1 and GLAST transporters are also present on the striatum of rats at later ages (Furuta et al., 1997).

An important result of the present investigation was that a low concentration of GA (10 nM) significantly reduced the efficacy (V_{MAX}), but not the affinity (K_D) of glutamate uptake in striatal synaptosomes. These findings suggest that glutamate uptake reduction induced by low concentration of GA is due non-competitive inhibition. Our results are in agreement with previous findings demonstrating that a high concentration of GA (1 mM) may reduce glutamate uptake in synaptosomes of whole brain (Porciúncula et al., 2000). Moreover, it was shown that 1 mM, but not 1 or 10 nM, of GA reduced the sodium-dependent glutamate binding in plasma membranes of whole brain, indicating that GA directly interacts with glutamate transporters (Porciúncula et al., 2000). In accordance with that idea that low concentration of GA did not directly interact with glutamate transporter, our kinetic data revelled that GA (10 nM) did not altered K_D value for glutamate uptake in striatal synaptosomes. Furthermore, the reduction in glutamate uptake produced by low concentrations of GA is mediated by a decrease in V_{Max}, which is consistent with a non-competitive inhibition of glutamate transporters. Thus, GA could reduce glutamate uptake directly by an interaction with an alosteric site in glutamate transporters (as acts reactive species). In fact, our experiments showed that the simultaneous addition of the glutamate transporter inhibitor PDC (50 μM) and GA (10 nM) did not alter the inhibitory effect on the glutamate uptake in striatal synaptosomes compared to the effect elicited by PDC alone, indicating that GA can be binding to glutamate transporters and that this disturbance of glutamatergic neurotransmission may explain, at least in part, cerebral damage observed in GA-I.

Other point to be considered is that glutamate transporter activity can be inhibited by oxidation (Volterra et al., 1994; Trotti et al., 1996, 1998; Nanitsos et al., 2004). Thus, we evaluated whether GA-induced glutamate uptake reduction can be due reactive species formation on the striatum synaptosomes of rats. Although trolox protects against GA-induced DCFH oxidation increase in all concentrations, it did not protect against GA-induced glutamate uptake reduction on the synaptosomes of cerebral structure studied, suggesting that ROS formation is a late event in the GA-induced neurotoxicity. Our results are in agreement with previous findings demonstrating that even though trolox may reduce DCFH increase to control levels (Dreiem and Seegal, 2007), it provides no protection against inhibition of aspartate uptake induced by methylmercury (MeHg) (Allen et al., 2001). Taken together these observations, our results suggest that the excitotoxicity induced by low GA concentration can be the initial mechanism of striatal damage and that free radical generation can occur secondarily to glutamatergic overstimulation, a fact that may be related to striatum degeneration observed in GA-I patients. Furthermore, since that a previous study showed

that GA inhibited synaptosomal glutamate uptake at 1 mM concentration (Porciúncula et al., 2000), it may be presumed that much lower intracellular concentrations of GA (10 nM) are sufficient to inhibit glutamate transport and increase oxidative stress. In line of this view, it has been reported that slight reduction on the excitatory neurotransmitter uptake can account an excitotoxic response (Allen et al., 2001).

Besides, we cannot rule out the possibility of that GA may also stimulate glutamate receptors since we found that DCFH oxidation increase provoked by GA was significantly attenuated by the non-NMDA receptor antagonist, but not by MK-801, suggesting that these receptors contributed, at least partly, to the GA-induced oxidative stress. Our present findings may possibly explain a previous *in vivo* report showing that the behavioral alterations and convulsions provoked by intrastriatal administration of GA, in the same dose that caused oxidative stress (Fighera et al., 2006), were prevented by the non-NMDA antagonist DNQX, but not by the NMDA antagonist MK-801 in adult rats (Lima et al., 1998). Reinforcing this point, a recent report also showed that GA ca bind to non-NMDA receptors in brain from rats (Porciúncula et al., 2004; Dalcin et al., 2007).

In addition, it has been demonstrated that glutamate transporters are mainly responsible for the maintenance of low extracellular glutamate concentrations (Rothstein et al., 1996; Danbolt, 2001; Amara and Fontana, 2002). Moreover, the transporter system present in synaptosomes has been considered as an important step for the modulation of the glutamatergic system by controlling the glutamate–glutamine cycle (Otis, 2001). Thus, inhibiting glutamate uptake, GA probably alters the synaptic turnover of glutamate, possibly leading to an increased cytosolic pool of the excitatory neurotransmitter, which in turn may result stimulation of glutamate receptors, causing intracellular Ca^{2+} increase, and leading to ROS generation (Volterra et al., 1994; Nanitsos et al., 2004). We hypothesize that this sequence of events is responsible for the GA-induced neurochemical alterations reported here. Taken together these observations and previous reports demonstrating that low GA concentration inhibits glutamate uptake (Porciúncula et al., 2004) and markedly reduces viability of neurons in culture via glutamate receptors (Kölker et al., 2000a,b), it is conceivable that our results may be related to these findings.

In this context, these mechanisms may explain the involvement of the glutamatergic system in the neuronal toxicity, convulsions and oxidative damage elicited by GA in rats (Kölker et al., 2001; Fighera et al., 2006; Magni et al., 2007; Rosa et al., 2007). Furthermore, the results presented in this report may be related to some of the pathological changes observed in patients with GA-I such as the post-synaptic vacuolization characteristic of excitotoxic neuronal death (Olney, 1980), which has been described in post-mortem examination of the brain of patients with GA-I (Amir et al., 1987). In fact, previous studies have demonstrated the presence of glutamate receptors in the basal ganglia and the characteristic lesions of these cerebral structures in GA-I (Amir et al., 1987; Goodman et al., 1995).

To determine precisely whether the induction of free radicals by GA occurs indirectly via its inhibitory activity on cellular metabolism, or whether it is a direct source of free radicals because of its chemical reactivity, we verified whether GA (10 nM) could increase chemiluminescence in cell-free system. We observed that GA did not show oxidant activity *per se* (data not shown), indicating that chemical reactivity of GA is not direct source of free radicals. In addition, these results reinforce the assumption that ROS generation elicited by this organic acid occur indirectly (Wajner et al., 2004) and that, in low concentration, some degree of cellular intactness is required to GA induce oxidative damage. In this context, it has been proposed that GA and 3-OHGA induce striatal degeneration by disrupting mitochondrial energy metabolism (Ullrich et al., 1999; Das et al., 2003; Ferreira et al., 2005), increasing glutamatergic neurotransmission (Wajner et al., 2004) and promoting oxidative stress (de Oliveira Marques et al., 2003; Latini et al., 2002, 2005b), which ultimately causes secondary excitotoxicity (Kölker et al., 1999, 2000a,b, 2002a,b; Porciúncula et al., 2004; Rosa et al., 2004).

Mechanisms other than glutamatergic facilitation have been claimed to account for the neurotoxic actions of GA, such as Na^+,K^+-ATPase activity and GABAergic mechanisms inhibition induced by GA (Fighera et al., 2006), inhibition of α-ketoglutarate dehydrogenase complex by glutaryl-CoA (Sauer et al., 2005) and bioenergetic impairment (Sauer et al., 2005). Therefore, we cannot rule out that the neurotoxic effects of GA on the glutamate uptake and free radicals production may be mediated, at least in part, by one of these mechanisms (Fighera et al., 2006; Sauer et al., 2006). However, specific studies are necessary to determine the involvement of these alternative mechanisms in the currently described excitotoxic effect of GA-induced on glutamate uptake and oxidative stress increase, and if these events are closely linked.

As regards to the physiological significance of our findings, although we cannot establish with certainty whether our *in vitro* data is related to the neurotoxicity observed in GA-I *in vivo*, it should be emphasized that the effects provoked by GA were observed with concentrations similar to those encountered in brain of glutaric acidemic patients (Goodman et al., 1977; Kölker et al., 2003; Külkens et al., 2005; Sauer et al., 2006). Furthermore, the degree of alterations of the glutamatergic system detected in our study is accepted to cause excitotoxicity in systems testing the effect of potential excitotoxins (Ozawa et al., 1998; Allen et al., 2001; Danbolt, 2001; Meldrum, 2002).

In conclusion, to our knowledge this is the first report showing a kinetic study to glutamate uptake in the presence GA in striatum synaptosomes of rats. Furthermore, taken together our results suggest that the inhibition of glutamate uptake on synaptosomes by the metabolite could result in elevated concentrations of the excitatory neurotransmitter in the synaptic cleft and secondary stimulation of glutamate receptors by GA (a glutamate structurally similar molecule). This glutamatergic neurotransmission increase can lead to reactive species formation and potentially causing excitotoxicity to neural cells, a fact that may be related to the brain damage characteristic of glutaric acidemia type I. The present data may explain, at least partly, initial striatal damage at birth, as evidenced by acute bilateral destruction of caudate and putamen observed in children with GA-I (Goodman, 2004). However, further investigation should be carried out to define initial mechanisms that can be involved on striatum degeneration in this disorder.

Acknowledgements

Work supported by CNPq (grant: 500120/2003-0), C.F. Mello, J. Ferreira and A.F. Furian are the recipients of CNPq fellowships. M. Schneider Oliveira and D.V. Magni are the recipients of CAPES fellowships.

References

Allen, J.W., Mutkus, L.A., Aschner, M., 2001. Methylmercury-mediated inhibition of ^{3}H-D-aspartate transport in cultured astrocytes is reversed by the antioxidant catalyse. Brain Res. 902, 92–100.

Amara, S.G., Fontana, A.C., 2002. Excitatory amino acid transporters: keeping up with glutamate. Neurochem. Int. 41, 313–318.

Amir, N., el-Peleq, O., Shalev, R.S., Christensen, E., 1987. Glutaric aciduria type I: clinical heterogeneity and neuroradiologic features. Neurology 37, 1654–1657.

Barnes, J.M., Dev, K.K., Henley, J.M., 1994. Cyclothiazide unmasks AMPA-evoked stimulation of [^{3}H]-L-glutamate release from rat hippocampal synaptosomes. Br. J. Pharmacol. 113, 339–341.
Bondy, S.C., Tseng, H., Orvig, C., 1998. Active oxygen species formation in synaptosomes exposed to an aluminum chelator. Neurotoxicol. Teratol. 20, 317–320.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Bridges, R.J., Kavanaugh, M.P., Chamberlin, A.R., 1999. A pharmacological review of competitive inhibitors and substrates of high-affinity, sodium-dependent glutamate transport in the central nervous system. Curr. Pharm. Des. 5, 363–379.
Conn, P.J., Pin, J.P., 1997. Pharmacology and functions of metabotropic glutamate receptors. Annu. Rev. Pharmacol Toxicol. 37, 205–237.
Dalcin, K.B., Rosa, R.B., Schmidt, A.L., Winter, J.S., Leipnitz, G., Dutra-Filho, C.S., Wannmacher, C.M., Porciúncula, L.O., Souza, D.O., Wajner, M., 2007. Age and brain structural related effects of glutaric and 3-hydroxyglutaric acids on glutamate binding to plasma membranes during rat brain development. Cell. Mol. Neurobiol. 27, 805–818.
Danbolt, N.C., 2001. Glutamate uptake. Prog. Neurobiol. 65, 1–105.
Das, A.M., Luche, T., Ullrich, K., 2003. Glutaric aciduria I: creatine supplementation restores creatine phosphate levels in mixed cortex cells from rat incubated with 3-hydroxyglutarate. Mol. Genet. Metab. 78, 108–111.
de Mello, C.F., Kölker, S., Ahlemeyer, B., de Souza, F.R., Fighera, M.R., Mayatepek, E., Krieglstein, J., Hoffmann, G.F., Wajner, M., 2001. Intrastriatal administration of 3-hydroxyglutaric acid induces convulsions and striatal lesions in rats. Brain Res. 916, 70–75.
de Oliveira Marques, F., Hagen, M.E., Pederzolli, C.D., Sgaravatti, A.M., Durigon, K., Testa, C.G., Wannmacher, C.M.D., de Souza Wyse, A.T., Wajner, M., Dutra-Filho, C.S., 2003. Glutaric acid induces oxidative stress in brain of young rats. Brain Res. 964, 153–158.
Dreiem, A., Seegal, R.F., 2007. Methylmercury-induced changes in mitochondrial function in striatal synaptosomes are calcium-dependent and ROS-independent. Neurotoxicology 28, 720–726.
Dunkley, P.R., Heath, J.W., Harrison, S.M., Jarvie, P.E., Glenfield, J.P., Rostas, J.A., 1988. A rapid Percoll gradient procedure for isolation of synaptosomes directly from an S1 fraction: homogeneity and morphology of subcellular fractions. Brain Res. 441, 59–71.
Ferreira, G.C., Viegas, C.M., Schuck, P.F., Tonin, A., Ribeiro, C.A.J., Coelho, D.M., Dalla-Costa, T., Latini, A., Wyse, A.T.S., Wannmacher, C.M.D., Vargas, C.R., Wajner, M., 2005. Glutaric acid administration impairs energy metabolism in midbrain and skeletal muscle of young rats. Neurochem. Res. 30, 1123–1131.
Fighera, M.R., Royes, L.F., Furian, A.F., Oliveira, M.S., Fiorenza, N.G., Frussa-Filho, R., Petry, J.C., Coelho, R.C., Mello, C.F., 2006. GM1 ganglioside prevents seizures, Na^+,K^+-ATPase activity inhibition and oxidative stress induced by glutaric acid and pentylenetetrazole. Neurobiol. Dis. 22, 611–623.
Flott-Rahmel, B., Falter, C., Schluff, P., Fingerhut, R., Christensen, E., Jakobs, C., Musshoff, U., Fautek, J.D., Deufel, T., Ludolph, A., Ullrich, K., 1997. Nerve cell lesions caused by 3-hydroxyglutaric acid: a possible mechanism for neurodegeneration in glutaric acidaemia I. J. Inherit. Metab. Dis. 20, 387–390.
Forstner, R., Hoffmann, G.F., Gassner, I., Heideman, P., De Klerk, J.B., Lawrenz-Wolf, B., Doringer, E., Weiss-Wichert, P., Tröger, J., Colombo, J.P., Plöchl, E., 1999. Glutaric aciduria type I: ultrasonographic demonstration of early signs. Pediatr. Radiol. 29, 138–143.
Freudenberg, F., Lukacs, Z., Ullrich, K., 2004. 3-Hydroxyglutaric acid fails to affect the viability of primary neuronal rat cells. Neurobiol. Dis. 16, 581–584.
Furuta, A., Rothstein, J.D., Martin, L.J., 1997. Glutamate transporter protein subtypes are expressed differentially during rat CNS development. J. Neurosci. 17, 8363–8375.
Goodman, S.I., Norenberg, M.D., Shikes, R.H., Breslich, D.J., Moe, P.G., 1977. Glutaric aciduria: biochemical and morphologic considerations. J. Pediatr. 90, 746–750.
Goodman, S.I., Kratz, L.E., DiGiulio, K.A., Biery, B.J., Goodman, K.E., Isaya, G., Frerman, F.E., 1995. Cloning of glutaryl-CoA dehydrogenase cDNA, and expression of wild type and mutant enzymes in *Escherichia coli*. Hum. Mol. Genet. 4, 1493–1498.
Goodman, S.I., Frerman, F.E., 2001. Organic acidemias due to defects in lysine oxidation: 2-ketoadipic acidemia and glutaric acidemia. In: Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (Eds.), The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill, New York, pp. 1451–1460.
Goodman, S.I., 2004. Development of pathogenic concepts in glutaryl-CoA dehydrogenase deficiency: the challenge. J. Inherit. Metab. Dis. 27, 801–803.
Haberny, K.A., Paule, M.G., Scallet, A.C., Sistare, F.D., Lester, D.S., Hanig, J.P., Slikker Jr., W., 2002. Ontogeny of the N-methyl-D-aspartate (NMDA) receptor system and susceptibility to neurotoxicity. Toxicol. Sci. 68, 9–17.
Hollmann, M., Heinemann, S., 1994. Cloned glutamate receptors. Annu. Rev. Neurosci. 17, 31–108.
Hoffmann, G.F., Zschocke, J., 1999. Glutaric aciduria type I: from clinical, biochemical and molecular diversity to successful therapy. J. Inherit. Metab. Dis. 22, 381–391.
Insel, T.R., Miller, L.P., Gelhard, R.E., 1990. The ontogeny of excitatory amino acid receptors in rat forebrain. I. N-methyl-D-aspartate and quisqualate receptors. Neuroscience 35, 31–43.
Jensen, F.E., 2002. The role of glutamate receptor maturation in perinatal seizures and brain injury. Int. J. Dev. Neurosci. 20, 339–347.
Koeller, D.M., Woontner, M., Crnic, L.S., Kleinschmidt-DeMasters, B., Stephens, J., Hunt, E.L., Goodman, S.I., 2002. Biochemical, pathologic and behavioral analysis of a mouse model of glutaric acidemia type I. Hum. Mol. Genet. 11, 347–357.
Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 1999. 3-Hydroxyglutaric and glutaric acids are neurotoxic through NMDA receptors in vitro. J. Inherit. Metab. Dis. 22, 259–262.
Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 2000a. Cerebral organic acid disorders induce neuronal damage via excitotoxic organic acids in vitro. Amino Acids 18, 31–40.
Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 2000b. Maturation-dependent neurotoxicity of 3-hydroxyglutaric and glutaric acids in vitro: a new pathophysiologic approach to glutaryl-CoA dehydrogenase deficiency. Pediatr. Res. 47, 495–503.
Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 2001. Contribution of reactive oxygen species to 3-hydroxyglutarate neurotoxicity in primary neuronal cultures from chick embryo telencephalons. Pediatr. Res. 50, 76–82.
Kölker, S., Kohr, G., Ahlemeyer, B., Okun, J.G., Pawlak, V., Horster, F., Mayatepek, E., Krieglstein, J., Hoffmann, G.F., 2002a. Ca^{2+} and Na^+ dependence of 3-hydroxyglutarate-induced excitotoxicity in primary neuronal cultures from chick embryo telencephalons. Pediatr. Res. 52, 199–206.
Kölker, S., Okun, J.G., Ahlemeyer, B., Wyse, A.T., Horster, F., Wajner, M., Kohlmuller, D., Mayatepek, E., Krieglstein, J., Hoffmann, G.F., 2002b. Chronic treatment with glutaric acid induces partial tolerance to excitotoxicity in neuronal cultures from chick embryo telencephalons. J. Neurosci. Res. 68, 424–431.
Kölker, S., Hoffmann, G.F., Schor, D.S., Feyh, P., Wagner, L., Jeffrey, I., Pourfarzam, M., Okun, J.G., Zschocke, J., Baric, I., Bain, M.D., Jakobs, C., Chalmers, R.A., 2003. Glutaryl-CoA dehydrogenase deficiency: region-specific analysis of organic acids and acylcarnitines in post mortem brain predicts vulnerability of the putamen. Neuropediatrics 34, 253–260.
Kölker, S., Koeller, D.M., Okun, J.G., Hoffmann, G.F., 2004a. Pathomechanisms of neurodegeneration in glutaryl-CoA dehydrogenase deficiency. Ann. Neurol. 55, 7–12.
Kölker, S., Koeller, D.M., Sauer, S., Hörster, F., Schwab, M.A., Hoffmann, G.F., Ullrich, K., Okun, J.G., 2004b. Excitotoxicity and bioenergetics in glutaryl-CoA dehydrogenase deficiency. J. Inherit. Metab. Dis. 27, 805–812.
Kölker, S., Sauer, S.W., Hoffmann, G.F., Müller, I., Morath, M.A., Okun, J.G., 2008. Pathogenesis of CNS involvement in disorders of amino and organic acid metabolism. J. Inherit. Metab Dis. 31, 194–204.
Külkens, S., Harting, I., Sauer, S., Zschocke, J., Hoffmann, G.F., Gruber, S., Bodamer, O.A., Kölker, S., 2005. Late-onset neurologic disease in glutaryl-CoA dehydrogenase deficiency. Neurology 64, 2142–2144.
Latini, A., Rosa, R.B., Scussiato, K., Llesuy, S., Bello-Klein, A., Wajner, M., 2002. 3-Hydroxyglutaric acid induces oxidative stress and decreases the antioxidant defenses in cerebral cortex of young rats. Brain Res. 956, 367–373.
Latini, A., Rodriguez, M., Rosa, R.B., Scussiato, K., Leipnitz, G., de Assis, D.R., Ferreira, G.C., Funchal, C., Jacques-Silva, M.C., Buzin, L., Giugliani, R., Cassina, A., Radi, R., Wajner, M., 2005a. 3-Hydroxygluraric acid moderately impairs energy metabolism in brain of young rats. Neuroscience 135, 111–120.
Latini, A., Scussiato, K., Leipnitz, G., Dutra-Filho, C.S., Wajner, M., 2005b. Promotion of oxidative stress by 3-hydroxyglutaric acid in rat striatum. J. Inherit. Metab. Dis. 28, 57–67.
Liesert, M., Zschocke, J., Hoffmann, G.F., Muhlhauser, N., Buckel, W., 1999. Biochemistry of glutaric aciduria type I: activities of in vitro expressed wild-type and mutant cDNA encoding human glutaryl-CoA dehydrogenase. J. Inherit. Metab. Dis. 22, 256–258.
Lima, T.T., Begnini, J., de Bastiani, J., Fialho, D.B., Jurach, A., Ribeiro, M.C., Wajner, M., de Mello, C.F., 1998. Pharmacological evidence for GABAergic and glutamatergic involvement in the convulsant and behavioral effects of glutaric acid. Brain Res. 802, 55–60.
Lipton, S.A., Rosenberg, P.A., 1994. Excitatory amino acids as a final common pathway for neurologic disorders. N. Engl. J. Med. 330, 613–622.
Lund, T.M., Christensen, E., Kristensen, A.S., Schousboe, A., Lund, A.M., 2004. On the neurotoxicity of glutaric, 3-hydroxyglutaric, and trans-glutaconic acids in glutaric acidemia type 1. J. Neurosci. Res. 77, 143–147.
Maragakis, N.J., Rothstein, J.D., 2001. Glutamate transporters in neurologic disease. Arch. Neurol. 58, 365–370.
Magni, D.V., Oliveira, M.S., Furian, A.F., Fiorenza, N.G., Fighera, M.R., Ferreira, J., Mello, C.F., Royes, L.F., 2007. Creatine decreases convulsions and neurochemical alterations induced by glutaric acid in rats. Brain Res. 1185, 336–345.
McDonald, J.W., Johnston, M.V., 1990. Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. Brain Res. Rev. 15, 41–70.
Meldrum, B.S., 2002. Implications for neuroprotective treatments. Prog. Brain Res. 135, 487–495.
Migues, P.V., Leal, R.B., Mantovani, M., Nicolau, M., Gabilan, N.H., 1999. Synaptosomal glutamate release induced by the fraction Bc2 from the venom of the sea anemone *Bunodosoma caissarum*. Neuroreport 10, 67–70.
Morton, D.H., Bennett, M.J., Seargeant, L.E., Nichter, C.A., Kelley, R.I., 1991. Glutaric aciduria type I: a common cause of episodic encephalopathy and spastic paralysis in the Amish of Lancaster County, Pennsylvania. Am. J. Med. Genet. 41, 89–95.
Nagy, A.K., Shuster, T.A., Delgado-Escueta, A.V., 1986. Ecto-ATPase of mammalian synaptosomes: identification and enzymic characterization. J. Neurochem. 47, 976–986.
Nakanishi, S., 1992. Molecular diversity of glutamate receptors and implications for brain function. Science 258, 597–603.
Nanitsos, E.K., Acosta, G.B., Saihara, Y., Stanton, D., Liao, L.P., Shin, J.W., Rae, C., Balcar, V.J., 2004. Effects of glutamate transport substrates and glutamate

receptor ligands on the activity of Na^+/K^+-ATPase in brain tissue in vitro. Clin. Exp. Pharmacol. Physiol. 31, 762–769.
Olney, J.W., 1980. Dietary MSG and behavior. Toxicol. Appl. Pharmacol. 30 (53), 177–178.
Otis, T.S., 2001. Vesicular glutamate transporters in cógnito. Neuron 29, 11–14.
Ozawa, S., Kamiya, H., Tsuzuki, K., 1998. Glutamate receptors in the mammalian central nervous system. Prog. Neurobiol. 54, 581–618.
Petralia, R.S., Esteban, J.A., Wang, Y.X., Partridge, J.G., Zhao, H.M., Wenthold, R.J., Malinow, R., 1999. Selective acquisition of AMPA receptors over postnatal development suggests a molecular basis for silent synapses. Nat. Neurosci. 2, 31–36.
Porciúncula, L.O., Dal-Pizzol Jr., A., Coitinho, A.S., Emanuelli, T., Souza, D.O., Wajner, M., 2000. Inhibition of synaptosomal [^{3}H]glutamate uptake and [^{3}H]glutamate binding to plasma membranes from brain of young rats by glutaric acid in vitro. J. Neurol. Sci. 173, 93–96.
Porciúncula, L.O., Emanuelli, T., Tavares, R.G., Schwarzbold, C., Frizzo, M.E., Souza, D.O., Wajner, M., 2004. Glutaric acid stimulates glutamate binding and astrocytic uptake and inhibits vesicular glutamate uptake in forebrain from young rats. Neurochem. Int. 45, 1075–1086.
Rosa, R.B., Schwarzbold, C., Dalcin, K.B., Ghisleni, G.C., Ribeiro, C.A., Moretto, M.B., Frizzo, M.E., Hoffmann, G.F., Souza, D.O., Wajner, M., 2004. Evidence that 3-hydroxyglutaric acid interacts with NMDA receptors in synaptic plasma membranes from cerebral cortex of young rats. Neurochem. Int. 45, 1087–1094.
Rosa, R.B., Dalcin, K.B., Schmidt, A.L., Gerhardt, D., Ribeiro, C.A., Ferreira, G.C., Schuck, P.F., Wyse, A.T., Porciúncula, L.O., Wofchuk, S., Salbego, C.G., Souza, D.O., Wajner, M., 2007. Evidence that glutaric acid reduces glutamate uptake by cerebral cortex of infant rats. Life Sci. 81, 1668–1676.
Robinson, M.B., Sinor, J.D., Dowd, L.A., Kerwin, J.F., 1993. Subtypes of sodium-dependent high-affinity L-[^{3}H]glutamate transport activity—pharmacologic specificity and regulation by sodium and potassium. J. Neurochem. 60, 167–179.
Rothstein, J.D., Dykes-Hoberg, M., Pardo, C.A., Bristol, L.A., Jin, L., Kuncl, R.W., Kanai, Y., Hediger, M.A., Wang, Y., Schielke, J.P., Welty, D.F., 1996. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16, 675–686.
Sauer, S.W., Okun, J.G., Schwab, M.A., Crnic, L.R., Hoffmann, G.F., Goodman, S.I., Koeller, D.M., Kölker, S., 2005. Bioenergetics in glutaryl-coenzyme A dehydrogenase deficiency: a role for glutaryl-coenzyme A. J. Biol. Chem. 280, 21830–21836.
Sauer, S.W., Okun, J.G., Fricker, G., Mahringer, A., Müller, I., Crnic, L.R., Mühlhausen, C., Hoffmann, G.F., Hörster, F., Goodman, S.I., Harding, C.O., Koeller, D.M., Kölker, S., 2006. Intracerebral accumulation of glutaric and 3-hydroxyglutaric acids secondary to limited flux across the blood–brain barrier constitute a biochemical risk factor for neurodegeneration in glutaryl-CoA dehydrogenase deficiency. J. Neurochem. 97, 899–910.
Silva, C.G., Silva, A.R., Ruschel, C., Helegda, C., Wyse, A.T., Wannmacher, C.M., Dutra-Filho, C.S., Wajner, M., 2000. Inhibition of energy production in vitro by glutaric acid in cerebral cortex of young rats. Metab. Brain Dis. 15, 123–131.
Strauss, K.A., Morton, H., 2003. Type I glutaric aciduria. Part 2. A model of acute striatal necrosis. Am. J. Med. Genet. 121, 53–70.
Strauss, K.A., Puffenberger, E.G., Robinson, D.L., Morton, H., 2003. Type I glutaric aciduria. Part 1. Natural history of 77 patients. Am. J. Med. Genet. 121, 38–52.
Tanaka, K., Watase, K., Manabe, T., Yamada, K., Watanabe, M., Takahashi, K., Iwama, H., Nishikawa, T., Ichihara, N., Kikuchi, T., Okuyama, S., Kawashima, N., Hori, S., Takimoto, M., Wada, K., 1997. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science 276, 1699–1702.
Tavares, R.G., Santos, C.E., Tasca, C.I., Wajner, M., Souza, D.O., Dutra-Filho, C.S., 2000. Inhibition of glutamate uptake into synaptic vesicles of rat brain by the metabolites accumulating in maple syrup urine disease. J. Neurol. Sci. 181, 44–49.
Trotti, D., Rossi, D., Gjesdal, O., Levy, L.M., Racagni, G., Danbolt, N.C., Volterra, A., 1996. Peroxynitrite inhibits glutamate transporter subtypes. J. Biol. Chem. 271, 5976–5979.
Trotti, D., Danbolt, N.C., Volterra, A., 1998. Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? Trends Pharmacol. Sci. 19, 328–334.
Ullensvang, K., Lehre, K.P., Storm-Mathisen, J., Danbolt, N.C., 1997. Differential development expression of the two rat brain glutamate transporter proteins GLAST and GLT. Eur. J. Neurosci. 9, 1646–1655.
Ullrich, K., Flott-Rahmel, B., Schluff, P., Musshoff, U., Das, A., Lucke, T., Steinfeld, R., Christensen, E., Jakobs, C., Ludolph, A., Neu, A., Roper, R., 1999. Glutaric aciduria type I: pathomechanisms of neurodegeneration. J. Inherit. Metab. Dis. 22, 392–403.
Volterra, A., Trotti, D., Tromba, C., Floridi, S., Racagni, G., 1994. Glutamate uptake inhibition by oxygen free radicals in rat cortical astrocytes. J. Neurosci. 14, 2924–2932.
Yildiz, G., Demiryürek, A.T., Sahin-Erdemli, I., Kanzik, I., 1998. Comparison of antioxidant of aminoguanidine, methylguanidine and guanidine by luminal-enhanced chemiluminescence. Br. J. Pharmacol. 124, 905–910.
Wajner, M., Kölker, S., Souza, D.O., Hoffmann, G.F., de Mello, C.F., 2004. Modulation of glutamatergic and GABAergic neurotransmission in glutaryl-CoA dehydrogenase deficiency. J. Inherit. Metab. Dis. 27, 825–828.