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Susceptibility of cerebellar granule neurons from GM2/GD2 synthase-null mice to apoptosis induced by glutamate excitotoxicity and elevated KCl: Rescue by GM1 and LIGA20

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Our previous study showed an impaired regulation of Ca^{2+} homeostasis in cultured cerebellar granule neurons (CGN) from neonatal mice lacking GM2, GD2 and all gangliotetraose gangliosides, due to disruption of the GM2/GD2 synthase (GalNAc-T) gene. In the presence of depolarizing concentration (55 mM) K^+, these cells showed persistent elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) leading to apoptosis and cell destruction. This was in contrast to CGN from normal littermates whose survival was enhanced by high K^+. In this study we demonstrate that glutamate has the same effect as K^+ on CGN from these ganglioside-deficient knockout (KO) mice and that apoptosis in both cases is averted by exogenous GM1. Even more effective rescue was obtained with LIGA20, a semi-synthetic derivative of GM1. LC_{50} of glutamate in the KO cells was 3.1 μM, compared to 46 μM in normal CGN. $[Ca^{2+}]_i$ measurement with fura-2 revealed no difference in glutamate-stimulated Ca^{2+} influx between the 2 cell types. However, reduction of $[Ca^{2+}]_i$ following application of Mg^{2+} was significantly impaired in the mutant CGN. The rescuing effects of exogenous GM1 and LIGA20 corresponded to their ability to restore Ca^{2+} homeostasis. The greater potency of LIGA20 is attributed to its greater membrane permeability with resultant ability to insert into both plasma and nuclear membranes at low concentration ($\leq 1\ \mu$M); GM1 at the same concentration was incorporated only into the plasma membrane and required much higher concentration to influence Ca^{2+} homeostasis and CGN viability.
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Keywords: **GM1 ganglioside, ganglioside, glutamate excitotoxicity, apoptosis, Ca^{2+} homeostasis, cerebellar granule neurons, nuclear envelop, nuclear calcium, Na^+/Ca^{2+} exchanger**

Abbreviations: **$[Ca^{2+}]_i$, intracellular Ca^{2+}; CGNs, cerebellar granule neurons; Ctx B; Cholera B subunit; Ctx B-HRP, Ctx B conjugated with horseradish peroxidase; Ctx B-FITC, Ctx B conjugated with fluorescein isothiocyanate; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; FDA, fluorescein diacetate; KO, knockout; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NCX, Na^+/Ca^{2+} exchanger; NE, nuclear envelop; PBS, phosphate-buffered saline; PI, propidium iodide; PM, plasma membrane; PSS, physiological saline solution. Ganglioside nomenclature is that of Svennerholm (*J Neurochem* 10, 613–23 (1963)).**

Introduction

The family of gangliotetraose gangliosides, consisting of GM1 and its polysialo-analogues, are major sialic acid-containing components of the neuronal glycocalyx [1,2]. In addition to their well known occurrence in the plasma membrane (PM) of these and other cells, they have also been found in various intracellular compartments including the nuclear envelope (NE). Their presence in the latter membrane was first discovered in neural cells, including neurons of both the CNS and PNS [3–7], and subsequently in certain non-neural cells [8]. The NE was shown to have a relatively simple ganglioside pattern, GM1 and GD1a being the major species with only trace quantities of others [3,9]. These studies revealed nuclear GM1 expression to increase dramatically with development, correlating specifically with axonal outgrowth. In regard to function,

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an initial finding indicated a role for nuclear GM1 in modulating nuclear Ca^{2+} homeostasis during neurite outgrowth [10]. The specific mechanism was subsequently shown to involve activation of a Na^+/Ca^{2+} exchanger (NCX) located in the inner membrane of the NE [11]. The NCX at that locus was found to be tightly associated with GM1, as seen in cohesion of the 2 components during SDS-PAGE; this contrasted with NCX in the PM which was not tightly associated with GM1, although a looser association that did not survive SDS-PAGE was suggested.

These findings provided another example of gangliosides serving, as one their many cellular functions, to modulate Ca^{2+} homeostasis, GM1 in particular accomplishing this through diverse mechanisms [12,13]. This had been suggested in a series of studies showing ganglioside protection of neuronal cultures subjected to glutamate excitotoxicity [14–17] and neuroblastoma cells treated with Ca^{2+} ionophore [18]. Additional evidence came from a study of cerebellar granule neurons (CGN) cultured from mice engineered to lack GM2/GD2 synthase (GalNAc-T), with resultant deficit of GM2, GD2, GM1 and all other gangliotetraose gangliosides [19]. These knockout (KO) cells showed relatively normal behavior when grown in the presence of physiological or moderately elevated K^+ but gradually degenerated in the presence of high K^+. This degeneration in depolarizing medium was accompanied by progressive elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and onset of apoptosis, phenomena not observed with normal cells. Since several gangliosides were deficient in these KO mice/cells it was not clear which one(s) was instrumental in restoring and maintaining Ca^{2+} homeostasis in that experiment. The present study provides data indicating GM1 as one likely candidate, based on its ability to rescue KO cells following glutamate-induced elevation of $[Ca^{2+}]_i$. Additional evidence is the observation that LIGA20, a membrane permeant analog of GM1, exerted even more striking neuroprotection in facilitating restoration of Ca^{2+} homeostasis, suggesting an intracellular locus of activity. We discuss reasons for proposing this intracellular site as the NE involving its complement of NCX isoforms complexed with GM1.

Materials and methods

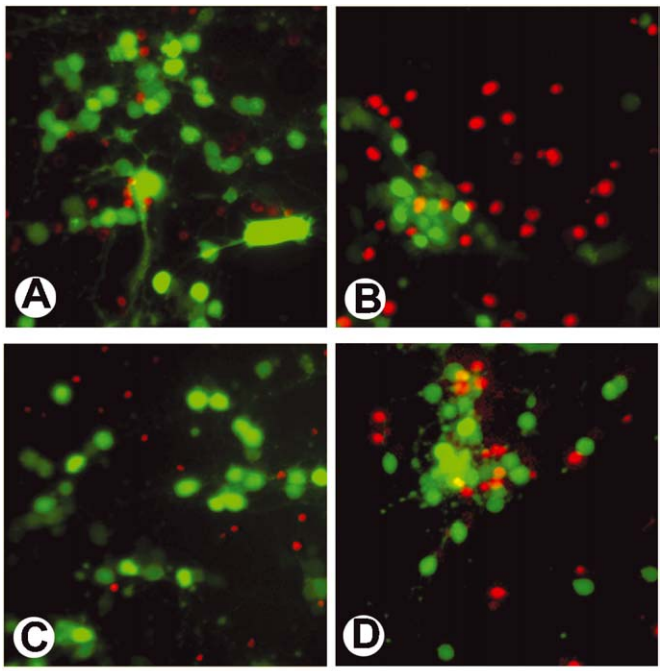
Materials

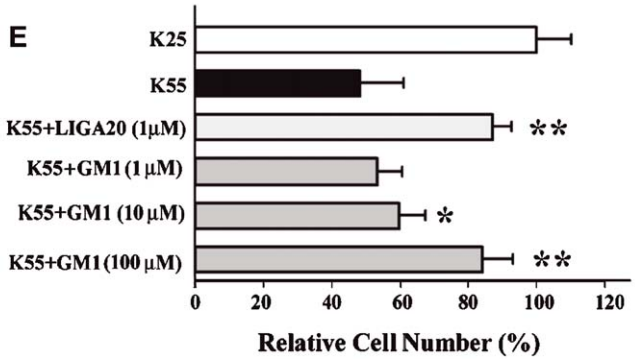
The following materials were obtained from the sources indicated. Dulbecco's modified Eagle medium (DMEM), heat-inactivated fetal bovine serum (FBS), N2 supplement and other chemicals for tissue culture from Invitrogen (Carlsbad, CA); Cholera B subunit conjugated with horseradish peroxidase (Ctx B-HRP) or fluorescein isothiocyanate (Ctx B-FITC) from List Biochemical (Campbell, CA); fura-2 AM and sulfinpyrazone from Molecular Probes (Eugene, OR); fluorescein diacetate (FDA), propidium iodide (PI), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), glutamate and other chemicals from SIGMA/RBI (St. Louis, MO); tissue culture plastic wares from Falcon. GM1 and LIGA20 were gifts from Fidia (Abano Terme, Italy). Stock solution preparations: GM1, LIGA20, PI, and glutamate in distilled water; FDA in acetone; fura-2 AM and sulfinpyrazone in dimethyl sulfoxide.

Animals and cell cultures

C57BL/6 mice with disrupted gene for GM2/GD2 synthase (GalNAc-$T^{-/-}$) were engineered by Proia and coworkers [20] and kindly supplied by Dr. Ronald Schnnar (Department of Pharmacology and Neuroscience, The John Hopkins University School of Medicine, Baltimore, MD). Knockout (KO) or heterozygote females were mated with heterozygote males and pups 6- to 7-days of age were used. Ganglioside phenotype was determined by staining blood cells from tail clips of pups with Ctx B-FITC. Ctx B positive phenotype consisted of normal and heterozygote genotypes, while Ctx B negative represented KO genotype. Since brain ganglioside profile and behavior of the cultured CGNs from the heterozygote mice were very similar to normal animals [19], we grouped these together as normal controls. In each experiment cerebella from a few mice of each group were collected and CGNs dissociated as previously described [5,21]. The isolated cells were suspended in DMEM supplemented with 10% heat inactivated FBS and antibiotics (50 μg/ml gentamycin, 50 units/ml penicillin and 50 μg/ml streptomycin), then seeded at a density of 2×10^5 cells /cm^2 onto 96-well plastic plates (for MTT assay) or 25-mm circular glass coverslips placed in 6-well plates (for calcium measurement and PI-FDA staining). Both plastic plates and coverslips were precoated with poly-L-lysine (0.5 mg/ml overnight at room temperature). After overnight incubation at 37°C in an incubator with 5% CO_2 and 95% humidified air, the media were replaced with fresh DMEM containing 10% FBS, N2 supplement, 80 μM 5-fluoro-2′-deoxyuridine, 25 mM KCl and above antibiotics. Small volume (one-tenth of starting volume) of additional medium was applied every 2 days. The cells were maintained for 5–7 days. In some cultures, medium KCl was increased to 55 mM and GM1 or LIGA20 at varied concentrations was co-applied. Viability of these cultures was determined after 5 days *in vitro* (DIV).

Cells cultured for 7 days were used for acute glutamate treatment. Part of the medium in each well was transferred to fresh plates (conditioned medium), and GM1 or LIGA20 was applied to the remaining medium to reach final designated concentrations, followed by incubation for one hour at 37°C. The cells were washed 2× in a Mg^{2+}-free, physiological saline solution (PSS) containing HEPES (2 mM, pH 7.2), 140 mM NaCl, 5 mM KCl, 2.3 mM $CaCl_2$, and 10 mM glucose, prewarmed at 37°C, and treated with designated amounts of glutamate in same PSS for 20 min at 37°C. The cells were then rinsed with PSS again, replaced to the conditioned medium, and cultured an additional 16 hrs followed by cell survival determination.

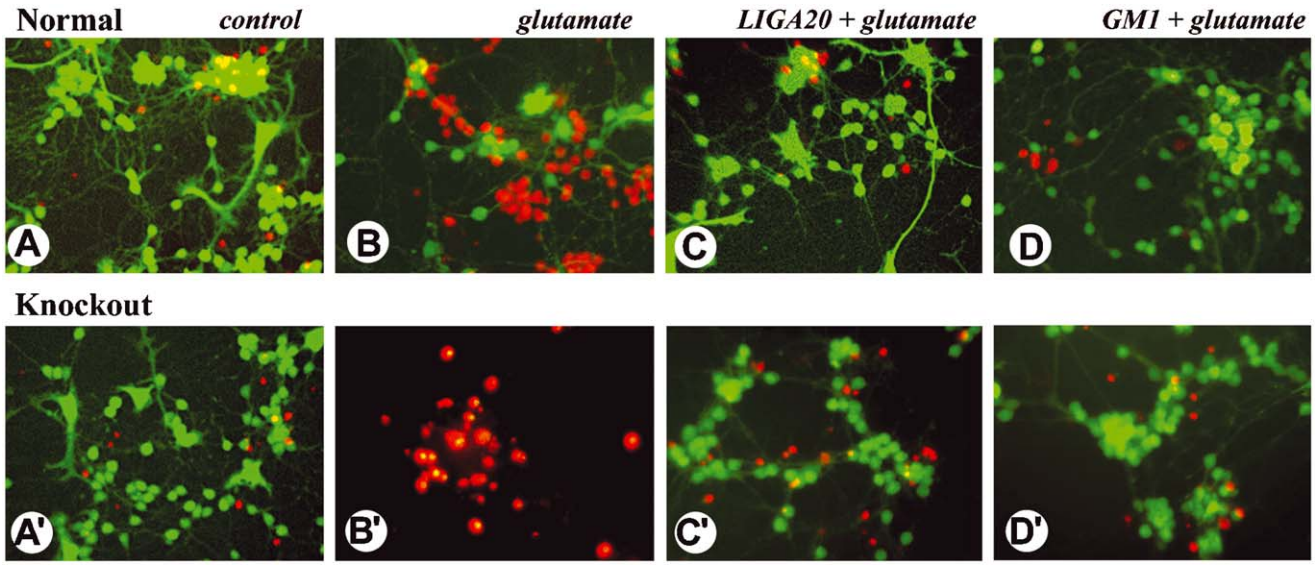




Cell survival determination

PI-FDA staining was employed to identify apoptotic vs. live cells, as described [22,23]. Briefly, the cells were incubated with PI (2 μg/ml) and FDA (8 μg/ml) in the medium for 5 min at 37°C. After removal of the medium, the cells were placed in fresh PSS. Fluorescent images were taken under a Nikon Diaphot microscope at $\times$200, with filters for rhodamine and fluorescein to illuminate PI and FDA staining respectively. The apoptotic cells were marked by red fluorescent PI in nuclei and the surviving cells by green fluorescent FDA in cytoplasm. Quantification of cell loss was achieved with MTT assay as described [18,21]; the resulting data ($OD_{570–640}$) were expressed relative to control cultures obtained with 25 mM KCl alone. All experiments were repeated at least three times, and LC_{50} of glutamate and EC_{50} of gangliosides from dose-response measurements were analyzed with Prism software. 2-tailed Student *t* test was used for statistical analysis.

Figure 1. Protection against KCl-induced apoptosis by LIGA20 and GM1. CGN from gangliotetraose deficient GalNAc-$T^{-/-}$ KO mice were cultured 5 DIV in media containing 25 mM KCl (K25, A), 55 mM KCl (K55, B), K55 plus 1 μM LIGA20 (C), and K55 plus 10 μM GM1 (D). Cell viability was determined by PI-FDA staining: apoptotic cells were stained with PI (red fluorescence) and live cells with FDA (green fluorescence). K55-induced cell death was largely prevented by 1 μM LIGA-20 (C) and partially by 10 μM GM1 (D). Analysis of cell survival with MTT assay (E) quantified the greater rescuing potency of LIGA20. Data are average $\pm$ SD from 3 independent experiments; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to K55 alone.



$[Ca^{2+}]_i$ measurement

To determine the effect of GM1 or LIGA20 on glutamate-induced $[Ca^{2+}]_i$ changes, the cells after 7 DIV were incubated in above DMEM medium with GM1 or LIGA20 for one hour at 37°C, then loaded with 5 μM of fura-2 AM plus 250 μM sulfinpyrazone for another hour. After rinsing with above Mg^{2+}-free PSS supplemented with sulfinpyrazone, and stabilized in the same buffer for 5 min, Ca^{2+} measurement was carried out with a MiraCal Interline Digital Ratio Imaging System with Olympix cooled CCD camera and xenon light source [19]. Fluorescence was measured at 510 nm emission with 350/380 nm dual excitation; $[Ca^{2+}]_i$ was represented by ratio of fluorescent intensity between the two excitation wavelengths (*i.e.* $R_{350/380}$), after correction for background fluorescence. $R_{350/380}$ for 20–30 cells in one field of each coverslip was averaged ($n = 1$). The measurement was started by recording basal level for 50 sec, followed by application of 25 μM glutamate that induced rapid elevation of $[Ca^{2+}]_i$ to a maximum level. After 200 sec of measurement, 5 mM of $MgCl_2$ was applied to block Ca^{2+} influx [24,25], resulting in decrease of $[Ca^{2+}]_i$. The recording was continued for another 250 sec until no further decrease of $[Ca^{2+}]_i$ was observed. $R_{350/380}$ reduction from the maximum to the final low level after Mg^{2+} application was used to quantify $[Ca^{2+}]_i$ decrease relative to the net increase of $R_{350/380}$ from basal level to the maximum after glutamate stimulation. All measurements were repeated at least 3 times; EC_{50} and statistical significance were analyzed as above.

Results

Subjection of CGN from KO mice to a highly depolarizing level (55 mM) of KCl over 4–5 DIV induced apoptosis, as seen with PI-FDA staining (Figure 1B), an indicator of apoptosis [23]. This was in contrast to similar cultures containing 25 mM KCl, which showed minimal cell loss (Figure 1A). Cell death was averted by LIGA20 at 1 μM (Figure 1C) and partially prevented by GM1 at 10 μM (Figure 1D). Analysis with MTT assay quantified this difference in potency: 1 μM LIGA20 increased survival from 46% (in 55 mM KCl) to 87%, whereas GM1 required much higher concentration (100 μM) to achieve equivalent protection (Figure 1E).

Similar phenomena were observed in more mature cultures (7 DIV) employing glutamate as Ca^{2+}-influx stimulant. A significant difference in viability between normal and knockout CGN was observed 16 hr after glutamate treatment (25 μM, 20 min in Mg^{2+}-free PSS). PI-FDA assay revealed that virtually none of the glutamate-treated KO neurons survived (Figure 2B′), whereas viability of normal neurons was about 50% (Figure 2B). LC_{50} of glutamate for normal CGN (Figure 3A) was ~15 fold higher than that for knockout cells (Figure 3B, Table 1A). However, apoptosis induced by glutamate in both cell types was greatly attenuated by preincubation with LIGA20 at 1 μM (Figure 2C and 2C′) or GM1 at 100 μM (Figure 2D and 2D′), resulting in rightward shift of glutamate dose-response curves (Figure 3A and B) and increase in LC_{50} values of glutamate (Table 1A). The greater potency of LIGA20 as rescuing agent was also revealed in experiments employing varied doses of GM1 or LIGA20 in CGN cultures treated with 25 μM glutamate (Figure 3C and D). EC_{50} values of GM1 were 131- and 68-fold higher than LIGA20 in normal and knockout cells, respectively (Table 1B). It was noteworthy that the LIGA20 effect was biphasic: protective at doses of 1 μM or less and cytotoxic at higher concentrations (Figure 3D).

In order to determine whether vulnerability of KO neurons to glutamate was due to Ca^{2+} regulatory dysfunction and whether exogenously applied LIGA20 and GM1 restored Ca^{2+} homeostasis, $[Ca^{2+}]_i$ was determined fluormetrically with fura-2. The basal level of $[Ca^{2+}]_i$ in mutant CGN was close to that in normal cells, and the plateau of elevated $[Ca^{2+}]_i$ in KO cells following glutamate application was only slightly higher (<10% in $R_{350/380}$) than in the normal CGN (Figure 4A and B). However,

Table 1. LC50 of glutamate and EC50 of GM1 or LIGA20

	Normal (μM)	*Knockout (μM)*
	A: LC50 of Glutamate	
Control	46 ± 11	3.1 ± 2.8^a
GM1 (100 μM)	115 ± 15^b	71 ± 12^c
LIGA20 (1 μM)	117 ± 19^b	73 ± 13^c
	B: EC50 of GM1 and LIGA20 on Cell Survival and $[Ca^{2+}]_i$ Reduction	
Cell survival		
GM1	74 ± 8.4	34 ± 11
LIGA20	0.56 ± 0.22^d	0.49 ± 0.18^d
$[Ca^{2+}]_i$ Reduction		
GM1	32 ± 15	48 ± 6.8
LIGA20	0.41 ± 0.18^d	0.44 ± 0.18^d

LC_{50} values in part A were from dose-response curves in Figure 3A and B. EC_{50} values in part B were from Figure 3C and D (cell survival) and Figure 4C and D ($[Ca^{2+}]_i$ reduction) respectively. Data are average ±SD from 3 experiments ($n = 3$). a, $p < 0.001$ compared to cells from normal animals; b, $p < 0.05$ compared to control normal cells; c, $p < 0.001$ compared to control knockout cells; d, $p < 0.001$ compared to GM1-treated cells.

Figure 2. Protection against glutamate-induced apoptosis by GM1 or LIGA20. CGN from normal (A–D) and KO (A′–D′) mice were cultured 7 DIV, then pretreated with LIGA20 (1 μM) or GM1 (100 μM) for one hr followed by glutamate treatment (25 μM, 20 min in Mg^{2+}-free buffer). After transferring to the conditioned medium and incubating 16 hr, cell viability was detected with PI-FDA staining. A, A′: Control; B, B′: glutamate on untreated cells; C, C′: glutamate on LIGA20 pretreated cells; D, D′: glutamate on GM1 pretreated cells. Virtually all cells from KO mice died, in contrast to cells from normal mice which showed ~50% mortality (B vs. B′). LIGA20 was significantly more effective than GM1 as rescuing agent.

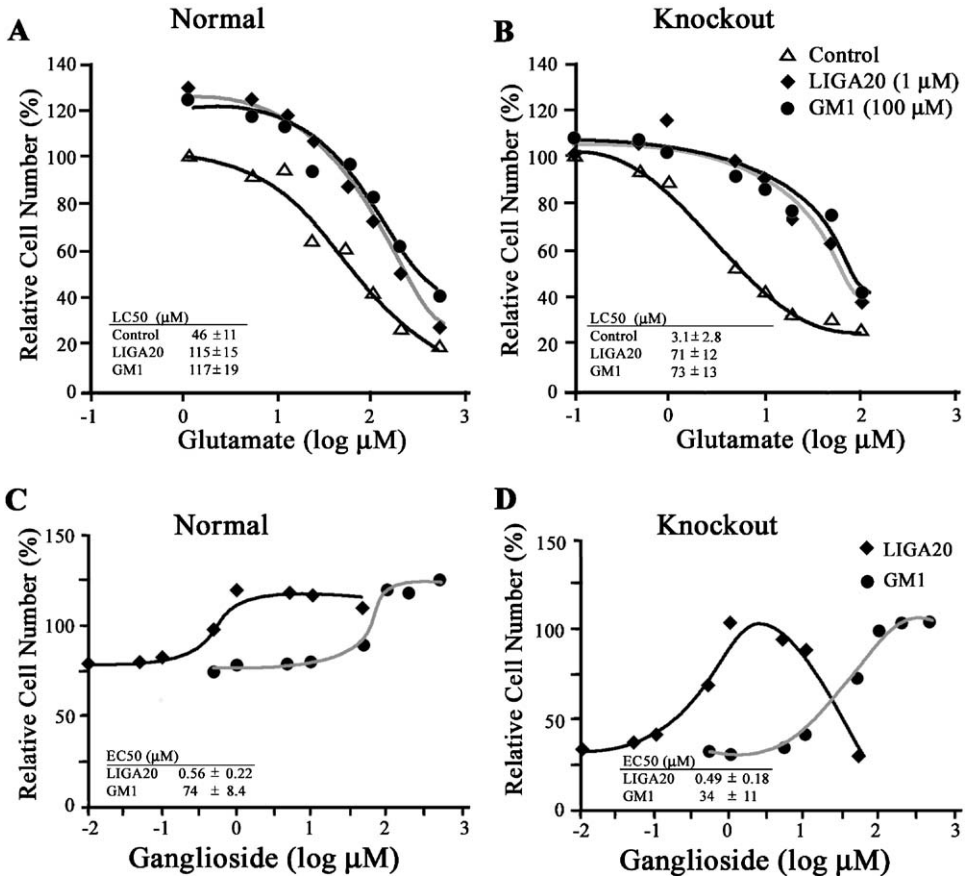


Figure 3. Quantitation of glutamate toxicity and neuroprotection by LIGA20 and GM1. CGN from normal and KO mice were cultured 7 DIV, pretreated with GM1 or LIGA20, and exposed to glutamate as in Figure 2. Cell survival was quantified with MTT assay. A and B: Dose-dependence of glutamate toxicity showed $LD_{50} \sim 15\times$ higher in CGN from normal (A) vs. KO (B) mice, with comparable rightward shifts for LIGA20 (1 μM) and GM1 (100 μM) pretreatment. C and D: Dose-dependence of GM1 and LIGA20 protective effects in CGN subjected to glutamate (25 μM), showing that both substances improved survival in the normal cells (C) and effectively rescued KO cells (D) from glutamate toxicity. The significantly higher potency of LIGA20 is indicated. Data are average of 3 independent experiments with standard error $<$10% of average.

a major difference was observed in $[Ca^{2+}]_i$ reduction following Mg^{2+} application, a treatment that blocked Ca^{2+} influx through NMDA channels. Taking the net glutamate-stimulated increase of $[Ca^{2+}]_i$ ($R_{350/380}$ between the basal and maximum levels) as 100%, the KO cells excluded only 36% of $[Ca^{2+}]_i$, significantly less than the normal cells that reduced $[Ca^{2+}]_i$ by 73%. Preincubation with LIGA20 significantly enhanced $[Ca^{2+}]_i$ reduction in both cell types in dose-dependent manner (Figure 4A and B). Optimal concentration of LIGA20 led to $[Ca^{2+}]_i$ below the original basal level. Pretreatment with GM1 also enhanced Ca^{2+} exclusion, but required much higher concentration (Figure 4C and D); EC_{50} of GM1 was 77- and 110-fold higher than that of LIGA20 in normal and KO cells, respectively (Table 1B). Unlike its effect on viability, LIGA20 promotion of Ca^{2+} extrusion in the KO cells was not biphasic (Figure 4D).

Intracellular location of GM1 ganglioside was revealed cytochemically with Ctx B-HRP. Cells from normal animals possessed endogenous GM1 in both PM and NE (Figure 5A), in contrast to CGN from KO mice which, as expected, showed no GM1 in any membrane (Figure 5B). However, one-hour incubation of the mutant cells with 1 μM LIGA20 resulted in positive staining in both membranes (Figure 5C). This differed from GM1 which, at 1 μM for the same period, associated only with the PM (Figure 5D). Nuclear membrane incorporation of exogenous GM1 could be achieved when the concentration of GM1 increased to 50–100 μM, accompanied by much heaver staining of the PM (Figure 5E).

Discussion

Mice engineered with a disrupted gene for GM2/GD2 synthase have provided a powerful tool to identify molecular mechanisms of gangliotetraose ganglioside functioning in neural systems. Following the initial report of this KO mouse showing moderate reduction in neural conduction velocity [26], subsequent investigations of similar mice [20] revealed deficient

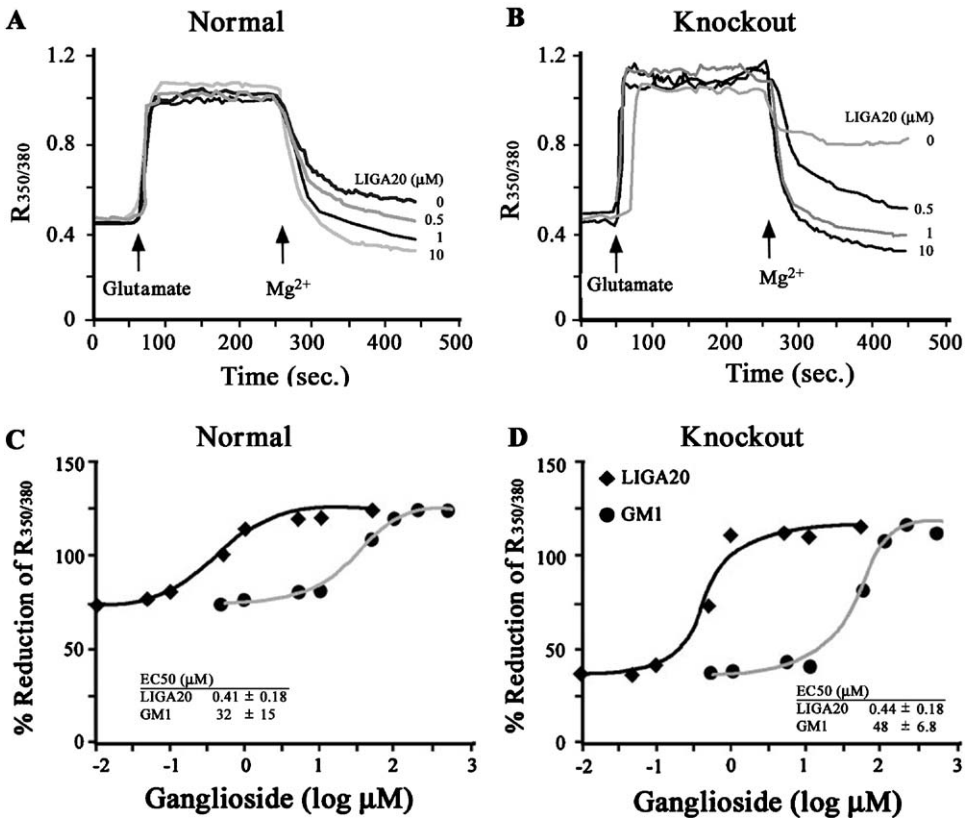


Figure 4. Facilitation of Ca^{2+} homeostasis by LIGA20 and GM1. Normal (A) and mutant (B) CGN were cultured 7 DIV, then treated with 25 μM glutamate in Mg^{2+}-free buffer with resultant $[Ca^{2+}]_i$ elevation that was measured ratiometrically with fura-2 fluorescence. Application of 5 mM $MgCl_2$ after 200 sec resulted in net $[Ca^{2+}]_i$ decrease that was recorded over another 200 sec. Whereas no significant difference was seen in basal level and glutamate-induced $[Ca^{2+}]_i$ elevation between two cell types, KO cells (B) showed impaired $[Ca^{2+}]_i$ reduction compared to normal CGN (A). Preincubation with LIGA20 dose-dependently restored Ca^{2+} homeostasis. C and D: Facilitation of $[Ca^{2+}]_i$ reduction dose-dependently by GM1 and LIGA20, showing higher potency of the latter in both cell types. Data are average of 3 independent experiments with standard error $<$10% of average.

myelination associated with axon degeneration [27], impaired motor coordination [28], and disrupted Ca^{2+} regulation in neuronal cell cultures challenged with depolarizing concentration of K^+ [19]. In the latter study exposure of CGN to 55 mM K^+ resulted in persistent and progressive elevation of $[Ca^{2+}]_i$ over days with gradual onset of apoptosis in virtually all cells; normal CGN, in contrast, survived well under similar conditions, suggesting a primary role in Ca^{2+} regulation for one or more of the deleted gangliosides. The present study extends those findings by demonstrating similar vulnerability of the gangliotetraose-deficient CGN to glutamate-induced excitotoxicity, a physiologically more relevant form of $[Ca^{2+}]_i$ elevation [29]. Overactivation of glutamate receptors leads to neuronal Ca^{2+} overload resulting in necrosis or apoptosis depending on intensity of the stimulus and the energy level of the affected cell [30]. Ionotropic glutamate receptors are classified into NMDA and non-NMDA subgroups, both of which possess intrinsic channels permeable to Ca^{2+} [31]. NMDA receptors have been shown to occur at both synaptic and extrasynaptic loci, the latter being coupled to cell death pathways [32]. These contribute importantly to such pathologies as epilepsy, brain trauma, and hypoxic-ischemic neuronal death [33,34].

The current findings have revealed the underlying cause of Ca^{2+} dysregulation in the mutant CGN to be failure to restore Ca^{2+} homeostasis following $[Ca^{2+}]_i$ elevation, rather than excessive elevation of Ca^{2+} influx (Figure 4). Elevation of $[Ca^{2+}]_i$ occurred to the same extent in normal and KO cells, and the fact that normal Ca^{2+} homeostasis was restored along with preserved cell viability by pre-incubation with GM1 pointed to this ganglioside as a factor in the relevant Ca^{2+} regulatory mechanisms. LIGA20, a semi-synthetic membrane permeant analog of GM1 [16], proved even more effective than GM1, showing similar activity at significantly lower concentrations. LIGA20 at 1 μM and GM1 at 100 μM were fully effective in preserving viability of normal CGN subjected to 10 μM glutamate, and 80–90% effective with KO cells under the same conditions; both treatments were only partially effective at higher glutamate (Figure 3). This ability of GM1 and LIGA20 to afford at least partial protection to normal neurons was well demonstrated in

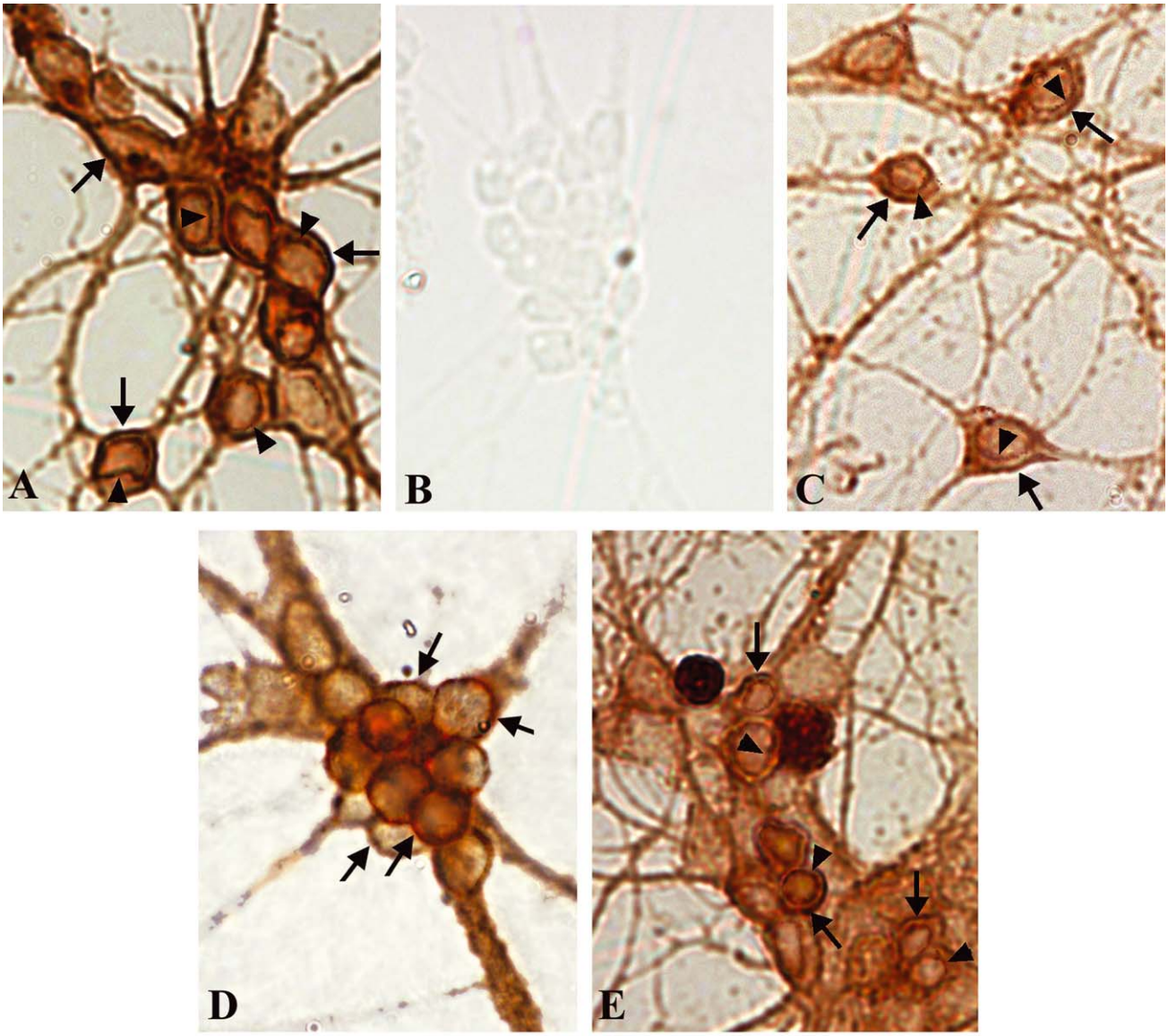


Figure 5. GM1 cytochemistry. Normal (A) and mutant (B–E) CGN cultured 7 DIV were untreated (A, B) or treated with 1 μM LIGA20 (C), 1 μM GM1 (D) or 100 μM GM1 (E). Ctx B-HRP staining of untreated normal CGN (A) revealed GM1 in PM (arrows) and NE (arrowheads), in contrast to KO cells which showed no GM1 staining (B). However, strong Ctx B-HRP staining was detected in both membranes of KO cells following incubation with LIGA20 at 1 μM (C). GM1 at the same concentration inserted only into the PM (D), whereas 100 μM GM1 inserted into both membranes (E).

previous cell culture studies [14–17,35,36]; the limited expression of GM1 (and other gangliotetraose gangliosides) in the cultured embryonic/neonatal neurons [37] could render them more vulnerable to glutamate exocytoxicity than mature neurons. NG-CR72 cells, a mutant line of NG108-15 deficient in GM1 synthase, were similar to CGN from KO mice in being highly vulnerable to elevated $[Ca^{2+}]_i$ and were also rescued by GM1 and LIGA20 [38]. That study revealed that in addition to its neuroprotective effects, LIGA20 proved toxic at higher concentrations, as also found in the current experiments (Figure 3D) and an earlier study [16].

Reduction of $[Ca^{2+}]_i$ is achieved by a combination of efflux through the PM and intracellular buffering. The latter mechanism includes sequestration into intracellular Ca^{2+} storage sites such as ER and the NE with which it is structurally and functionally connected. Movement of Ca^{2+} from cytoplasm into ER/NE is facilitated by the Ca^{2+}-ATPase pump (SERCA), which is present in the outer membrane of the NE [39] but apparently not the inner nuclear membrane [40]. However, another system was recently discovered that could facilitate such transfer, a Na^+/Ca^{2+} exchanger (NCX) present in the inner membrane of the NE that moved Ca^{2+} from nucleoplasm to NE lumen in

the presence of a Na^+ gradient [11]. An unusual feature of this NCX that distinguished it from similar exchangers in the PM was association with GM1 [11]. This association was unusually tight, surviving SDS-PAGE, and caused potentiation of nucleoplasmic Ca^{2+} exchange into the NE. Owing to the presence of the nuclear pore complexes over the entire NE with resultant equilibration of Ca^{2+} between nucleoplasm and cytosol under most conditions [41], enhanced removal of nucleoplasmic Ca^{2+} would be expected to facilitate reduction of $[Ca^{2+}]_i$ as seen in Figure 4.

LIGA20 is a derivative of natural GM1 in which stearic acid joined in amide linkage to the 2-amino group of sphingosine is replaced by dichloroacetyl; the oligosaccharide and sphingosine moieties remain unchanged [16]. This structural modification, while retaining many bioactive properties of GM1, renders LIGA20 less aggregative in forming micelles and more membrane permeant [42]. LIGA-20 was reported to undergo transport from gut to vascular system and to penetrate the blood-brain barrier more readily than GM1 when administrated orally to rats [43,44]. Utilization of KO cells deficient in GM1 for intracellular localization revealed exogenous LIGA20 applied at 1 μM to enter the NE in addition to PM, whereas GM1 at the same concentration entered only the PM; much higher concentrations (50–100 μM) were required for penetration of GM1 to the NE (Figure 5). Similar results were obtained with NG-CR72 cells [38]. It was noteworthy that nuclear insertion of LIGA20 and GM1 was correlated to their effects on cell survival and $[Ca^{2+}]_i$ reduction (Figures 3 and 4). This may explain the neuroprotective efficacy of these substances, especially LIGA20, in animal models of neurodegenerative disorders related to glutamate excitotoxicity [43,45,46]. A similar finding was recently made for kainate-induced seizures [47].

The widespread occurrence of NCX/GM1 complexes in the NE of neural and non-neural cells [11,48,49] suggests this may be a common safeguard mechanism in nature to protect cells from the potentially harmful effects of excessive nucleoplasmic Ca^{2+}[50]. Despite the existence of nuclear pore complexes, recent studies suggest the possibility of independent regulation of nucleoplasmic and cytoplasmic Ca^{2+} [51–53]. However, this may not be the only mechanism by which GM1 and LIGA20 promote neuronal survival. The studies of Costa and coworkers showed that these substances inhibit activation of protein kinase C by blocking its sustained translocation from cytosol to PM induced by glutamate [14,16,54]. Such translocation was suggested to be pivotal in the disruption of $[Ca^{2+}]_i$ homeostasis [17], perhaps owing to suppression of the Na^+/Ca^{2+} exchanger in the PM [55,56]. Protein kinase C has been reported in the nucleus of various cells [57] but it remains to be determined whether this exerts a similar inhibitory effect on the nuclear exchanger. It is not known whether GM1 or LIGA20 activate the Na^+/Ca^{2+} exchanger in the PM; although these isoforms of NCX are not tightly associated with GM1 as they are in the NE, there was an indication of looser binding [11] which may have functional implications. The neuroprotection afforded by GM1, in addition to requiring an order of magnitude higher concentration than LIGA-type compounds, was shown to be non-specific in that other members of the gangliotetraose family were equally or more effective [14,58]. This suggests a more general pharmacological effect, perhaps based on membrane perturbation phenomena, in contrast to interaction with a specific protein as seems more likely with LIGA20. Different mechanisms for these 2 glycolipids were also indicated in a study with NIH-3T3 fibroblasts in which high concentrations of GM1 released neurotrophin-3 as opposed to release of brain-derived neurotrophic factor by lower concentrations of LIGA20 [59]. Finally, a possible effect of GM1 and its analogs on Ca^{2+}-ATPase in the PM has been proposed [17], based on the affinity of gangliosides for calmodulin [60] which was shown to modulate this enzyme. The many known ways in which gangliosides regulate Ca^{2+} changes and homeostasis [13] suggest a complexity of interactive mechanisms will be needed to fully explain neuroprotective effects of the kind observed in this study.

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