Excitotoxicity Induces Changes in Rat Brain Gangliosides

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The behavior of major gangliosides in rat hippocampus and olfactory bulbs after microinjections of Ibotenic acid, an NMDA receptor agonist was examined in vivo. Four days after injection in the olfactory bulbs under free-movement conditions, the ratio of gangliosides in rats injected with the the excitatory amino acid increased significantly for GQ1b, GT1b and GD1b in hippocampus and a decreased for GQ1b, GT1b, GD1b, GD1a and GM1 in the olfactory bulbs. The alterations in gangliosides were minimal one day after injection. Five weeks after, the amount of GD1a and GM1 in the hippocampus remained at normal levels while GQ1b, GT1b and GD1b dramatically decreased. In the olfactory bulbs ganglioside recovery was observed. Experiments with Beta-N-oxalylamino-L-alanine (L-BOAA), an AMPA receptor agonist corroborated the suggestion that gangliosides play an active role during excitotoxicity. The changes of hippocampal gangliosides could be related with a compensatory mechanisms. Experiments with PBS confirmed that the changes observed were due to the action of the excitotoxicity. The results obtained explain in part, the data published in recent reports concerning the effectiveness of ganglioside in the treatment of degenerated brain cells due to excitoxicity.

Introduction

Hippocampal gangliosides have been the focus of interest in several reports because of the fundamental role that hippocampus plays in memory formation and its vulnerability to several neurological diseases[1,2,3]. Glutamate excitotoxicity seems to play an important role as a factor in many acute and chronic neurologic diseases[3,4], glutamatergic pathway predominates in the hippocampus[5] and exogenous gangliosides promote changes on glutamate receptors[1]. Taking in account these three facts, the authors utilized the signal transmission pathway between olfactory system and hippocampus[6,7,8] in order to clarify the effect of excitotoxity on endogenous gangliosides. First, Ibotenic acid and beta-N-oxalylamino-L-alanine (L-BOAA) which are glutamate-like excitatory aminoacids (NMDA and AMPA glutamate receptor agonists respectively)[9,10,11,12], were microinjected into the olfactory bulbs of rats under free movement conditions in order to observe the behavior of gangliosides in this organ. Second, the possible effect of induced excitatory signals from

the olfactory bulb on hippocampal gangliosides was investigated. Besides the effect of excitoxicity in the total neuraminic acid amount in hippocampus was determined.

Materials and Methods

Microinjections

Rats (male, 10 weeks) were anesthetized with pentobarbital (50mg/kg) and placed in a stereotaxic apparatus. A guide-cannula was permanently implanted to a depth of 2.5 mm in the left olfactory bulb (6.2 mm forward and 0.9 mm lateral from bregma)[13]. Two days after the implantation procedure, the animals were injected under free-movement conditions with 4 uL 0.01M Ibotenic acid in PBS (pH 7.4) at a flow rate of 1 uL/min using the micro-syringe pump and Hamilton syringes. The rats were decapitated 1, 4 or 35 days after the injection and the left and right hippocampus and olfactory bulbs were dissected. Non-injected rats were used as control. The same procedure was repeated with injections of 1 uL of 0.05M L-BOAA in PBS (pH 7.4) at a flow rate of 0.1 uL/min or 4 uL of PBS (pH 7.4) at a flow rate of 1uL/min flow rate. Four days after the injection, the rats were decapitated and the left and right hippocampi and olfactory bulbs were dissected.

Ganglioside extraction:

The left and right hippocampi and olfactory bulbs were processed and prepared separately. Approximately 25 mg of wet tissue was homogenized in 4 mL of chloroform/methanol/water (1.5:3:0.4), centrifuged at 2500 rpm for 10 min, and the supernatant was collected. The pellet was re-homogenized in chloroform/methanol (0.75:1.5) and centrifuged at 2500 rpm for 30 min. The supernatants were then combined and ran through a DEAE Sephadex column (0.5 id×3 cm). The neutral lipids were washed out with 5 mL of chloroform/methanol/water (30:60:8). The acidic lipids were eluted with 5 mL of chloroform/methanol/2M sodium acetate (30:60:8). The acidic fractions were then evaporated to remove the solvents and redissolved in 2 mL of 0.1N potassium hydroxide in methanol. After 2 hours of hydrolysis at 37°C, the reaction was stopped with 2 mL of 0.1N acetic acid, the solvent removed by evaporation, and the hydrolisate dialyzed against water for 1 day. The solution was then evaporated to remove the water, the sample redissolved in chloroform/methanol (9:1), and put through an IATROBEADS column (0.5 id×3 cm). The first fraction eluted with 5 mL of chloroform/methanol (85:15) was discarded. The second fraction eluted with 5 mL of chloroform/methanol (3:7) was collected, evaporated to remove the solvent, redissolved in 1.5 mL of chloroform/methanol (1:1), and dried under nitrogen stream. Finally, chloroform/methanol (3:7) was added to obtain concentrations of 1mg/uL that corresponded to the initial wet weight of the tissues, for the samples of Ibo after 35 days the prepared concentrations were of 2mg/uL.

TLC:

Ganglioside composition was determined by TLC-densitometry. Calibration curves were drawn using an authentic ganglioside mixture. This mixture was composed of known amounts of GM1, GD1a, GD1b, GT1b, and GQ1b. In addition, a bovine ganglioside mixture was used as standard. The correlation coefficients for linearity of calibration curves of standards were between 0.98 and 0.99 ($p<0.01$). Spots of 2 uL of samples were applied to the TLC plates, using a Hamilton syringe and developed for 20 min in chloroform/methanol/0.2% calcium chloride (55:45:10). Standards samples were developed in parallel with the test samples. Gangliosides were visualized by the resorcinol-HCL reagent and measured at 580 nm using the scanner.

Ganglioside change determination:

Ganglioside changes in olfactory bulbs were calculated as follow:

Ganglioside ratio (GgR)=amount of neuraminic acid in gangliosides of left olfactory bulb (injected side of olfactory bulb)/amount of neuraminic acid in gangliosides of right olfactory bulb (non-injected side of olfactory bulb).

GgR changes in hippocampi were calculated as follow:

Ganglioside ratio (GgR)=amount of neuraminic acid in gangliosides of left hippocampus (hippocampus corresponding to injected side of olfactory bulb)/amount of neuraminic acid in gangliosides of right hippocampus (hippocampus corresponding to non-injected side of olfactory bulb).

For the statistical analysis of the results were used first ANOVA and then a multiple comparison Dunett test ($p<0.05$).

Total neuraminic acid determination:

The rats were microinjected and the hippocampus dissected. The hippocampal tissues were homogenized in 300 uL of 0.1N H_2SO_4, hydrolyzed for 2 hours at 80°C, centrifuged at 3000 rpm for 20 min at 4°C, and the supernatants were collected. Next, 20 uL of 0.1N H_2SO_4 and 50 uL of 7mM DMB in solution containing 1.0 M 2-mercaptoethanol and 18mM Na_2SO_4 were added to 30 uL of supernatant. The samples were heated for 2.5 hours at 60°C, purified on a reverse phase chromatography column using chloroform/methanol/water (25:4:91) as an effluent at a rate of 1.2 mL/min, and the neuraminic acid was analyzed with the fluorometer. For the statistical analysis of the results ANOVA was used ($p<0.01$).

Results

The gangliosides from the hippocampus were purified separately and developed by TLC 1, 4 and 35 days after Ibotenic acid injection (Fig. 1).

Hippocampal GgR were calculated and compared with GgR of normal rat brain. The results showed that one and four days after injection the ganglioside ratio in the rats injected with the excitatory aminoacid increased, significantly for GQ1b, GT1b and four GD1b days after injection. Five weeks after, the ratio of GD1a and GM1 remained at normal levels, but the amount of GQ1b, GT1b and GD1b decreased significantly from the initial stage ($p<0.05$) (Fig. 2).

The analysis of the gangliosides purified from the olfactory bulbs was performed in the same manner as the hippocampal gangliosides (Fig. 3). The results showed that there was no significant changes one day after injection of Ibotenic acid. However, after four days, compared with the ganglioside ratio in the normal rat olfactory bulb, the ratio in rats injected with the excitatory amino acid decreased, particularly with respect to GQ1b, GT1b, GD1b, GD1a and GM1 levels. Five weeks after, the gangliosides recovered to normal rat brain levels ($p<0.05$) (Fig.4).

Four days after injection fundamental changes induced by Ibotenic acid were observed in both, hippocampus and olfactory bulbs, therefore the subsequent experiments, considered this interval of time to investigate the effect of L-BOAA injections on the GgR in olfactory bulbs and hippocampi. The gangliosides were purified separately and developed by TLC and the respective GgR calculated. The results obtained with L-BOAA were compared with the GgR of rats injected with PBS. The results obtained for Ibotenic acid four days after injections were also compared in order to determine whether the microsurgery and injection affect the results (Fig 5 and Fig. 6)

We found for rats injected with L-BOAA, significant increases in hippocampal GgR in terms of GT1b, GD1b and GM1 and significant decreases in GgR of olfactory bulbs, in particular for GT1b, GD1a and GM1. In rats injected with Ibotenic acid hippocampal GgR showed significant increases for GT1b and GD1b (GQ1b was not calculated and for olfactory bulbs there were observed significant decreases for GD1a and GM1 when compared with rats injected with PBS ($p<0.05$).

Moreover some significant differences were found when the results of rats injected with L-BOAA and rats injected Ibotenic acid were compared. Hippocampal GgR showed significant differences for GM1, due to the higher increment of GM1 in rats injected with L-BOAA. For GgR in olfactory bulbs significant differences were observed for GT1b and GD1a due to the greater decline in GgR of those gangliosides in rats injected with L-BOAA ($p<0.05$).

There were no observable changes in the total hippocampal neuraminic acid content of rats that had received L-BOAA and Ibotenic acid injections in the olfactory bulbs when compared with non-operated rats and rats injected with PBS ($P<0.01$) (Table 1).

Dicussion

The excitotoxicity is characterized by the increase of intracellular Ca^{2+} and excessive glutamate release that triggers a cascade of process, many of which remain activated long time

after the initial stimulus is removed, leading to neuronal cell injury, even death. Our results show that excitotoxicity induced by injections of Ibotenic acid in the rat olfactory bulbs promote transient changes of ganglioside amount in olfactory bulb and permanent changes in hippocampus. The decreases of GgR in olfactory bulbs are due to neuronal injury caused by the direct effect of excitotoxicity. The observed increase of hippocampal pathway B gangliosides suggests the action of a compensatory process to increase the number of glutamate receptor that decreased due to neuronal loss as a result of the induced excitoxicity from the olfactory bulb. The results obtained with L-BOAA strengthen the results observed in the experiments with Ibotenic acid. The recovery of GgR in the olfactory bulbs five weeks after the injection of Ibotenic acid could be related with the capacity of regeneration of olfactory neurons. The non-recovery of hippocampal polysialo-gangliosides GgR to normal levels indicates that these are the most involved gangliosides in the protective response to counteract the effect of excitotoxicity. The changes in the hippocampus were permanent due to the higher sensibility of hippocampus to the excitotoxicity. Our results demonstrate that gangliosides have an active role during excitotoxicity as modulation molecules and explain, in part, the published data concerning the effectiveness of ganglioside in the treatment of degenerated brain cells due to excitoxicity.

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Table 1
Effect of injections of L-BOAA and Ibotenic acid into olfactory bulb on total neuroaminic acid in rat hippocampus.

Parameter measured	*Control*	*PBS*	*L-BOAA*	*Control*	*PBS*	*Ibotenic acid*
Total neuroaminic acid	3.8 ± 0.20	3.7± 0.3	3.63 ± 0.2	4.7± 0.3	4.8 ± 0.1	4.7 ± 0.1

Values are mean ± S.E.M (nmol/mg wet tissue: brain) from 3 animals in each group. *PBS* are the data of the rats injected with PBS, *L-BOAA* are the data of the rats injected with L-BOAA, *Ibotenic acid* are the data of the rats injected with Ibotenic acid and *Control* are the data of not operated rats. Values were compared by ANOVA ($P<0.01$).

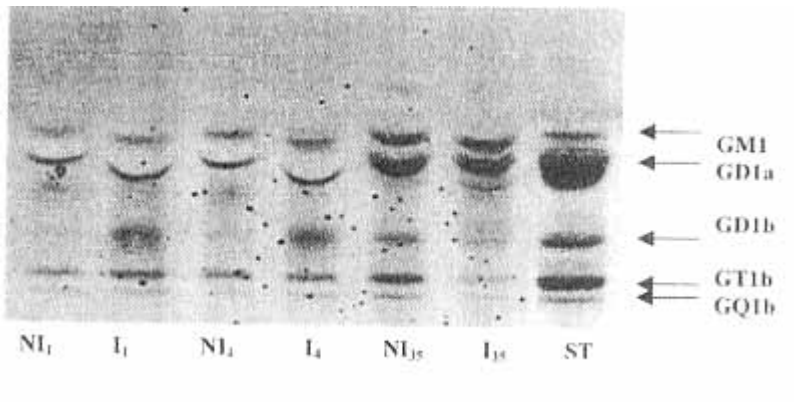

Fig. 1. Thin layer chromatogram of rat hippocampal gangliosides after injection with Ibotenic acid. NI, hippocampus corresponding to the side of olfactory bulb that was not injected. I, hippocampus corresponding to the injected side of olfactory bulb. Subscripts 1, 4 and 35 different days after injection. ST is the standard.

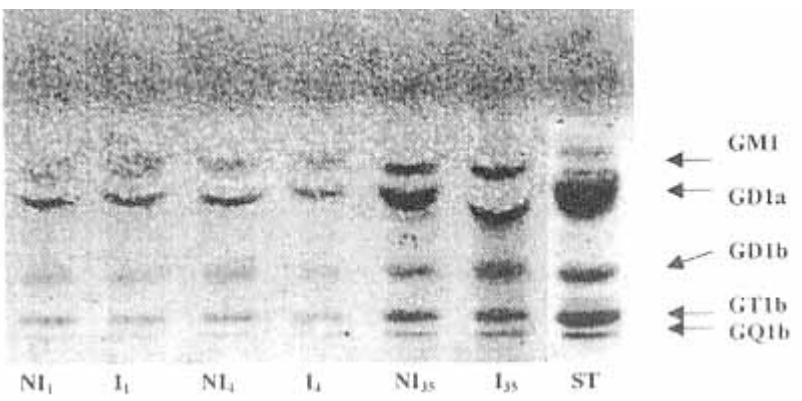

Fig. 2. Time-dependent changes of Hippocampal ganglioside ratio (GgR) after injections with Ibotenic acid. NB are non-injected rats. Rats were sacrificed 1, 4 or 35 days after injections. Values are mean ± S.E.M. from 3 animals. The data were evaluated by ANOVA and then by a multiple comparison Dunett test ($p<0.05$).

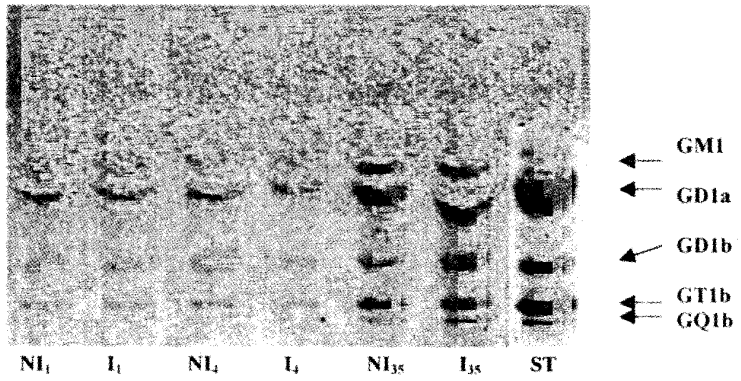

Fig. 3. Thin layer chromatogram of gangliosides of rat olfactory bulbs after injection with Ibotenic acid. NI, refers to the no injected olfactory bulb. I, refers to the injected olfactory bulb. Subscripts 1, 4 and 35 represent different days after injection and subscript ST represent the standard.

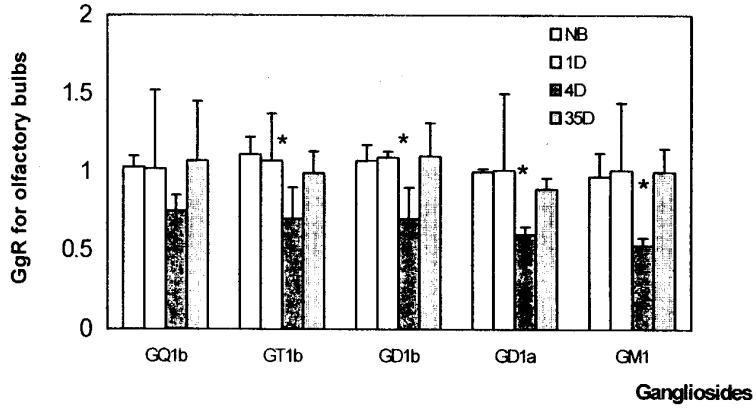

Fig. 4. Time-dependent changes of ganglioside ratio (GgR) in olfactory bulbs after injections with Ibotenic acid. NB are non-injected rats. Rats were sacrificed 1, 4 or 35 days after injections. Values are mean ± S.E.M. from 3 animals. The data were evaluated by ANOVA and then by a multiple comparison Dunett test ($p<0.05$).

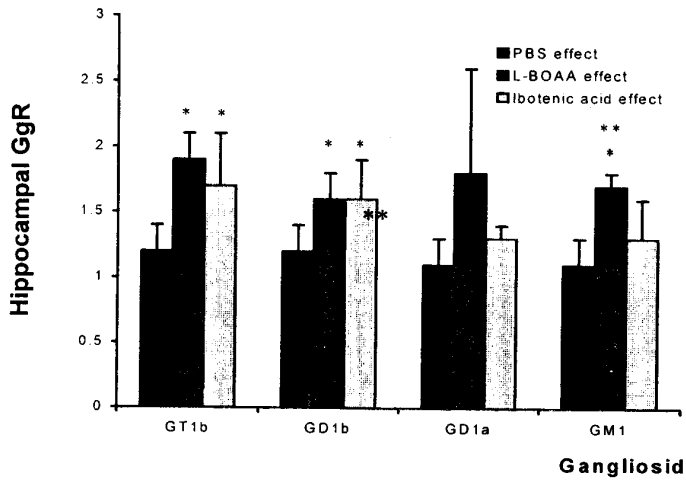

Fig. 5. Hippocampal ganglioside ratio (GgR) for rats injected with L-BOAA or Ibotenic acid. PBS are the data of rats injected with PBS. Rats were sacrificed 4 days after injections. Values are mean ± S.E.M. from 3 animals. The data were evaluated by ANOVA and then a multiple comparison Dunett test. *significance for comparison with PBS, **significance for comparison between L-BOAA and Ibotenic acid ($p<0.05$).

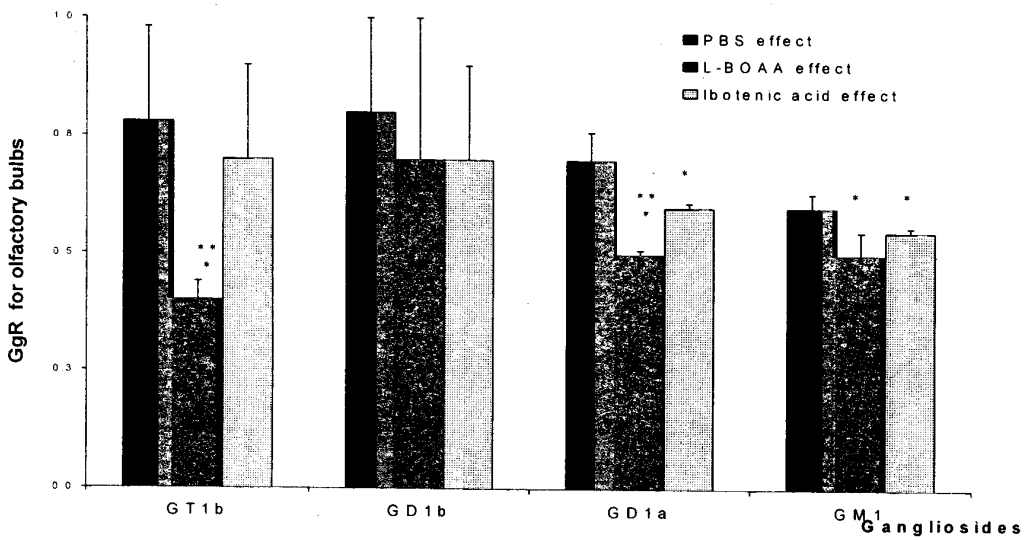

Fig. 6. Ganglioside ratio (GgR) in olfactory bulbs for rats injected with L-BOAA or Ibotenic acid. PBS are the rats injected with PBS. Rats were sacrificed 4 days after injections. Values are mean ± S.E.M. from 3 animals. The data were evaluated by ANOVA and then a multiple comparison Dunett test. *significance for comparison with PBS, **significance for comparison between L-BOAA and Ibotenic acid. ($p<0.05$).