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Pretreatment with Monosialoganglioside GM1 Protects the Brain of Fetal Sheep against Hypoxic-Ischemic Injury without Causing Systemic Compromise

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ABSTRACT. The purpose of this study was to determine whether prophylaxis with monosialoganglioside GM1 can protect the fetus from hypoxic-ischemic encephalopathy *in utero*. Because some protective strategies can compromise the fetus, the effect of GM1 treatment on metabolic status and blood pressure was also evaluated. Chronically instrumented near-term fetal sheep (119–133 d) were subjected to 30 min of severe cerebral ischemia. Six were given 30 mg/kg GM1 through the umbilical vein 2 h before insult followed by continuous infusion of 30 mg/kg/d over the next 60 h, and these were compared with seven vehicle-treated control sheep. The time course of electrocorticographic activity and cytotoxic edema within the parasagittal cortex were determined with real-time spectral analysis and continuous impedance measurements, respectively. Histologic outcome was assessed 72 h later. Pretreatment with GM1 improved recovery of primary edema, reduced the duration of epileptiform activity (15 ± 2 *versus* 31 ± 5 h; $p < 0.05$) and the magnitude of secondary edema ($p < 0.05$). At 72 h, histologic damage was reduced, particularly in the cortex ($p < 0.05$) and hippocampus ($p < 0.01$), and residual electrocorticographic activity was increased in the GM1-treated group (−5 ± 1 *versus* −9 ± 3 dB, $p < 0.01$). GM1 infusion did not alter arterial blood pressure or metabolic status. These results indicate that GM1 can protect the fetal brain against hypoxic-ischemic injury without causing hypotension or metabolic compromise. (*Pediatr Res* 34: 18–22, 1993)

Abbreviations

HI, hypoxic-ischemic
ECoG, electrocorticographic
EAA, excitatory amino acid

A number of strategies have been suggested to protect the CNS against HI injury. This range of approaches reflects the multiplicity of neurotoxic processes initiated by such insult. Particular attention has focused on the use of calcium channel antagonists, glutamate antagonists, and free radical scavengers. However, the potential application of many proposed treatments in the perinatal period is limited. For example, calcium channel antagonists, although neuroprotective, can cause hypotension and compound the injury (1–3). Glutamate antagonists can have a number of side effects (4, 5). Increasingly, it is recognized that perinatal asphyxial encephalopathy can originate from prenatal insults (6–9). Although urgent delivery and postasphyxial approaches to therapy may well be the mainstay of obstetric management, the potential for intrauterine prophylaxis of the fetus at risk while awaiting delivery should not be ignored.

The gangliosides are a family of glycosphingolipids that are endogenous components of plasma membrane. Exogenous monosialoganglioside GM1 has been shown to be neuroprotective (10–14) and can cross the placenta (15). We recently described an experimental preparation in which a chronically instrumented fetal sheep is subjected to transient cerebral ischemia; this procedure induces an encephalopathy showing characteristics similar to HI brain damage of some asphyxiated term infants (16). We have used this preparation to examine whether GM1 is neuroprotective *in utero* and whether it causes hypotension or compromises metabolic status.

MATERIALS AND METHODS

Surgical procedures. Nineteen Romney/Suffolk fetal sheep from 119 to 133 d of gestation were operated on under halothane anesthesia (2%) using sterile techniques as previously described (16). Briefly, the head, neck, and forelimbs of the fetus were externalized and catheters were inserted into the ascending aortic arteries, umbilical vein, brachial vein, and amniotic cavity. Three pairs of shielded stainless steel electrodes were placed over the parietal dura, two at 10 mm lateral to the bregma and 5 mm and 15 mm anterior, and the third at 15 mm lateral and 10 mm anterior to the bregma. A pair of electrodes was also sewn into the paraspinal nuchal muscle to record electromyographic activity. The vertebral-occipital anastomoses between the carotid arteries and vertebral arteries were ligated bilaterally to eliminate vertebral blood supply to the brain. Inflatable occluder cuffs were placed around both carotid arteries. The fetus was returned to the uterus and the uterine and abdominal walls were closed. After the operation, the ewe was housed in a metabolic cage at constant temperature (20°C) and humidity (50%) and given free access to hay and water, supplemented by sheep nuts and alfalfa. Antibiotics (gentamicin, 80 mg; penicillin, 500 mg) were administered to the ewe daily.

Recordings. A four-electrode technique was used to measure changes in impedance associated with changes in extracellular space that occur concomitantly with cytotoxic edema (17). The

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impedance signal, ECoG, nuchal electromyographic, and fetal arterial blood pressure corrected for amniotic fluid pressure were recorded on an analog chart running at 5 mm/min. ECoG intensity spectra were analyzed on-line as previously described (16).

Experimental procedures. Experiments were performed 72 h after surgery. Fetal arterial samples were obtained before the start of each experiment and only fetuses with normal arterial blood gases (pH >7.32 and arterial oxygen pressure >2.26 kPa) were used. After a baseline recording period of 12 h, the carotid cuffs were inflated with saline solution for 30 min. Successful occlusion was confirmed by an isoelectric ECoG. Six animals were rejected from the analysis, four due to poor baseline arterial blood gases and two (one was infused with GM1 and the other was a vehicle-treated control) due to incomplete suppression of the ECoG during occlusion presumably reflecting inadequate ligation of the vertebral-occipital anastomoses. Animals were assigned to two groups, with one group receiving GM1 (Fidia Research Laboratories, Abano Terme, Italy) ($n = 6$) and the control group ($n = 7$) receiving equivalent volumes of phosphate buffer infusion only. In the former group, 30 mg/kg of GM1 dissolved in phosphate buffer (pH = 7.4) was administered systemically through the umbilical vein as a bolus 2 h before the ischemia followed by a continuous infusion of 30 mg/kg/d administered at a rate of 1.2 mL/h using a syringe pump (Harvard Apparatus, Millis, MA) over the next 60 h postinsult. These experiments were approved by the Animal Ethics Committee of the University of Auckland.

Histology. Each sheep was killed by pentobarbital injection 3 d after the ischemia. The fetal brain was immediately perfused through the carotid arteries with 500 mL of physiologic saline followed by 500 mL of FAM (formaldehyde, acetic acid, methanol; 1:1:8). The fixed brain was removed and placed in FAM for at least 24 h. After processing and wax-embedding in paraffin, coronal subserial sections were cut 8 μm thick and then stained with thionin-acid fuchsin (18). Every 40th section was examined by light microscopy by two independent assessors, one of whom was blinded to the experiment. The correlation coefficient between scores obtained by the two observers was 0.88 ($p < 0.01$). Neurons with ischemic cell change, consisting of acidophilic (red) cytoplasm and contracted nuclei or with just a thin rim of red cytoplasm with pyknotic nuclei, were assessed as dead, whereas all others were considered viable (19). Each region was scored for the proportion of dead neurons as follows: 0 = no dead neurons, 5 = 0 to 10%, 30 = 10 to 50%, 70 = 50 to 90%, 95 = 90 to 99%, and 100 = 100%. Each score corresponds with the midpoint of its range (16).

Analysis. The ECoG intensity was presented in dB [log(x)·10] because this transformation gives a better approximation to the normal distribution (20). The intensity was then normalized with respect to the 12-h reference period before occlusion; thus, these measurements are expressed as a log ratio of the baseline period. A digital Blackman low-pass filter with a cutoff of 0.1 cycles/point was applied to the log-transformed ECoG intensity data to minimize short-term fluctuations of less than 20 min. Similarly, a cutoff of 0.25 cycles/point was applied to the impedance time series. The final ECoG intensity was calculated as the average of the final 4-h period, from 68 to 72 h. The duration of the depressed period was measured as the time after insult that the ECoG was more than 8 dB below the baseline intensity. The onset of epileptiform activity was defined as the time after insult when smoothed ECoG intensity increased above −5 dB of the preocclusion intensity, which corresponds to the development of low median frequency spike-wave or polyspike activity (21). We have previously shown that this activity is associated with electromyographic evidence of seizure activity (16). The impedance was expressed as a percentage of baseline levels and reflects changes in extracellular space (17). Onset of the acute and delayed increase in impedance was determined by the 10% rise above baseline. The time course of ECoG intensity and impedance in the two groups and arterial blood pressure before and during GM1 infusion were compared using two-way analysis of variance with time as a repeated measure, followed by unpaired t tests with Bonferroni's correction for multiple comparisons. All results are presented as mean ± SEM.

Changes in histologic outcome were then compared by two-way analysis of variance with regions as a repeated measure and then by t test with Bonferroni's correction for comparisons of the degrees of damage in the different regions.

RESULTS

The gestational age and physiologic parameters before insult were similar for the treatment and control groups (Table 1). During GM1 infusion, there was no significant change in arterial blood pressure or alteration in the metabolic parameters compared with the controls (Table 1).

Thirty min of bilateral carotid occlusion induced complete suppression of the ECoG during ischemia (Fig. 1). The drop in ECoG intensity was not significantly different between the treated (−12.4 ± 1.8 dB) and control (−13.3 ± 1.9 dB) animals. The ECoG remained depressed after the insult. GM1-treated fetuses showed earlier recovery of ECoG intensity to −8 dB (4.8 ± 0.7 h) than controls (8.5 ± 1.1 h, $p < 0.05$). In both groups of animals, ECoG then progressively increased in intensity (Figs. 1 and 2). This was followed by a shift to a state of low median frequency epileptiform activity that corresponded with spike-wave or polyspike activity on the raw ECoG and increased nuchal muscle activity (Fig. 3). The duration of epileptiform activity was shorter in the treated group (15 ± 2 h) than in the controls (31 ± 5 h, $p < 0.05$), although the peak and onset time of the epileptiform activity were not significantly different. From 10 to 30 h after ischemia, the ECoG of treated animals showed a significantly higher median frequency of 10.1 ± 0.3 Hz compared with controls (8.8 ± 0.5 Hz, $p < 0.05$). At 72 h after ischemia was induced, the residual ECoG intensity of GM1-treated fetuses was higher (−5 ± 1 dB) than that of the controls (−9 ± 3 dB, $p < 0.01$).

Cortical impedance rapidly increased from 5 ± 2 min after occlusion and peaked at 3 ± 2 min after release of the clamps (Fig. 1). The initial peak of impedance in the treated group (144.3 ± 5.5%), which was not significantly different from that in the controls (143.6 ± 4.7%, $p = 0.92$), partially resolved with some residual impedance after the insult (Fig. 4). At 3 h after ischemia, the residual impedance was greater in the controls (105.1 ± 0.7%) than in the treatment group (101.0 ± 1.4%, $p < 0.01$). There was a secondary rise in impedance coincident with the onset of epileptiform activity (Figs. 1 and 2). The secondary increase in impedance in the controls occurred earlier (10% rise at 8.9 ± 0.6 h) than that in the treated animals (13.9 ± 1.9 h, $p < 0.05$) and gradually reached a peak of 133.1 ± 5.1% in the former as compared with 118.2 ± 3.4% ($p < 0.05$) in the latter group, although the secondary peak occurred at a similar time (36.9 ± 3.8 h). The residual increase in impedance at 72 h was lower in the treated group (105.5 ± 1.9%) than in the controls (120.3 ± 4.7%, $p < 0.05$).

There was an overall reduction in neuronal loss in the treated animals ($p < 0.05$) with greater improvement ($p < 0.01$) in the lateral (temporal) cortex and CA4 subfield of the hippocampus. Relative protection was conferred ($p < 0.05$) in the parasagittal cortex, thalamus, and the dentate gyrus, CA1, 2, and 3 regions of the hippocampus (Fig. 5).

DISCUSSION

HI encephalopathy in the fetal or perinatal period is believed to be an important cause of stillbirth and frequently leads to neurologic sequelae including cerebral palsy, sensory deficits, and learning disorders. Our study demonstrates that GM1 improved outcome as indicated by reduced neuronal loss, particu-

Table 1. *Changes in fetal blood pressure and metabolic status in response to infusion**

	Vehicle-treated controls (n = 7, gestation = 125 ± 3 d)		GM1-treated fetuses (n = 6, gestation = 126 ± 2 d)	
	Baseline	Infusion	Baseline	Infusion
BP (kPa)	6.2 ± 0.5	6.1 ± 0.5	6.0 ± 0.8	6.1 ± 0.8
pH	7.38 ± 0.01	7.36 ± 0.01	7.37 ± 0.01	7.36 ± 0.01
Pa_{O_2} (kPa)	2.93 ± 0.16	2.75 ± 0.15	3.01 ± 0.17	2.98 ± 0.18
Pa_{CO_2} (kPa)	6.18 ± 0.27	6.48 ± 0.22	6.51 ± 0.23	6.56 ± 0.23
Lactate (mmol/L)	0.9 ± 0.2	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1
Glucose (mmol/L)	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1

* Mean ± SEM. Baseline = 2-h period before any intervention. Infusion = 2-h period before occlusion. BP, blood pressure; Pa_{O_2}, arterial oxygen pressure; Pa_{CO_2}, arterial carbon dioxide pressure.

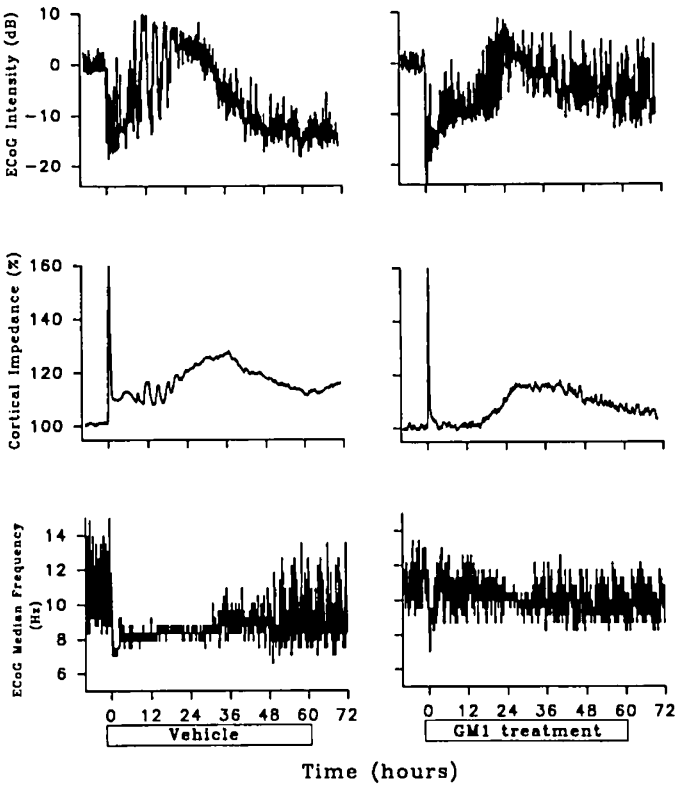

Fig. 1. Time course of changes in ECoG intensity (*upper panels*), cortical impedance (*middle panels*), and ECoG median frequency (*lower panels*) in a representative GM1-treated fetal sheep (*right*) and a control animal (*left*) after 30 min of cerebral ischemia. GM1 (30 mg/kg) was given 2 h before the insult and continuously (30 mg/kg/d) for 60 h after ischemia. Time is shown as hours postischemia. Note that after depression of the ECoG after the insult, intense low-frequency epileptiform activity developed in both GM1-treated and control animals. GM1 treatment improved recovery of primary edema and reduced the duration of epileptiform activity and magnitude of secondary edema. At 72 h, residual ECoG intensity was higher in the GM1-treated group.

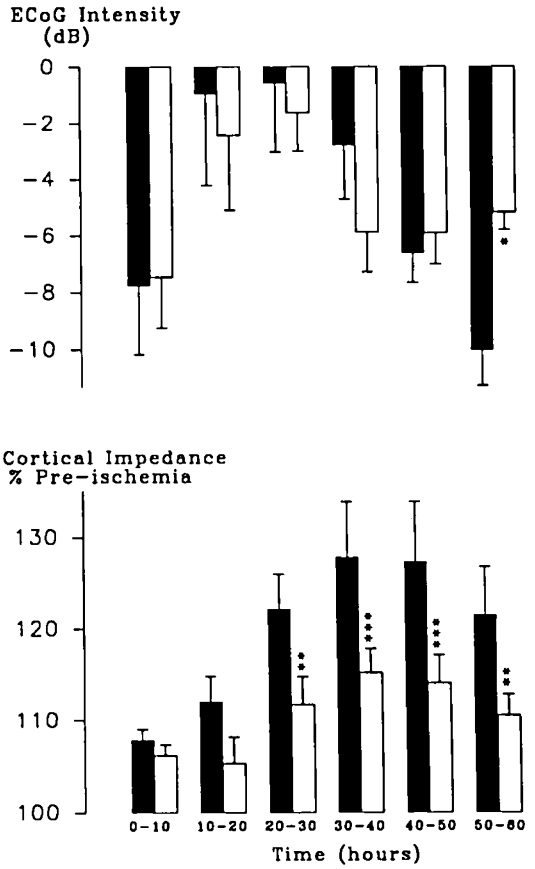

Fig. 2. Bar graphs comparing the time course of changes in mean ECoG intensity (*upper panel*) and mean cortical impedance (*lower panel*) after 30 min of cerebral ischemia in GM1-treated fetuses (*white bars*) and vehicle-controls (*black bars*). GM1 (30 mg/kg) was given 2 h before the insult and continuously (30 mg/kg/d) for 60 h after ischemia. Onset of the secondary increase in impedance coincided with the onset of epileptiform activity. Time is shown as hours postischemia. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (repeated-measures analysis of variance).

larly in the cortex and hippocampus, and greater residual ECoG activity. GM1 therapy may therefore confer worthwhile *in utero* neuroprotection of the fetus against asphyxial insults.

Our findings are consistent with previous reports in adult animals demonstrating that systemic GM1 treatment can reduce morphologic, biochemical, neurophysiologic, and behavioral manifestations of HI brain damage (22–28). However, the concept of *in utero* neuroprotection has not been extensively considered. In the fetal rat subjected to umbilical cord occlusion, prophylactic maternal administration of nimodipine reduces postnatal histochemical derangement and behavioral impairment (29). Previous studies have reported that treatment with the calcium channel antagonists nicardipine or flunarizine can cause cardiac depression and death at high doses in fetal sheep (1, 2) or human neonates (3). Our data showed that GM1 did not alter fetal arterial blood pressure or metabolic status and confirmed previous findings in adults (30) supporting the potential application of this agent.

GM1-treated fetuses in our study showed less residual cytotoxic edema after ischemia. This suggests that neuroprotection is conferred during the acute phase of the ischemic injury and reperfusion. This early action in the cascade of events responsible for neuronal damage may account for the subsequent delay in the onset and magnitude of secondary edema and the shorter duration of low-frequency epileptiform activity, thereby resulting in greater ECoG intensity and improved histologic outcome at 72 h postischemia. Several mechanisms have been proposed to explain the capability of GM1 to limit neuronal damage associ-

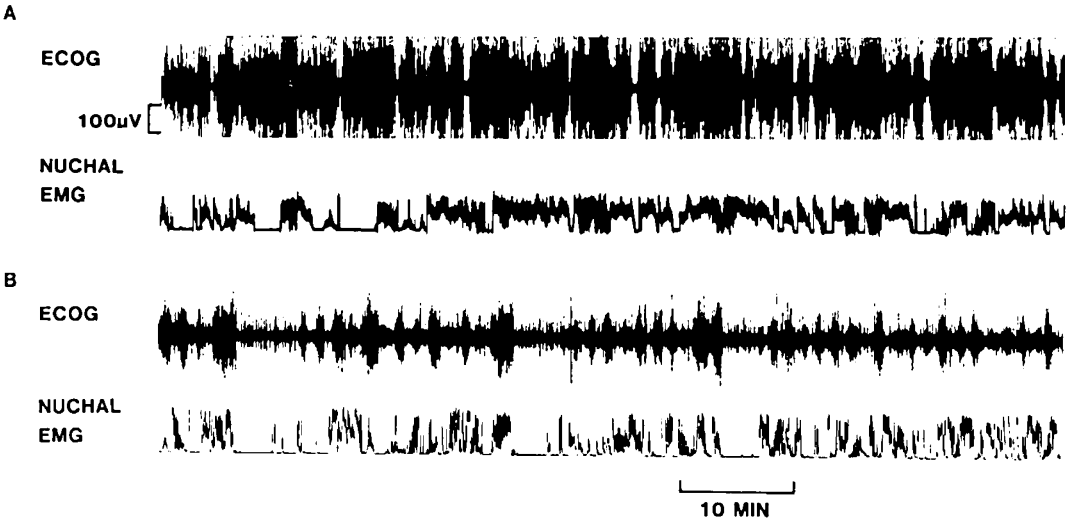

Fig. 3. Representative chart recordings showing raw ECoG and nuchal electromyographic (*EMG*) activity from 10 h after insult in control (*A*) and GM1-treated (*B*) animals. The intense low-frequency epileptiform activity was recorded as spike-wave or polyspike activity and increased nuchal activity. In *B*, GM1 (30 mg/kg) was given at 2 h before the insult and continuously (30 mg/kg/d) for 60 h after ischemia.

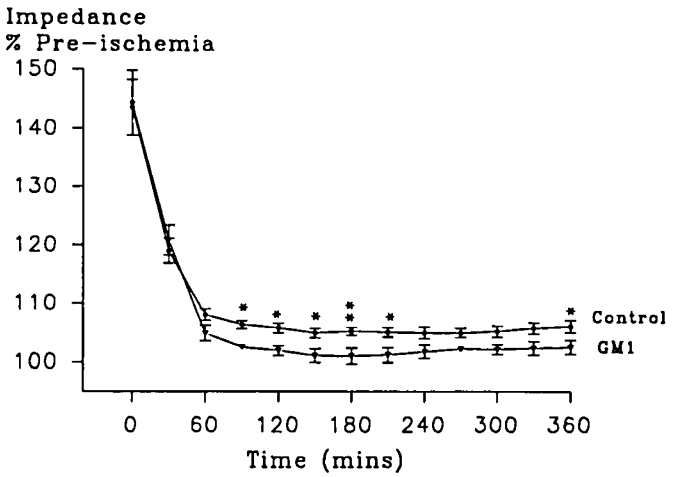

Fig. 4. Graph comparing the time course of change in impedance after 30 min of cerebral ischemia in control (♦) and GM1-treated animals (▽). The initial peak of impedance partially resolved with some residual impedance after the insult. The residual impedance was significantly greater in the controls than in the treatment group. Time is shown as hours postischemia. *, $p < 0.05$; **, $p < 0.01$.

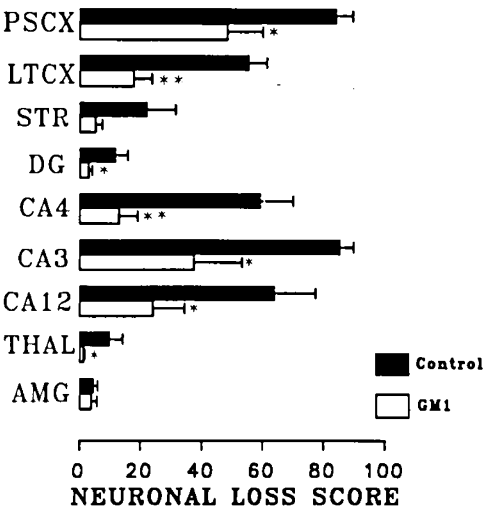

Fig. 5. Histologic demonstration of neuronal damage in various brain regions at 72 h after 30 min of cerebral ischemia. Damage scores are on a linearized scale, 0 to 100. 0: no neuronal loss; 100: total necrosis. Values are means ± SEM. GM1-treated animals showed significantly less damage (*, $p < 0.05$). *PSCX*, parasagittal cortex; *LTCX*, lateral cortex; *STR*, striatum; *DG*, dentate gyrus; *CA12, 3, 4*, cornu ammonis 1, 2, 3, and 4 of the hippocampus; *THAL*, thalamus; *AMG*, amygdala.

ated with cerebral HI insult. These include a direct membrane effect that involves incorporation of GM1 into plasma membranes and results in a stabilization of structure and function. When added to cultured neuronal cells, GM1 is incorporated into the plasma membrane in a time- and concentration-dependent manner (31, 32). A similar uptake occurs in the CNS after the systemic administration of GM1 *in vivo* (33). The reduction in losses of enzymes and fatty acids from plasma membranes (25) supports the hypothesis that GM1 has a direct role in the protection of membrane structure and function.

GM1 can protect neurons against excitotoxicity. Studies have shown that GM1 attenuates EAA-related neurotoxicity in neuronal cell cultures (34–39). GM1, when administered systemically to neonatal or adult rodents, reduces brain neuronal damage induced by exogenous excitotoxins (11, 40, 41). The antiexcitotoxic effects of GM1, unlike those of EAA antagonists, appear to occur by inhibition of intracellular toxic-related events resulting from excessive activation of EAA receptors (38, 42). GM1 was reported to inhibit glutamate-mediated translocation of protein kinase C from cytosol to cell membranes involved in Ca^{2+} influx, thereby limiting the increase in intracellular free Ca^{2+} in primary granular cerebellar cultures (38, 43, 44) and in fetal rat brain (15). Alternatively, GM1 has been shown to potentiate the action of neuronotrophic factors both *in vitro* (45–47) and *in vivo* (47–50) that may improve the survival rate of injured neurons.

In summary, these observations suggest that pretreatment with GM1 protects the brain of fetal sheep during the acute phase of HI injury. GM1 can cross the placental barrier (15) and lacks systemic side effects that are observed with other potential prophylactic agents such as glutamate and calcium antagonists. Thus, prophylactic therapy *in utero* with GM1 is a potential strategy to protect the CNS of fetuses at risk of HI injury.

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