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Cerebral organic acid disorders induce neuronal damage via excitotoxic organic acids *in vitro*

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Summary. Glutaryl-CoA dehydrogenase deficiency (GDD), which is one of the most frequent organic acid disorders, is characterized by a specific age- and regional-dependent neuropathology. We hypothesized that the distinct brain damage in GDD could be caused by the main pathologic metabolites, the organic acids glutaric (GA) and 3-hydroxyglutaric (3-OH-GA) acids, through an excitotoxic sequence. Therefore, we investigated the effects of 3-OH-GA and GA on primary neuronal cultures from chick embryonic telencephalons. Here we report that 3-OH-GA and GA decreased cell viability concentration- and time-dependently, which could be only totally prevented by preincubation with MK-801, ifenprodil and NR2B antibodies. Furthermore, cell viability decreased in parallel with the increasing expression of NR2B subunit on cultured neurons from 2nd to 6th DIV. We conclude that GA and 3-OH-GA act as excitotoxic organic acids (EOA) specifically through NR1/NR2B and that the extent of induced neurotoxicity is dependent on NR1/NR2B expression during maturation.

Keywords: Amino acids – Organic acids – Glutaryl-CoA dehydrogenase deficiency – Excitotoxicity – NMDA receptor

Introduction

GDD (synonymous: glutaric acidura type I; McKusick 231670) is the 'classic' representative of cerebral organic acid disorders, a distinct group of metabolic diseases that are characterized by a distinct (progressive) neuropathology (Hoffmann et al., 1991, 1994). The autosomal recessive inherited deficiency of the mitochondrial enzyme glutaryl-CoA dehydrogenase (EC 1.3.99.7) in the catabolic pathway of lysine, hydroxylysine and tryptophan causes an accumulation of GA, 3-OH-GA and glutaconic acids in body fluids as well as in brain tissue (Goodman et al., 1977; Baric et al., 1998). Typically between the 6th and 18th month of age an unspecific illness or vaccination triggers an acute

encephalitis-like encephalopathic crisis leading to an irreversible bilateral destruction especially of the striatum and consequently to a severe dystonic-dyskinetic disorder with loss of previously acquired motor skills (Hoffmann et al., 1991, 1996). The risk of severe brain damage declines with age: no acute encephalopathic crises have been reported in children with GDD who had survived the first 5–6 years unharmed.

The mechanism of the age-specific destruction of specific cerebral structures in GDD has been subject of intense debates and different hypotheses (Stokke et al., 1976; Heyes, 1977). Substantial evidence points to an excitotoxic sequence. Both, GA and 3-OH-GA exhibit structural similarities to the excitotoxic amino acid glutamate. *Post mortem* examination of the basal ganglia and cortex in GDD patients has revealed postsynaptic vacuolation similar to that of glutamate-mediated damage (Goodman et al., 1977).

Excitotoxicity is transmitted primarily through NMDA receptors consisting of a Na^+, K^+ and Ca^{2+} ion channel formed by assembly of the obligatory NR1 subunit and any one of four modulatory NR2 subunits (NR2A-D) (Choi, 1988; Scatton, 1993). The two major NR2 subunits, NR2A and NR2B, reveal different physiological and pharmacological characteristics (Williams, 1993; Priestley et al., 1994). Studies in rats have shown that NR2B is the prevailing subunit in the rat brain during prenatal and early postnatal development and is highly expressed in forebrain areas (striatum, cortex), whereas NR2A is predominantly present in the adult brain and ubiquitously expressed (Portera-Cailliau et al., 1996; Wenzel et al., 1997). Stimulated NR1/NR2B mediates longer duration excitatory postsynaptic potentials than NR1/NR2A (Tsumoto et al., 1987; Carmignoto and Vicini, 1992; Hestrin, 1992; Monyer et al., 1994; Ramoa and McCormick, 1994). As a consequence the NR1/NR2B-dominated immature brain is more susceptible to excitotoxicity than the NR1/NR2A-dominated adult brain (McDonald et al., 1988, 1997).

We hypothesize that the age- and regional-specific brain damage observed in GDD is caused by NMDA receptor-mediated excitotoxic neuronal damage and investigated the effects of GA and 3-OH-GA on cultured neuronal cells and especially the interaction with NR1/NR2B.

Material and methods

Cell cultures

Primary neuronal cultures from 7-day-old chick embryo telencephalons were prepared as previously described by Pettmann et al. (1979). Briefly, cerebral hemispheres were dissociated through nylon meshes (48μm mesh width). The suspension was seeded onto poly-L-lysine (Sigma, Deisenhofen, Germany)-coated culture flasks (Nunc, Hamburg, Germany) with a density of 4×10^4 cell/cm^2. Cell cultures were maintained in Dulbecco's modified Eagle medium supplemented with 20% fetal bovine serum at 37°C and 5% CO_2 in a humidified atmosphere (DMEM and FBS; Life Technologies, Eggenstein, Germany). Every 2 days the medium was changed and the cells were used for the experiments between 2nd to 6th days *in vitro* (DIV).

Drug treatment

Glutaric acid (Sigma) and 3-hydroxyglutaric acid (Prof. Buckel, Institute of Microbiology, Philipps University of Marburg, Germany) were dissolved in the respective culture media, and the antagonists ifenprodil (Sigma), MK-801, CNQX, L-AP3 (Tocris, Bristol, U.K.), L-NAME, and CGS-19755 (RBI, Natick, MA, U.S.A.) in PBS. Cell cultures were incubated for 1–24 h with different concentrations (0.1–5 mM) of 3-OH-GA and GA after 5 DIV. Antagonists were added 1 h prior to the application of GA and 3-OH-GA.

Trypan-blue exclusion method

Cell viability was determined by trypan blue (0.4% in PBS) exclusion method. The uptake of trypan blue indicates membrane leakage, an endpoint of neuronal degeneration. Cells were washed once with PBS and were then exposed to trypan blue for a period of 5 min. The number of stained (non-viable) and unstained (viable) cells were counted under a microscope and the level of cell viability was expressed as percent ratio of unstained vs. total number of cells. Only darkly stained neurons were considered damaged. A total number of 500–600 neurons were counted in 8 randomly chosen subfields per group. Cell counts were performed without knowledge of the preceding treatment.

LDH release

Cell viability was also determined by LDH release (Koh and Choi, 1987). After exposure to 0.1–5 mM GA or 3-OH-GA for 24 h the intra- and extracellular LDH activity was determined by a LDH assay kit (Sigma) based on the optimized method as recommended by the German Society for Clinical Chemistry. LDH release was calculated as percent ratio of $\Delta\varepsilon$/min of the extra-vs. the intracellular LDH activity. Controls were taken as 100 per cent.

Protein gel electrophoresis and Western blotting

Cells were rinsed with PBS and lysed in Tris-buffered saline containing SDS, glycerin, and protease inhibitors. Protein contents of samples (n = 3 culture flasks per group) which were collected on DIV 2–6 and after long-term exposure (2 and 5 days) to 0.01–0.1 mM 3-OH-GA or GA, respectively, were determined using a protein assay kit (Sigma) based on the Lowry method. Samples were supplemented with 2-mercaptoethanol and denaturated at 95°C for 5 min. SDS-PAGE was performed according to the technique of Laemmli (1970) using 7.5% gels with 30 μg protein per lane which were transferred to nitrocellulose membranes (Protran BA 83; Schleicher & Schüll, Dassel, Germany). To ensure that equivalent amounts of protein were loaded in each lane and that transfer was comparable, membranes were stained with Ponceau S. Non-specific binding was blocked with blocking buffer containing 5% milk powder, 2% BSA and 0.02% Tween 20 in PBS at room temperature for 3 h. Subsequently, membranes were incubated with polyclonal rabbit anti-rat NR2B antibodies (Molecular Probes, Eugene, OR, U.S.A.), diluted 1:200 in blocking buffer, overnight at 4°C. The next day, membranes were washed in washing buffer (0.05% Tween 20 in PBS) and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Promega, Madison, WI), diluted 1:2,500 in blocking buffer, at room temperature for 1 h. After several rinses in washing buffer, immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (SuperSignal; Pierce, Rockford, IL, U.S.A.).

Results

Incubation with 0.1–5 mM GA and 3-OH-GA decreased cell viability in chick embryonic neuronal cultures, depending on metabolite concentration

Table 1. Concentration-dependent decrease in neuronal cell viability induced by 3-OH-GA and GA after an incubation period of 24 h

	Cell viability (%)
Control	92.9 ± 1.9
3-OH-GA 0.1 mM, 24 h	87.8 ± 3.0
3-OH-GA 0.5 mM, 24 h	76.3 ± 5.8*
3-OH-GA 1.0 mM, 24 h	60.9 ± 6.3*
3-OH-GA 5.0 mM, 24 h	54.1 ± 5.9*
GA 0.1 mM, 24 h	81.5 ± 3.1*
GA 0.5 mM, 24 h	75.7 ± 3.8*
GA 1.0 mM, 24 h	67.1 ± 4.6*
GA 5.0 mM, 24 h	65.6 ± 4.4*

All values are given as means ± S.D. of eight experiments. *$P < 0.001$ (3-OH-GA or GA vs. control; ANOVA-1 followed by Scheffé's test).

Table 2. Time-dependent decrease in neuronal cell viability induced by 3-OH-GA and GA after incubation periods of 1 to 24 h

	Cell viability (%)	
Control	91.9 ± 2.6	
3-OH-GA 1.0 mM, 1 h	75.1 ± 5.2*	
3-OH-GA 1.0 mM, 4 h	71.9 ± 4.2*	]*]*
3-OH-GA 1.0 mM, 24 h	61.9 ± 3.6*	
GA 1.0 mM, 1 h	79.6 ± 3.6*	
GA 1.0 mM, 4 h	76.0 ± 2.7*	]*]*
GA 1.0 mM, 24 h	69.2 ± 2.1*	

All values are given as means ± S.D. of eight experiments. *$P < 0.001$ (3-OH-GA or GA vs. control or as indicated; Student's t test).

(Table 1) and length of incubation period (Table 2). By the trypan blue exclusion method, a significant decrease in cell viability was observed after 1 h at concentrations of ≥0.5 mM GA or ≥0.75 mM 3-OH-GA, and after 24 h at concentrations of ≥0.1 mM GA and ≥0.5 mM 3-OH-GA, respectively. Maximum decrease of cell viability was found at 5 mM for both metabolites, but there was only little further reduction in cell viability when concentrations of GA or 3-OH-GA were raised above 1 mM, at incubation times of either 1 or 24 h. A direct comparison of GA or 3-OH-GA in sister cultures (incubation time 24 h) showed that 3-OH-GA induced a more pronounced decrease in cell viability at concentrations of ≥1 mM than GA ($P < 0.001$, 3-OH-GA vs. GA, Student's t test). The difference between 3-OH-GA and GA concerning neurotoxicitiy was confirmed by two-way analysis of variance ($P < 0.001$; 3-OH-GA vs. GA). By LDH release it was shown that GA- and 3-OH-GA-

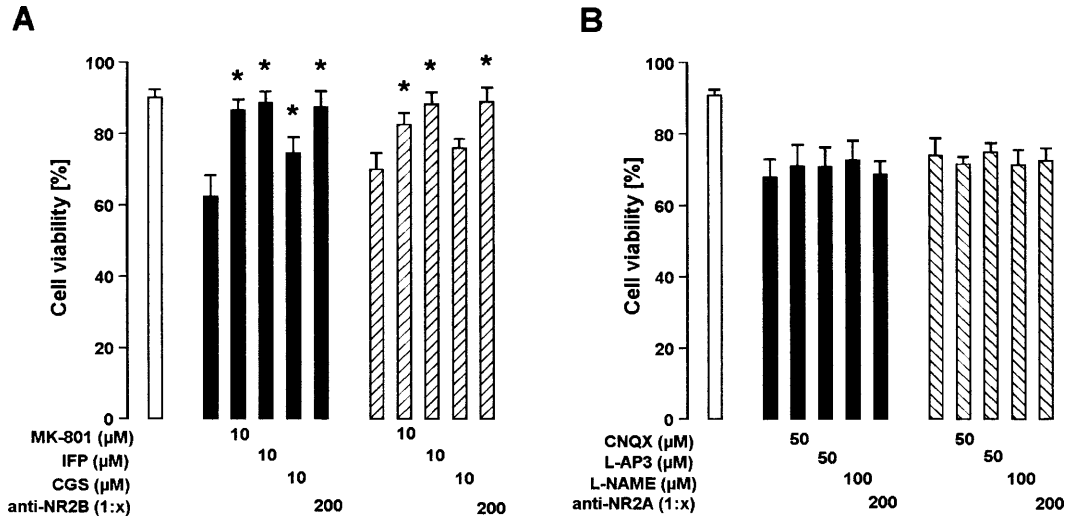

Fig. 1. Effect of different antagonists and antibodies on 3-OH-GA- and GA-induced neuronal damage. All antagonist and antibodies were preincubated 1 h before 1 mM 3-OH-GA or GA were added to cultured chick embryonic neurons. Cell viability was determined by trypan blue exclusion method. **A** Effect of different NMDA receptor antagonists and NR2B antibodies. Solid bars indicate effect on 3-OH-GA-induced, hatched bars the effect on GA-induced neuronal damage. *$P < 0.001$ (1 mM 3-OH-GA or GA vs. antagonists plus 1 mM 3-OH-GA or GA, ANOVA-1 followed by Scheffé's test) *IFP* ifenprodil, *CGS* CGS 19755. **B** Effect of other antagonists and NR2A antibodies. Solid bars indicate effect on 3-OH-GA-induced, hatched bars the effect on GA-induced neuronal damage. No significant inhibition could be found (1 mM 3-OH-GA or GA vs. antagonists plus 1 mM 3-OH-GA or GA, ANOVA-1 followed by Scheffé's test)

induced efflux of LDH increased concentration-dependently at concentrations of ≥0.5 mM GA (138.8 ± 7.3%) or 3-OH-GA (141.2 ± 22.3%), respectively, after 24 h in comparison to control (100 ± 10.4%), reaching a maximum at 5 mM 3-OH-GA (210.6 ± 5.3%) as well as 5 mM GA (185.9 ± 7.3%).

To determine whether 3-OH-GA- and GA-induced neuronal damage was mediated through NMDA receptors, we preincubated primary neuronal cultures from chick embryonic telencephalons with different antagonists and antibodies 1 h prior to the application of 1 mM 3-OH-GA or GA. Cell viability was measured 24 h later. Both 3-OH-GA- and GA-mediated neuronal damage could be completely blocked by MK-801 (unspecific NMDA receptor antagonist), ifenprodil (NR2B-specific antagonist, Williams, 1993; Priestley et al., 1994) and NR2B antibodies, and only partially with CGS-19755 (NMDA receptor antagonist that is 3- fold more selective for NR2A than for NR2B, Priestley et al., 1994) (Fig. 1A). It was not prevented by NR2A antibodies, CNQX (AMPA receptor antagonist), L-AP3 (metabotropic glutamate receptor antagonist), or L-NAME (NO-synthase inhibitor) (Fig. 1B). CGS-19755 only blocked 3-OH-GA-induced but not GA-induced neuronal damage and was less potent in inhibiting 3-OH-GA- and GA-induced neuronal

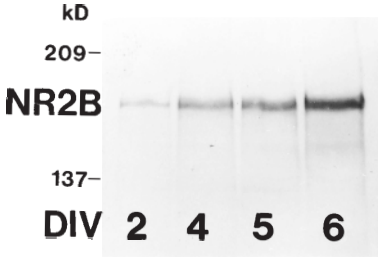

Fig. 2. Expression of NR2B subunit from 2nd to 6th DIV. Expression of NR2B subunit increased from 2nd to 6th DIV as determined by SDS-PAGE (7.5% gel) and Western blotting with polyclonal NR2B anti-bodies (1:200) and anti-rabbit IgG coated with horseradish-peroxidase (1:2,500). NR2B subunit was visualized by enhanced chemiluminescence

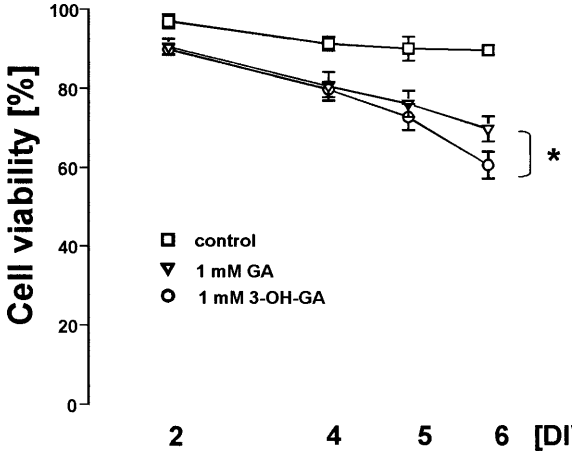

Fig. 3. Maturation-dependent susceptibility to 3-OH-GA- and GA-induced neuronal damage. 3-OH-GA- and GA-induced neuronal damage increased from 2nd to 6th DIV. Cultured chick embryonic neurons were incubated with 1 mM 3-OH-GA (open circles) or GA (open triangles) on different DIV. On each DIV 3-OH-GA- and GA-induced neuronal damage was different from control (open squares) ($P < 0.001$, 3-OH-GA or GA vs. control, Student's t test). Decrease in cell viability was more pronounced after an incubation with 1 mM 3-OH-GA than 1 mM GA (*$P < 0.001$, 3-OH-GA vs. GA, ANOVA-2)

damage than MK-801 and ifenprodil ($P < 0.001$, MK-801 or ifenprodil vs. CGS-19755, Student's t test). Ifenprodil, MK-801 and CGS-19755 inhibited 3-OH-GA- and GA-induced cell damage in a concentration-dependent manner. 3-OH-GA- and GA-induced increases in LDH release were likewise prevented by preincubation with ifenprodil (data not shown).

We investigated the expression of NR2B receptor subunits on cultured chick embryonic neurons from 2nd to 6th DIV. The NR2B subunit was expressed on cultured neurons and its expression increased strongly from 2nd to 6th DIV (Fig. 2). Exposition of cultured chick embryonic neurons to 1 mM GA or 3-OH-GA at different DIV showed a marked increase of susceptibility to 3-OH-GA- and GA-induced neuronal damage from 2nd to 6th DIV (Fig. 3).

This change was more pronounced for 3-OH-GA than for GA ($P < 0.001$, 3-OH-GA vs. GA, ANOVA-2).

Discussion

The major findings of the present study are the induction of neuronal damage by the organic acids 3-OH-GA and GA, acting as false neurotransmitters through NMDA receptors *in vitro*, and the dependency of induced neuronal damage on the extent of NR2B subunit expression.

Concentrations of 3-OH-GA and GA which were shown to induce neuronal damage in cell culture systems in vitro are similar to those found in body fluids of patients with GDD, in whom 3-OH-GA and GA levels in plasma and cerebrospinal fluid are usually between 5 and 400 μM (Hoffmann et al., 1991; 1996; Merinero et al., 1995) but may rise considerably higher during encephalopathic crises (Goodman et al., 1977; Land et al., 1992). This indicates that primary neuronal cultures from chick embryonic telencephalons (Pettmann et al., 1979) is a valid in vitro model system for the investigation of the effects of 3-OH-GA and GA on neurons. Previously, in organotypic brain slice cultures a neurotoxic effect of 3-OH-GA could only be demonstrated at irrelevant high concentrations and none of GA, suggesting an inadequate sensitivity to 3-OH-GA and GA of this system (Flott-Rahmel et al., 1997). However, our results indicate that both metabolites and not only 3-OH-GA are relevant neurotoxins in GDD.

Neuropathological mechanisms previously suggested an inhibition of brain glutamate decarboxylase by 3-OH-GA and GA resulting in an impaired production of GABA, a reduction of glutamate uptake into synaptosomes or unspecific neurotoxic mechanisms (Bennett et al., 1973; Stokke et al., 1976; Flott-Rahmel et al., 1997). We now demonstrate a specific inhibition of 3-OH-GA- and GA-induced neuronal damage after preincubation with MK-801, ifenprodil and NR2B antibodies, but not with CNQX, L-AP3, L-NAME or NR2A antibodies. This finding is consistent with the hypothesis that 3-OH-GA and GA act as false neurotransmitters – instead of glutamate – specifically through NR1/NR2B, and is in line with the previous observation that histological abnormalities found in in the basal ganglia of GDD patients *post mortem* resemble glutamate-mediated damage (Goodman et al., 1977).

In previous studies it has been shown that the expression of different NR2 subunits is age- and regional-dependent (Monyer et al., 1994; Portera-Cailliau et al., 1996; Wenzel et al., 1997). The NR1/NR2B-dominated immature brain is more susceptible to NMDA-induced neurotoxicity than the NR1/NR2A-dominated mature brain (McDonald et al., 1988, 1997). Therefore, our findings of an increase in 3-OH-GA- and GA-induced neurotoxicity together with an increasing expression of NR2B subunit on neurons during the culture period is in line with the assumption of maturation-dependent neurotoxicity of 3-OH-GA and GA. Why the striatum is the main structure involved in acute encephalopathic crises remains to be clarified. One possible explanation is a high expression of NR1/NR2B during a vulnerable period of time (Monyer et al., 1994; Portera-Cailliau et al., 1996; Wenzel et al., 1997; Kosinski

et al., 1998). It appears of relevance that the striatum has a high blood supply and metabolic rate, indicating a high demand for ATP (Hawkins et al., 1983; Cunningham et al., 1986). Therefore, the striatum is supposed to be at particular risk for limitations of function in energy metabolism, e.g. in disorders of oxidative phosphorylation or after administration of 3-nitropropionic acid and methylmalonic acid (Ludolph et al., 1991; Wajner et al., 1997). Acute encephalopathic crises in GDD typically precipitate from a catabolic state and can be often prevented by sufficient glucose and carnitine supply (Hoffmann et al., 1996). The addition of both, high NR1/NR2B expression and high energy demand, may lead to an amplification of neuronal damage through primary and secondary excitotoxic mechanisms in the striatum (Ludolph et al., 1993). Our study provides for the first time evidence that an age- and regional-specific pathology observed in a neurological disease may be related to ontogenetic expression of a particular neurotransmitter receptor subtype. Our findings of specific, NR1/NR2B-mediated neurotoxicity of GA and 3-OH-GA implicate that the severe age- and regional-specific neuronal damage observed in GDD is related to a high ontogenetic expression of NR1/NR2B in the forebrain (striatum, cortex) during a vulnerable period of brain development (Kosinski et al., 1998; Stocca and Vicini, 1998). Furthermore, we showed that also organic acids can act as specific excitotoxic agents. We assume that 3-OH-GA and GA are representatives of a new group of excitotoxic agents, which could be called – in analogy to excitotoxic amino acids (EAA)-excitotoxic organic acids (EOA).

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