Striatal neuronal death mediated by astrocytes from the Gcdh-/- mouse model of glutaric acidemia type I

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ABSTRACT

Glutaric acidemia type I (GA-I) is an inherited neurometabolic childhood disorder caused by defective activity of glutaryl CoA dehydrogenase (GCDH) which disturb lysine (Lys) and tryptophan catabolism leading to neurotoxic accumulation of glutaric (GA) and related metabolites. However it remains unknown whether GA toxicity is due to direct effects on vulnerable neurons or mediated by GA-intoxicated astrocytes that fail to support neuron function and survival. As damaged astrocytes can also contribute to sustain high GA levels, we explored the ability of Gcdh -/- mouse astrocytes to produce GA and induce neuronal death when challenged with Lys. Upon Lys treatment, Gcdh-/- astrocytes synthetized and released GA and 3-hydroxyglutaric acid (3HGA). Lys and GA treatments also increased oxidative stress and proliferation in Gcdh-/- astrocytes, both prevented by antioxidants. Pretreatment with Lys also caused Gcdh-/- astrocytes to induce extensive death of striatal and cortical neurons as compared to milder effect in WT astrocytes. Antioxidants abrogated the neuronal death induced by astrocytes exposed to Lys or GA. In contrast, Lys or GA direct exposure on Gcdh-/- or WT striatal neurons cultured in the absence of astrocytes was not toxic, indicating that neuronal death is mediated by astrocytes. In summary, GCDH-defective astrocytes actively contribute to produce and accumulate GA and 3HGA when Lys catabolism is stressed. In turn, astrocytic GA production induces a neurotoxic phenotype that kills striatal and cortical neurons by an oxidative stress-dependent mechanism. Targeting astrocytes in GA-I may prompt the development of new antioxidant-based therapeutical approaches.
INTRODUCTION

Glutaric acidemia type I (GA-I, MIM# 231670) is an inherited neurometabolic and degenerative disease of early childhood caused by lack of function mutations in the mitochondrial enzyme glutaryl-CoA dehydrogenase (GCDH, MIM# 608801, E.C. 1.3.99.7). Reduced GCDH activity alters L-tryptophan and L-lysine (Lys) catabolism (1-4), resulting in accumulation of glutaric (GA) and 3-hydroxyglutaric (3HGA) acids in brain and body fluids. Increased concentration of GA-I metabolites is thought to trigger the clinical features of GA-I, characterized by acute “encephalopathic crises”, and the installation of subsequent chronic motor and neurological sequels (4, 5). GA-I pathological features include acute loss of striatal neurons, progressive cortical neurodegeneration and white matter diffuse alterations (1, 6).

In an effort to understand the pathophysiological mechanisms of neuronal death in GA-I, a Gcdh-/- mouse model of the disease, which lacks GCDH activity and consequently accumulates high levels of GA-I metabolites in tissues and fluids (7, 8), was developed. Interestingly, Gcdh-/- mice do not show spontaneous striatal neurodegeneration or relevant neurological symptoms, unless they are fed with a high Lys diet to stimulate the production of GA-I metabolites (7-9). Thus, while neurological damage in GA-I seems to be dependent on GA and 3HGA-mediated neurotoxicity (2, 10), the vulnerability of striatal neurons to GA-I metabolites appears to be less marked. For instance, cultured neurons from either mouse (11, 12) or rats (13) appear non responsive to pathophysiological concentrations of GA. Moreover, Gcdh-/- neuronal cultures did not show affected survival in basal conditions (14) implying that GCDH absence might not be sufficient to elicit neuron death.

Growing evidence suggest that astrocytes may play a pivotal role in GA-I pathology (13-18).
Astrocytes play key homeostatic and metabolic roles in the CNS and have the ability to uptake GA (14). Upon GA and 3HGA pretreatment, cultured astrocytes react with a phenotypic change that includes increased proliferation, expression of S100β and mitochondrial dysfunction (13, 15, 16). In turn, such reactive astrocytes mediate the death of striatal neurons (13), suggesting a mechanism by which GA-damaged astrocytes follow a profound and long-lasting functional change that kills vulnerable neurons. In accordance, Jafari et al. (17) reported that GA-induced astrocyte damage results in decreased ability of astrocytes to produce glutamine, leading to hyperammonemia and depletion of neuronal glutamate reservoirs and thus, promoting neuronal and oligodendrocyte death. Other report has showed that Gcdh-/- astrocytes treated with GA or 3HGA have a reduced $[^{14}C]$-succinate efflux (14), further suggesting a defective metabolic supply to neurons. Thus, GA-I accumulated metabolites may cause astrocytes to be less supportive to maintain synaptic and trophic activities necessary for the healthy brain function.

In the present study we show evidence that Gcdh-/- astrocytes produce and accumulate GA and 3HGA in conditions of increased Lys catabolism. We also show that astrocytes isolated from WT and Gcdh-/- mouse pups induce neuronal death in a cell culture system where neurons are maintained on top of astrocytic feeder layers that were previously challenged with Lys or GA. Furthermore, these effects were prevented by antioxidant pre-treatment in Gcdh-/- astrocytes. Conversely, neither Lys nor GA at the concentrations employed produced significant effects on neuronal survival.

**RESULTS**

**Astrocytes from Gcdh-/- mice generate GA and 3HGA when exposed to Lys**

Although the neuronal expression of GCDH is consensually recognized (6, 14, 18, 19), we assessed whether this enzymatic pathway is part of the astrocyte signaling repertoire to
maintain CNS homeostasis (19-21). The analysis of GCDH expression by using a polyclonal anti-GCDH in astrocyte cultures from WT mice evidenced a punctate pattern suggesting a mitochondrial expression of this enzymatic protein. In co-cultures, GCDH immunoreactivity in neurons was much higher than that of astrocytes (Fig. 1A). The ability of WT and Gcdh-/- astrocytes isolated from the cerebral cortex to produce GA and 3HGA was then assessed by using gas chromatography coupled to mass spectrometry (GC/MS). GC/MS analyses show that Gcdh-/- astrocytes secreted low levels of GA-I metabolites in basal conditions and that levels increased several times when astrocytes were exposed to Lys (Fig. 1B). Quantitative results indicate that in basal conditions Gcdh-/- astrocytes released 11 times more GA (1.94 \( \mu M \) vs 0.17 \( \mu M \)) and 2.5 fold more 3HGA (0.22 \( \mu M \) vs 0.10 \( \mu M \)) than WT astrocytes (Table I). In presence of Lys, Gcdh-/- astrocytes released 15 times more GA (64 \( \mu M \) vs 1.94 \( \mu M \)) and 2 times more 3HGA (0.49 \( \mu M \) vs 0.22 \( \mu M \)) than WT astrocytes (Table I). Interestingly, for both astrocyte backgrounds, Lys exposition increased around 30-40 times the amount of GA released and 2-3 times that of 3HGA (Table I). Variable amounts of GA (ranging from 3.68 to 5.51 \( \mu M \) and 1.4 to 22.6 \( \mu M \)), and 3HGA (ranging from 0.28 to 0.39 \( \mu M \) and 0.37 to 0.57 \( \mu M \)), were also detected in cell homogenates from WT and Gcdh-/- astrocytes, respectively; thus indicating that part of the GA and 3HGA synthetized by astrocytes remained inside the cells.

**Astrocytes exposed to Lys undergo oxidative stress and increased proliferation**

Neurotoxic levels of GA have been showed to promote sub-lethal toxicity in astrocytes, characterized by mitochondrial dysfunction, increased expression of S100β and exacerbated proliferation (13, 15, 16). Similarly, a single exposure to 10 mM Lys induced a significant degree of sub-lethal damage in Gcdh-/- cortical astrocytes when compared to those maintained in low Lys concentrations. Ten mM Lys elicited oxidative stress as estimated by carboxy-H2DCFDA (DCF), diminution of glutathione (GSH) levels and increased levels of...
Thiobarbituric acid reactive substances (TBARS) (Fig. 2A, B), which was associated with increased number of S100β expressing astrocytes and proliferating cells label with 5-bromo-3′-deoxyuridine (BrdU). In comparison, WT astrocytes that express functional GCDH also reacted to Lys overload with a comparable increased cell proliferation, S100β expression and oxidative stress, although the amplitude of the response was less prominent than Gcdh-/- astrocytes (Fig. 2A, B). Lys induced astrocyte damage was not observed at lower concentrations (Supplementary Table I).

Exposure of Gcdh-/- astrocyte to 5 mM GA induced similar effects that Lys, suggesting Lys toxicity could be at least partially mediated by the production of cytotoxic levels of GA. In comparison, WT astrocytes that express functional GCDH also reacted to Lys overload with a comparable increased cell proliferation, S100β expression and oxidative stress, although the amplitude of the response was less prominent than Gcdh-/- astrocytes (Fig. 2A, B).

Astrocytes exposed to Lys caused death of striatal neurons

To determine whether Lys exposure to Gcdh-/- astrocytes could modulate their ability to support neuronal growth in co-culture, astrocytes were exposed to 10 mM Lys for 24 h and, after washing, neurons were seeded for 3 days on the top of astrocyte monolayer for evaluation of survival and morphology. For comparison, similar experiments were performed using 5 mM GA instead of Lys. WT and Gcdh-/- astrocyte feeder layers equally supported the survival of striatal neurons in the absence of Lys or GA challenge (Fig. 3A). Exposure to Lys or GA caused astrocytes to become toxic for striatal neurons causing a 35-45% decreased survival (Fig. 3B) and decreased body size and simpler pattern of primary dendrites in either condition (Table II). Astrocyte-mediated neuronal toxicity induced by Lys was not significantly different between WT and Gcdh-/- astrocytes. Similarly, striatal neurons from both WT and Gcdh-/- mouse embryos were equally vulnerable to astrocytes.
challenged with Lys, although Gcdh-/- neurons appeared with more limited growth potential and more sensitive to astrocyte-induced damage (Fig. 3B).

Cortical but not hippocampal neurons are also vulnerable to Lys stimulated-astrocytes

The vulnerability of other neuronal populations to the toxicity exerted by astrocytes challenged by Lys was also tested. Neurons from cerebral cortex or hippocampus seeded on the top of non-stimulated WT or Gcdh-/- astrocytes developed normally, both in number and size (Fig. 4A). Astrocytes stimulated with Lys or GA caused a 25-35% decrease in survival of cortical neurons (Fig. 4B), as well as a reduction in body size and primary neurites (Table III). Compared to cortical neurons, hippocampal neurons showed a less marked and non-significant vulnerability to astrocytes stimulated with Lys or GA (Fig. 4A, B). As described for striatal neurons, cortical and hippocampal Gcdh-/- neurons appeared more vulnerable than those from WT animals, although the difference was not statistically significant (Fig. 4B).

Antioxidants prevented Lys- and GA-astrocyte damage and dependent neuronal death

Because Lys or GA potently induced oxidative stress, we evaluated whether antioxidant exposure or induction of antioxidant/cytoprotective defenses could prevent Lys and GA induced damage to astrocytes, and further protect co-cultured neurons. Lipid peroxidation and/or decrease in GSH levels elicited by Lys or GA in Gcdh-/- astrocytes were prevented by the antioxidants Melatonin (MEL) (22, 23) and Trolox (24); as well as by tert-butylhydroquinone (tBHQ), an inducer of the protective Nuclear Factor Erythroid 2-related Factor 2 (Nrf2)/antioxidant response element (ARE) pathway (25-27) (Supplementary Fig. S1).

Remarkably, Fig. 5 shows that both strategies applied on astrocytes before Lys or GA challenge and co-culturing with striatal neurons prevented most of the neuronal death
induced by the direct exposure of Gcdh-/- astrocytes to Lys or GA (Fig. 5). Similar preservation of neuron survival was observed with antioxidants treatments on WT astrocytes (Supplementary Fig. S2). MEL showed a slightly minor protection than tBHQ (Fig. 5, S2) which is likely related to the induction of the neuroprotective Nrf2/ARE pathway as shown in Fig. S1 for Gcdh-/- astrocytes.

Lys exposure does not induce cell death in Gcdh-/- striatal neurons

Since metabolic overload with Lys could be cytotoxic for neurons via a direct effect (28, 29) or by producing increased levels of metabolites such as GA, we determined the effect of a 72h exposure of 3-4 DIV striatal embryonic neuronal cultures to 10 mM Lys or 5 mM GA. Neither Lys nor GA caused alterations in neurons survival or growth (Fig. 6A). Gcdh-/- striatal neurons exposed to Lys showed a moderate but non-significant decreased survival (Fig. 6B). Remarkably, Lys or GA did not cause significant effects on the survival of E18 cortical and hippocampal neurons.

DISCUSSION

Recent evidences indicate that glial cells play important pathogenic roles in neurodegeneration through non-cell autonomous mechanisms (20, 30, 31). In different genetic models, specific populations of vulnerable neurons can degenerate when astrocytes become dysfunctional, affecting the clearance of extracellular glutamate and/or the release of neurotrophic factors or compounds fueling neuron metabolism (19, 32, 33). In accordance, the reduced activity of GCDH or the direct exposure of astrocytes to GA or 3HGA has been found to induce astrocyte dysfunction and subsequent astrocyte-mediated neuronal death (13, 15, 16). In the present study, we have used Gcdh-/- astrocytes to further investigate the contribution of astrocytes to striatal neuronal death in a cell culture system where neurons were maintained on the top of the astrocytic feeder layer. We found evidence that GCDH is
expressed in cortical astrocytes and that Gcdh-/- astrocytes maintained in a high Lys medium can accumulate and release GA and 3HGA in a much higher extent as compared to WT astrocytes exposed to Lys. Moreover, Lys exposure strongly stimulated oxidative stress and proliferation in WT and Gcdh-/- astrocytes, and caused them to become neurotoxic for cocultured neurons. Remarkably, exposure of isolated neuronal cultures to Lys or GA did not induce any apparent death, suggesting astrocytes as the key pathogenic cell type mediating neuron loss in GA-I.

We demonstrated for the first time that WT astrocytes express low levels of the enzyme GCDH displaying a perinuclear punctuate cellular distribution reminiscent of mitochondria. This result suggests that astrocytes can detoxify to a certain extent the GA produced locally during Lys or tryptophan catabolism. Remarkably, incubation of WT or Gcdh-/- astrocytes with high Lys concentration stimulated the production and accumulation of GA and also 3HGA in the culture medium, being several fold greater (especially for GA) in Gcdh-/- than WT astrocytes. These data support the view that astrocytic GCDH has the capacity to handle GA accumulation, although the metabolic pathway can be saturated by high Lys concentration. Moreover, while most GA (and 3HGA) produced by astrocytes appears to diffuse extracellularly, a substantial amount remains inside the cells, potentially reaching cytotoxic concentrations responsible for mitochondrial dysfunction or switch of signaling favoring cell proliferation (13, 15). We also showed here that exposure of either Gcdh-/- or WT astrocytes to high Lys or GA provoked a reactive response, characterized by oxidative stress, increased proliferation rate of around 50% and 70% for WT and Gcdh-/- astrocytes, respectively, as well as increased number of S100β positive cells. The reactive response was more pronounced in Gcdh-/- astrocytes, as expected for a reduced ability to handle intracellular GA. Taken together, our results indicate that astrocytes can be a source of GA in high catabolic states and that in turn, GA accumulation can impact or damage key
astrocytic functions. Instead of triggering necrosis or apoptosis, astrocytes respond to GA by increasing proliferation and phenotypic changes that might be relevant to understand GA-I pathophysiology.

Remarkably, WT and Gcdh-/- astrocytes became neurotoxic for striatal and cortical neurons when incubated with high Lys or GA concentrations. Data obtained indicate that oxidative stress plays a leading role on Lys- and GA-dependent astrocyte toxicity. Remarkably, the solely preservation of astrocyte against oxidative damage was enough to protect against neuronal death. Astrocyte preservation was achieved either by direct scavenger antioxidant properties or by induction of own antioxidant responses such as the Nrf2/ARE pathway. Activation of the astrocytic Nrf2/ARE pathway and downstream cascades were previously shown as neuroprotective in different damaging conditions (25-27).

On the other hand, the absence of GCDH had little effect enhancing the astrocyte-mediated neuronal death. This result could be explained by the fact that the Lys and GA concentration used in WT astrocytes largely surpassed the capacity to metabolize GA, thus triggering similar response in both astrocyte types. Gcdh-/- and WT astrocytes exposed to Lys not only induced neuronal death but also reduced neuronal size and neurite growth, suggesting insufficient trophic or metabolic support preceding neuronal death. This is in agreement with previous reports showing that Gcdh-/- astrocytes exposed to GA have a defective export of tricarboxylic acid cycle intermediates (14). WT astrocytes exposed to GA showed a decreased ability to produce glutamine (17), both pathways potentially leading to neuronal metabolic starvation and also to death by hyperammonemia (17). Striatal neurons were the most affected to Lys or GA treated astrocytes, when compared to cortical and hippocampal neurons. This is in accordance with the higher vulnerability of striatal neurons in GA-I patients (3, 6, 9, 34). In this context, the worse detrimental neuronal effects were found in
the experimental condition combining Gcdh-/- neuron and astrocyte cultures, suggesting a synergistic detrimental effect of reduced GCDH activity in interacting neural cells.

Regarding the understanding of GA-I pathophysiology, our data suggest that GA and 3HGA can be produced locally in the striatum in conditions of GCDH genetic defects. While GCDH levels seem to be higher in neurons than astrocytes, the later can critically contribute to GA detoxification in normal conditions. On the other hand, astrocytes probably have the ability to uptake GA (14), therefore contributing to buffering the compound or its effects up to certain levels. Intact WT astrocytes can protect against GA neurotoxic effects (35-37), reducing the concentrations of glutamate in the synaptic cleft or releasing glutathione to reduce oxidative stress (19, 20). However, upon metabolic damage with GA, astrocytes proliferate and lose their homeostatic normal functions (20) adopting a phenotype less supportive or directly toxic to neurons. Astrocyte dysfunction elicited by Lys or GA may also impair blood brain barrier homeostasis as well as oligodendrocyte generation (16, 38), leading to the additional neurological damage characteristic of GA-I patients (39-41).

Taken together, our data strongly suggest that dysfunctional Gcdh-/- astrocytes play a crucial role in GA-I neuropathology, and that preserving protective astrocyte phenotype is critical to maintain neuron survival. Data showing that Lys and GA-dependent astrocyte neurotoxicity is mediated by oxidative stress and abrogated by antioxidants; strongly suggest that inhibition of oxidative stress may be a valuable therapeutic strategy for GA-I patients.

MATERIAL AND METHODS

Ethical statement

This study was carried out in strict accordance with the National law and Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA). All efforts were made to minimize suffering, discomfort, stress and number of animals necessary to produce
reliable scientific data.

Chemicals
Dulbecco’s modified Eagle's medium (DMEM), Neurobasal medium, B27, glutamine, foetal bovine serum (FBS), penicillin/streptomycin, trypsin, DCF, poly-D-lysine and DAPI were purchased from Invitrogen (Carlsbad, CA, USA). Lys, GA, BrdU, β-tubulin, tBHQ, MEL, Trolox, glial acidic fibrillar protein (GFAP) and S100β antibodies, as well as all other chemicals of analytical grade were obtained from Sigma (St Louis, MO, USA). Anti-GCDH antibody was kindly provided by Jonna Westover (Utah State University, USA). The anti-BrdU antibody was purchased to Dako (Carpinteria, CA, USA). The anti heme-oxigenase 1 (HO-1) antibody was purchased to Stressgen (San Diego, CA, USA). HRP or fluorescent secondary antibodies were obtained from Invitrogen Molecular Probes. Deuterium-labeled internal standards for GA (d4-GA) and HGA (d5-HGA) were obtained from MDS Isotopes, Montreal, Canada.

Animals
Gcdh−/− and WT mice of 129SvEv background were generated by crossing heterozygotes at the Unidade Experimental Animal of the Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil). Pregnant females were kept in plastic cages on a 12:12 h light/dark cycle (lights on 07.00– 19.00 h) with constant temperature (22±1°C), and water and 20% w/w protein commercial chow ad libitum. Cultures of astrocytes were made from 1-2 days old rat pups from 7 whole litters. Neurons were obtained from E17.5-18 embryos after anesthetizing and sacrificing seven females. To avoid cross contamination between WT and Gcdh−/− animals, in all conditions, animals were processed separately and cultures made independently and with different dissection materials. All experiments were performed at least three independent times in duplicates or triplicates.
Isolated neuronal cultures

Neurons from the striatum, fronto-parietal cortex or CA1-CA3 hippocampal regions were prepared from E17-18 embryos according to Ventimiglia et al. (42) with minor modifications. Briefly, pregnant females were euthanized and both uterus horns removed. Five to seven embryos were obtained in aseptic conditions and washed in sterile phosphate buffered saline solution (PBS). Then each correspondent brain region was dissected, immersed in fresh Neurobasal medium containing 2% B27 and 1 mM glutamine, cleaned, minced and mechanically dissociated to obtain isolated cells. Around 300,000 cells (3.2–3.4x10^4 cells/cm^2 density) were seeded onto 35 mm or 24 multiwell plates pre-covered with 0.1 mg/ml poly-D-lysine and pre-washed with sterile water. At 3-4 days in vitro (DIV), neurons were treated with 10 mM Lys or 5 mM GA for 72 h, then fixed with 4% paraformaldehyde (PFA) and imaged by light microscopy or immunostained against β-tubulin to evaluate viability and morphological parameters.

Astrocyte cultures and treatments

Primary astrocyte cultures were prepared from whole cortices of 1–2 day-old Gcdh-/- and WT mice. Briefly, cortices from 4-5 pups were placed in sterile PBS, cleaned from meninges, cut in small pieces and incubated in 0.05% trypsin-EDTA for 25 min at 37 ºC. Trypsin was then blocked with astrocyte culture medium (DMEM supplemented with 10% FBS, 3.6 g/l HEPES, 1.2 g/l NaHCO₃, 100 IU/ml penicillin, and 100 μg/ml streptomycin), tissue homogenized by repeated pipetting, and then spun 10 min at 1000 rpm (CL-2, Sigma Centrifuge). The resulting pellet was re-suspended in DMEM-10% FBS and cells plated in culture bottles at a density of 2x10^4 cells/cm^2 in astrocyte culture medium. Whole media was changed every day. Once in confluence, cultures were enriched in astrocytes by shaking for 48 h at 250 rpm and 37 ºC. This procedure allows obtaining astrocyte monolayers at least
98% GFAP positive and devoid of OX42 microglial cells and GalC oligodendrocytes. After a week of enrichment, astrocytes were trypsinized for 3-7 min and then plated on sterile 35 mm Petri dishes or 24 multiwell plates until confluence (around 1 week after plating). Before each treatment, astrocytes were incubated with DMEM-2% FBS during 24 h and then exposed to 10 mM Lys or 5 mM GA for another 24 h (13, 15). Appropriate aliquots of a 500-1000 mM Lys or GA stock solution were prepared in 10 mM PBS, and the pH adjusted to 7.4 immediately prior to use. In some experiments the antioxidants MEL (1 µM) and Trolox (soluble α-tocopherol, 10 µM) or tBHQ (20 µM), prepared in DMSO (vehicle); were used to pre-incubate Gcdh-/- astrocytes for 1 (MEL, Trolox) or 6 h (tBHQ); respectively. Then, 10 mM Lys or 5 mM GA was added to culture medium. After 24 h, proliferation and oxidation parameters were analyzed. In other batch of experiments, WT and Gcdh-/- astrocytes were pretreated with 1 µM MEL or 20 µM tBHQ for 1 or 6 h before adding 10 mM Lys or 5 mM GA. 24 h later, co-cultures with striatal WT or Gcdh-/- neurons were performed.

**Astrocyte proliferation assay**

Proliferation rate was assessed by determining the percentage of cells that incorporated BrdU related to total DAPI positive cells. This compound was added at the beginning of each experimental situation (10 µM, 24 h) and then was recognized by immunocytochemistry (15). Briefly, cultured astrocytes were fixed in ice-cold 4% PFA (20 min, room temperature, RT), permeabilized with 0.1% Triton X-100 (20 min, RT) and denatured with 2N HCl (37 °C, 30 min). After extensive washes with 10 mM, borate buffer, pH 8, the non-specific binding was blocked with 5% bovine serum albumin (BSA, 60 min, RT). After that, cells were incubated with a 1:800 dilution of anti-BrdU antibody in 5% BSA (overnight, wet chamber, 4 °C), washed again and finally incubated with 1:500 anti-mouse Alexa 488 secondary antibody (25 °C, 90 min). Cells were mounted with glycerol containing...
1 µg/ml DAPI and imaged in an Olympus FV300 laser scanning confocal microscope. Astrocytes that show green signal in at least 70% of the whole nucleus were considered BrdU positive.

Co-cultures of neurons on astrocyte feeder layers

Confluent astrocyte monolayers were incubated with DMEM-2% FBS during 24 h and then exposed to 10 mM Lys or 5 mM GA for another 24 h. After that, the medium was completely removed and fresh astrocyte culture medium was added to wash cells and avoid any remaining Lys or GA contact neurons. After 6 h, the medium was completely removed and each neuron suspension added to astrocyte monolayers. Fifty or 300 µl of a 3 x 10⁵ neurons/ml dilution were seeded on top of confluent astrocyte monolayers grown in 24 multiwell plates or 35 mm Petri dishes, respectively. After 30 min of rest to facilitate neuron attachment to astrocyte feeder layer, the volume was completed to 400 or 1500 µl with Neurobasal medium-2% B27 and 2% FBS. 3-4 days later, co-cultures were fixed with 4% PFA and submitted to light imaging and neuronal immunostaining.

Immunocytochemistry

This approach was performed in neuronal and astrocyte cultures as well as co-cultures. The procedures were similar in all cases (15). Briefly, cells were fixed with 4% PFA, permeabilized with 0.1% Triton X-100 (20 min, RT), blocked with 5% BSA (60 min, RT) and incubated (wet chamber, overnight, 4 °C) with one or two of the following antibodies: 1:400 anti-GFAP, 1:500 anti-S100β or 1:500 anti-β-tubulin. Cells were then washed and incubated with correspondent 1:500 dilutions of secondary antibodies conjugated to Alexa Fluor 488 or 546 (90 min, RT). After 3 washes with PBS, cells were mounted on glass slides with 50% glycerol-PBS containing 1 µg/ml DAPI. All images were taken with a confocal Olympus FV300 microscope maintaining equal parameters in all conditions analyzed.
Cell counting and statistical analysis

The number of viable neurons was estimated by immunostaining against β-tubulin or MAP-2 or bright field imaging. All cells positive to β-tubulin or MAP2 were counted regardless of its appearance in at least 75% of each whole area seeded. From light images, neurons counted were those with a clear morphology and bearing cell processes with a length at least equal to the body size. All samples underwent same parallel procedures. Measurement of size and number of neurites was done by using the free ImageJ (NIH, USA) software. At least 150 neurons were analyzed per experimental condition.

Organic acid analysis

Astrocytes from WT and Gcdh-/- mice were cultivated in the presence of 10 mM Lys for 72h, the culture media collected and organic acid analysis was carried out according to Sweetman et al (43) with slight modifications (44). First, culture media were acidified to pH 1.5–2.0 and mixed with 100 µl hexadecane (internal standard). Ammonium chloride was then added and the organic acids were extracted twice with ethylacetate and the organic phases were pooled. Five hundred milligrams of sodium sulfate was added to each preparation, the mixture stayed for at least 60 min at RT, then passed through 0.2 µm filters and evaporated in N₂ at 60 °C. A hundred microliter of ethanol was added, homogenized, centrifuged for 10 min and finally evaporated in N₂. Derivatization to allow the analysis of organic acids as trimethylsylil compounds was performed by adding 27.5 µl of BSTFA (bis-(trimethylsylil) trifluoracetamide) and 1% TMCS (trimethylchlorosilane), and allowed to react (60 min, 60 °C). Half microliter of each derivatized sample was then injected into Varian Saturn 2000 GC/MS equipment with a CP-Sil 8 CB capillary column (length 30 m, internal diameter 0.25 mm, film 0.25 µm), an open split injector and helium as the carrier gas. The GC/MS temperatures were as follows: injector 250 °C, column 90 °C to 280 °C.
with an increment of 3 °C per min, transfer line 280 °C, ion source 150 °C and mass analyzer 35 °C. The total run time was 75 min. Finally, the mass spectrometer was programmed from m/z 10–650 at the rate of 0.6 Hz.

Quantitative measurement of GA and 3HGA in culture medium and astrocyte homogenates was performed by gas chromatography–mass spectroscopy using stable-isotope dilution (6). Deuterium-labeled internal standards for GA (d4-GA, 0.05 mg/ml) and HGA (d5-HGA, 0.05 mg/ml) were added at 0.05 ml each to 1.0 ml cultured medium or 0.3 ml cell homogenates. Sulfosalicylic acid at 0.15 ml of 9.33% or 70 mg was added to the samples. Organic acids were extracted twice with 3 ml diethyl ether and 1.5 ml ethyl acetate. Combined organic phases were dried at 30°C under nitrogen and derivatized with 0.05 ml BTSFA/1% TMCS [N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane] for 20 minutes at 80°C. Injected volume of 0.001 ml was analyzed using an Agilent Technologies 6890N Gas Chromatograph equipped with a 5973N Mass Selective Detector. The mass spectrometer monitored ions at 265/261 (with check of ratio at 237/233) for GA and 188/185 (check ratio at 262/259) for HGA in separate runs.

Assessment of oxidative levels in living astrocytes

Carboxy-H2DCFDA (DCF) probe in astrocytes

Oxidative activity was measured in controls and Lys or GA-treated WT and Gcdh-/- astrocytes with the cell-permeant carboxy-H2DCFDA probe (Invitrogen). According to the manufacturer’s instructions, living cells were washed and incubated with 5 µM carboxy-H2DCFDA in 10 mM PBS containing 20 mM glucose (1 h, 37 °C). Then, 1 µg/ml DAPI was added, cells rinsed and each fluorescence emission immediately imaged or measured after excitations of 405 and 488 nm in a Varioskan spectrophotometer, respectively. All data were expressed as percent of respective control values obtained for WT and Gcdh-/- astrocytes.
Malondialdehyde (TBA-RS) and reduced glutathione (GSH) concentrations in astrocyte homogenates

Confluent fresh astrocyte monolayers seeded on two 35 mm Petri dishes were scraped in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl, and then were centrifuged at 750 g (10 min, 4 °C) to discard nuclei and cell debris. The pellet was discarded and the supernatant, a suspension of preserved organelles, including mitochondria, was used to measure lipid peroxidation and reduced glutathione concentrations. The protein content was determined by the method of Lowry et al. (46), using BSA as the standard.

MDA levels were determined according Yagi (47) with slight modifications. Briefly, 200 µL of 10 % trichloroacetic acid and 300 µL of 0.67 % thiobarbituric acid in 7.1 % sodium sulfate were added to 100 µL of cell supernatants and incubated for 2 h in a boiling water bath. The mixture was allowed to cool in running tap water for 5 min. The resulting pink-stained complex was extracted with 300 µL of butanol. Fluorescence of the organic phase was read with 515 nm excitation and 553 nm emission wavelengths. MDA levels, determined in triplicate for each experimental condition, were calculated as nmol MDA / mg protein using a calibration curve determined with 1,1,3,3-tetramethoxypropane.

GSH concentrations were measured according to Browne and Armstrong (48) with minor modifications. Supernatants (0.3-0.5 mg of protein/mL) were first deproteinized with metaphosphoric acid, centrifuged at 7,000 x g for 10 min and immediately used for GSH quantification. One hundred and eighty-five microliters of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM ethylenediaminetetraacetic acid, and 15 µL of o-phthalaldehyde (1 mg/mL) were added to 30 µL of supernatant previously deproteinized. This mixture was incubated at RT in a dark room for 15 min. Fluorescence was measured
using excitation and emission wavelengths of 350 nm and 420 nm, respectively. The calibration curve was prepared with standard GSH (0.001-1 mM) and the concentrations, determined in triplicate for each experimental condition, and referred as nmol GSH/mg protein.

**Western blotting assays**

To assess the Nrf2/ARE dependent expression of HO-1 (23-25); confluent Gcdh-/- astrocyte cultures were pretreated with 1 µM MEL or 20 µM tBHQ, then challenged to 10 mM Lys. 24 h later, astrocytes of all conditions were scrapped in cell lysis buffer, sonicated and the protein concentration determined by the bicinchoninic acid procedure. Denatured samples were seeded and a typical SDS-PAGE electrophoresis and blotting was performed (23). Proteins that were transferred to a PVDF membrane were incubated overnight with a 1:1000 dilution of anti-HO-1 or with 1:4000 of anti-βactin antibody that was used as a protein loading control. After 1 h of incubation with HRP-conjugated secondary antibodies, the PVDF membrane was incubated with a commercial ECL kit (Pierce, Rockford, IL) and bands were analyzed with the Image J (NIH, USA) gel analyzer tool. Data were referred as the percentage of βactin expression in each corresponding sample.

**Statistical analysis**

All values shown are the mean ± SEM of at least 3 to 5 independent experiments performed in triplicates. Data analysis was performed using standard statistical packages (SigmaStat 2.0 and Origin 8.1). To determine statistical difference among groups we use a two-factor analysis of variance (ANOVA). $p<0.05$ was considered statistically significant, and $p<0.1$ marginally significant.
ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST STATEMENT

The authors declare no possible conflict of interest in the conduct and reporting of research.

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LEGEND TO FIGURES

Fig. 1: Astrocytes express GCDH in cultures and co-cultures with neurons. A) GCDH immunoreactivity in WT astrocyte cultures shows a perinuclear punctate specific expression. The same pattern was maintained in astrocytes co-cultured with WT neurons in spite of the astrocytic signal (short arrow) that was much lower than that of neurons (long arrow). In co-cultures of Gcdh-/-astrocytes (KO) with WT neurons the high neuronal GCDH expression (long arrows) coexists with a very low no specific binding in astrocytes (short arrow). Calibration bars: 25 μm. B) CG/MS chromatograms showing the presence of detectable levels of GA (arrows) in the culture medium of WT and Gcdh-/- astrocytes under basal conditions or exposed to 10 mM Lys during 72 h.

Fig. 2: Astrocytes increase proliferation and oxidative stress in response to Lys and GA. A) The exposure of WT and Gcdh-/- astrocytes to 10 mM Lys or 5 mM GA during 24 h caused an increased BrdU positive proliferation (cyan, arrows); a moderate increase in S100ß (green) immunoreactivity accompanied by a decrease in GFAP signal (red), as well as an increased green carboxy-H2DCFDA (DCF) fluorescence. Nuclei were stained with DAPI (blue). Calibration bars: 50 μm for upper and mid pictures and 40 μm for bottom image, respectively. B) Quantitation of proliferation rate and S100ß and GFAP positive cells upon Lys and GA treatments. All values were referred as a percentage of total cells positive to DAPI. C) Quantitation of the oxidative stress induced by Lys or GA after measuring DCF emission, lipid peroxidation (B) and GSH levels. Data are mean ± S.E.M of 3 separate experiments. * p<0.05 indicates statistical difference related to WT astrocytes under basal conditions.

Fig. 3: Vulnerability of GCDH-/- striatal neurons to astrocytes pretreated with Lys or GA. A) Representative panoramic light images and β-tubulin (green) immunofluorescences
of all of the background combinations in astrocyte-neuron co-cultures 4 days after plating neurons on astrocytes pretreated with vehicle (V), 10 mM Lys or 5 mM GA during 24 h. Note the significant decreased number and simpler morphology of neurons seeded on top of Lys or GA pre-treated astrocytes. In all conditions, Gcdh-/- neurons appeared more affected. Scale bar = 75 μm for light images and 40 μm for fluorescent pictures, respectively. **B)** Quantitation of neuronal survival denoting significant effects of Lys and GA. All values were related to WT neurons seeded on top of control WT astrocytes. * p<0.05 indicates statistical signification related to WT astrocytes.

**Fig. 4: Differential vulnerability of cortical and hippocampal neurons to astrocytes pretreated with Lys or GA.** **A)** Representative images of β-tubulin positive 4 DIV cortical and hippocampal neurons plated on top of WT or Gcdh-/--astrocytes pre-treated during 24 h with vehicle (V), 10 mM Lys or 5mM GA. Note the significant effects on the morphology of cortical neurons when seeded on top of Lys or GA-pretreated astrocytes in clear contrast with the very healthy appearance of hippocampal neurons in all conditions. **B)** Quantitation of survival of cortical and hippocampal neurons co-cultured on WT or Gcdh-/-- astrocyte feeder layers. Note as the viability of cortical neurons significantly decreased when co-cultured on top or WT or Gcdh-/- astrocytes pretreated with Lys or GA; whereas that of hippocampal neurons was significantly unaffected. Values were referred as the percentage of the viability determined in WT neurons co-cultured on control WT astrocytes. Data are media ± SEM from 3 separated experiments. *p<0.05 indicates statistical difference related to WT astrocytes.
Fig 5: Antioxidants abrogated neuronal death induced by astrocyte pretreatment with Lys or GA. A) Light images showing the protection that 1 μM MEL and 20 μM tBHQ caused on the number and gross morphology of striatal neurons co-cultured on top of Gcdh-/ astrocyte feeder layers challenged with 10 mM Lys or 5 mM GA. Also note that upon Lys and GA astrocyte challenge, the frequency of dead neurons (black arrows) on top of astrocyte monolayers seemed increased. Magnification: 40x. Calibration bar = 25 μm. B) Quantitation of survival of WT and Gcdh-/- neurons upon the treatment of Gcdh-/ astrocytes with in the different experimental conditions. Values were related to the number of surviving neurons seeded on top of astrocytes treated with vehicle (V). *p<0.05 means statistical differences related to each respective control.

Fig. 6: Absence of direct effects of Lys and GA on 3-4 DIV embryonic E18 striatal neurons obtained from WT and Gcdh-/ mice. A) Bright field images showing absence of significant morphological effects of Gcdh-/ striatal neurons submitted to 10 mM Lys or 5 mM GA during 72 h. Scale bar = 20 μm. B) Quantitation of survival of WT and Gcdh-/- neurons after Lys and GA treatments. Values were related to vehicle-treated neurons (V). No significant decreases were found, except a marginally significant decrease (#, p<0.1) in Gcdh-/ striatal neurons submitted to Lys. Data are mean ± S.E.M of 2-3 separated experiments.
**Table I:** Glutaric and 3-hydroxyglutaric acids concentrations (µM) in culture medium and cell homogenates after exposure of WT and Gcdh -/- astrocytes to 10 mM Lys for 72 hours.

### Glutaric Acid

<table>
<thead>
<tr>
<th></th>
<th>WT astrocytes</th>
<th>Gcdh -/- astrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>10 mM Lys</td>
</tr>
<tr>
<td>Culture medium</td>
<td>0.17 (0.13 – 0.19)</td>
<td>4.17 (3.50-4.87)</td>
</tr>
<tr>
<td>Cell homogenate</td>
<td>0.60 (0.24-1.61)</td>
<td>4.85 (3.68-5.51)</td>
</tr>
</tbody>
</table>

### 3-Hydroxyglutaric Acid

<table>
<thead>
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<th>Gcdh -/- astrocytes</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>10 mM Lys</td>
</tr>
<tr>
<td>Culture medium</td>
<td>0.10 (0.10-0.11)</td>
<td>0.30 (0.24-0.39)</td>
</tr>
<tr>
<td>Cell homogenate</td>
<td>0.12 (0.10-0.17)</td>
<td>0.33 (0.28-0.39)</td>
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</table>

Data are expressed as median and range of the values (n=3).
Table II: Effects of Lys (10 mM) and GA (5 mM) astrocyte pre-treatment on body size and number of primary dendrites of co-cultured striatal neurons.

<table>
<thead>
<tr>
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<th>Gcdh-/– astrocytes</th>
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<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Lys</td>
</tr>
<tr>
<td><strong>WT neurons</strong></td>
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<td></td>
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<tr>
<td>Body size</td>
<td>100±6</td>
<td>77±4</td>
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<tr>
<td>1st order dendrite</td>
<td>100±7</td>
<td>74±8*</td>
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<tr>
<td><strong>Gcdh-/– neurons</strong></td>
<td></td>
<td></td>
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<tr>
<td>Body size</td>
<td>96±6</td>
<td>73±4*</td>
</tr>
<tr>
<td>1st order dendrite</td>
<td>100±7</td>
<td>72±6*</td>
</tr>
</tbody>
</table>

Values were obtained as the percentage of body size and number of first order dendrites shown by WT neurons seeded on top of control WT astrocytes (Vehicle). Data represented are mean ± SEM obtained from 3 separated experiments performed by triplicate or quintuplicate. *p<0.05, relative to WT neurons seeded on top of control WT astrocytes.
Table III: Effects of Lys (10 mM) and GA (5 mM) astrocyte pre-treatment on body size and number of primary dendrites of co-cultured cortical neurons.

<table>
<thead>
<tr>
<th></th>
<th>WT astrocytes</th>
<th></th>
<th>Gcdh/-/- astrocytes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Lys</td>
<td>GA</td>
<td>Vehicle</td>
</tr>
<tr>
<td><strong>WT neurons</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body size</td>
<td>100±12</td>
<td>80±12</td>
<td>85±12</td>
<td>100±8</td>
</tr>
<tr>
<td>1st order dendrite</td>
<td>100±9</td>
<td>95±8</td>
<td>76±8*</td>
<td>100±12</td>
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<tr>
<td><strong>Gcdh/-/- neurons</strong></td>
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</tr>
<tr>
<td>Body size</td>
<td>93±5</td>
<td>75±8*</td>
<td>80±10</td>
<td>78±12</td>
</tr>
<tr>
<td>1st order dendrite</td>
<td>83±12</td>
<td>64±10*</td>
<td>68±10*</td>
<td>75±13</td>
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</table>

Values were obtained as the percentage of body size and number of first order dendrites shown by WT neurons seeded on top of control WT astrocytes (Vehicle). Data represented are the mean ± SEM obtained from 3 separated experiments performed by triplicate or quintuplicate. *p<0.05, relative to WT neurons seeded on top of control WT astrocytes.
ABBREVIATIONS

BSA  Bovine serum albumin
BrdU  5-bromo-3'-deoxyuridine
DAPI  4,6-diamino-2-phenylindole
DCF  Carboxy-H2DCFDA
DMEM  Dulbecco's modified Eagle's medium
FBS  Foetal bovine serum
GA  Glutaric acid
GA-I  Glutaric acidemia type I
GCDH  Glutaryl CoA dehydrogenase
GFAP  Glial acidic fibrillar protein
GSH  Reduced glutathione
3HGA  3-hydroxyglutaric acid
HO-1  Heme-oxygenase 1
Lys  Lysine
MEL  Melatonin
Nrf2  Nuclear erythroid related factor 2
PAF  Paraformaldehyde
PBS  Phosphate buffered saline solution
RT  Room temperature
TBARS  Thiobarbituric acid reactive substances
tBHQ  tert-butyl-hydroquinone
WT  Wild type