THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 20, pp. 17777–17784, May 20, 2011
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Glutaric Aciduria Type 1 Metabolites Impair the Succinate Transport from Astrocytic to Neuronal Cells*[S]

Received for publication, February 23, 2011, and in revised form, March 27, 2011 Published, JBC Papers in Press, March 29, 2011, DOI 10.1074/jbc.M111.232744

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The inherited neurodegenerative disorder glutaric aciduria type 1 (GA1) results from mutations in the gene for the mitochondrial matrix enzyme glutaryl-CoA dehydrogenase (*GCDH*), which leads to elevations of the dicarboxylates glutaric acid (GA) and 3-hydroxyglutaric acid (3OHGA) in brain and blood. The characteristic clinical presentation of GA1 is a sudden onset of dystonia during catabolic situations, resulting from acute striatal injury. The underlying mechanisms are poorly understood, but the high levels of GA and 3OHGA that accumulate during catabolic illnesses are believed to play a primary role. Both GA and 3OHGA are known to be substrates for Na^+-coupled dicarboxylate transporters, which are required for the anaplerotic transfer of the tricarboxylic acid cycle (TCA) intermediate succinate between astrocytes and neurons. We hypothesized that GA and 3OHGA inhibit the transfer of succinate from astrocytes to neurons, leading to reduced TCA cycle activity and cellular injury. Here, we show that both GA and 3OHGA inhibit the uptake of [^{14}C]succinate by Na^+-coupled dicarboxylate transporters in cultured astrocytic and neuronal cells of wild-type and *Gcdh*$^{-/-}$ mice. In addition, we demonstrate that the efflux of [^{14}C]succinate from *Gcdh*$^{-/-}$ astrocytic cells mediated by a not yet identified transporter is strongly reduced. This is the first experimental evidence that GA and 3OHGA interfere with two essential anaplerotic transport processes: astrocytic efflux and neuronal uptake of TCA cycle intermediates, which occur between neurons and astrocytes. These results suggest that elevated levels of GA and 3OHGA may lead to neuronal injury and cell death via disruption of TCA cycle activity.

Glutaric aciduria type 1 (GA1)[3] is caused by deficiency of the mitochondrial matrix protein glutaryl-CoA dehydrogenase (GCDH). This enzyme catalyzes the oxidative decarboxylation of glutaryl-CoA in the degradative pathway of the amino acids lysine, hydroxylysine and tryptophan. GCDH deficiency leads to the accumulation of the dicarboxylic acids (DC) glutaric acid (GA) and 3-hydroxyglutaric acid (3OHGA) in tissues and body fluids. Affected patients during a time window from birth to 36 months of age are at risk for the development of encephalopathic crises triggered by catabolic situations such as infectious diseases, fever, vomiting or diarrhea, accompanied with a further increase of GA and 3OHGA concentrations (1, 2). These encephalopathic crises lead to the selective destruction of striatal neurons with a subsequent irreversible disabling movement disorder.

The pathophysiologic mechanisms underlying the cytotoxic effects of GA and 3OHGA are not fully understood. In cultured primary neuronal cells prepared from rat and chicken brains the activation of *N*-methyl-D-aspartate (NMDA) receptors has been reported upon incubation with GA and 3OHGA *in vitro* (3, 4), which could not be confirmed in other studies (5, 6). Furthermore, inhibition of γ-aminobutyric acid (GABA) synthesis and the impairment of mitochondrial energy production due to inhibition of the α-ketoglutarate dehydrogenase complex and depletion of creatine phosphate are suggested to be relevant for neuronal death (3, 4, 7–9). In addition, it has been shown that GA and 3OHGA impair the integrity of endothelial barriers *in vitro* and *in vivo* (10). The physiologic significance of these studies, however, is unclear, because they have been performed with cells exhibiting enzymatically active endogenous GCDH.

Gcdh$^{-/-}$ mice display a biochemical phenotype similar to human GA1 patients with an accumulation of GA and 3OHGA in tissues and body fluids (11). The administration of a high protein diet to young *Gcdh*$^{-/-}$ mice leads to the induction of an encephalopathic crisis accompanied by vacuolation in the brain and death within 3–5 days. Under these conditions, a further increase of GA and 3OHGA concentrations in urine, serum, and tissues is observed (12, 13).

Recently, we identified the sodium-dependent dicarboxylate co-transporter 3 (NaC3) and organic anion transporters (OAT) 1 and 4 as low and high affinity transporters, respectively, for the translocation of GA and 3OHGA through membranes (14, 15). The concerted action of NaC3, OAT1, and OAT4 in renal proximal tubule cells may be important for the urinary excretion of GA and 3OHGA (16). How these metabolites are transported in the brain, however, and which transporters are involved are still unclear. In addition to renal proximal tubule cells, NaC3 and OAT1 have been reported to be expressed in murine and human choroid plexus, respectively (17, 18). More recently, it has been concluded from mRNA and immunocyto-

* This work was supported by Deutsche Forschungsgemeinschaft Grants MU1778/2-1 and 2-2 (to C. M., B. K., and J. L.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5 and Table 1.

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[3] The abbreviations used are: GA1, glutaric aciduria type 1; DC, dicarboxylic acid; GA, glutaric acid; GCDH, glutaryl-CoA dehydrogenase; GFAP, glial fibrillary acidic protein; 3OHGA, 3-hydroxyglutaric acid; L2OHGA, L- 2-hydroxyglutaric acid; NaC, sodium-dependent dicarboxylate co-transporter; NeuN, neuron-specific nuclear protein; OAT, organic anion transporter; qRT-PCR, quantitative RT-PCR; TCA, tricarboxylic acid.

chemical expression studies in cultured primary cells from rat brain that NaC3 is expressed in astrocytes, whereas in neurons NaC2 is present (19). Both transporters have a broad substrate specificity and translocate dicarboxylates such as succinate, α-ketoglutarate, malate, and fumarate with high affinity (17, 20). Neurons depend on the anaplerotic supply of these dicarboxylates from astrocytes required as substrates for the tricarboxylic acid (TCA) cycle to maintain energy metabolism and the synthesis of the neurotransmitters glutamate and GABA (21, 22), which might involve sodium-dependent dicarboxylate co-transporters (19, 23). Based on the ability of NaC3 to transport GA and 3OHGA (14), we hypothesized that the increased concentrations of GA and 3OHGA in the brain of GA1 patients during an encephalopathic crisis, and induced in *Gcdh*$^{-/-}$ mice, impair the anaplerotic supply of TCA cycle intermediates from astrocytes to neurons in a competitive manner.

In the present study, we have demonstrated for the first time that GA and 3OHGA inhibit the uptake of [^{14}C]succinate into primary neuronal cells of wild-type and *Gcdh*$^{-/-}$ mice. Most important, the efflux of [^{14}C]succinate from *Gcdh*-deficient astrocytic cells has been found to be strongly retarded compared with wild-type cells.

EXPERIMENTAL PROCEDURES

Materials—GA and succinate were purchased from Fluka (Taufkirchen, Germany). 3OHGA was synthesized as described previously (14). 1,4-[^{14}C]-Labeled succinate was obtained from Moravek Biochemicals (Brea, CA). DNase I, papain, and AraC were purchased from Sigma. Minimal essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), neurobasal A medium, and horse serum were from Invitrogen. Fetal calf serum (FCS) was from PAA Laboratories (Pasching, Austria), and B27 supplement was obtained from Invitrogen. All other chemicals were of analytical grade or higher.

Antibodies—Rabbit anti-glial fibrillary acidic protein (GFAP) antibody was purchased from DAKO Cytomation (Glostrup, Denmark), and mouse anti-neuron-specific nuclear protein (NeuN) antibody was from Millipore. Peroxidase-conjugated goat anti-rabbit IgG was from Dianova (Hamburg, Germany). Sheep anti-mouse IgG coupled to fluorescein isothiocyanate (FITC) and anti-rabbit IgG-Cy3 were from Sigma.

Animals—*Gcdh*$^{-/-}$ or wild-type mice were bred from homozygous (*Gcdh*$^{-/-}$ or *Gcdh*$^{+/+}$, respectively) parents and sacrificed at P0–P2. The genetic background in all mice groups used in this study was C57BL6/SJ129 hybrid. Genotypes were confirmed by PCR and measurement of glutarylcarnitine concentration in dried blood spots as described previously (13). Mice were housed in the animal facility of the University Medical Center with a 12-h light-dark cycle and allowed water and food *ad libitum*. Animal care and experiments were carried out in accordance with institutional guidelines as approved by local authorities.

Cell Culture and Preparation of Primary Cells—The immortalized murine astrocytoma cell line 11$^{+/+}$ was cultured as described previously (24). For the preparation of primary neuronal cells from mouse cortex, *Gcdh*$^{-/-}$ or wild-type pups were sacrificed at P0–P1. Preparation and maintenance of cells were performed according to Ref. 25. After decapitation and removal of vessels and meninges, cortices were removed and washed in Hanks' buffered salt solution (1 mM $MgCl_2$, 5.5 mM glucose, 137 mM NaCl, 5.4 mM KCl, 0.4 mM KH_2PO_4, 2.7 mM $Na_2HPO_4{\cdot}2H_2O$, 4.2 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 1.7 mM CaCl) and subsequently incubated in papain solution (10 mM phosphate-buffered saline (PBS), 10 mM glucose, 50 μl of DNase I, 2.5 mg of papain) for 30 min at 37 °C on a shaker. Afterward, cortices were washed three times in plating medium (0.6% glucose, 10% horse serum in MEM) followed by homogenization of tissue by repeated resuspension through yellow pipette tips. Subsequently, cells were counted and plated at a density of ~500 cells/mm^2 on glass coverslips coated with poly-L-lysine, 12-well plates, or 35-mm dishes. After 4 h, medium was changed to medium 1 (neurobasal A medium supplemented with 2% B27, 0.5 mM glutamine, 25 μM glutamate, 20 units/ml penicillin, and 20 μg/ml streptomycin). Cells were grown in medium 1 for 3 days, maintained another 4 days in medium 2 (medium 1 without glutamate) supplemented with 10 μM AraC, and another 3 days in medium 2 without AraC. Primary neuronal cells were used for experiments after a total of 10 days in culture.

For preparation of astrocytic cells, whole brains of *Gcdh*$^{-/-}$ or wild-type mice at P0–P2 were excised, vessels and meninges removed, and brains washed three times in Hanks' buffered salt solution. Subsequently, brains were dissected in small pieces and homogenized in 5 ml of medium (DMEM supplemented with 10% FCS, 0.6% glucose, 25 mM $NaHCO_3$, 200 nM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin) at 37 °C by passing the tissue repeatedly through a Pasteur pipette. Cells were separated by sequentially passing the cell suspension through 180-, 140-, and 30-μm nylon net filters (Millipore) with a 10-ml syringe. Finally, cells were plated at a density of ~1,000 cells/mm^2. Medium was changed every 2–3 days, and cells were used for experiments 7 days after preparation.

[^{14}C]Succinate Uptake Assay—For uptake experiments, either primary neuronal cells cultured for 10 days or astrocytic cells cultured for 7 days on 12-well plates were used. After washing with prewarmed (37 °C) transport buffer (25 mM HEPES, pH 7.4, containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose), cells were incubated for 10 or 20 min at 37 °C/5% CO_2 with 0.3 ml of uptake buffer (transport buffer supplemented with 0.1 μCi of [^{14}C]-labeled succinate in the absence or presence of 2 mM effectors GA, 3OHGA, or nonlabeled succinate). After removal of uptake buffer, cells were immediately washed three times with ice-cold transport buffer and lysed in 0.2 M NaOH. The amount of cell-associated radioactivity was determined by scintillation counting (Tri-Carb 2900TR liquid scintillation analyzer; Packard, Groningen, The Netherlands or Rotiszint eco plus liquid scintillation mixture; Roth, Karlsruhe, Germany) and related to the protein content (Roti-Quant protein assay; Roth).

[^{14}C]Succinate Efflux Assay—Cells were incubated with [^{14}C]succinate for 20 min as described above. After removal of the radioactive buffer, cells were further incubated in transport buffer for the indicated times. The supernatant was collected, and cells were lysed in 0.2 M NaOH. Radioactivity was determined in supernatant and cell lysates and related to cellular protein content.

HPLC Analyses—The content of cell pellets and supernatants of [^{14}C]succinate efflux assays was determined by anion exchange HPLC analyses using a Mono Q PC 1.6/5 column connected to a SMART system (GE Healthcare). A mobile phase consisting of 10 mM Tris-HCl, pH 8.5, operated at a flow rate of 100 μl/min at ambient temperature was utilized with a gradient of 0–0.25 M NaCl within 20 min. Supernatants from efflux experiments were centrifuged at 20,000× *g* for 10 min at 4 °C to remove cell debris and divided into four aliquots of 50 μl. Cell pellets from efflux experiments were sonicated for 3 × 20 s, and proteins were denatured by incubation of samples for 5 min at 95 °C. Cell debris and denatured proteins were sedimented by centrifugation at 20,000× *g* for 10 min at 4 °C, and the resulting cell extract was divided into four aliquots of 50 μl each. Aliquots of efflux supernatants and cell extracts were diluted 1:20 in mobile phase buffer (10 mM Tris, pH 8.5) and loaded onto the HPLC column. Bound anions were eluted by an NaCl gradient, and fractions of 100 μl were collected. Radioactivity in pooled HPLC fractions of four sequential HPLC runs was determined by liquid scintillation counting. Elution profiles of unlabeled standard anions (1–100 nmol; aspartic acid, fumaric acid, glutamic acid, α-ketoglutaric acid, oxaloacetic acid, sodium citrate, sodium succinate; Sigma) were recorded at 210 nm.

RNA Extraction, cDNA Preparation, and Quantitative RT-PCR (qRT-PCR)—Total RNA was prepared from cell pellets with a TRI®-Reagent RNA preparation kit (Sigma). RNA (1 μg) was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). For qRT-PCR, 6-carboxy-fluorescein dye-labeled murine TaqMan MGB probes (Applied Biosystems) were used in 96-well optical reaction plates, and triplicates were quantified in an Mx3000P® qRT-PCR system (Stratagene). TaqMan assay ID numbers are as follows: NaC2, Mm01334459_m1; NaC3, Mm00475280_m1; Oat1, Mm00456258_m1; β-actin, Mm00607939_s1; GAPDH, Mm99999914_g1. The relative level of each mRNA was determined using the DART-PCR method and software (26).

Other Methods—Protein concentration was determined with the Roti-Quant protein assay (Roth). For double immunofluorescence microscopy, primary astrocytic or neuronal cells were grown on poly-L-lysine-coated glass coverslips, fixed, permeabilized, and stained using rabbit anti-GFAP (1:250) and mouse anti-NeuN (1:50) as primary antibodies as described previously (27). For staining of nuclei with 4,6-diamidino-2-phenylindol (DAPI; Sigma), cells were washed with PBS and subsequently incubated with 200 μl of 5 μg/ml DAPI in PBS for 5 min.

Data Analysis—Data were analyzed using either one-way analysis of variance followed by Scheffé's test or unpaired two-tailed Student's *t* tests as applicable. Significance was accepted at $p < 0.05$. Calculations were operated using SPSS® 15.0 (SPSS, Chicago, IL) and Microsoft® Office Excel 2003 software.

RESULTS

[^{14}C]Succinate Uptake into Astrocytic Cells—Astrocytic cells were isolated from wild-type mouse brain and cultured for 7 days. Double immunofluorescence staining of cultures with astrocyte-specific GFAP and NeuN antibodies was performed.

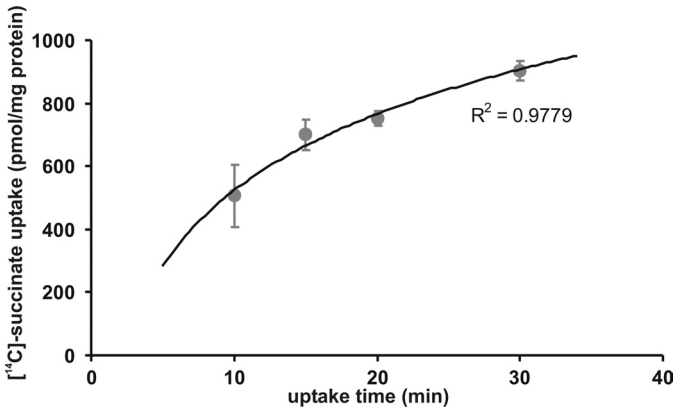

FIGURE 1. **Time course of uptake of [^{14}C]succinate into primary astrocytic cells.** The uptake of [^{14}C]succinate into primary astrocytic cells derived from wild-type mice was measured after the indicated times. *Gray circles* and *bars* represent mean ± S.D., respectively, of two independent experiments performed with cells from two different preparations. Curve fitting (*black line*) was performed by logarithmic regression analysis with a resulting coefficient of determination $R^2 = 0.9779$.

About 80–90% of cells were GFAP-positive, and no anti-NeuN immunoreactivity was observed (supplemental Fig. S1). Staining of primary astrocytic cells from *Gcdh*$^{-/-}$ mice showed similar results (data not shown).

Incubation of primary astrocytic cells prepared from wild-type mice with [^{14}C]succinate revealed a time-dependent internalization of radioactivity (Fig. 1). Logarithmic regression analysis generated a fitted curve, where the time point of 10 min was located in the linear slope region of the curve, whereas the rate of [^{14}C]succinate internalized after 30 min reached the saturation level. Further uptake experiments were performed by incubating cells with [^{14}C]succinate for 10 and/or 20 min as indicated.

The uptake of [^{14}C]succinate into wild-type astrocytic cells required the presence of Na^+ ions (supplemental Fig. S2*A*). An excess of unlabeled succinate (2 mM) inhibited the uptake of [^{14}C]succinate both into wild-type and *Gcdh*$^{-/-}$ astrocytic cells to 8–10% of control during an incubation time of 10 min (Fig. 2*A*), indicating the specificity of [^{14}C]succinate transport into these cells. In the presence of GA (40 μM), succinate (25 μM), and the GA derivative L-2-hydroxyglutaric acid (L2OHGA, 267 μM), concentrations corresponding to the K_m values of NaC3 for these effectors (15), the uptake of [^{14}C]succinate was reduced to 55, 44, and 56%, respectively, of control as expected (Table 1 and supplemental Fig. S2*B*). In contrast, incubation of cells in the presence of 930 μM 3OHGA (corresponding to K_m of 3OHGA for NaC3) showed an inhibition of [^{14}C]succinate uptake to 77.3 ± 22.1% of control. Therefore, the concentration-dependent inhibition of [^{14}C]succinate into primary astrocytic cells derived from wild-type mice in the presence of 3OHGA was tested. The strongest inhibition of [^{14}C]succinate uptake (approximately 50%) was observed in the presence of 2 mM 3OHGA (supplemental Fig. S2*A*). In the presence of 2 mM GA, the uptake of [^{14}C]succinate into wild-type and *Gcdh*$^{-/-}$ astrocytic cells was significantly reduced to 14–20% of controls (Fig. 2*A*). These data suggest that uptake of [^{14}C]succinate into astrocytic cells is sodium-dependent and can be inhibited in a competitive manner by dicarboxylates such as succinate, GA, and 3OHGA.

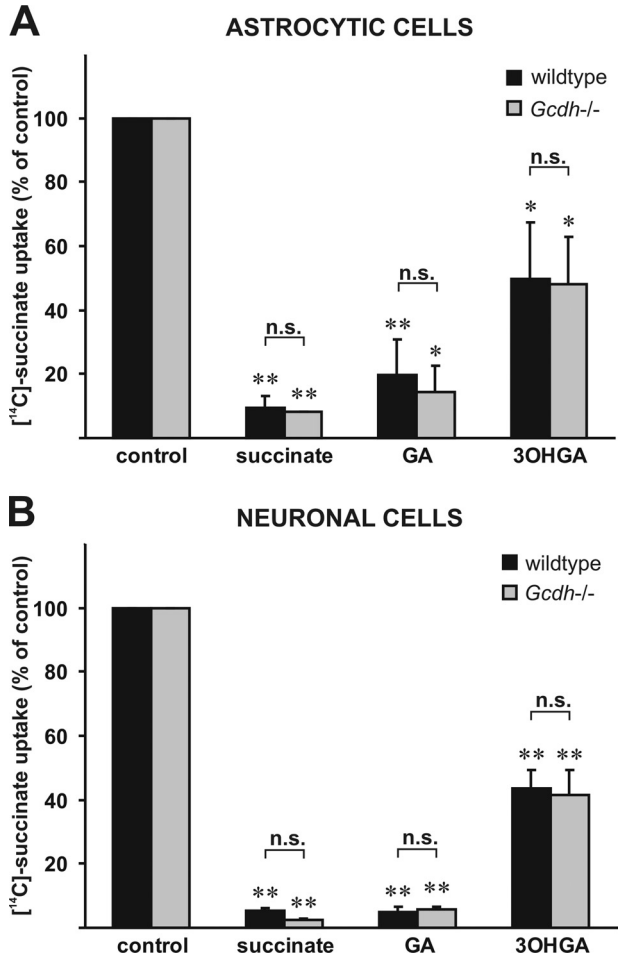

FIGURE 2. **Uptake of [^{14}C]succinate into primary astrocytic and neuronal cells.** Primary cultured astrocytic and neuronal cells derived from wild-type (*black bars*) or *Gcdh*$^{-/-}$ mice (*gray bars*) were incubated with [^{14}C]succinate in the absence (control) and presence of each 2 mM succinate, GA, and 3OHGA. Columns represent mean values ± S.D. of three experiments performed by using cells from three independent cell preparations. Significance was tested by one-way analysis of variance followed by Scheffé's test and accepted at $p < 0.05$. *n.s.*, not significant; *, $p < 0.05$; **, $p < 0.001$ *versus* respective control.

TABLE 1

Dicarboxylate-dependent inhibition of [^{14}C]succinate uptake into astrocytic cells

Compound	K_m[a]	[^{14}C]Succinate uptake[b]
	mM	% of control
GA	0.04 ± 0.02	54.9 ± 12.9
3OHGA	0.93 ± 0.25	77.3 ± 22.1
Succinate	0.025	44.1 ± 9.3
L2OHGA	0.267 ± 0.048	55.9 ± 12.2

[a] According to Ref. 15.
[b] [^{14}C]succinate uptake was determined in the absence (control) or presence of dicarboxylates at concentrations corresponding to their K_m values for NaC3.

GA and 3OHGA Inhibit the Uptake of Succinate into Primary Neuronal Cells—Next, the uptake of [^{14}C]succinate was determined in neuronal cells. Primary neuronal cells prepared from wild-type and *Gcdh*$^{-/-}$ mice cultured for 10 days were 80–90% NeuN-positive (supplemental Fig. S1). The uptake of [^{14}C]succinate into neuronal cells during an incubation time of 10 min was strongly inhibited by an excess of unlabeled succinate and GA to 2–6% of controls (Fig. 2*B*), whereas 2 mM 3OHGA reduced the uptake only to 42–44%. There were no significant differences between astrocytic or neuronal cells from wild-type and *Gcdh*$^{-/-}$ mice in the relative (percent of respective control, Fig. 2) or absolute amount of radioactivity (cpm/mg of protein, data not shown) accumulated.

Efflux of [^{14}C]Succinate Is Retarded in Gcdh$^{-/-}$ Astrocytic Cells—To investigate the efflux of [^{14}C]succinate from the intracellular compartment, primary wild-type or *Gcdh*$^{-/-}$ astrocytic or neuronal cells were preincubated with [^{14}C]succinate for 20 min. After washing, cells were incubated in fresh nonradioactive medium for various time periods. Subsequently, radioactivity was measured in medium and cell lysates (Fig. 3). [^{14}C]Succinate disappeared from primary astrocytic cells of wild-type mice with a half-efflux time ($t_{½}$ efflux) of 5.6 ± 2.6 min upon incubation at 37 °C (Fig. 3*A*). Between 20 and 25% of the initial radioactivity remained intracellular, and 75 to 80% was released into the medium. In contrast, the $t_{½}$ efflux rate of [^{14}C]succinate in astrocytic cells of *Gcdh*$^{-/-}$ mice was significantly retarded to 20.2 ± 8.6 min ($p < 0.05$; Fig. 3*B*). In addition, after 30 min of incubation, only 60% of the initial cell-associated radioactivity was released into the medium. The $t_{½}$ efflux rates of [^{14}C]succinate from neuronal cells prepared from wild-type and *Gcdh*$^{-/-}$ mice were not significantly different (13.5 ± 9 min *versus* 12.6 ± 2.7 min, respectively; Fig. 3, *C* and *D*). Of note, the percentage of radioactivity remaining in neuronal cells after 30 min comprised approximately 40% in both wild-type and *Gcdh*$^{-/-}$ cells, respectively. The direct comparison of $t_{½}$ efflux rates of [^{14}C]succinate from astrocytic and neuronal cells of wild-type and *Gcdh*$^{-/-}$ mice is summarized in supplemental Fig. S3.

Effused Material Represents [^{14}C]Succinate—To elucidate whether the measured radioactivity effused from astrocytic and neuronal cells represents [^{14}C]succinate or metabolized [^{14}C]-labeled compounds, HPLC analyses of efflux media and cell extracts were performed. First, anionic exchange HPLC conditions were established allowing the separation of the TCA cycle intermediates succinate (fractions 9 and 10), α-ketoglutarate (fractions 11 and 12), fumarate (fraction 13), and citrate (fractions 15 and 16) as well as aspartate/glutamate (both co-eluting in overlapping fractions 4 and 5; supplemental Fig. S4). [^{14}C]Succinate co-eluted with unlabeled succinate in fractions 9 and 10.

HPLC analyses of cell extracts of efflux experiments from wild-type astrocytic cells revealed that after loading (efflux t = 0 min) 69% of radioactivity bound to the column represented intracellular [^{14}C]succinate, whereas 17, 2, and 5% of total column-bound radioactivity co-eluted with aspartate/glutamate, fumarate, and citrate, respectively. These data indicate that intracellular [^{14}C]succinate was partially metabolized to these compounds (Fig. 4*A*). After 10 and 20 min (data not shown) and 30 min of efflux (Fig. 4*B*), the amount of intracellular radioactivity co-eluting with succinate was reduced to 33–39% of total column-bound radioactivity, whereas the proportion of bound radioactivity co-eluting with aspartate/glutamate, fumarate, and citrate was 35–43, 3, and 7%, respectively. HPLC analyses of efflux media from wild-type astrocytic cells after 10- and 20-min (data not shown) and 30-min efflux (Fig. 4*C*) showed that the majority (86–91%) of radioactivity co-eluted with unlabeled succinate in fractions 9 and 10. These data provide evi-

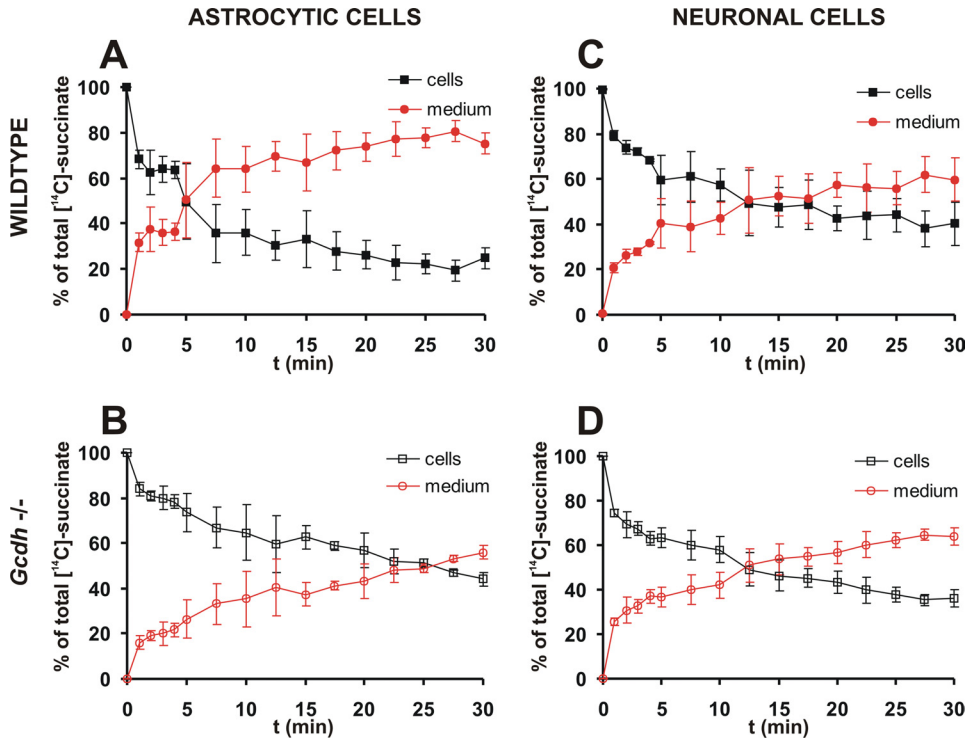

FIGURE 3. **Efflux of [^{14}C]succinate from wild-type and *Gcdh*$^{-/-}$ astrocytic and neuronal cells.** Primary astrocytic cells prepared from wild-type (*A*) and *Gcdh*$^{-/-}$ (*B*) mice were first loaded with [^{14}C]succinate for 20 min. After removal of radioactive medium, cells were either harvested ($t = 0$ min) or further incubated in unlabeled medium for the indicated times. Primary neuronal cells prepared from wild-type (*C*) and *Gcdh*$^{-/-}$ (*D*) mice were analyzed as described above. The radioactivity was measured in cells (■, wild-type; □, *Gcdh*$^{-/-}$) and medium (*red* ●, wild-type; *red* ○, *Gcdh*$^{-/-}$), respectively, and expressed as percentage of total radioactivity in cells and medium. The diagrams show mean values ± S.D. of four (time points 1, 2, 3, and 4 min), or six (time points 0 and 5–30 min) experiments performed by using cells from four or six independent cell preparations.

dence that virtually all of the effused radioactivity represents [^{14}C]succinate. HPLC analyses of media and cell pellets from *Gcdh*$^{-/-}$ astrocytic cells revealed similar results (data not shown).

HPLC analyses of efflux media and cell extracts from wild-type neuronal cells revealed that the majority of effused radioactivity in the media after 10 and 20 min (data not shown), and 30 min (Fig. 4*F*) corresponds to [^{14}C]succinate, representing 67–82% of total column-bound radioactivity. Compared with wild-type astrocytic cells, a higher proportion of effused radioactivity co-eluted with aspartate/glutamate (neuronal cells, Fig. 4*F*: 9–15%, *versus* astrocytic cells, Fig. 4*C*: 3–6% of column-bound radioactivity). Additionally, the proportion of cellular radioactivity at 0 min (Fig. 4*D*), after 10 and 20 min (not shown) and 30 min of efflux (Fig. 4*E*) co-eluting with aspartate/glutamate was higher (59–71% of column-bound radioactivity) compared with wild-type astrocytic cells (16–43% of column-bound radioactivity; Fig. 4, *A* and *B*). This indicates that compared with astrocytic cells, neuronal cells display a higher rate of intracellular metabolism of [^{14}C]succinate. It is unclear whether the increased level of metabolites (aspartate/glutamate) in the efflux media from neuronal cells results from an increased capacity for transport of these substrates, or merely reflects their increased rate of intracellular production. Analyses of efflux media and cell extracts of *Gcdh*$^{-/-}$ neuronal cells provided results similar to those of wild-type neuronal cells (data not shown).

Expression of NaC2, NaC3, and Oat1 in Primary Astrocytic and Neuronal Cells—It has previously been reported that NaC3 and NaC2 are only expressed in primary cultures of rat cerebrocortical astrocytes and neurons, respectively (19). Therefore, we tested the expression of NaC2- and NaC3-specific mRNA in primary cultured murine astrocytic and neuronal cells used in the [^{14}C]succinate uptake and efflux experiments. The mRNA expression of NaC2 as well as NaC3 was comparable between wild-type astrocytic and neuronal as well as *Gcdh*$^{-/-}$ astrocytic cells (supplemental Fig. S5). In contrast, the mRNA expression of both NaC2 and NaC3 was 2.2- and 4.2-fold up-regulated, respectively, in *Gcdh*$^{-/-}$ neuronal cells compared with wild-type neuronal cells. Of note, in the primary brain cell model used in this study, both astrocytic and neuronal cells displayed mRNA expression of NaC2 as well as NaC3. The murine astroglial cell line 11$^{+/+}$ was used as a control and displayed neither NaC2- nor NaC3-specific mRNA expression (supplemental Fig. S5). The organic anion transporter Oat1 is described to be expressed in both kidney and brain tissue (18, 28, 29, supplemental Table 1). Using quantitative RT-PCR, no Oat1 transcripts were detected in wild-type or *Gcdh*$^{-/-}$ astrocytic cells whereas both wild-type and *Gcdh*$^{-/-}$ neuronal cells express similar levels of Oat1 mRNA (supplemental Table 1).

DISCUSSION

Production of neurotransmitters glutamate and GABA in neurons requires a continuous supply of TCA cycle intermediates such as α-ketoglutarate and succinate. However, due to a

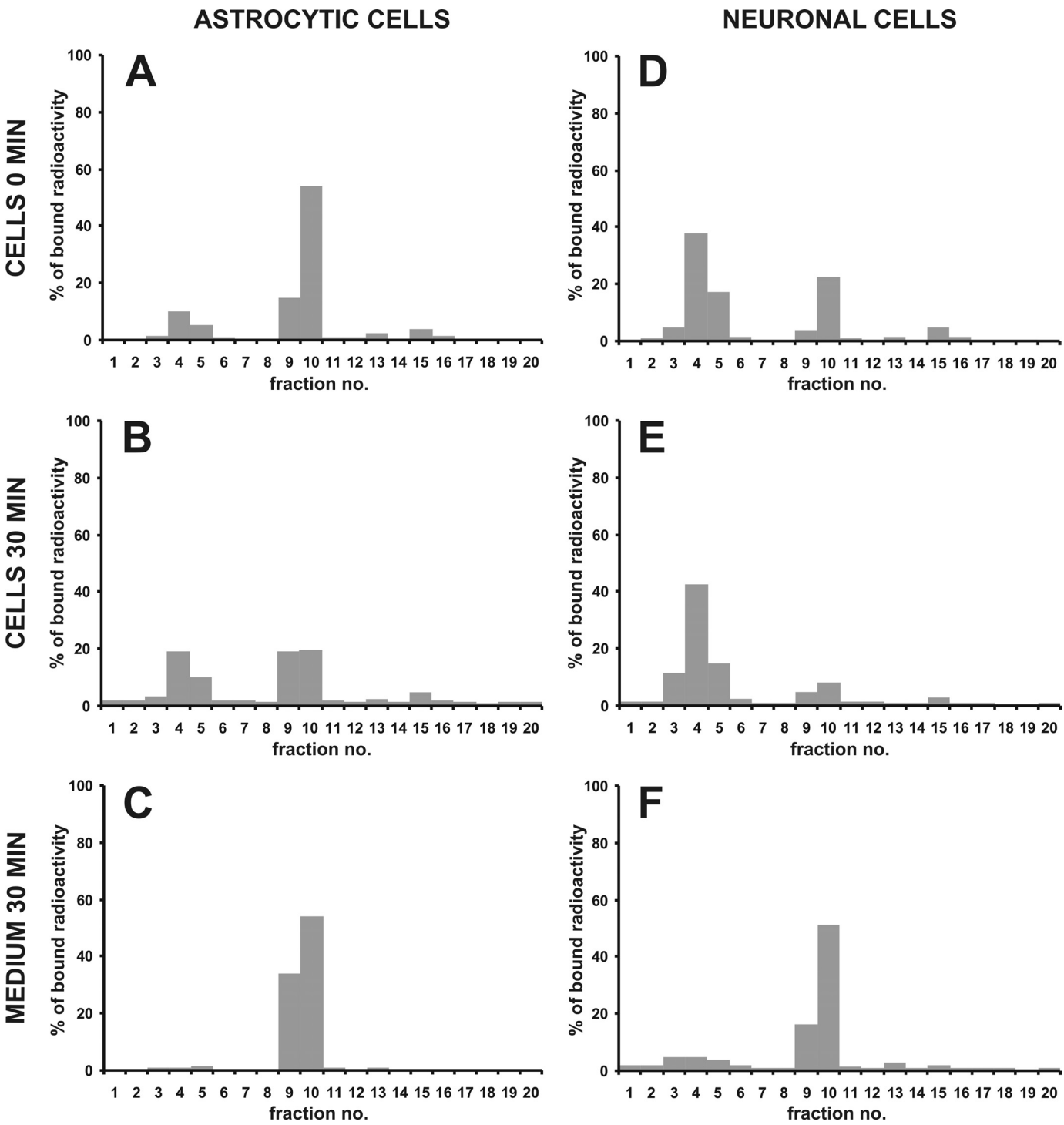

FIGURE 4. **HPLC analyses of cell extracts and media from efflux experiments with wild-type astrocytic and neuronal cells.** Efflux experiments were performed as described under "Experimental Procedures" and in the legend to Fig. 3. Extracts of wild-type astrocytic cells at $t = 0$ (*A*) and after 30 min (*B*) of efflux experiments and the corresponding medium after 30 min of efflux (*C*) were fractionated by anionic exchange HPLC, and the eluted radioactivity was determined in each fraction and expressed as percentage of total radioactivity bound to the column. The majority of effused radioactivity in the medium co-elutes with succinate, whereas the proportion of radioactivity co-eluting with aspartate/glutamate remains intracellular. Extracts of wild-type neuronal cells at $t = 0$ (*D*) and after 30-min incubation (*E*) and corresponding medium after 30 min of efflux (*F*) were analyzed identically.

lack of pyruvate carboxylase activity neurons are unable to produce these metabolites endogenously. As a result, normal neuron function is dependent on the import of metabolites produced by astrocytes to replenish TCA cycle intermediates (anaplerosis). The import of these TCA cycle intermediates by neurons is dependent on the Na^+-coupled transporters NaC2 and NaC3 (17, 23, 30).

Recently, we have shown that NaC3 is capable of transporting the dicarboxylates GA and 3OHGA (14, 15), pathologic metabolites that accumulate in body fluids and tissues, espe-

cially in the brain, of GCDH-deficient patients and *Gcdh*$^{-/-}$ mice (1, 11). Because the presence of GA and 3OHGA could be of importance for the anaplerotic supply of TCA cycle intermediates by astrocytic cells, their potential interference with [^{14}C]succinate transport was investigated in cultured neuronal and astrocytic cells of wild-type and *Gcdh*$^{-/-}$ mice.

To establish the experimental conditions for *in vitro* [^{14}C]succinate transport into cultured brain cells, the inhibitory effects of various dicarboxylates were tested at concentrations corresponding to their K_m values for NaC3 (15). GA, succinate, and L2OHGA inhibited [^{14}C]succinate uptake into astrocytic cells as expected (55, 44, and 56%, respectively), inhibition by 3OHGA was less than expected (23%). Dose-response experiments revealed that uptake of [^{14}C]succinate into astrocytic cells was inhibited by succinate and GA in a concentration-dependent manner, and to a lesser extent by 3OHGA (50% inhibition at 2.1-fold K_m value). An 80- and 50-fold molar excess of the K_m value for succinate and GA, respectively, led to an almost complete inhibition of [^{14}C]succinate uptake into astrocytic cells. Therefore, the inhibition of [^{14}C]succinate uptake by the structurally similar dicarboxylates GA, succinate, and 3OHGA can be considered as competitive. To facilitate the comparison between uptake experiments in wild-type and *Gcdh*$^{-/-}$ neuronal and astrocytic cells, 2 mM GA and 3OHGA were used in the *in vitro* transport assays. This level of GA is similar to cerebral concentrations reported in *post mortem* biopsy material from GA1 patients and the brains of *Gcdh*$^{-/-}$ mice (500–5,000 μM (11, 31), whereas the 3OHGA concentration used in the present study is 10-fold higher than reported in GA1 brain tissue (31). The conditions of these short term *in vitro* transport assays are not directly comparable with what occurs *in vivo*, and therefore the significance of the differences between the level 3OHGA used in these experiments and those seen *in vivo* is uncertain. The levels of GA and 3OHGA are known to increase during periods of catabolism, but there are very few data on specific brain levels at such times.

The major finding of the present study is that both GA and 3OHGA inhibit the sodium-dependent uptake of [^{14}C]succinate by cultured mouse neuronal and astrocytic cells, suggesting that the neurotoxicity in GA1 may in part result from disruption of anaplerosis by the high levels of GA and 3OHGA that accumulate in the brains of affected patients (1). Recently, we have shown that NaC3 is capable of transporting both GA and 3OHGA (14, 15). The ability of these metabolites to inhibit sodium-dependent [^{14}C]succinate uptake in our assays suggests that NaC3 or the closely related NaC2 may mediate this transport. Quantitative RT-PCR demonstrated that in neuronal cells derived from *Gcdh*$^{-/-}$ mice the expression of NaC3 mRNA was significantly higher than in cells from wild-type animals, similar to previous observations in the kidney (14). The up-regulation of NaC3 in brain cells from *Gcdh*$^{-/-}$ mice may represent an adaptive response to a reduced supply of TCA cycle intermediates resulting from inhibition of their uptake by elevated GA and 3OHGA. Despite these differences in NaC3 expression the extent of inhibition of sodium-dependent [^{14}C]succinate uptake by GA and 3OHGA was similar in cells from wild-type and *Gcdh*$^{-/-}$ mice.

The second important result of this report is the reduced efflux rate of [^{14}C]succinate from *Gcdh*$^{-/-}$ astrocytic cells, which may be of physiological relevance for anaplerotic reactions to replenish TCA cycle intermediates in neurons. Due to the inwardly directed sodium gradient NaC2 and/or NaC3 can be excluded to mediate the succinate efflux from astrocytic cells. This is supported by an observation showing that intracellular injected [^{14}C]succinate is not transported outward in NaC3-expressing oocytes.[4] Although the identity of the transporter mediating the efflux of succinate from astroglial cells is not known, a member of the organic anion transporter (OAT) family, Oat1, was considered a potential candidate (32). Expression studies provided evidence that Oat1 transports both succinate as well as GA and 3OHGA across membranes (15, 33) and is expressed in the brain (34–36). Quantitative mRNA expression analyses, however, in primary cultured brain cells from wild-type and *Gcdh*$^{-/-}$ mice demonstrated that Oat1 was expressed in neuronal cells only. Therefore, it appears unlikely that Oat1 plays a role in the efflux of TCA cycle intermediates from astrocytic cells, and further studies are necessary to identify the responsible transporter.

The reduced efflux rate of succinate from *Gcdh*$^{-/-}$ astrocytic cells might be either the result of a *cis*-inhibition by the dicarboxylates GA and/or 3OHGA accumulating intracellularly (15), or of the reduction of the transporter number at the plasma membranes of *Gcdh*$^{-/-}$ astrocytic cells. The molecular mechanisms responsible for the reduced export of [^{14}C]succinate from astrocytic cells of *Gcdh*$^{-/-}$ mice remain to be further investigated.

The biphasic shape of the [^{14}C]succinate efflux curve in astrocytic as well as neuronal cells appears to be composed by a rapid initial [^{14}C]succinate efflux rate ending in a reduction of intracellular radioactivity and the intracellular metabolization of [^{14}C]succinate into other compounds such as glutamate and aspartate less capable to effuse from the cells, paralleled with a corresponding second efflux phase of continuous low release of [^{14}C]succinate.

Taken together, our results show that the export of the TCA cycle intermediate succinate from *Gcdh*$^{-/-}$ astrocytic cells is impaired. Furthermore, its subsequent uptake into neuronal cells, which is most likely mediated by NaC2 and/or NaC3 (17, 23, 30; this report), can be inhibited by GA and 3OHGA accumulating in the extracellular fluid. These data suggest that the increased levels of GA and 3OHGA in the brain of *Gcdh*$^{-/-}$ mice affect the anaplerotic supply of TCA cycle intermediates by astrocytic cells and their neuronal uptake. It is tempting to speculate that a subsequent depletion of TCA cycle intermediates may impair the generation of both ATP and neurotransmitters, finally causing cell death. The present data provide an experimental explanation for pathogenic biochemical alterations in patients with GA1 that lead to neuronal degeneration during encephalopathic crises, a condition with increased demands of energy-rich substrates. In agreement with our results, strongly reduced levels of cortical ATP and GABA have been observed during diet-induced encephalopathic crises in young *Gcdh*$^{-/-}$ mice (37).

[4] B. C. Burckhardt, unpublished observation.

Acknowledgment—We thank R. Kleene (Center for Molecular Neurobiology, University of Hamburg) for advice in astrocytic cell preparation.

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