Monosialoganglioside protected ischemic rat hippocampal slices through stabilizing expression of *N*-methyl-*D*-aspartate receptor subunit

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ABSTRACT

AIM: To determine direct protective effect of monosialoganglioside (GM1) on hippocampal slices after oxygen-glucose deprivation and reperfusion (OGD/RP), and investigate the influence on the expression of *N*-methyl-*D*-aspartate receptor subunit 1 (NMDAR1) in those hippocampal slices. **METHODS:** Injury of hippocampal slices and protective effects of GM1 were detected by 2,3,5-triphenyltetrazolium chloride (TTC) staining, toluidine blue staining, and transmission electron microscopy of rat hippocampal slices. Expression of NMDAR1 was detected by Western blot. **RESULTS:** (1) GM1 at 1.0 μmol/L was the most effective concentration to preserve the TTC staining of the hippocampal slices after OGD/RP ($P<0.05$), and the next was GM1 at 10.0 μmol/L ($P<0.05$). (2) Toluidine blue staining and transmission electron microscopy showed GM1 protected the injuried hippocamal slices after OGD/RP. (3) GM1 downregulated the temporally high expression of NMDAR1 in the hippocampal slices immediately after a 25-min OGD and prevented the over low expression of NMDAR1 after a 30-min reperfusion. **CONCLUSION:** GM1 could protect injuried rat hippocampal slices after OGD/RP through stabilizing the expression of NMDAR1.

INTRODUCTION

Monosialoganglioside (GM1) can alleviate cerebral edema and reduce cerebral infarct volume in *in vivo* animal models[1-3], and can prevent death of cultured granule cells *in vitro* from anoxia as well[4]. However, meaning of data derived from a neural preparation without a vasculature and mammalian systemic interaction is limited. The direct protective effect of GM1 on ischemic brain slices has not been reported. In present study, we evaluated the direct effect of GM1 on rat hippocampal slices by detecting 2,3,5-triphenyltetrazolium chloride (TTC) staining, toluidine blue staining, and ultramicrostructure of rat hippocampal slices after oxygen-glucose deprivation and reperfusion (OGD/RP).

N-Methyl-*D*-aspartate receptor (NMDAR), a kind of ionotrophic glutamate receptor, is a receptor-gated Ca^{2+} channel and extensively distributed in the central nervous system (CNS). NMDAR subunit is coded by two gene families: NMDAR1 and NMDAR2. The latter has 4 independent genes: NMDAR2A, 2B, 2C, and 2D. In adult rat, NMDAR1, NMDAR2A, and NMDAR2B

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subunits are mainly distributed in the forebrain[5], and NMDAR2C and NMDAR2B subunits are only found in the hindbrain[6]. In the mammalian CNS, NMDAR serves prominent roles in many physiological and pathophysiological processes including cerebral ischemia during which NMDAR becomes over excited and causes neural injury[7,8].

Previous studies showed GM1 could reduce neural injury induced by toxicity of excitatory amino acid via NMDAR, meanwhile GM1 did not suppress the function of NMDAR-gated Ca^{2+} channel directly[9,10]. Is it possible that the suppressive effect of GM1 on excitatory amino acid toxicity is via influence of GM1 on the expression of NMDAR? Brandoli *et al*[11] found BDNF elicited a time-dependent decrease in the expression of NMDAR2A and NMDAR2C subunits and reduced the NMDA-mediated $[Ca^{2+}]_i$ increase with a time dependency that correlated with their abilities to decrease NMDAR2A and NMDAR2C subunit expression, suggesting that BDNF may protect cerebellar granule cells against excitotoxicity by altering the NMDAR-Ca^{2+} signaling via a downregulation of NMDAR subunit expression. GM1 can enhance the protective effect of BDNF and nerve growth factor (NGF) on neurons[12-14]. While it is unclear whether GM1 can suppress the expression of NMDAR subunits in ischemic neurons. This study was designed to determine whether GM1 could modulate the expression of NMDAR1 in hippocampal slices after OGD/RP as well.

MATERIALS AND METHODS

Slice preparation The experiments were approved by the Institutional Animal Care and Use Committee. Transverse hippocampal slices were prepared from 3 adult male Sprague-Dawley rats (weighing 150 to 200 g, bought from Medical Institute of Zhejiang Province Certificate No 2001001). Rats were anesthetized with 10 % hydrochloride (400 mg/kg, ip) before decapitation[15].

To prepare for TTC staining, we divided the slices into 5 groups (each group had about 10 slices). Slices of each group were preincubated in a beaker containing 25 mL oxygenated normal artificial cerebrospinal fluid (nACSF in mmol/L: NaCl 125, KCl 5, $NaHCO_3$ 25.7, $MgSO_4$ 2, NaH_2PO_4 1.2, Glucose 10, pH 7.4), which was equilibrated with a gas mixture of 95 % O_2 and 5 % CO_2 at 35 ºC for 30 min. GM1 (Manufactured by TRB PHAMA SA of Argentina, Certificate No 38837) at 0 μmol/L (control group), 0.01 μmol/L (group A), 0.1 μmol/L (group B), 1.0 μmol/L (group C), and 10.0 μmol/L (group D) was added into each beaker, and the slices were incubated for another 30 min. After that, the slices were moved to corresponding beaker containing 25 mL deoxygenatd glucose-free nACSF (in mmol/L: NaCl 125, KCl 5, $NaHCO_3$ 25.7, $MgSO_4$ 2, NaH_2PO_4 1.2, Sucrose 10, pH 7.4) equilibrated with a gas mixture of 95 % N_2 and 5% CO_2 at 35 ºC for 25 min OGD. At last, the slices of each group were reperfused for 30 min in the former beaker containing oxygenated nACSF and GM1 in various concentrations[15]. Slices after reperfusion were prepared for TTC staining. The experiment was repeated for 3 times.

To determine the influence of incubating time on the expression of NMDAR1 in hippocampal slices, those slices in different groups were incubated in oxygenated nACSF for 1.0, 1.5, 2.0, 3.0, and 4.0 h respectively. After that, slices of each group were considered as one sample and detected by Western blot (It is the same hereinafter). The experiment was repeated for 3 times.

To determine the influence of reperfusion time on the expression of NMDAR1, slices were incubated for 1 h in the oxygenated nACSF and underwent OGD for 25 min, then slices of each group were reperfused for 0, 30, 60, and 120 min, respectively. Slices of the control group were incubated in oxygenated nACSF for 85 min. The experiment was repeated for 4 times.

To determine the influence of GM1 on the expression of NMDAR1 immediately after OGD, slices were incubated for 30 min in oxygenated nACSF, then GM1 was added into each incubator at 0.0, 0.1, 1.0, and 10.0 μmol/L respectively before slices were incubated for another 30 min. At last, the slices underwent OGD for 25 min. Slices of the control group were incubated in oxygenated nACSF for 85 min. The experiment was repeated for 6 times.

Slices were incubated for 30 min in the oxygenated nACSF, then GM1 was added into each incubator medium at 0.0, 0.1, 1.0, and 10.0 μmol/L, respectively before slices were incubated for another 30 min. At last, slices underwent OGD for 25 min and reperfusion for 30 min. Slices of the control group were incubated in oxygenated nACSF for 115 min. The experiment was repeated for 5 times.

TTC staining Slices after reperfusion were incubated in 0.5 % TTC at 37 ºC for 30 min before washed with PBS and dried with filter paper. Each slice was put into 150 μL raffinate (ethanol:dimethylsulfoxide=

1:1) for 24 h. Therein, 100 μL extractant was moved to 96-well plate, OD_{490} was detected with spectrophotometry. OD_{490} of every gram slice tissue (OD_{490}/g) was calculated ($OD_{490}/g=OD_{490}\cdot(\text{neat weight of each slice})^{-1}$)[15].

Toluidine blue staining Slices were treated as those prepared for TTC staining, and 3 groups were included: Group A, without OGD; Group B, underwent a 25-min OGD and a 30-min reperfusion; Group C, underwent a 25-min OGD and a 30-min reperfusion with 1.0 μmol/L GM1 administrated during ODG. Slices were 10-μm thicknessly frozen-sectioned for toluidine blue staining[16].

Transmission electron microscopy Slices were treated as those for TTC staining. After hippocampal slices were fixed in 2 % precold glutaraldehyde for 30 min, 1×1×0.4 mm^3 blocks in hippocampal CA1 were cut for observation. The samples for transmission electron microscopy were prepared according to standard procedures.

Western blotting Samples were washed twice in PBS and pelleted. Preparation of cell membrane protein, detection of protein concentrations, procedure of SDS-PAGE and Western blot, and source of NMDAR1 antibody were described before[17,18]. Blots from SDS/7.5 % PAGE gels were probed with antibodies to NMDAR1 and reacted for chemiluminesence. Protein of bilateral hippocampi of a normal rat was set as the control. Ratio of band density of each sample to that of the control was calculated as the relative OD.

Statistical analysis Results were presented as mean±SD. Value of pH, PO_2, Pco_2, TTC staining (OD 490/g), and band density (Relative OD) was analyzed statistically by one-way analysis of variance (ANOVA) with SPSS 10.0. $P<0.05$ was considered significant.

RESULTS

Physiological data PH, partial pressure of O_2 (pO_2), and CO_2 (pCO_2) were measured in both nACSF and glucose-free nACSF. The pO_2 of deoxygenated nACSF and deoxygenated glucose-free nACSF was significantly lower than that of oxygenated nACSF and oxygenated glucose-free nACSF, there was no significant difference in pCO_2 or pH among four groups (Tab 1).

Influence of GM1 on TTC staining of rat hippocampal slices after OGD/RP OD_{490}/g of group C (1.0 μmol/L GM1) was significantly higher than that of the control, group A (0.01 μmol/L GM1), and group B (0.1 μmol/L GM1). OD_{490}/g of group D (10.0 μmol/L GM1) was significantly higher than that of the control and group A (Tab 2).

Toluidine blue staining Neurons of the group A had large cell bodies, clear seen Nissl bodies, and normal contour. Neurons of the group B had small cell bodies, condensed nuclei, and few Nissl bodies. Neurons of group C had larger cell bodies, more Nissl bodies and less condensed nuclei when compared with that of group B (Fig 1).

Transmission electron microscopy Neurons of group B had small nucleus, crescent-like condensed chromatin, and disappearance of nucleolus. Neurons of another 2 groups had larger nucleus, existence of nucleolus, and no condensed chromatin (Fig 2).

Influence of incubating time on the expression of NMDAR1 Incubation for less than 3 h in the oxygenated nACSF did not influence the expression of NMDAR1. After the slices were incubated for 4 h, the expression of NMDAR1 began to decrease, and incubating time in three following experiments was less than 3 h (Fig 3).

Influence of reperfusion time on the expression of NMDAR1 and the influence of GM1 on the expression of NMDAR1 after OGD or after OGD/RP The expression of NMDAR1 reached the peak immediately after OGD, and decreased significantly after

Tab 1. pH, pO_2, pCO_2, and glucose concentrations in oxygenated and deoxygenated ACSF. n=8. Mean±SD. $^cP<0.01$ *vs* the groups of oxygenated nACSF.

Group		pO_2/mmHg	pCO_2/mmHg	pH	Glucose/mmol·L^{-1}
Oxygenated	nACSF	203±1.5	41.8±0.8	7.34±0.02	9.62±0.47
	Glucose-free nACSF	203±1.8	41.8±0.6	7.35±0.02	Not detected
Deoxygenated	nACSF	14.4±1.2^c	41.0±0.7	7.34±0.02	9.49±0.61
	Glucose-free nACSF	12.6±1.4^c	42.5±1.3	7.36±0.01	Not detected

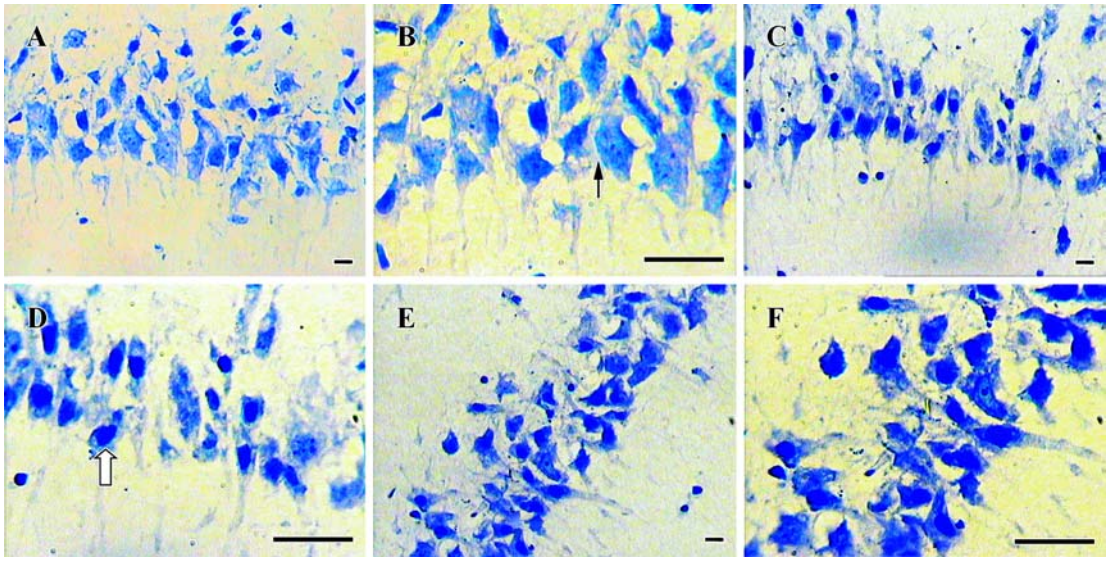

Fig 1. Influence of GM1 on toluidine blue staining of neurons in CA1 area of rat hippocampal slices after OGD/RP. Group A (A, B): Neurons have normal morphology with large cell body and clear seen Nissl bodies (Arrow). Group B (C, D): Most of neurons shrinking with condensed nuclei and sparse Nissl bodies (Hollow arrow). Group C (C, D): Neurons had larger cell bodies than that of group B, most of neurons had Nissl bodies and milder condensed nuclei compared with that of group B. A, C, E: Low magnification; B, D, F: High magnification. Bar=50 μm. The experiment was repeated 6 times with similar results.

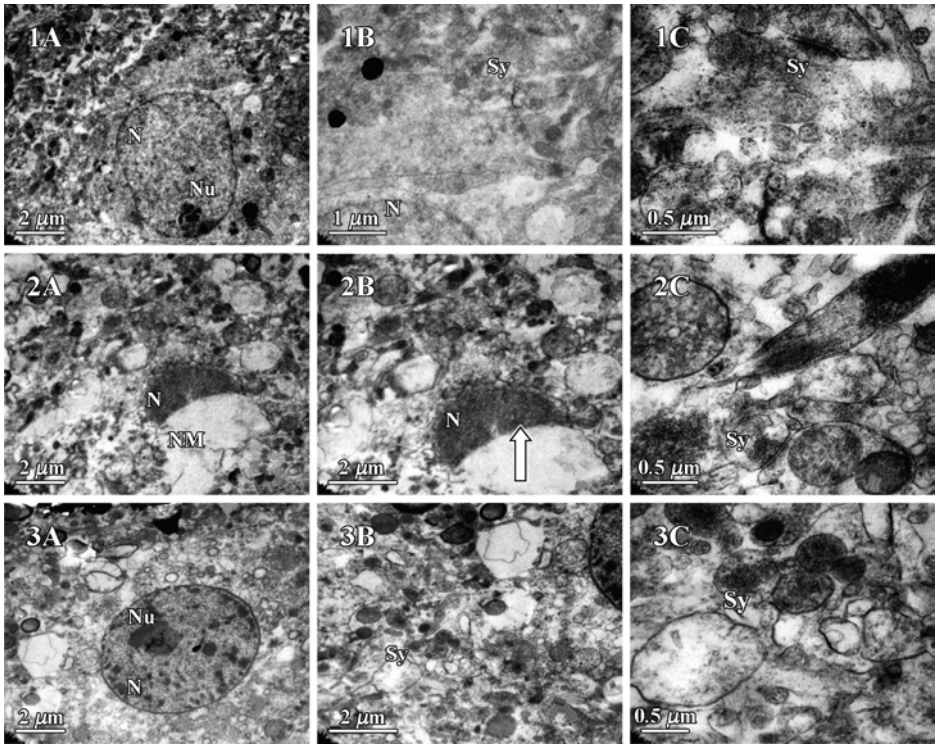

Fig 2. Influence of GM1 on ultramicroscostructure of neurons of hippocampl slices after OGD/RP. Group A (1A-1C): Neurons had larger nucleus with existence of nucleolus and without condensed chromatin; Group B (2A-2C): Neurons had small nucleus, crescent-like condensed chromatin, and disappearance of nucleolus (Hollow arrow); Group C (3A-3C): Nucleus had similar morphology as that of group A, but had small spots of condensed chromatin in the nucleus. (There were many synapses around those observed cells, which showed that those cells were neurons. N: nucleus; Nu: nucleolus; Sy: synapse; NM: nuclear membrane). The experiment was repeated for 6 times with similar results.

Tab 2. Influence of GM1 on TTC staining (OD 490/g) of rat hippocampal slices after a 25-min OGD and a 30-min reperfusion. Mean±SD. $^{b}P<0.05$ *vs* control, group A, and B; $^{e}P<0.05$ *vs* control and group A.

Group	OD490/g
Control (n=12)	6.88±0.71
GM_1 0.01 (n=14)	6.89±0.72
GM_1 0.1 (n=10)	7.46±2.23
GM_1 1.0 (n=11)	10.19±2.45^{b}
GM_1 10.0 (n=13)	9.18±2.49^{e}

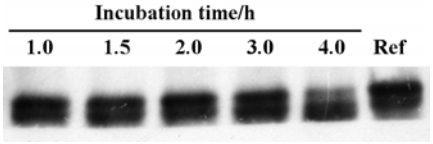

Fig 3. Influence of incubating time on the expression of NMDAR1 of rat hippocampal slices. Ref: Reference. The experiment was repeated for 3 times with similar results.

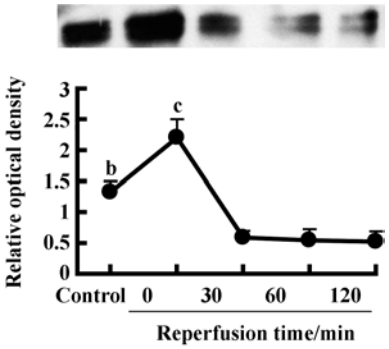

Fig 4. Influence of reperfusion time on the expression level of NMDAR1 of rat hippocampal slices. n=4. $^{b}P<0.05$ *vs* groups at 30, 60, and 120 min after reperfusion. $^{c}P<0.01$ *vs* other groups.

a 30-min reperfusion (Fig 4). GM1 at 0.1, 1.0, and 10.0 μmol/L inhibited the peak expression of NMDAR1 immediately after OGD, and the decreased expression of NMDAR1 at 30 min after a reperfusion concentration-dependenthy (Fig 5).

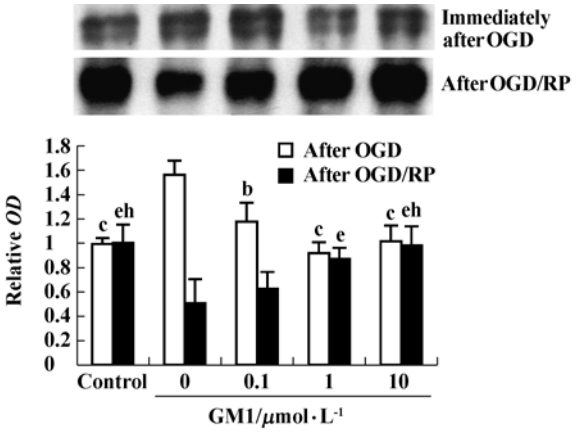

Fig 5. Influence of GM1 on the expression of NMDAR1 immediately after OGD (n=6) and after OGD/RP (n=5) in the hippocampal slices. $^{b}P<0.05$, $^{c}P<0.01$, $^{e}P<0.05$ group treated with 0.0 μmol/L GM1. $^{b}P<0.05$ *vs* group treated with 0.1 μmol/L GM.

DISCUSSION

Direct protective effect of GM1 on ischemic hippocampal slices *in vitro* has not been reported. We evaluated the protective effect of GM1 on hippocampal slices after OGD and reperfusion. Preston *et al*[19] reported a new method to evaluate neural viability after ishemia. Incubation of fresh brain tissue with TTC will produce crystal of red formazan, which can be quantificationally detected to evaluate the neural viability. According to this rational, we designed the method to evaluate the protective effect of GM1 on ischemic hippocampal slices *in vitro*. We found GM1 could protect hippocampal slices after OGD/RP with a concentration-related manner. GM1 at 1.0 μmol/L was the most potent.

Morphological evidences also showed the protective effect of GM1 on hippocampal slices after OGD/RP, neurons of whose CA1 area became apoptotic with condensed nuclei and crescent-shaped chromatin marginally located, however, GM1 could inhibit the apoptotic changes of the neurons.

Cerebral ichemia could cause change of the expression of NMDAR subunits in the gerbil and rat brain *in vivo*[20-22]. In the present study, we found the expression of NMDAR1 of hippocampal slices reached the peak level immediately after OGD, and decreased significantly after 30 min reperfusion. GM1 can inhibit the temporally peak expression of NMDAR1 of hippocampal slices after OGD, and help neurons to preserve appropriate NMDAR1 expression level for normal physiological process after a 30 min-reperfusion. That is, GM1 could stabilize the expression of NMDAR1 in the ischemic neurons of hippocampal slices, which conformed the results of previous studies that GM1 could inhibit exitoxicity of amino acid via NMDA re-

ceptor during cerebral ischemia, but did not block the NMDA-gated Ca^{2+} ion channel[11].

In conclusion, GM1 can protect hippocampal slices after OGD/RP *in vitro* through stabilizing the expression of NMDAR1.

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