



Induction of Neuroinflammatory Response and Histopathological Alterations Caused by Quinolinic Acid Administration in the Striatum of Glutaryl-CoA Dehydrogenase Deficient Mice

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Abstract

Glutaric acidemia type I (GA I) is an inherited neurometabolic disorder caused by a severe deficiency of the mitochondrial glutaryl-CoA dehydrogenase (GCDH) activity. Patients usually present progressive cortical leukodystrophy and commonly develop acute bilateral striatal degeneration mainly during infections that markedly worsen their prognosis. A role for quinolinic acid (QA), a key metabolite of the kynurenine pathway, which is activated during inflammatory processes, on the pathogenesis of the acute striatum degeneration occurring in GA I was proposed but so far has not yet been evaluated. Therefore, we investigated whether an acute intrastriatal administration of quinolinic acid (QA) could induce histopathological alterations in the striatum of 30-day-old wild-type (WT) and GCDH knockout (*Gcdh*^{-/-}) mice. Striatum morphology was evaluated by hematoxylin and eosin, T lymphocyte presence (CD3), and glial activation (GFAP and S100 β) by immunohistochemistry and 3-nitrotyrosine (YNO2) by immunofluorescence. QA provoked extensive vacuolation, edema, and especially lymphocyte infiltration in the striatum of *Gcdh*^{-/-}. QA also enhanced CD3 staining and the number of YNO2 positive cells in *Gcdh*^{-/-} mice, relatively to WT, indicating T lymphocyte infiltration and nitrosative stress, respectively. QA-treated WT mice also showed an increase of GFAP and S100 β staining, which is indicative of reactive astrogliosis, whereas the levels of these astrocytic proteins were not changed in *Gcdh*^{-/-} QA-injected mice. The present data indicate that QA significantly contributes to the histopathological changes observed in the striatum of *Gcdh*^{-/-} mice.

Keywords Glutaric acidemia type I · Acute striatum degeneration · Neuroinflammation · Quinolinic acid · Histopathology · Nitrosative stress

Introduction

Glutaric acidemia type I (GA I, OMIM #231670) is a neurometabolic disorder caused by deficiency of glutaryl-CoA dehydrogenase (GCDH, EC 1.3.99.7) activity, that is involved in

the catabolic pathways of lysine (Lys), hydroxylysine, and tryptophan. The disorder is biochemically characterized by accumulation of glutaric (GA) and 3-hydroxyglutaric (3HGA) acids in

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tissues and biological fluids (Brismar and Ozand 1995; Goodman and Frerman 2001). Clinical symptomatology is basically neurologic. A considerable number of untreated patients develop acute irreversible striatal degeneration between 3 months and 4 years of life during or following acute encephalopathic crises, which are precipitated by catabolic events, mainly infectious diseases. Thereafter, developmental delay, dystonia, dyskinesia, hypotonia, seizures, and spasticity commonly affect these patients (Boy et al. 2016; Goodman et al. 1977; Hoffmann and Zschocke 1999; Neumaier-Probst et al. 2004). Chronic presentations characterized by insidious progressive striatum abnormalities and cortical atrophy associated with demyelination of the central nervous system are also commonly found (Neumaier-Probst et al. 2004; Harting et al. 2009; Funk et al. 2005; Garbade et al. 2014; Strauss et al. 2007).

Little is known about the mechanisms underlying the neuropathology of this disease. However, growing evidence obtained in rat brain with normal GCDH activity indicates that the major organic acids accumulating in GA I, namely GA and 3HGA, disturb redox and energy homeostasis besides inducing excitotoxicity in rat brain (de Oliveira Marques et al. 2003; Figuera et al. 2003; Jafari et al. 2011; Kolker et al. 1999; Kolker et al. 2001; Kolker et al. 2002; Latini et al. 2002; Magni et al. 2009; Olivera et al. 2008; Olivera-Bravo et al. 2011; Porciuncula et al. 2004; Rosa et al. 2007; Wajner et al. 2004).

A genetic knockout mice model of GA I was developed with complete loss of GCDH activity (Koeller et al. 2002) in order to get a better insight on the neuropathology of this disease. GCDH deficient mice (*Gcdh*^{-/-}) had increased cerebral, blood, and urine GA and 3HGA concentrations and displayed vacuolation in the **frontal cortex** (spongiform **leukoencephalopathy**), but did not present striatum damage. Exposing these mice to a high protein or Lys chow provoked neuronal loss, **myelin** disruption, and **gliosis**, mostly in the deep cortex and **striatum**, and induced higher brain GA concentrations from Lys (Zinnanti et al. 2006; Zinnanti et al. 2007). Furthermore, disturbances of energy metabolism, redox homeostasis, and of the glutamatergic and GABAergic systems have been also demonstrated in the brain of *Gcdh*^{-/-} mice exposed to a short-term or long-term Lys overload (Amaral et al. 2012a; Amaral et al. 2012b; Amaral et al. 2015; Busanello et al. 2014; Lagranha et al. 2014; Rodrigues et al. 2015; Seminotti et al. 2012; Seminotti et al. 2013; Seminotti et al. 2014; Vendramin Pasquetti et al. 2017). Behavioral alterations were also observed in adult *Gcdh*^{-/-} animals reflecting a permanent brain damage (Busanello et al. 2013).

However, the pathogenesis of the acute degeneration of the striatum that takes place in GA I patients during episodes of metabolic decompensation triggered by infections or immunization is not yet fully established. It is of note that the kynurenine pathway (KP) is activated during these crises by inflammatory cytokines, leading to overproduction of

kynurenic acid, kynurenine, and particularly quinolinic acid (QA). Although these compounds are important for normal brain functioning (Maddison and Giorgini 2015; Muhl and Pfeilschifter 2003; Reyes Ocampo et al. 2014; Schwarcz et al. 2012), their excess may be toxic to the central nervous system. In fact, QA induces neurotoxic effects through different mechanisms (Lugo-Huitron et al. 2013), including activation of N-methyl-D-aspartate receptors (NMDAR), leading secondarily to oxidative damage and mitochondrial dysfunction (Perez-De La Cruz et al. 2012; Ribeiro et al. 2006).

Regarding to GA I, it was previously postulated that QA may contribute to the striatal pathogenesis in this disease potentially precipitating severe neurological symptoms (Varadkar and Surtees 2004), but so far, little has been done to test this hypothesis. In this scenario, a recent report has shown that intracerebral injection of QA induces inflammatory response and oxidative stress and disturbs bioenergetics in the brain of *Gcdh*^{-/-} mice (Seminotti et al. 2016). In the present work, we investigated whether an acute intrastriatal administration of QA could provoke histopathological changes in the striatum of developing *Gcdh*^{-/-} mice.

Material and Methods

Chemicals

All chemicals were of analytical grade and purchased from Sigma (St Louis, MO, USA) unless otherwise stated. Solutions were prepared on the day of the experiments and the pH was adjusted to 7.2–7.4 in the appropriate buffer for each analysis.

Animals

Wild-type (WT) and *Gcdh*^{-/-} littermates, both of C129SvEv background, were generated from heterozygotes and maintained at Unidade de Experimentação Animal from Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil). The animals were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in air-conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and 20% (w/w) protein commercial chow containing 0.9, 2.8, or 4.7% Lys (Nuvilab, Porto Alegre, RS, Brazil). Thirty-day-old WT and *Gcdh*^{-/-} mice were used in all experiments.

This study was performed in strict accordance with the Principles of Laboratory Animal Care, National Institute of Health of United States of America, NIH, publication no. 85-23, revised in 2011, the *International Guiding Principles for Biomedical Research Involving Animals*, and approved by the Ethical Committee for the Care and Use of Laboratory Animals of the Hospital de Clínicas de Porto Alegre (no. 140544). All efforts were made to minimize suffering,

discomfort, stress, and the number of animals necessary to produce reliable scientific data.

High Dietary Lysine Treatment and Quinolinic Acid Striatal Injection

Thirty-day-old WT and *Gcdh*^{-/-} mice were first submitted to a high dietary Lys intake (4.7% Lys) for 48 h prior to QA intrastriatal administration in order to mimic the human GA I pathology of increased brain GA and 3HGA concentrations in the *Gcdh*^{-/-} mice (Zinnanti et al. 2007). After 48 h of high Lys dietary intake (4.7%), WT and *Gcdh*^{-/-} mice were anesthetized with isoflurane (45 mg/kg) and placed on a stereotaxic frame (Stoelting Co., Wood Dale, IL, USA), using the following coordinates: 0.0 mm anteroposterior to bregma, +2.6 mm lateral to bregma, and -2.4 mm dorsoventral to the dura (Paxinos and Franklin 2012). They were then injected with 1 μ L of phosphate-buffered saline (PBS), pH 7.4, or of 25 or 50 mM QA solution (25 and 50 nmol, respectively) dissolved in PBS into the striatum of both hemispheres during 3 min using a 10- μ L Hamilton microsyringe. QA doses correspond to 2.8 and 5.6 nmol/g, respectively. The needle was left in place for an additional 1 min after the injection and then slowly withdrawn. Thereafter, the animals received a moderately increased Lys (2.8%) chow that was previously shown not to provoke mortality in the animals (Seminotti et al. 2013). WT and *Gcdh*^{-/-} mice were euthanized 48 or 96 h after QA or PBS infusion. The doses of QA used were based on previous studies (Ignarro et al. 2013; Seminotti et al. 2016). Treatment protocol is illustrated in Fig. 1.

Sample Preparation for Histological Analyses

For immunohistochemical and hematoxylin and eosin (HE) staining analyses, WT and *Gcdh*^{-/-} mice were euthanized 48 or 96 h after PBS or QA (25 or 50 nmol) intrastriatal administration and brains were removed and post-fixed in 10% formaldehyde buffered solution (pH 7.00–7.05) for 48 h at room temperature and processed for paraffin-embedded sectioning. The brain was sectioned transversely (3 μ m thick) at the level of dorsal striatum on a microtome (MICROM HM 360). The sections utilized for the histological analyses were equidistant to the lesion at 100 μ m anterior and 100 μ m posterior to the needle tract and obtained from three to four WT and *Gcdh*^{-/-} mice. Three sections were prepared per animal and five fields per section were analyzed (a total of 15 fields were observed). Medians obtained from five randomly selected fields per animal were used for the statistical calculations. Quantification of histological data was analyzed with $\times 400$ magnification.

For immunofluorescent analyses, WT and *Gcdh*^{-/-} mice were anesthetized 48 h after PBS or QA (50 nmol) intrastriatal administration with isoflurane (45 mg/kg) and intracardially

perfused with 4% paraformaldehyde (PAF) in 0.01 M PBS, pH 7.4. After fixation, brains were quickly removed, maintained overnight at 4 °C in 10% PAF, and changed to PBS until sectioning. A 1000S Leica vibratome was used to obtain 30–50 μ m thick consecutive coronal series. Striatal sections were stored either free-floating at 4 °C or mounted on gelatin-coated slides for analysis.

Hematoxylin and Eosin Staining

Striatal sections were stained in hematoxylin solution for 5 min and then washed in running tap water. The sections were then placed in eosin solution for 3 min, dehydrated with 100% ethanol, and cleared with xylene for 2 min. Image analysis (magnification of $\times 100$ and $\times 400$) was performed using the Q Capture Pro Software (Olympus). Image J software was utilized to count the numbers of vacuoles (magnification of $\times 400$). Three to four mice were used per group.

Immunohistochemistry

Three-micrometer-thick transverse striatal sections obtained with a microtome (MICROM HM 360) were deparaffinized and immersed in a mixture of 3% hydrogen peroxide and 10% methanol for 15 min to inhibit the endogenous peroxidase activity. After washing with PBS, tissue was blocked with common horse serum for 1 h and then incubated with the primary antibody at 4 °C in a wet chamber. The following primary antibodies were used: anti-GFAP (glial fibrillary acidic protein, 1:400 dilution, Cell Marque, clone: EP672Y) incubated for 32 min; anti-S100 β (S100 calcium-binding protein B, 1:100 dilution, Sigma, clone SH-B1) incubated for 16 min; anti-CD3 (cluster of differentiation 3, Roche, clone: 2GVA) incubated for 16 min. After incubation with the primary antibodies, the slides were washed three times with PBS and stained by using ultraView Universal DAB Detection Kit according manufacturer's instructions (Ventana). The slices were counterstained with hematoxylin for 10 s. All procedures were performed with negative and positive controls (da Rosa et al. 2015). Incubation times with primary antibodies were employed according to manufacturer's instructions. Quantification of S100 β , GFAP (reactive astrocytes), and CD3 (T lymphocytes) was performed using the Image J software by counting the numbers of immunoreactive cells (magnification of $\times 400$). Three to four mice were used per group.

Immunofluorescence

To perform immunofluorescence assays, free-floating striatal slices were washed with PBS, submitted to antigen retrieval by boiling in 10 mM pH 6.0 sodium citrate during 10 min, and then washed twice for 10 min. After that, slices were permeabilized with PBS plus 0.1–0.3% Triton X-100 (PBST) and

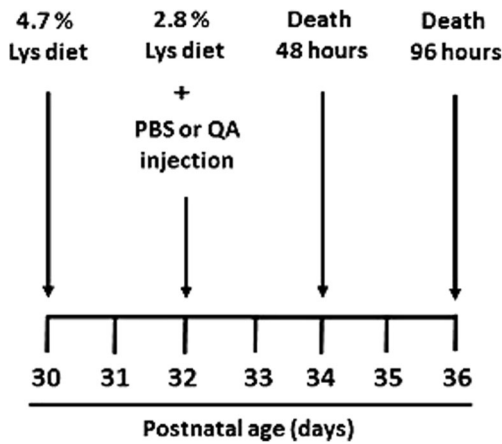
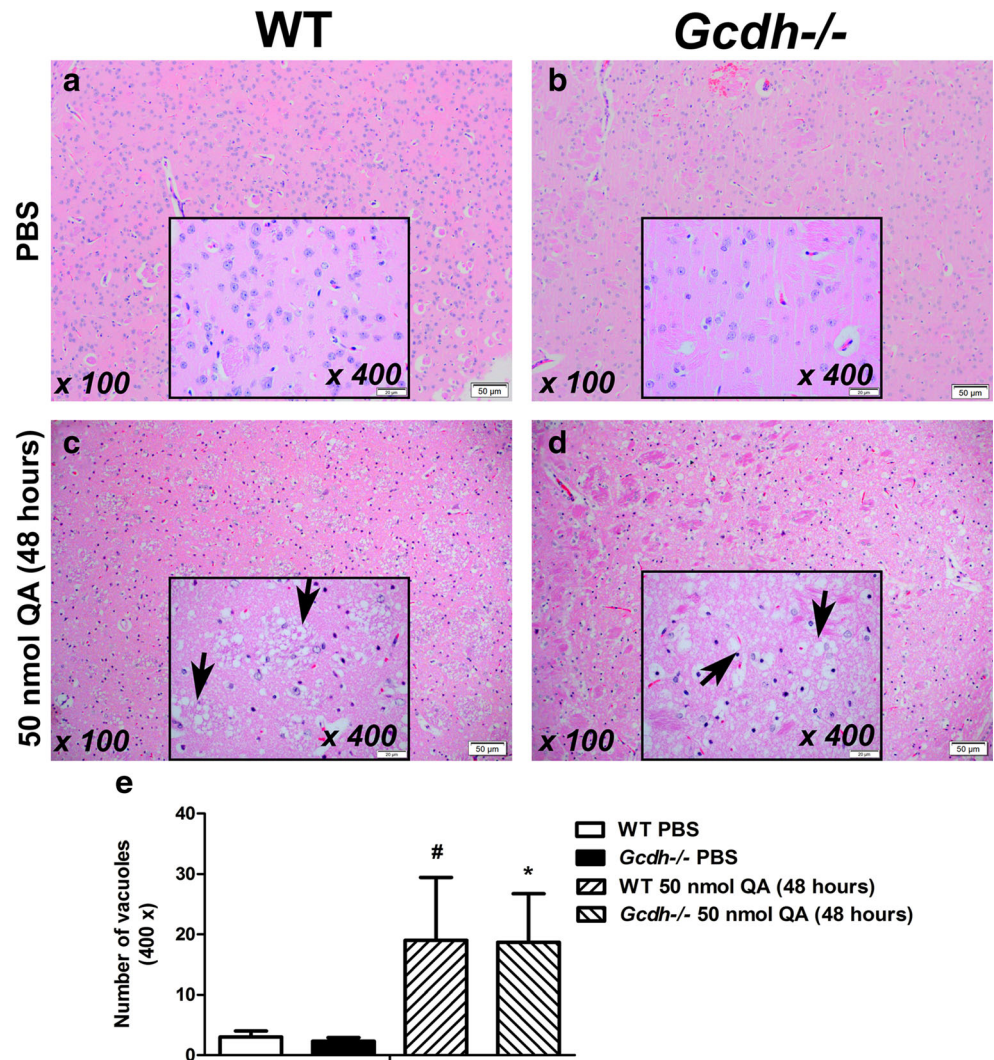


Fig. 1 Thirty-day-old wild-type (WT) and glutaryl-CoA dehydrogenase deficient (*Gcdh*^{-/-}) mice were fed a 4.7% lysine (Lys) diet for 48 h prior to PBS or quinolinic acid (QA, 25 or 50 nmol) intrastratial injection. Animals were then fed a 2.8% Lys diet and euthanized 48 or 96 h afterwards

Fig. 2 Light microscopy images showing hematoxylin and eosin (HE) staining of the striatum from adolescent (30-day-old) wild-type (WT) (a, c) and glutaryl-CoA dehydrogenase deficient (*Gcdh*^{-/-}) (b, d) mice submitted to an intrastratial injection of PBS (a, b) or 50 nmol quinolinic acid (QA) (c, d). Mice were sacrificed 48 h after intrastratial administration. Representative images were obtained from three mice per group. HE staining with magnification of $\times 100$ and $\times 400$. Arrows in panels c and d indicate vacuolation. e Quantification of the number of vacuoles was performed with $\times 400$ magnification by using the median of five randomly selected fields from three striatal sections per brain. Values are mean \pm standard deviation for three mice per group. Two-way ANOVA was performed as described in the text. # $P < 0.05$ compared to PBS-injected WT mice; * $P < 0.05$ compared to PBS-injected *Gcdh*^{-/-} mice (Student's *t* test)

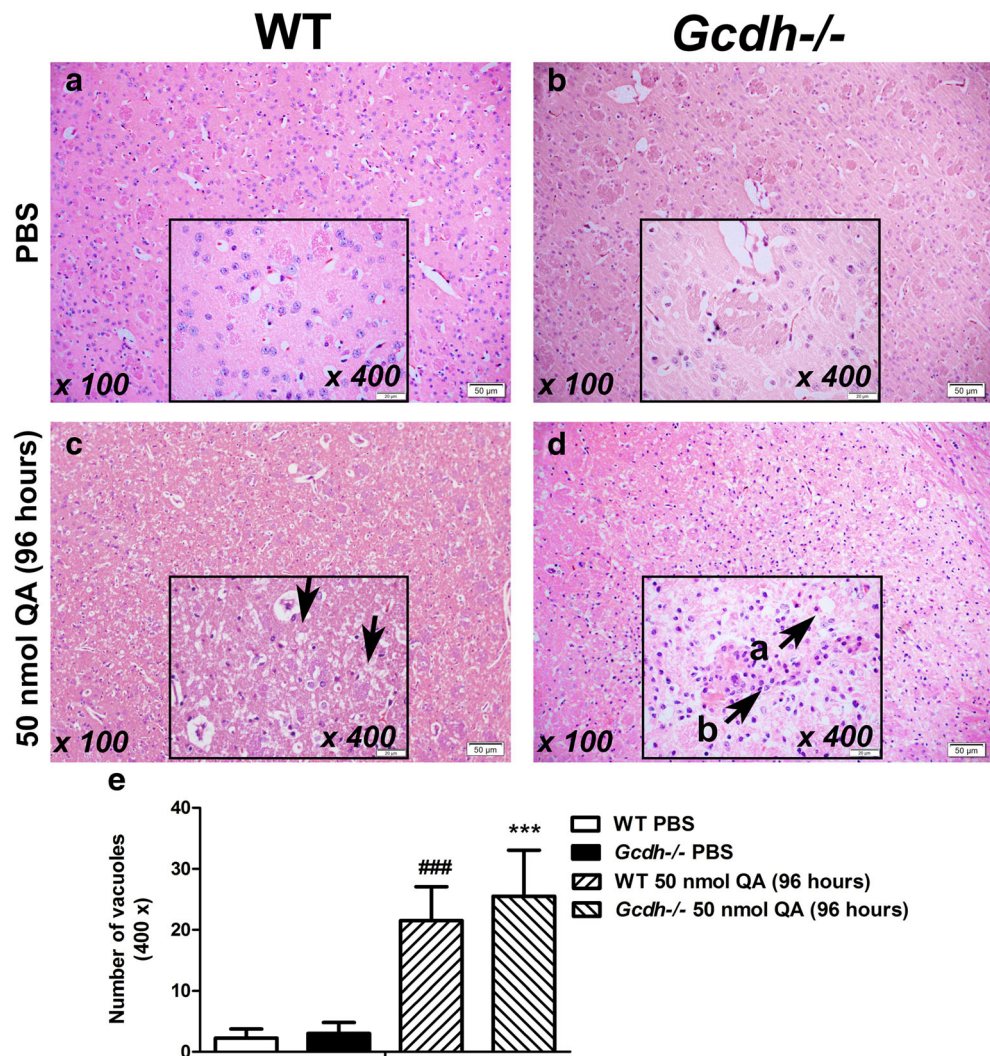


treated with blocking buffer (PBS + 0.3% Triton X-100 + 5% bovine serum albumin) for 30 min and incubated with anti-nitrotyrosine (YNO2, Millipore, 1:300). All dilutions were made in PBST. After a 4 °C overnight incubation, sections were rinsed in PBS and incubated at room temperature for 90 min with 1:800 dilutions of the secondary antibody conjugated to fluorescent probes (Molecular Probes). Sections were then washed, mounted in glycerol, and imaged in a FV300 Olympus confocal microscope provided with 405, 488, 546, and 633 nm lasers. Primary or secondary antibodies were omitted in negative controls (Olivera-Bravo et al. 2011). Three mice were used for group.

Statistical Analysis

Unless otherwise stated, results are presented as mean \pm standard deviation. Data were analyzed by two-way ANOVA, considering the factors (1) genotype: WT or *Gcdh*^{-/-}; (2)

Fig. 3 Histopathological changes observed in the striatum of adolescent (30-day-old) wild-type (WT) (a, c) and glutaryl-CoA dehydrogenase deficient (*Gcdh*^{-/-}) (b, d) mice submitted to an intrastriatal injection of PBS (a, b) or 50 nmol quinolinic acid (QA) (c, d). Representative images obtained with hematoxylin and eosin (HE) staining with magnification of $\times 100$ and $\times 400$ from four mice per group 96 h after intrastriatal administration. Arrows in panel c and arrow *a* in panel d indicate vacuolation, whereas arrow *b* in panel d indicates inflammatory infiltration. **e** Quantification of the number of vacuoles was performed with $\times 400$ magnification by using the median of five randomly selected fields from three striatal sections per brain. Values are mean \pm standard deviation for four mice per group. Two-way ANOVA was performed as described in the text. ### $P < 0.001$ compared to PBS-injected WT mice; *** $P < 0.001$ compared to PBS-injected *Gcdh*^{-/-} mice (Student's *t* test)



treatment: PBS or QA; (3) interaction between genotype x treatment. *F* values are presented when differences between groups were rated significant at $P < 0.05$. Post hoc Duncan's multiple range test was performed when means from three or more groups were compared within the same genotype, whereas the Student's *t* test for unpaired samples was used to compare means between two different groups. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

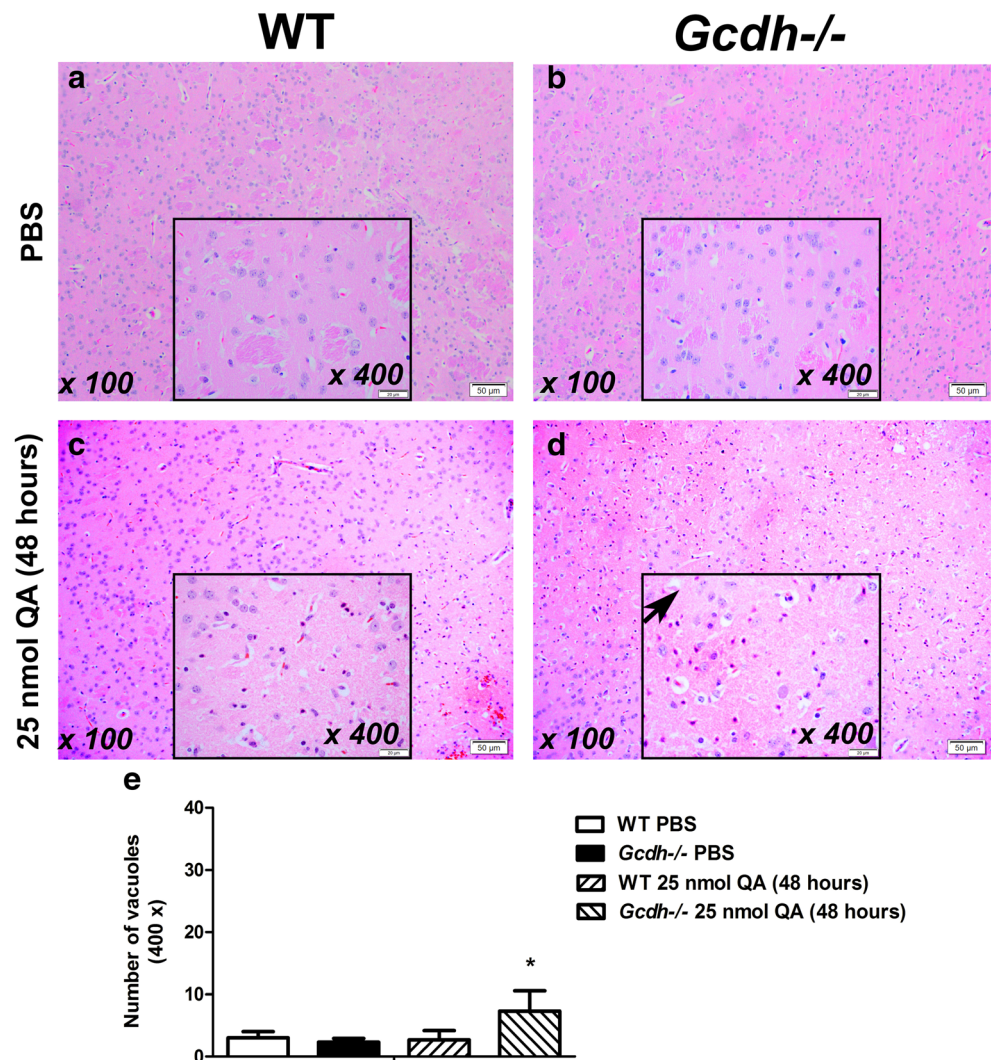
Results

Quinolinic Acid Provokes Extensive Vacuolation and Inflammatory Infiltration in the Striatum of *Gcdh*^{-/-} Mice

We initially observed that approximately 20% of *Gcdh*^{-/-} mice injected intrastrially with QA (50 nmol) and submitted

to high Lys (4.7%) chow became hypoactive and died 48 h after injection, whereas 25 nmol QA injection provoked no symptomatology or mortality in these animals. Furthermore, WT mice were asymptomatic after receiving 25 or 50 nmol QA. Thereafter, we analyzed the histopathological findings in the striatum of asymptomatic WT and *Gcdh*^{-/-} mice. Statistical analysis by two-way ANOVA of HE staining revealed intense vacuolation and edema (up to eightfold) at 48 h after 50 nmol QA injection (Fig. 2) (QA treatment, $F_{(1,11)} = 17.854$; $P < 0.01$), which was more marked (up to 9.6-fold) at 96 h after QA injection (Fig. 3) (QA treatment, $F_{(1,15)} = 73.976$; $P < 0.001$) in the striatum from WT and *Gcdh*^{-/-} mice, without significant influence of genotype or interaction between treatment x genotype. Furthermore, inflammatory infiltration was observed only in the striatum of *Gcdh*^{-/-} mice administered with QA 96 h after injection (Fig. 3). We also found that a lower dose of QA (25 nmol) caused a significant increase in the number of vacuoles (up to 3.2-fold) at 48 h after QA injection only in the striatum of *Gcdh*^{-/-} mice

Fig. 4 Vacuolation provoked by quinolinic acid (QA, 25 nmol) in the striatum from adolescent (30-day-old) glutaryl-CoA dehydrogenase deficient (*Gcdh*^{-/-}) mice 48 h after intrastriatal injection. Light microscopy images were obtained by hematoxylin and eosin (HE). Wild-type (WT, **a**) and *Gcdh*^{-/-} (**b**) submitted to an intrastriatal injection of PBS or quinolinic acid (QA) (**c**, **d**). Representative images were obtained from three mice per group. HE staining with magnification of $\times 100$ and $\times 400$. Arrow in panel **d** indicates vacuolation. **e** Quantification of the number of vacuoles was performed with $\times 400$ magnification by using the median of five randomly selected fields from three striatal sections per brain. Values are mean \pm standard deviation for three mice per group. Two-way ANOVA was performed as described in the text. * $P < 0.05$ compared to PBS-injected *Gcdh*^{-/-} mice (Student's *t* test)



(interaction between QA treatment \times *Gcdh*^{-/-} genotype, $F_{(1,11)} = 6.095$; $P < 0.05$) (Fig. 4), with no impact of genotype or treatment separately on the histopathological changes observed. However, vacuolation was seen in the striatum (up to 3.7-fold) of both WT and *Gcdh*^{-/-} mice after 96 h of 25 nmol QA administration (QA treatment, $F_{(1,13)} = 14.721$; $P < 0.01$) (Fig. 5), without significant influence of genotype or interaction between treatment \times genotype.

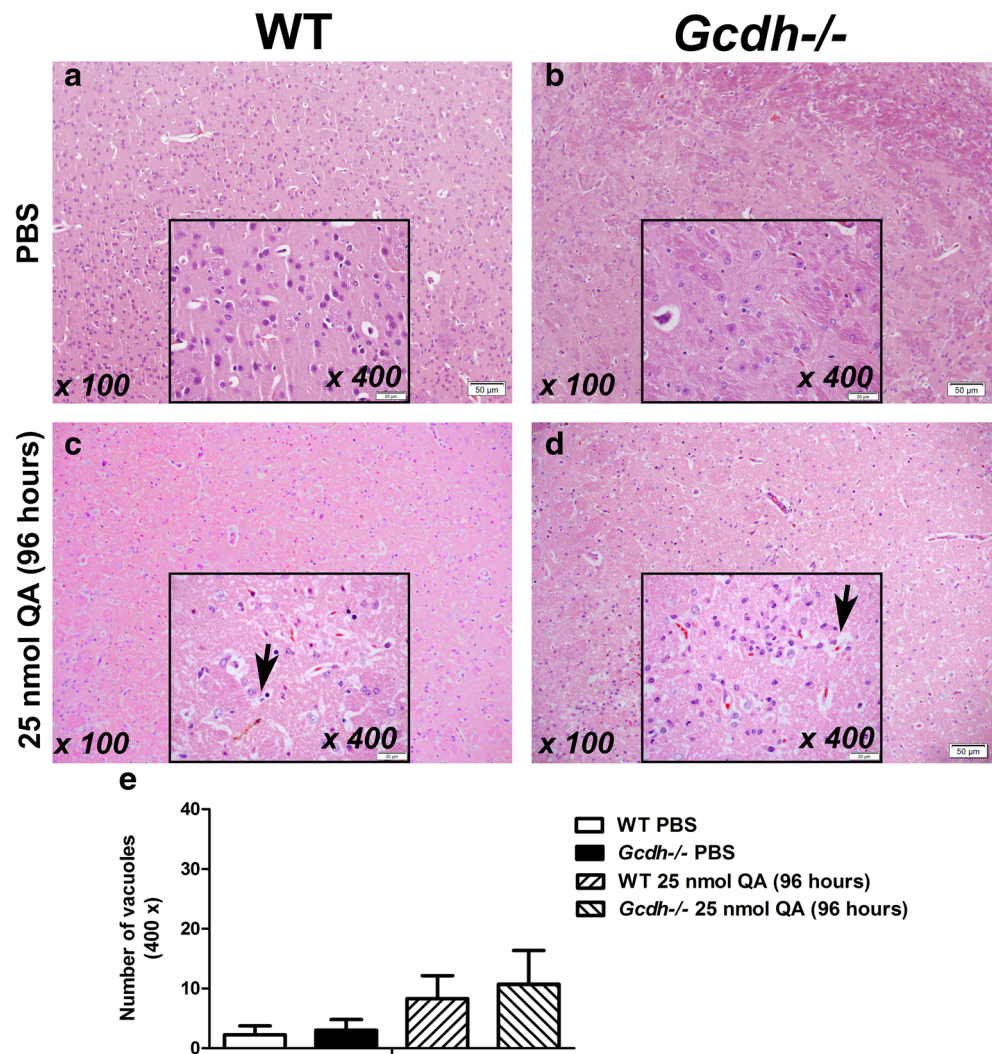
Another interesting finding of the present study was an increase of CD3 staining in the striatum of *Gcdh*^{-/-} mice 96 h after 50 nmol QA intrastriatal injection (3.75-fold), as compared to QA-injected WT mice (interaction between QA treatment \times *Gcdh*^{-/-} genotype, $F_{(1,11)} = 22.261$; $P < 0.01$) (Fig. 6), indicating T lymphocyte infiltration and corroborating with the data obtained with HE staining. Furthermore, statistical analysis revealed separate influences of *Gcdh*^{-/-} genotype ($F_{(1,11)} = 25.130$; $P < 0.01$) and QA treatment ($F_{(1,11)} = 58.783$; $P < 0.001$) in the increased CD3 staining (Fig. 6). Taken together, these data indicate that QA behaved

as a neuroinflammatory agent and that the striatum of *Gcdh*^{-/-} mice was more susceptible to this neurotoxin as compared to WT.

Quinolinic Acid Induces Astrocyte Activation in the Striatum of WT Mice

Next, we determined GFAP and S100 β content in the striatum of WT and *Gcdh*^{-/-} mice after intrastriatal QA injection (50 nmol). QA significantly increased GFAP staining at 48 (up to eightfold) and 96 h (up to 16.5-fold) after injection in the striatum of WT mice (interaction between QA treatment \times WT genotype, $F_{(2,21)} = 24.186$; $P < 0.001$), but not in the striatum of *Gcdh*^{-/-} mice (Fig. 7). Similar results were obtained when analyzing S100 β staining, which increased 2-fold after 48 and 3.5-fold at 96 h after QA injection in WT mice (interaction between QA treatment \times WT genotype, $F_{(2,17)} = 6.500$; $P < 0.05$) (Fig. 8). Statistical analysis also showed an influence of WT genotype (GFAP, $F_{(1,21)} = 61.738$; $P < 0.001$;

Fig. 5 Histopathological changes obtained by hematoxylin and eosin (HE) staining in the striatum from adolescent (30-day-old) wild-type (WT) (a, c) and glutaryl-CoA dehydrogenase deficient (*Gcdh*^{-/-}) (b, d) mice submitted to an intrastriatal injection of 25 nmol quinolinic acid (QA) (c, d). Mice were sacrificed 96 h after intrastriatal administration. PBS intrastriatal injection was also carried out (a, b). Representative images were obtained from four mice per group. HE staining with magnification of $\times 100$ and $\times 400$. Arrows in panel c and d indicate vacuolation. e Quantification of the number of vacuoles was performed with $\times 400$ magnification by using the median of five randomly selected fields from three striatal sections per brain. Values are mean \pm standard deviation for four mice per group. Two-way ANOVA is described in the text. No significant differences were detected (Student's *t* test)



S100 β , $F_{(1,17)} = 28.571$; $P < 0.001$) and QA treatment (GFAP, $F_{(2,21)} = 31.368$; $P < 0.001$; S100 β , $F_{(2,17)} = 4.786$; $P < 0.05$) on the increased GFAP and S100 β staining (Figs. 7 and 8). Considering that GFAP secretion is increased in activated astrocytes and that S100 β is released by astrocytes during proliferation, our data indicate that QA induced astrogliosis only in WT mice, whereas astrocytes of *Gcdh*^{-/-} mice were not able to respond to the toxicity provoked by QA. It can be also seen in Figs. 7 and 8 that GFAP and S100 β staining were minimum in the striatum of WT and *Gcdh*^{-/-} mice not injected with QA.

Quinolinic Acid Causes Nitrosative Stress in the Striatum of *Gcdh*^{-/-} Mice

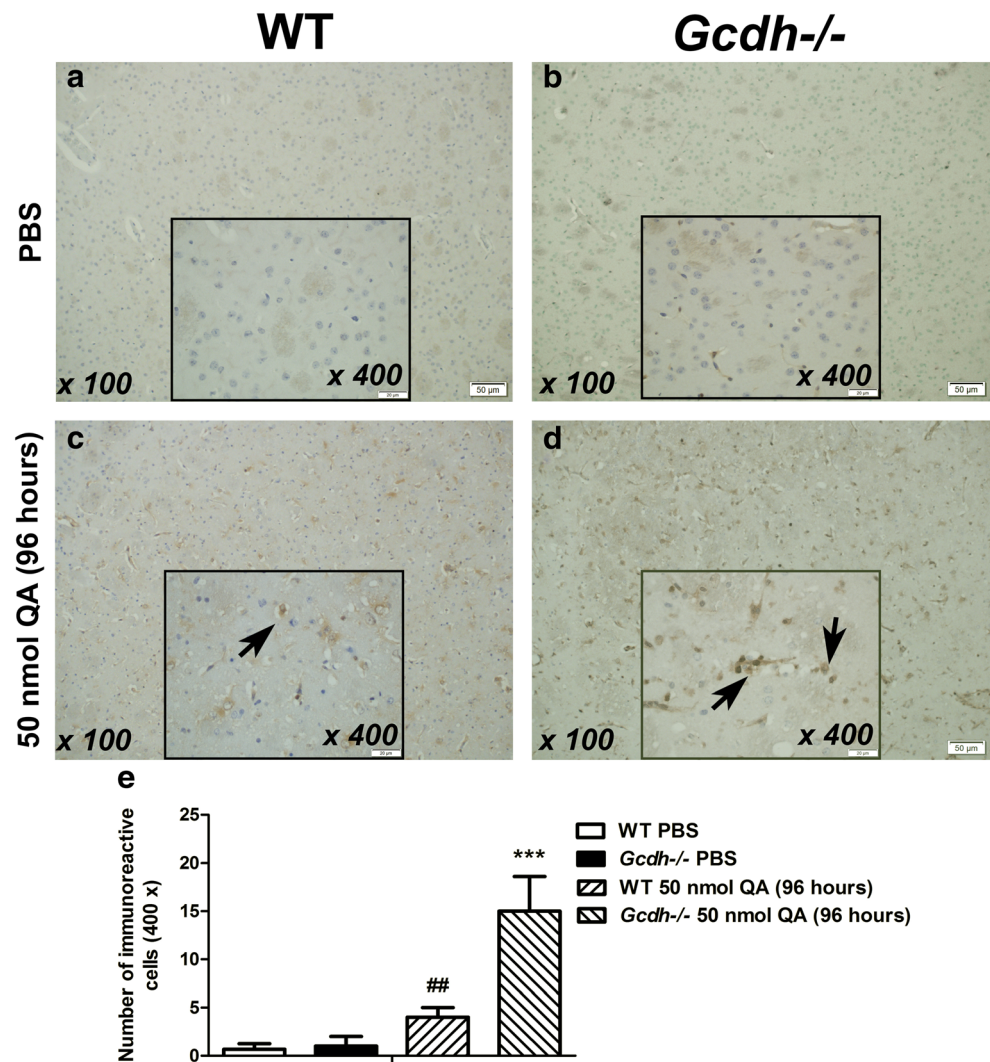
We finally examined the involvement of nitrosative damage in the histopathological alterations provoked by QA in the striatum of these animals. It was observed that there is a large increase of YNO2 staining in *Gcdh*^{-/-} striatum but not in

WT mice at 48 h after QA administration, indicating the involvement of nitrosative damage in the striatum of *GCDH* deficient submitted to QA injection (Fig. 9).

Discussion

Although the pathogenesis of the brain damage in GA I has been extensively investigated, the exact underlying mechanisms responsible for the acute striatal degeneration that severely compromises the outcome of affected patients remain to be elucidated. It is of note that basal ganglia degeneration in GA I patients occurs during encephalopathic crises triggered by infections or immunizations both associated with inflammatory processes (Goodman and Frerman 2001; Jafari et al. 2011). In this scenario, it has been previously postulated that high QA production may contribute to the acute neuropathological abnormalities observed in GA I (Varadkar and Surtees 2004). Noteworthy, QA was demonstrated to provoke

Fig. 6 Quinolinic acid (QA) injection provokes T lymphocyte infiltration in the striatum of glutaryl-CoA dehydrogenase deficient (*Gcdh*^{-/-}) mice 96 h after its administration. Light microscopy images showing CD3 immunohistochemistry staining with magnification of $\times 100$ and $\times 400$ in the striatum from adolescent (30-day-old) wild-type (WT) (a, c) and *Gcdh*^{-/-} (b, d) mice submitted to an intrastriatal injection of PBS (a, b) or 50 nmol quinolinic acid (QA) (c, d). Representative images were obtained from three mice per group. Arrows in panels c and d indicate presence of T lymphocytes. e Quantification of the number of immunoreactive cells (T lymphocytes) was performed with $\times 400$ magnification by using the median of five randomly selected fields from three striatal sections per brain. Values are mean \pm standard deviation for three mice per group. Two-way ANOVA was performed as described in the text. ##*P* < 0.01 compared to PBS-injected WT mice; ****P* < 0.001 compared to PBS-injected *Gcdh*^{-/-} mice (Student's *t* test)

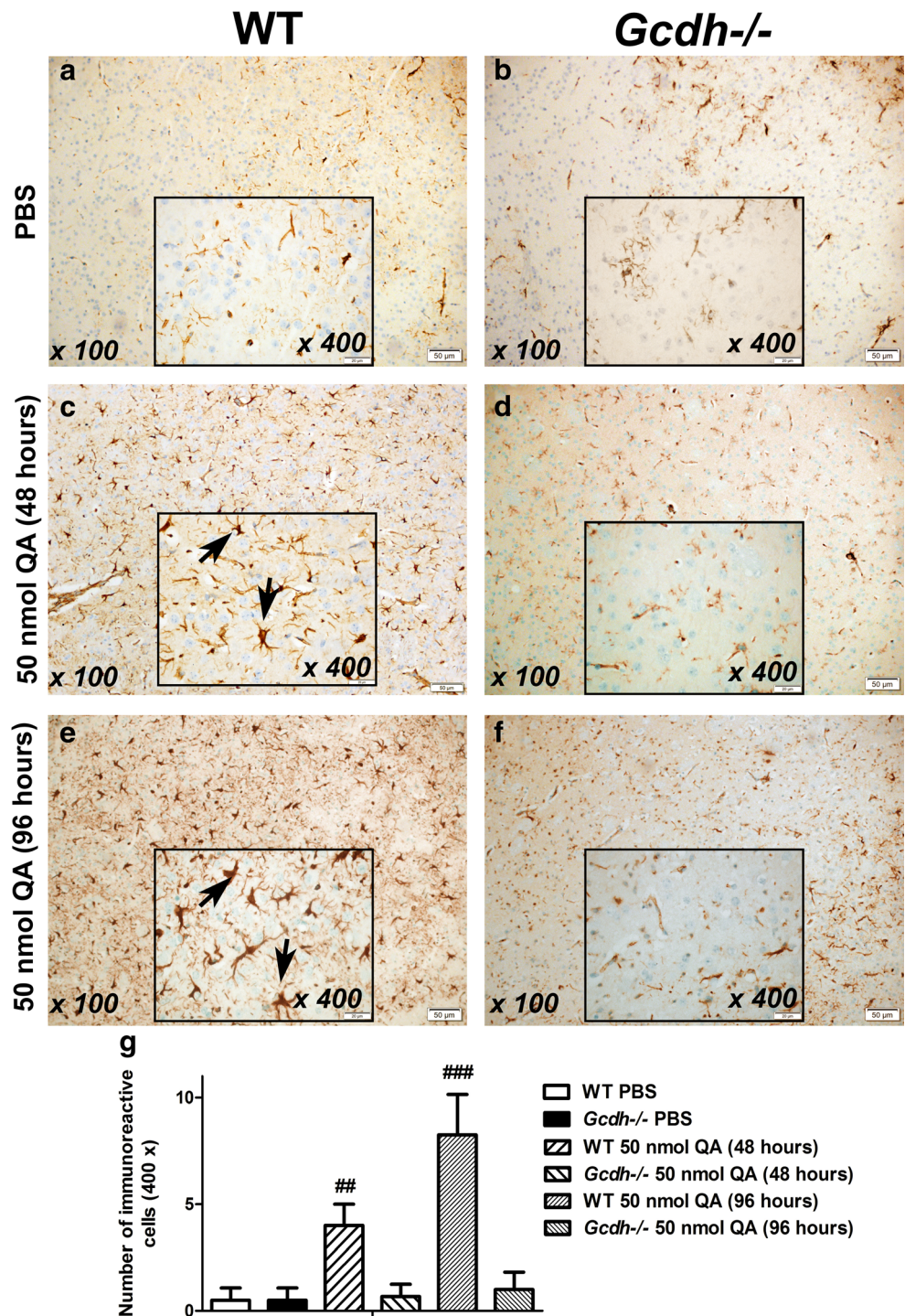


neurodegeneration and disturb energy metabolism and redox status, particularly in the striatum and possibly due to overstimulation of NMDAr (Perez-De La Cruz et al. 2012). It was also demonstrated that intrastriatal injection of QA to *Gcdh*^{-/-} mice under Lys overload induces oxidative stress through modulation of distinct signaling pathways and transcription factors (Seminotti et al. 2016). Furthermore, brain morphological alterations were observed in *Gcdh*^{-/-} mice fed a high Lys chow (Zinnanti et al. 2006; Amaral et al. 2015; Keyser et al. 2008) that better mimics the in vivo human condition of GA I, but to the best of our knowledge, no study investigated whether high striatum QA concentrations could contribute to cerebral histopathological changes in *Gcdh*^{-/-} mice. Therefore, in the present study, we aimed to evaluate whether a single intrastriatal injection of QA that induces a neuroinflammatory response (Perez-De La Cruz et al. 2012; Guillemain 2012; Jamwal et al. 2015; Lim et al. 2016) could alter the morphology of the striatum of developing *Gcdh*^{-/-} mice (Zinnanti et al. 2006; Zinnanti et al. 2007).

We initially observed that approximately one fifth of *Gcdh*^{-/-} mice became hypoactive and died 48 h after 50 nmol intrastriatal QA injection, whereas these mice presented no symptomatology or mortality when injected with 25 nmol QA, clearly demonstrating a selective genotype dose-dependent QA effect on morbidity and mortality. Furthermore, WT mice receiving 25 or 50 nmol QA presented no morbidity or mortality, indicating a higher vulnerability of *Gcdh*^{-/-} mice to the QA toxicity.

Regarding to the histopathological findings, it was found that the striatum of *Gcdh*^{-/-} mice injected with the higher QA dose (50 nmol) presented an intense and progressive vacuolation, edema, and T lymphocyte infiltration along time up to 96 h after injection, whereas WT striatum did not present the inflammatory infiltrate and showed less vacuolation. Furthermore, 25 nmol QA caused a moderate vacuolation and edema only in *Gcdh*^{-/-} mice as compared to WT mice at 48 h after injection, indicating a higher vulnerability of *Gcdh*^{-/-} striatum to QA-induced

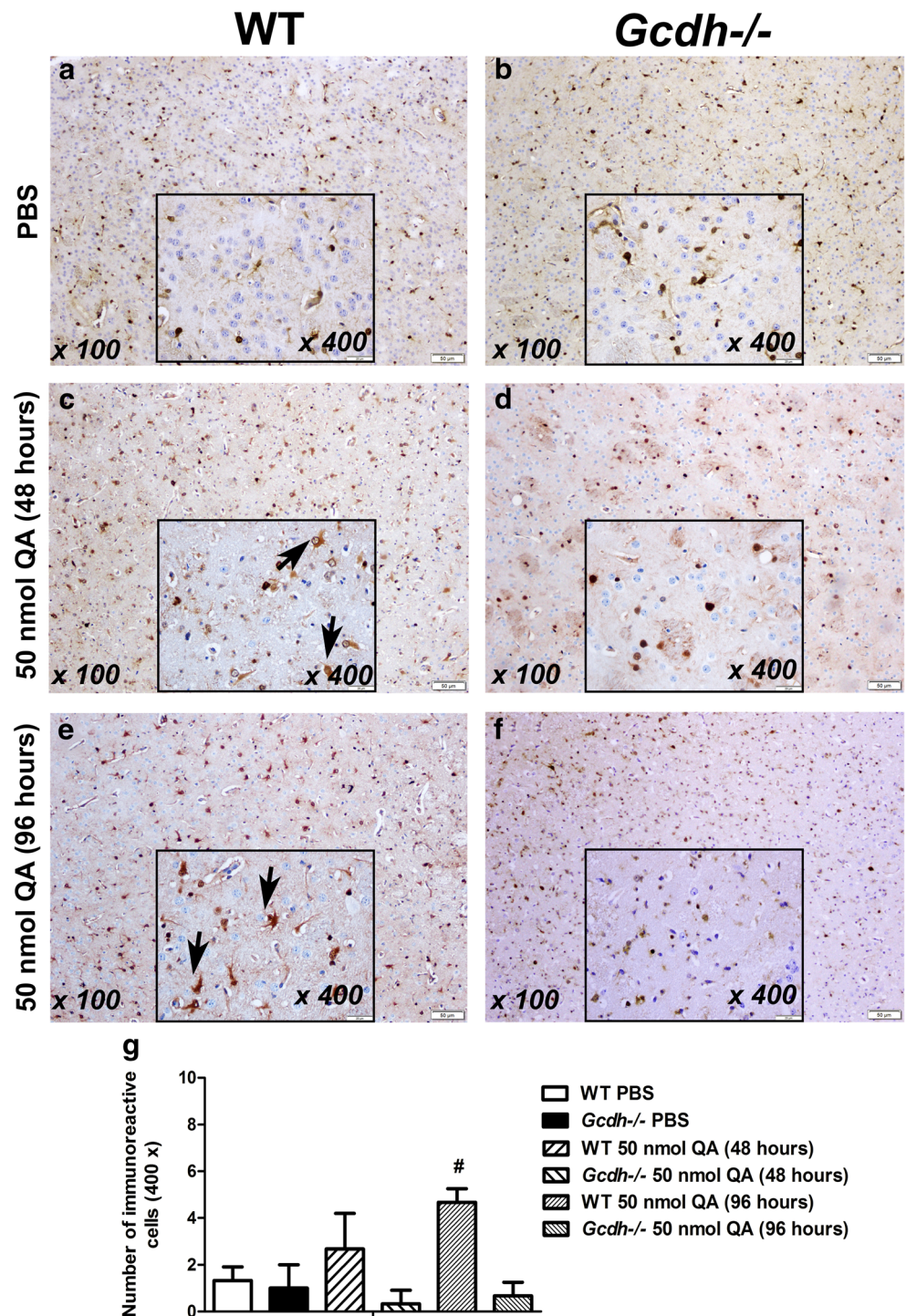
Fig. 7 Light microscopy images showing GFAP immunohistochemistry staining in the striatum from adolescent (30-day-old) wild-type (WT) (a, c, e) and glutaryl-CoA dehydrogenase deficient (*Gcdh*^{-/-}) (b, d, f) mice submitted to an intrastriatal injection of PBS (a, b) or 50 nmol quinolinic acid (QA) (c–f). Mice were sacrificed 96 h (a, b and e, f) or 48 h (c, d) after intrastriatal administration. Representative images were obtained from three to four mice per group. GFAP immunohistochemistry staining with magnification of ×100 and ×400. Arrows in panels c and e indicate presence of reactive astrocytes. **g** Quantification of the number of immunoreactive cells (reactive astrocytes) was performed with ×400 magnification by using the median of five randomly selected fields from three striatal sections per brain. Values are mean ± standard deviation for three to four mice per group. Two-way ANOVA was performed as described in the text. ##*P* < 0.01, ###*P* < 0.001 compared to PBS-injected WT mice (Duncan multiple range test)



neuroinflammatory response. Notably, similar vacuolation attributed to glutamate-mediated excitotoxicity was found in post-mortem examination of the basal ganglia and cerebral cortex of patients with GA I (Goodman et al. 1977). Therefore, we presume that the vacuolation provoked by QA, a known NMDAR agonist, observed in our present study may be possibly related to the striatum damage in GA I patients. However, we cannot rule out that the

histopathological alterations found in the striatum of *Gcdh*^{-/-} mice were caused by synergistic effects of increased brain concentrations of QA, GA, and 3HGA (Zinnanti et al. 2007) since GA and 3HGA were previously shown to induce excitotoxicity in vitro and in vivo (Kolker et al. 2002; Rosa et al. 2007; Wajner et al. 2004; Lagranha et al. 2014; Busanello et al. 2014; Rosa et al. 2004). Our present data indicate that a neuroinflammatory insult may

Fig. 8 Light microscopy images showing S100 β immunohistochemistry staining in the striatum from adolescent (30-day-old) wild-type (WT) (a, c, e) and glutaryl-CoA dehydrogenase deficient (*Gcdh*^{-/-}) (b, d, f) mice submitted to an intrastriatal injection of PBS (a, b) or 50 nmol quinolinic acid (QA) (c–f). Mice were sacrificed 96 h (a, b and e, f) or 48 h (c, d) after intrastriatal administration. Representative images were obtained from three mice per group. S100 β immunohistochemistry staining with magnification of $\times 100$ and $\times 400$. Arrows in panels c and e indicate presence of reactive astrocytes. **g** Quantification of the number of immunoreactive cells (reactive astrocytes) was performed with $\times 400$ magnification by using the median of five randomly selected fields from three striatal sections per brain. Values are mean \pm standard deviation for three mice per group. Two-way ANOVA was performed as described in the text. [#] $P < 0.05$ compared to PBS-injected WT mice (Duncan multiple range test)

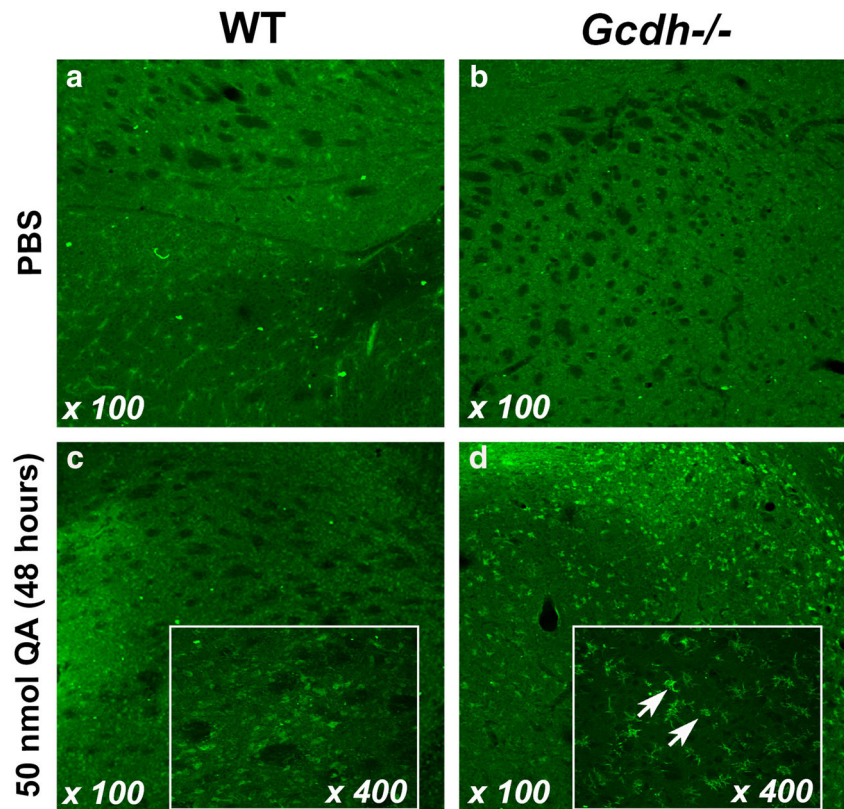


contribute to the striatal damage observed in GA I patients following infections (Varadkar and Surtees 2004).

It was also observed that GFAP and S100 β content was significantly increased reflecting astrogliosis only in the striatum of WT mice 48 or 96 h after QA injection with no increase of GFAP and S100 β staining in the striatum of *Gcdh*^{-/-} mice. In this particular, it is possible that *Gcdh*^{-/-} astrocytes were unable to proliferate and/or being

activated when submitted to the pro-inflammatory insult induced by QA. Noteworthy, normal astrocytes promptly react to injury by increasing proliferation and being a source of multipotent cells for the injured brain, providing substrates for neuronal intermediary metabolism, antioxidant balance, neurotransmitter synthesis, and myelination (Buffo et al. 2008; Maragakis and Rothstein 2006; Moore et al. 2011; Nash et al. 2011; Sloan and Barres 2014). We

Fig. 9 Immunofluorescence images showing 3-nitrotyrosine staining in the striatum of adolescent (30-day-old) wild-type (WT) (a, c) and glutaryl-CoA dehydrogenase deficient (*Gcdh*^{-/-}) (b, d) mice submitted to an intrastriatal injection of PBS (a, b) or 50 nmol quinolinic acid (QA) (c, d). Mice were sacrificed 48 h after intrastriatal administration. Arrows in panel d indicate the presence of 3-nitrotyrosine (YNO₂). Representative images were obtained from three independent experiments (animals) per group. YNO₂ immunofluorescence staining with magnification of ×100 and ×400



cannot exclude the possibility of astrocytic death in the striatum of *Gcdh*^{-/-} mice exposed to QA. This is in line with a study showing that exposition of cultured astrocytes of *Gcdh*^{-/-} mice under the same conditions, i.e., to the pro-inflammatory agent menadione resulted in significant cell death relatively to WT astrocytes (Rodrigues et al. 2016). Astrocytes were also demonstrated to be the most vulnerable neural cells to GA and 3HGA exposition in 3D organotypic brain rat cultures, highlighting their importance in GA I neuropathology (Jafari et al. 2013). It is also of note that astrocyte dysfunction has been associated with various neurodegenerative diseases, including Alzheimer's disease (Kamphuis et al. 2015; Kulijewicz-Nawrot et al. 2012; Lanciotti et al. 2013; Olabarria et al. 2010; Pekny et al. 2016).

Another novel finding of the present study was that QA injection markedly increased YNO₂ staining, providing a role for nitrosative stress in the QA-induced toxicity in the striatum of *Gcdh*^{-/-} mice. These data agree with a previous report demonstrating increased nitrite and nitrate concentrations in the striatum of *Gcdh*^{-/-} mice receiving an intrastriatal injection of QA (Seminotti et al. 2016). This is also in line with another work showing decreased mitochondrial functions and oxidative stress induction in rat brain caused by a synergism between QA, GA, and 3HGA, which were partially prevented by the nitric oxide synthase (NOS) inhibitor nitro-L-arginine methyl ester (Colin-Gonzalez et al. 2015). It is therefore

feasible that overstimulation of NMDAr by QA leads to Ca²⁺ influx and activation of nitric oxide synthase resulting in nitric oxide production that may underlie at least partly the effects elicited by QA, contributing to the striatum damage found in *Gcdh*^{-/-} mice.

In conclusion, the present study demonstrated for the first time a higher susceptibility of the genetic mouse model of GA I to QA, as reflected by the marked histopathological alterations in the striatum of these animals relatively to the WT mice. Intense vacuolation, edema, and particularly T lymphocyte infiltration that characterize an inflammatory response were observed in these animals. Astrocytic dysfunction and nitrosative stress were also verified in the striatum of the *Gcdh*^{-/-} mice. In case these findings can be extrapolated to the human condition, it is presumed that these pathomechanisms may be involved in the acute degeneration of the basal ganglia that takes place in GA I patients during infections and/or inflammation associated with encephalopathic crises.

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Compliance with Ethical Standards This study was performed in strict accordance with the Principles of Laboratory Animal Care, National Institute of Health of United States of America, NIH, publication no. 85-23, revised in 2011, the *International Guiding Principles for Biomedical Research Involving Animals*, and approved by the Ethical Committee for the Care and Use of Laboratory Animals of the Hospital de Clínicas de Porto Alegre (no. 140544).

Conflicts of Interest The authors declare that they have no conflict of interest.

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