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Ca^{2+} and Na^{+} Dependence of 3-Hydroxyglutarate-Induced Excitotoxicity in Primary Neuronal Cultures from Chick Embryo Telencephalons

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ABSTRACT

Glutaryl-CoA dehydrogenase deficiency (also known as glutaric aciduria type I) is an autosomal, recessively inherited neurometabolic disorder with a distinct neuropathology characterized by acute encephalopathy during a vulnerable period of brain development. Neuronal damage in this disease was demonstrated to involve *N*-methyl-D-aspartate (NMDA) receptor-mediated neurotoxicity of the endogenously accumulating metabolite 3-hydroxyglutarate (3-OH-GA). However, it remained unclear whether NMDA receptors are directly or indirectly activated and whether 3-OH-GA disturbs the intracellular Ca^{2+} homeostasis. Here we report that 3-OH-GA activated recombinant NMDA receptors (*e.g.* NR1/NR2A) but not recombinant α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors (*e.g.* GluR-A/GluR-B) in HEK293 cells. Fluorescence microscopy using fura-2 as Ca^{2+} indicator revealed that 3-OH-GA increased intracellular Ca^{2+} concentrations in the presence of extracellular Ca^{2+} in cultured chick neurons. Similar to glutamate-induced cell damage, 3-OH-GA neurotoxicity was modulated by extracellular Na^{+}. The large cation *N*-methyl-D-glucamine, which does not permeate NMDA receptor channels, enhanced 3-OH-GA-induced Ca^{2+} increase and cell damage. In contrast, 3-OH-GA-induced neurotoxicity was reduced after replacement of Na^{+} by Li^{+}, which permeates NMDA channels but does not affect the Na^{+}/Ca^{2+} exchanger in the plasma membrane. Spectrophotometric analysis of respiratory chain complexes I–V in submitochondrial particles from bovine heart revealed only a weak inhibition of 3-OH-GA on complex V at the highest concentration tested (10 mM). In conclusion, the present study revealed that NMDA receptor activation and subsequent disturbance of Ca^{2+} homeostasis contribute to 3-OH-GA-induced cell damage. **(*Pediatr Res* 52: 199–206, 2002)**

Abbreviations

3-OH-GA, 3-hydroxyglutarate
AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt
D-AP5, (D)-(−)-2-amino-5-phosphonopentanoic acid
GCDH, glutaryl-CoA dehydrogenase
GDD, glutaryl-CoA dehydrogenase deficiency
HBS, HEPES-buffered saline
MK-801, (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine
NaCaX, Na^{+}/Ca^{2+} exchanger of the plasma membrane
NMDA, *N*-methyl-D-aspartate
NMG, *N*-methyl-D-glucamine
PM, plasma membrane
ROS, reactive oxygen species

GDD [also known as glutaric aciduria type I; MIM #23170 (1)] is an autosomal, recessively inherited neurometabolic disorder (2). So far, more than 70 disease-causing mutations have been identified in the GCDH gene (3, 4), localized on chromosome 19p13.2 (5). The mitochondrial enzyme GCDH (EC 1.3.99.7) catalyzes the reaction of glutaryl-CoA to crotonyl-CoA in the catabolic pathways of L-tryptophan, L-lysine, and L-hydroxylysine (6).

Deficiency of GCDH results in an accumulation of upstream metabolites and their derivatives, mainly glutarate, 3-OH-GA, and glutaconate (7). Most of the affected children, if untreated,

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develop acute encephalopathic crises, usually around age 6–12 mo (8), typically precipitated by acute intercurrent illnesses (7). These episodes usually result in irreversible neurodestruction of vulnerable brain regions, *i.e.* striatum and cortex (9). The structural similarity of the accumulating organic acids to glutamate led to the suggestion that excitotoxic cell damage is involved in the neuropathogenesis of GDD (10, 11).

Excitotoxicity is a pathogenetic concept that has been considered as major mechanism in many neurologic diseases (12). Although the molecular basis of glutamate toxicity is still uncertain, a Ca^{2+}-dependent component is undisputed (13). The NMDA subtype of glutamate receptors plays a key role in mediating excitotoxic damage, owing to its high Ca^{2+} permeability (14). Apart from this, primary inhibition of mitochondrial respiratory chain activity has been shown to indirectly induce NMDA receptor stimulation and excitotoxic damage, and therefore has been termed "secondary" excitotoxicity (15, 16).

Recently, it was shown that 3-OH-GA-induced neuronal damage involved the NMDA subtype of ionotropic glutamate receptors *in vitro* (10, 11) and *in vivo* (17), suggesting 3-OH-GA as the main neurotoxin in GDD. Increased formation of ROS (18) and a decreased energy-buffering capacity may also contribute to this process (10). However, although previous studies have strongly suggested an involvement of excitotoxic pathways in 3-OH-GA-induced neuronal damage, it remained open whether NMDA receptors are directly or indirectly activated. Therefore, the present study focuses on the initiation of excitotoxic neuronal damage by the organic acid 3-OH-GA, investigating the effects on recombinant ionotropic glutamate receptors, intracellular Ca^{2+} homeostasis, and mitochondrial respiratory chain function.

METHODS

Cell transfection and electrophysiology. Transient NMDA receptor subunit expression and electrophysiology was performed in HEK293 cells as previously described (19). In brief, a vector carrying a cloned cDNA for the green fluorescent protein was co-transfected to visualize expressing cells 48 h after transfection by standard epifluoresence optics. Cells were continuously perfused with nominally Mg^{2+}-free external solution containing 135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 10 μM glycine, and 5 mM HEPES (pH 7.25, NaOH). The pipette solution contained 140 mM CsCl, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, and 4 mM Mg^{2+}-ATP (pH 7.25, CsOH). After the establishment of the whole-cell configuration of the patch-clamp technique, cells were lifted from the bottom of the dish to ensure rapid and complete solution exchange when applying agonists *via* a piezoelectric double-barreled pipette. The solution exchange time, measured with an open patch pipette, was 100–200 μs.

Cell cultures. Primary neuronal cultures from chick embryo telencephalons, known to be susceptible to excitotoxic cell damage, were prepared as previously described (11). Briefly, neuronal cultures were maintained in Dulbecco's modified Eagle medium supplemented with 20% fetal bovine serum and penicillin/streptomycin (all obtained from Invitrogen, Eggenstein, Germany) until they were used for the experiments after 6 d *in vitro*. Animal care followed the official governmental guidelines and was approved by the government ethics committee.

Treatment protocol. Neuronal cultures were exposed to 1 mM 3-OH-GA (the kind gift of W. Buckel, Department of Microbiology, University of Marburg, Germany) or with 1 mM glutamate (Sigma Chemical, St. Louis, MO, U.S.A.) for 1 h, diluted in standardized Mg^{2+}- and Zn^{2+}-free HBS (sHBS), containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2 \times 2$ H_20, 10 mM D-glucose, 10 mM HEPES, and 10 μM glycine in deionized water (all adjusted to pH 7.4) as previously described (18). The effect of Ca^{2+} and Na^+ on 3-OH-GA-induced cell damage was investigated by varying the contents of these cations in HBS. We used Ca^{2+}-free (CaF-HBS) and regular sHBS to differentiate between exogenous and endogenous Ca^{2+} sources. Extracellular Na^+ was present (sHBS) or was replaced by equimolar concentrations of NMG (NMG-HBS) or Li^+ (Li-HBS). The large cation NMG^+ is nearly unable to permeate NMDA receptor channels, whereas Na^+ and Li^+ permeate and depolarize the PM. However, only Na^+ enhances Ca^{2+} entry *via* the NaCaX located in the PM (20–22). This experimental design was expected to discriminate the modulatory effect of Na^+ on cytosolic Ca^{2+} concentrations after NMDA receptor activation, involving PM depolarization and NaCaX activation. The effect of 3-OH-GA on ionotropic glutamate receptors was investigated after pretreatment for 1 h with the competitive NMDA receptor antagonist D-AP5 (50 μM; #A8054, Sigma Chemical), the noncompetitive NMDA receptor antagonist MK-801 (#M107, Sigma Chemical), or the competitive non-NMDA receptor antagonist CNQX (50 μM; #C239, Sigma Chemical).

Measurement of $[Ca^{2+}]_i$. Fura-2 acetoxymethylester (5 μM in HBS; Sigma Chemical) was bath-loaded into cultured neurons for 30 min in the presence of 0.1% Pluronic F-127 (Sigma Chemical), followed by washing and another 30 min incubation in HBS at 37°C (23). The intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, was measured using an inverted microscope (Axiovert 100; Carl Zeiss GmbH, Jena, Germany), equipped with a 40× fluorescence objective (Carl Zeiss GmbH), a charge-coupled device camera, an image processor and controller (all purchased from Hamamatsu, Herrsching, Germany) as described previously (24). Fura-2 fluorescence was recorded in single neurons ($n = 60–90$; excitation wavelengths: 340 nm and 380 nm; emission wavelength: 510 nm). The 340/380 nm ratios were converted to $[Ca^{2+}]_i$ as previously described (23, 24). The application of the calcium ionophore ionomycin (10 μM; Sigma Chemical) at the end of measurement confirmed that cultured neurons revealed a similar responsiveness.

Cell viability assays. Cell viability was determined by trypan blue (0.4% in PBS) exclusion method 24 h after 3-OH-GA or glutamate exposure as previously described (11). A total number of 600–800 neurons were counted in randomly chosen subfields ($n = 8$) without knowledge of treatment.

Measurement of single complex activities. Protein was determined according to Lowry *et al.* (25). Submitochondrial particles (SMP) were prepared from bovine heart as described (26). After preparation, SMP were dissolved in 250 mM

sucrose, 50 mM KCl, 5 mM $MgCl_2$, and 20 mM Tris/HCl (adjusted to pH 7.4). Steady-state activities of single respiratory chain complexes I–V were recorded on a computer-tunable spectrophotometer (Versamax Microplate Reader, Molecular Devices, Sunnyvale, CA, U.S.A.) operating in the dual-wavelength mode. All activities were measured at pH 7.4 according to standard protocols (26–30). Standard activities (units per milligram total protein) were calculated and were expressed as percentage of control activity (normalized to 100%). Standard respiratory chain inhibitors [2-n-decyl-quinazolin-4-yl-amine (1 μM; the kind gift of U. Brandt, Department of Biochemistry I, Molecular Bioenergetics, University of Frankfurt, Germany), thenoyltrifluoroacetone (8 mM; #T9888, Sigma Chemical), antimycin A (1 μM; #A8674, Sigma Chemical), NaCN (2 mM; #S3296, Sigma Chemical), and oligomycin (80 μM; #O4876, Sigma Chemical)] were added as positive controls.

Data analysis. All data are expressed as mean ± SEM. Figures show representative experimental data of one of at least three independent experiments. A p value of <0.05 was considered significant. A t test (two groups) or one-way ANOVA followed by Scheffé's test (three or more groups) was used to determine the statistical significance of any difference (SPSS Version 10.0, SPSS Inc., Chicago, IL, U.S.A.).

RESULTS

3-OH-GA activates recombinant NMDA but not AMPA receptors. Previous studies demonstrated that 3-OH-GA-induced cell damage was mediated *via* NMDA but not *via* AMPA/kainate receptors *in vitro* and *in vivo* (10, 11, 17). To find out whether 3-OH-GA activates ionotropic glutamate receptors, we expressed NMDA receptor channels of the NR1/NR2A and NR1/NR2B subtype in HEK293 cells (19) and applied the potential agonist for 200 ms at −60 mV in the constant presence of the NMDA receptor co-agonist glycine (10 μM). Glutamate (1 mM) activated currents were 818 ± 228 pA (n = 6) in NR1/NR2A and 67 ± 32 pA (n = 4) in NR1/NR2B channels. Compared with glutamate, 3-OH-GA (1 mM) activated currents were 3.5 ± 1.0% (*i.e.* 24 ± 6 pA; n = 6) in NR1/NR2A (Fig. 1*A*) and not detected (n = 4) in NR1/NR2B channels, likely due to our signal-to-noise ratio

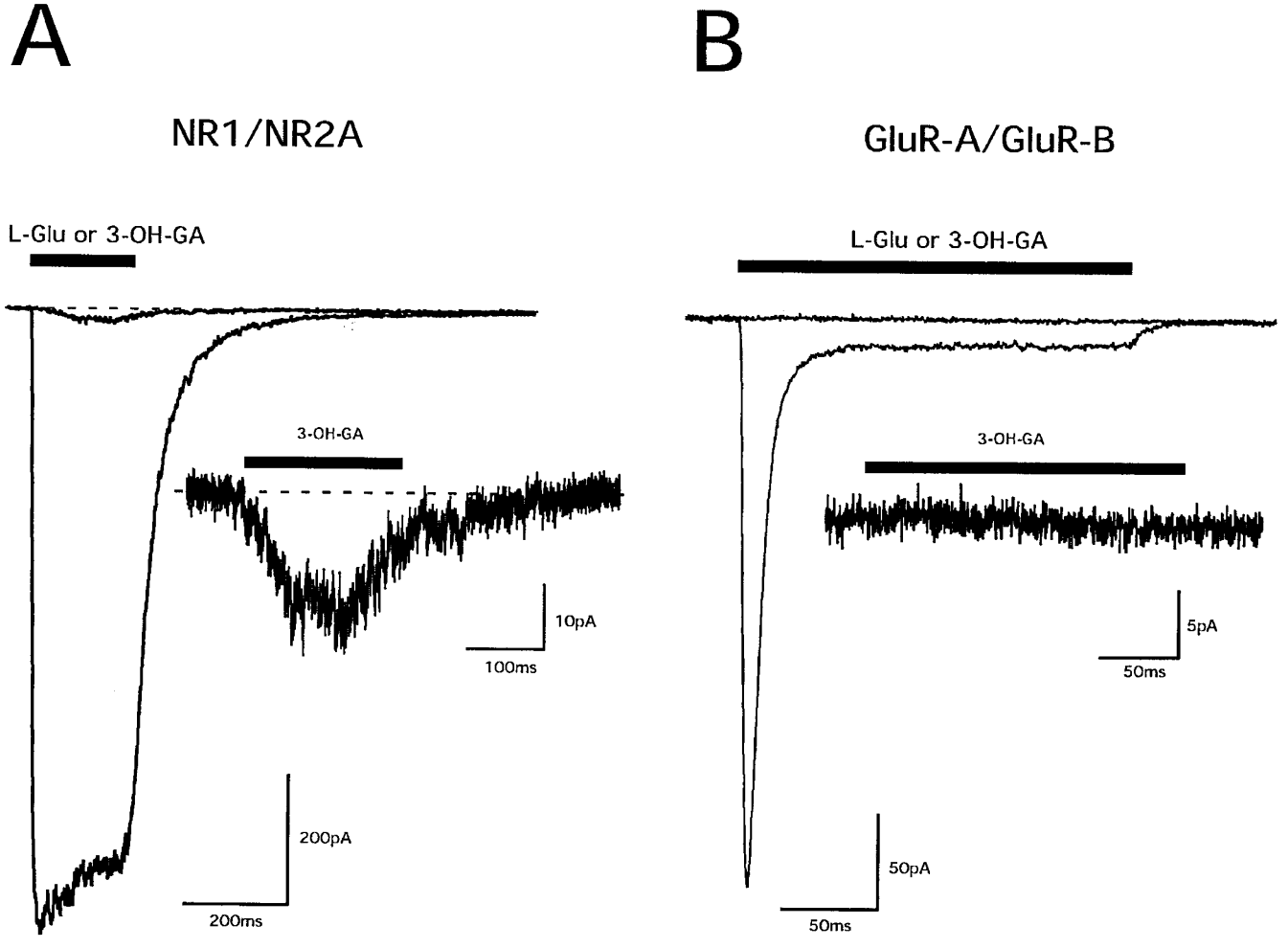

Figure 1. 3-OH-GA activates recombinant NMDA but not AMPA receptors. (*A*) Glutamate and 3-OH-GA (both 1 mM; 200 ms) were applied to HEK293 cells expressing NR1/NR2A channels at −60 mV. Compared with glutamate, 3-OH-GA-activated whole-cell currents were 3.5 ± 1.0% (n = 6) in NR1/NR2A. (*B*) In contrast, 3-OH-GA (1 mM, 200 ms, −60 mV) did not activate whole-cell currents in HEK 293 expressing the recombinant AMPA receptors GluR-A/GluR-B, which could be activated by glutamate. *Insets:* Enlargement of 3-OH-GA-mediated current traces.

(Fig. 1*A*, *inset*). As expected for NMDA receptor-activated currents, 3-OH-GA-activated currents were blocked in the presence of the NMDA receptor antagonist D-AP5 (50 μM; not shown). In contrast to NR1/NR2A, 3-OH-GA did not activate recombinant AMPA receptors, GluR-A (not shown), or GluR-A/GluR-B ($n = 2$; Fig. 1*B*).

3-OH-GA induces Ca^{2+} influx* via *NMDA receptors. Next, we investigated the effect of 3-OH-GA on intracellular Ca^{2+} concentrations, $[Ca^{2+}]_i$, by fluorescence microscopy using fura-2 as Ca^{2+} indicator. 3-OH-GA (1 mM) raised $[Ca^{2+}]_i$ moderately in chick neurons (Fig. 2*A*), which was only observed in the presence of extracellular Ca^{2+} (sHBS) but not in the absence of Ca^{2+} (CaF-HBS). Comparability of $[Ca^{2+}]_i$ measurements in our model system was confirmed by final application of the Ca^{2+} ionophore ionomycin (Fig. 2*A*, *right panel*). 3-OH-GA-induced $[Ca^{2+}]_i$ increase was prevented by the competitive NMDA receptor antagonist D-AP5 (50 μM), but not by the competitive AMPA/kainate receptor antagonist CNQX (50 μM; Fig. 2*B*). Combined determination of $[Ca^{2+}]_i$ and cell viability in single neurons after exposure to 1 mM 3-OH-GA revealed a close relationship between $[Ca^{2+}]_i$ increase and neuronal damage (Fig. 2*C*). NMDA receptor blockade by 10 μM MK-801 (95 $\pm$ 3% of control viability) or 50 μM D-AP5 (97 $\pm$ 5%) prevented 3-OH-GA (1 mM) induced neuronal cell damage, in line with previous studies (11, 17).

Modulation of 3-OH-GA-induced $[Ca^{2+}]_i$ increase and cell damage by monovalent cations. Besides being highly permeable to Ca^{2+} (14), NMDA receptors are also permeable to Na^+

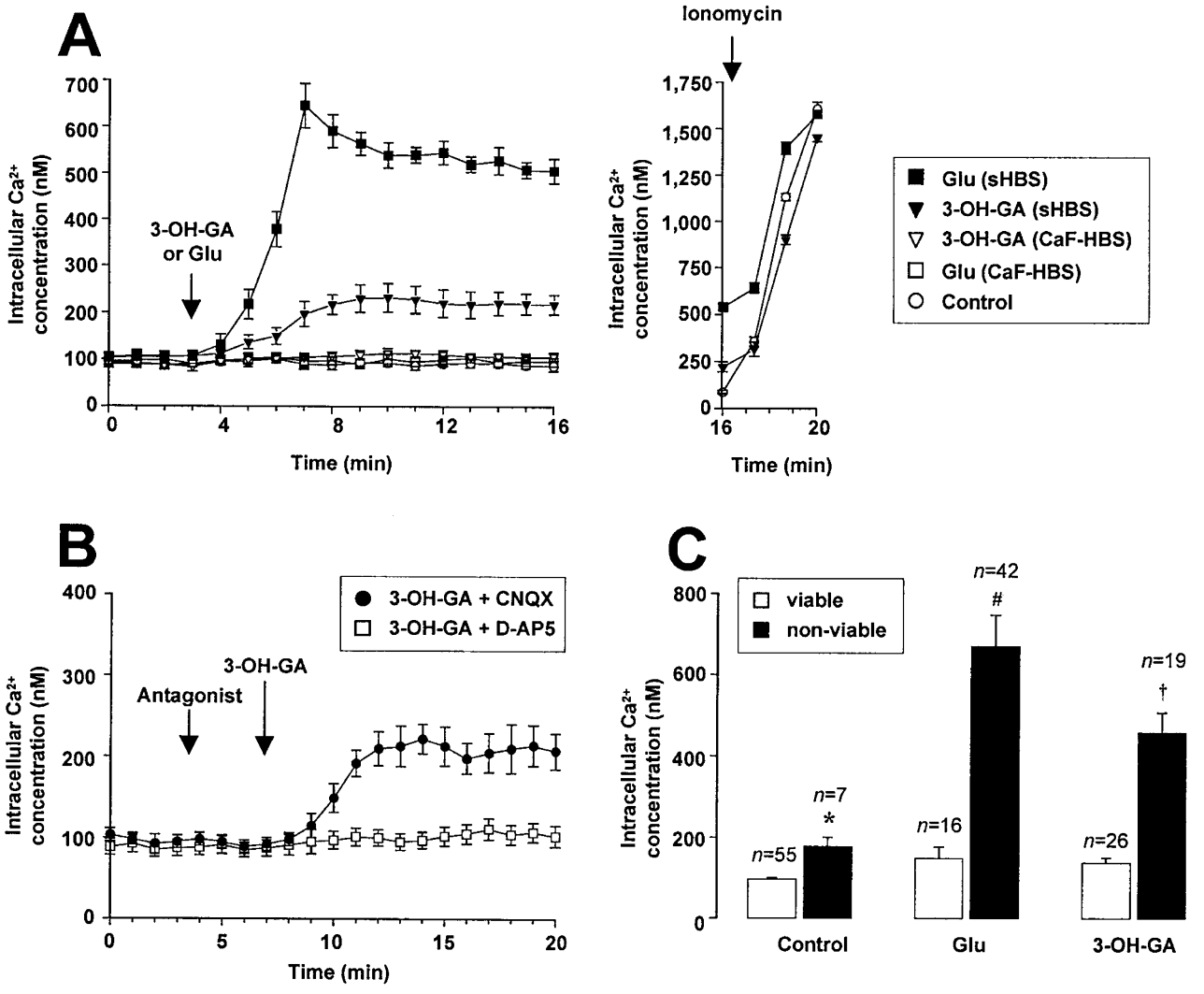

Figure 2. 3-OH-GA induces Ca^{2+} influx *via* NMDA receptors in chick neuronal cultures. (*A*) 3-OH-GA (1 mM) induced a Ca^{2+} increase in chick neurons in the presence (sHBS, *solid triangle*) but not in the absence of extracellular Ca^{2+} (CaF-HBS, *open triangle*) or in the control (*open circle*). Glutamate (*Glu*; 1 mM, *open square*) induced a stronger Ca^{2+} increase than 3-OH-GA (1 mM). Administration of the Ca^{2+} ionophore ionomycin (10 μM) at the end of each measurement showed a homogenous increase in $[Ca^{2+}]_i$ with or without previous 3-OH-GA or glutamate treatment (*right panel*). (*B*) NMDA receptor blockade by D-AP5 (50 μM, *solid circle*) but not AMPA receptor blockade by CNQX (50 μM, *open square*) prevented an increase in $[Ca^{2+}]_i$ induced by 3-OH-GA (1 mM, diluted in sHBS). (*C*) Combined fura-2 measurement and trypan blue exclusion after exposure to glutamate (1 mM, 1 h, diluted in sHBS), 3-OH-GA (1 mM, 1 h, diluted in sHBS), or vehicle revealed a close relationship between $[Ca^{2+}]_i$ increase and cell viability in viable (*open bars*) and damaged (*solid bars*) neurons. $[Ca^{2+}]_i$ was determined 10 min after application of glutamate, 3-OH-GA, or vehicle; cell viability 24 h after the incubation with glutamate, 3-OH-GA, or vehicle. **vs* viable (control), $t(60) = 20.059$, $p < 0.001$; $^{\#}$*vs* viable (Glu), $t(56) = 13.090$, $p < 0.001$; †*vs* viable (3-OH-GA), $t(43) = 8.139$, $p < 0.001$ (*t* test).

(31). Influx of Na^+, Cl^-, and water leads to neuronal death (32) but also modulates NMDA receptor-mediated Ca^{2+} influx in neurons by PM depolarization and by activating the reverse operation of NaCaX (21, 22). To differentiate the effects of Na^+ on 3-OH-GA-mediated Ca^{2+} influx, we replaced Na^+ (sHBS) with NMG^+ (NMG-HBS) or Li^+ (Li-HBS). Application of 3-OH-GA (1 mM) in NMG-HBS enhanced both $[Ca^{2+}]_i$ increase (Fig. 3*A*) and neuronal damage (Fig. 3*B*). In contrast, incubation of 3-OH-GA in Li-HBS decreased Ca^{2+} influx (Fig. 3*A*) and neuronal damage (Fig. 3*B*) compared with sHBS. As expected, no Ca^{2+} increase or cell damage was observed in the absence of Ca^{2+} (Fig. 3, *A* and *C*). These results are in line with previous reports on the modulation of NMDA receptor-mediated cell damage by the monovalent ions Na^+, NMG^+, and Li^+ (20–22).

3-OH-GA does not affect respiratory chain complexes I–IV but weakly inhibits complex V. Inhibition of the respiratory chain by mitochondrial toxins induces neuronal damage, involving indirect excitotoxic mechanisms (15, 16). However, 3-OH-GA weakly affected complex V activity only at a concentration of 10 mM in SMP (Fig. 4) but had no effect on complexes I–IV. The reliability of our system was confirmed by application of standard respiratory chain inhibitors, 2-n-decyl-quinazolin-4-yl-amine, thenoyltrifluoroacetone, antimy-

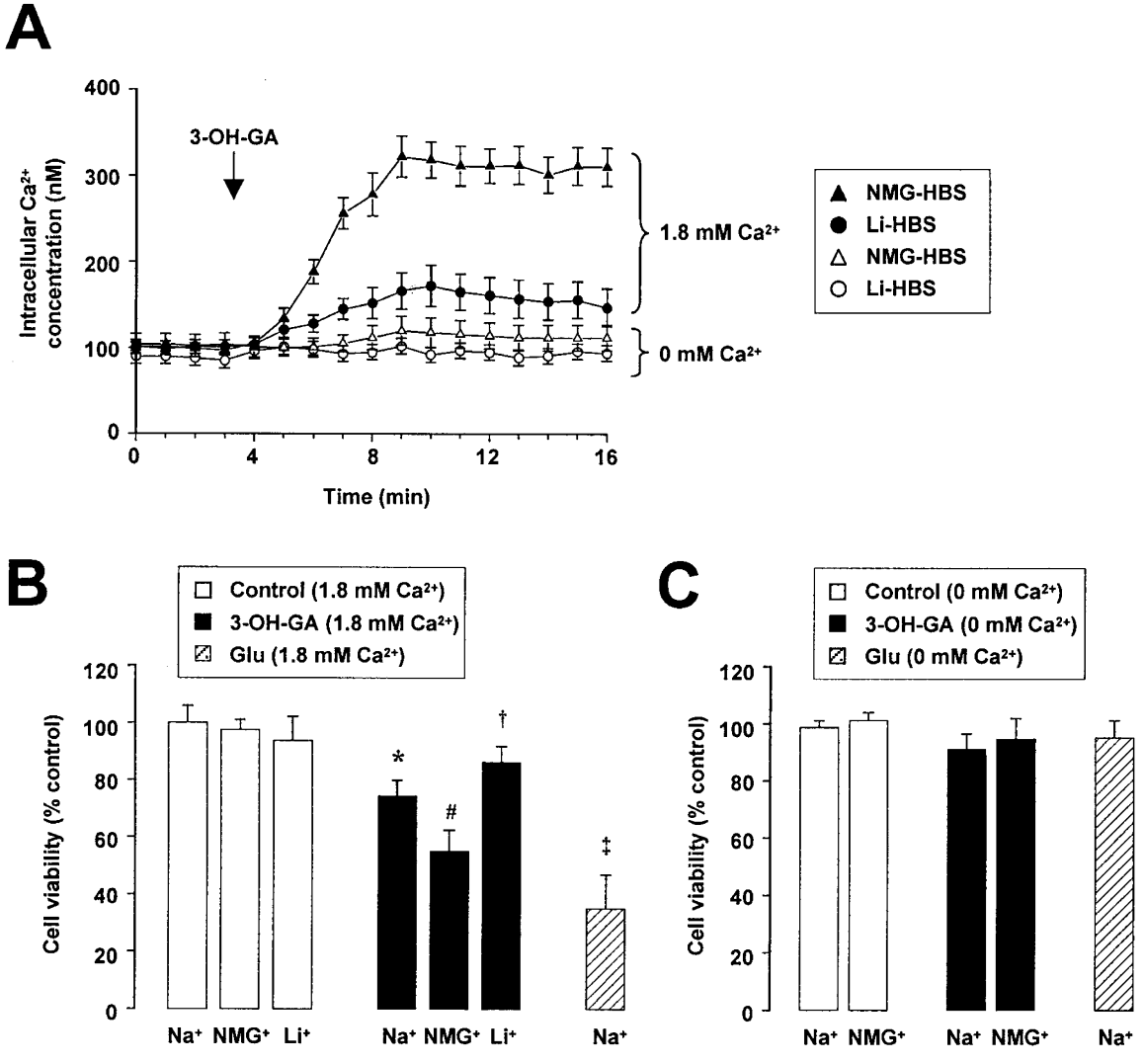

Figure 3. Modulation of 3-OH-GA-induced Ca^{2+} influx and cell damage by monovalent cations in chick neurons. (*A*) NMG^+ enhanced 3-OH-GA (1 mM) induced $[Ca^{2+}]_i$ in the presence (*solid triangle*) but not in the absence of extracellular Ca^{2+} (*open triangle*). In contrast, replacement of Na^+ by Li^+ (Li-HBS, *solid circle*) revealed a decrease in $[Ca^{2+}]_i$. Thus, 3-OH-GA-induced $[Ca^{2+}]_i$ increases in the presence of Na^+ were interposed between those induced in the presence of NMG^+ or Li^+ (see also Fig. 2*A*). Again, 3-OH-GA-induced Ca^{2+} influx was not observed in the absence of extracellular Ca^{2+} (*open circle*). (*B*) In parallel to the effects on $[Ca^{2+}]_i$, NMG^+ enhanced, whereas Li^+ reduced, the neurotoxic effect of 1 mM 3-OH-GA. After glutamate treatment (*Glu*; 1 mM, 1 h, diluted in sHBS), cell viability decreased stronger than after administration of 3-OH-GA. Statistical analysis was performed by one-way ANOVA ($F_{6,49} = 93.713$, $p < 0.001$) and *a posteriori* Scheffé's test: *3-OH-GA (Na^+) *vs* control (Na^+), $p < 0.001$; #3-OH-GA (NMG^+) *vs* 3-OH-GA (Na^+), $p = 0.001$; †3-OH-GA (Li^+) *vs* 3-OH-GA (Na^+), $p = 0.021$; ‡glutamate (Na^+) *vs* 3-OH-GA (Na^+), $p < 0.001$. (*C*) 3-OH-GA (1 mM) and glutamate (Glu; 1 mM) induced no significant cell damage in Ca^{2+}-free media, confirming the importance of extracellular Ca^{2+} in this process. Statistical analysis was performed by one-way ANOVA ($F_{4,35} = 4.097$, $p = 0.008$) and *a posteriori* Scheffé's test: 3-OH-GA (Na^+) *vs* control (Na^+), $p = 0.196$; 3-OH-GA (Na^+) *vs* 3-OH-GA (NMG^+), $p = 0.931$; 3-OH-GA (Na^+) *vs* glutamate (Na^+), $p = 0.883$.

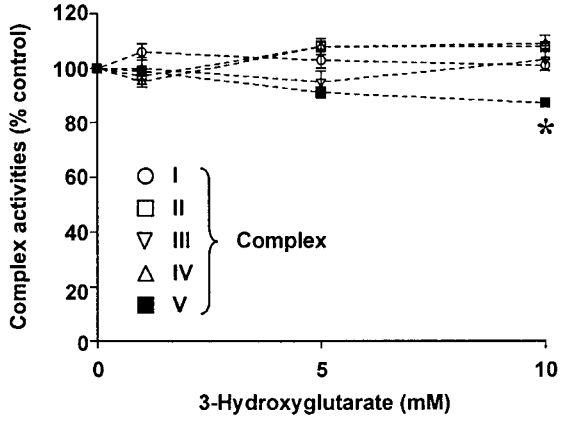

Figure 4. Effect of 3-OH-GA on respiratory chain complexes I–V in submitochondrial particles from bovine heart. Steady-state activities (units per milligram protein) of single respiratory complexes I–V were recorded on a computer-tunable spectrophotometer operating in the dual-wavelength mode. Control activity was normalized to 100%. Activities of the electron-transferring complexes I (*open circle*), II (*open square*), III (*open triangle, point down*), or IV (*open triangle, point up*) were not affected by 3-OH-GA (1–10 mM), whereas a weak inhibition of complex V activity was revealed at a concentration of 10 mM (*solid square*). Statistical analysis was performed by one-way ANOVA ($F_{3,32} = 13.784$, $p < 0.001$) and *a posteriori* Scheffé's test: *3-OH-GA *vs* control; $p < 0.001$.

cin A, NaCN, and oligomycin, which revealed an inhibition of 90–100%. However, an inhibitory effect of 3-OH-GA on the associated proteins of the mitochondrial respiratory chain cannot be completely excluded by this model (33).

DISCUSSION

The major findings of the present study are the activation of recombinant NMDA receptors and the disturbance of the intracellular Ca^{2+} homeostasis by the endogenously accumulating organic acid 3-OH-GA as underlying neurotoxic mechanism in GDD.

Neurotoxicity of 3-OH-GA and a pathomechanistic involvement of NMDA receptors but not of AMPA receptors was previously demonstrated in different neuronal culture systems (10, 11) as well as after intrastriatal administration in rats (17). In this study, we demonstrated that 3-OH-GA activated NMDA but not AMPA receptors, confirming our previous hypothesis. Although a former study demonstrated a prevention of 3-OH-GA-induced neuronal damage by MK-801 in organotypic hippocampal and corticostriatal slice cultures from 6-d-old rats, these authors, however, failed to demonstrate an activation of NMDA receptors in *Xenopus laevis* oocytes that were injected with mRNA from neocortex of 21-d-old rats (10).

Based on cell viability data, we originally hypothesized that the NR1/NR2B receptor subtype might be of particular importance for 3-OH-GA-induced neuronal damage (11). However, here we excluded that this effect was mediated by a subtype-selective stimulation of NMDA receptors by 3-OH-GA. In fact, activation of the NR1/NR2A receptor subtype by 3-OH-GA was even more pronounced than that of NR1/NR2B. This was unexpected, inasmuch as the NR1/NR2B receptor subtype was the predominant receptor subtype in our neuronal culture system at the time point of 3-OH-GA exposure (11) as well as in immature brain (34), resulting in a high susceptibility to excitotoxic damage (35). Furthermore, because NR1/NR2B is highly expressed in neostriatal medium spiny neurons (36), which are particularly damaged during acute encephalopathy in GDD patients (37), this particular receptor subtype is still of pathophysiologic interest for GDD.

In the present study, 3-OH-GA induced an influx of Ca^{2+} *via* NMDA receptors, which was dependent on the availability of extracellular Ca^{2+}. Although Ca^{2+} increase after 3-OH-GA exposure was moderate, a neuronal Ca^{2+} load, which is one of the most important mechanisms in NMDA receptor-mediated neurotoxicity (38), seems likely to contribute to 3-OH-GA neurotoxicity. The excitotoxic mechanism could be facilitated by 3-OH-GA-induced inhibition of glutamate decarboxylase, the key enzyme in γ-aminobutyric acid formation (39). Reduced γ-aminobutyric acid levels in the CNS could indirectly enhance the excitatory influence of its physiologic counterplayer glutamate on susceptible neurons. In addition, inhibition of glutamate uptake—as demonstrated for the structurally related compound glutarate—may also contribute to 3-OH-GA-induced neuronal damage (40, 41). Although previous studies demonstrated a positive correlation between Ca^{2+} influx and cell death in NMDA receptor-mediated neuronal damage (38), the above-mentioned additional mechanisms may amplify the adverse effects of 3-OH-GA-induced NMDA receptor activation. On the other hand, even small NMDA receptor-mediated Ca^{2+} increases may be responsible for significant cell death, especially because 3-OH-GA is persistently present in affected patients. It has been demonstrated that NMDA receptor activation can initiate specific subcellular signaling under physiologic and pathophysiological conditions (42–44). Also, the precise relationships between NMDA receptor activation, cytosolic Ca^{2+} increase, and mitochondrial Ca^{2+} uptake are unknown. Therefore, cytosolic Ca^{2+} concentrations may not reflect the Ca^{2+} accumulation in mitochondria, to which Ca^{2+} after NMDA receptor activation shows a privileged access (45). These possibilities highlight the importance of Ca^{2+} influx *via* NMDA receptors for the induction of cell damage by 3-OH-GA.

NMDA receptors are permeable to Ca^{2+} but also to Na^+, and increased intracellular Na^+ concentrations enhance Ca^{2+} influx *via* reverse activation of the NaCaX in the PM (21). To investigate the relationship between extracellular Na^+ and 3-OH-GA-induced damage, we replaced Na^+ with NMG^+ or Li^+. Unlike Na^+, neither NMG^+ nor Li^+ support Ca^{2+} transport *via* the NaCaX, whereas Na^+ and Li^+ but not NMG^+ permeate NMDA receptor channels and depolarize the PM (22). In the present study, we found that replacement of Na^+ by NMG^+ enhanced Ca^{2+} increase and neuronal damage after 3-OH-GA exposure. In contrast, replacement of Na^+ with Li^+ reduced it. These results are in line with previous studies, revealing an enhancement of NMDA receptor-mediated Ca^{2+} influx and cell damage by NMG^+ and a reduction by Li^+ (20, 22). These results underline the role of Na^+ influx in excitotoxicity: 1) a limiting role, due to the depolarization-dependent decrease in the electrochemical driving force for Ca^{2+} (defined as the difference between the PM potential and the Ca^{2+}

equilibrium potential); and 2) a promoting role, due to Ca^{2+} influx *via* reverse operation of NaCaX (22) and inhibition of Ca^{2+} extrusion by the NaCaX (20). Taken together, we suggest that Ca^{2+} influx is modulated by Na^+ in 3-OH-GA-induced cell damage.

Inhibition of the respiratory chain can impair the ability of neurons to maintain normal resting membrane potential due to ATP depletion and can decrease the activity of Na^+/K^+-ATPases (46). In addition, ATPase activity can be reduced by a ROS-dependent mechanism as previously described for glutarate (47). Consequently, membrane depolarization, in general, and the removal of the voltage-dependent Mg^{2+} block of NMDA receptors, in particular, results in an unimpeded influx of Ca^{2+} and Na^+ into neurons (15, 48). Furthermore, increased $[Na^+]_i$ up-regulates NMDA receptor activity *via* an enhancement of Src kinase activity (49). However, because we found only a weak inhibition of complex V by 3-OH-GA at a high *milli*molar concentration (10 mM) and because 3-OH-GA did not affect intracellular ATP levels in a previous study (10), we suggest that a direct inhibition of the mitochondrial respiratory chain is only of minor importance for 3-OH-GA neurotoxicity. However, we cannot completely exclude that 3-OH-GA affects the associated proteins of the mitochondrial respiratory chain, the Krebs cycle, or the mitochondrial β-oxidation of fatty acids.

In conclusion, we confirmed that 3-OH-GA-induced cell damage is primarily mediated *via* NMDA receptors (11, 17, 18, 50), resulting in disturbed Ca^{2+} homeostasis. Because susceptibility to encephalopathic crises declines with age in GDD (7), ontogenetic factors may open a window of vulnerability for 3-OH-GA toxicity during infancy and early childhood. In particular, ontogenetic changes in the excitatory glutamatergic system should be considered (51–53). However, the neurotoxic properties of 3-OH-GA might not be sufficient to fully explain the onset of acute encephalopathy during a vulnerable period of brain development in GDD patients. Thus, the existence of additional potentiating factors has been suggested, such as catabolism and inflammatory cytokines. Catabolism, *e.g.* during intercurrent illness, is thought to enhance the accumulation of organic acids (37, 54) and to impair energy metabolism. Furthermore, inflammatory cytokines have been shown to potentiate 3-OH-GA neurotoxicity *via* induction of astrocytic iNOS (50) and are suggested to stimulate the intracerebral formation of the NMDA receptor agonist quinolinic acid, an alternative intermediate product of the tryptophan metabolism (55). Furthermore, data obtained in the present study clearly demonstrate that 3-OH-GA-induced cell damage might critically be modulated by Na^+. Inasmuch as the final Ca^{2+} load, which determines whether neurons live or die, may be regulated by two opposite effects of Na^+, *i.e.* PM depolarization and reverse activation of NaCaX (21, 22), and because electrolyte infusions with Na^+ and Cl^- are broadly implemented in current emergency treatment protocols with the intention to reduce cerebral edema (56), our results urge re-evaluation of the efficiency of this strategy in the light of neuroprotection.

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