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Ganglioside Content and Composition in Rat Cerebellum After Prolonged Diazepam Treatment

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Summary

The main purpose of this study was to determine the content and composition of cerebellar gangliosides after prolonged diazepam treatment and their possible recovery after diazepam withdrawal. Male Wistar rats were administered diazepam in a dose of 10 mg/kg/day in drinking water for 3, 5 or 6 months. A additional group of rats had a one-month recovery period after five months of diazepam treatment. Control animals were age-matched and pair-fed. At the end of the experiment, the animals were sacrificed and the total cerebellar contents of ganglioside-NeuAc as well as its content in particular ganglioside fractions were estimated. After three months of diazepam consumption, no changes of ganglioside-NeuAc in investigated fractions (G_{Q1b}, G_{T1b}, G_{D1b}, G_{D1a}, G_{M1}, G_{M2}, and G_{M3}) were observed. Five months of diazepam treatment caused a significant decrease in the total amount of gangliosides, which was evident in most of the investigated fractions, with the exception of the monosialoganglioside G_{M2}. Six months of treatment induced a generalized decrease in all the investigated ganglioside fractions. The diazepam-induced ganglioside reduction found after five months of treatment was also present after a one-month recovery period. The only fraction, which recovered and reached its control value ,was monosialoganglioside G_{M3}.

Key words
Cerebellum • Ganglioside • Diazepam • Recovery • Rat

Introduction

Most of current research in CNS pharmacology has been focused on adaptive changes elicited in the brain by chronic treatments with various drugs (Bloom 1985). However, no evidence has been published that benzodiazepines may modify brain lipid composition. The aim of our previous investigations (Vrbaški *et al.* 1989) was to determine the biochemical alterations of brain lipids in rats after six months of diazepam treatment (10 mg/kg/day in drinking water) as well as to draw some conclusions with regard to the role of brain lipid composition in relation to benzodiazepine action. It was found that the content of phospholipids, phosphatidylethanolamine and phosphatidylserine, monogalactosyl glycolipids, hydroxy and nonhydroxy

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fatty acyl galactocerebroside and gangliosides G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}, were significantly reduced in the brain of diazepam-treated rats. A decrease of monosialoganglioside G_{M1} and an increase of disialoganglioside G_{D1b} content were observed in the striatum of diazepam-treated rats (Vrbaški and Kostic 1990). These results suggest that changes in the brain lipid content may reflect the adaptive changes that occur after prolonged exposure to diazepam. Furthermore, the changes in lipid composition also appeared to be important in connection with the prolonged administration of these drugs in clinical practice, e.g. diazepam addiction.

It is well known that the cerebellum is particularly sensitive to benzodiazepines as compared to other brain regions (Haefely *et al.* 1983). The main purpose of this study was to determine the content and composition of cerebellum gangliosides during and after diazepam treatment.

Methods

Twenty-four 2-month-old male Wistar rats (Institute colony, Belgrade, Yugoslavia) weighing approximately 200 g, were divided into four groups of six animals each. All animals were fed a nutritionally and energetically adequate diet (protein 21 %, carbohydrate 62 %, fat 5 %, vitamin premix 0.25 %, mineral mixture 2.25 %, Veterinarski Zavod, Subotica, Yugoslavia). They were individually housed in cages for daily determination of fluid and food consumption. The average food intake per rat during the study was 18 g per day, in both control and experimental groups. The control animals were matched for initial body weight and weighed at weekly intervals thereafter.

The control groups received tap water *ad libitum* as the only drinking fluid. The daily fluid intake of the rats drinking diazepam solution was found to be equal to the daily water intake consumed by the control groups.

Diazepam (KRKA, Novo Mesto, Slovenia) dissolved in tap water was offered daily as the sole drinking solution to all experimental groups. The diazepam-treated groups consumed 10 mg of diazepam/kg of body weight daily, and this amount remained relatively constant throughout each period of treatment. The dose of 10 mg of diazepam/kg/day had been established as optimal in preliminary experiments, because it induced physical dependence without causing toxic or other side effects during chronic administration (Fuch *et al.* 1984).

The rats were randomly divided into four groups designated III, V, VI and V+1. The designation III, V and VI indicates the duration in months of diazepam treatment, while V+1 indicates that after 5 months of diazepam treatment the animals were switched to tap water as the only available source of drinking fluid and this was continued for another month of recovery (I-REC).

At the end of experiment, the animals were killed by CO_2 inhalation. Their brains were rapidly removed, the cerebella were excised and processed for biochemical assays.

Table 1. Mean cerebellar weight (g) and relative cerebellar weight (g/100g BW)

Group	III	V	VI	V+1
Controls				
(g)	0.263±0.011	0.260±0.031	0.283±0.014	0.286±0.021
(g/100g BW)	0.071±0.003	0.073±0.006	0.067±0.003	0.067±0.005
Diazepam (10 mg/kg/d)				
(g)	0.254±0.012	0.274±0.021	0.270±0.032	0.276±0.012
(g/100g BW)	0.072±0.005	0.075±0.004	0.068±0.003	0.069±0.004

Results are expressed as means ± SD, BW – body weight.

Ganglioside extraction and determination of ganglioside classes

Total gangliosides were extracted according to Harth *et al.* (1978) and total ganglioside-NeuAc (N-acetylneuraminic acid) was determined by Svennerholm's resorcinol method (1957), as modified by Miettinen and Takki-Luukkainen (1959). We applied monodimensional ascending chromatography for the ganglioside separation, which was carried out on Silica G HPTLC plates (10x10 cm) according to the method of Harth *et al.* (1978) using successively three different solvent systems: 1) chloroform to the top; 2) chloroform–methanol–water, 70:30:4 (v/v); 3) chloroform–methanol–0.25 % $CaCl_2$, 50:42:11 (v/v). Ganglioside-NeuAc in the fractions obtained by TLC was determined according to the method of Horgan (1981).

Statistical analysis

The data were analyzed for main effects using a one-way analysis of variance (ANOVA), followed by a multiple comparison to corresponding controls by Dunnett's test (Cheung and Holland 1991). The Dunnett's test was performed only if the analysis of variance showed a significant difference between the groups.

Table 2. Total cerebellar ganglioside-NeuAc content (μmol/g tissue) in rats after increasing duration of diazepam treatment (10 mg/kg/d) and one month after diazepam exposure was discontinued

Group	III	V	VI	V+1
Controls	1.767±0.146	1.809±0.233	1.836±0.177	1.836±0.177
Diazepam	1.836±0.294	1.421±0.101*	0.996±0.101**	
I-REC				1.553±0.154*

Results are expressed as means ± SD (n=6). Asterisks indicate significant differences from corresponding control values ($p<0.05$; ** $p<0.01$).*

Results

The weight of the cerebellum in diazepam-treated groups did not differ from the controls (Table 1).

The total cerebellar ganglioside-NeuAc content after five and six months of diazepam treatment was significantly decreased compared to corresponding controls, while it was unchanged in group III (Table 2). The total cerebellar ganglioside content in the group V+1 that had undergone a period of recovery (I-REC) showed a slight but non-significant increase (1.553±0.154 μmol/g tissue) when compared to the diazepam-treated group V (1.421±0.101 μmol/g tissue) (Table 2).

We also analyzed the content of ganglioside fractions (G_{Q1b}, G_{T1b}, G_{D1b}, G_{D1a}, G_{M1}, G_{M2}, and G_{M3}) after prolonged diazepam treatment (Table 3). After three months of diazepam consumption, no significant changes in ganglioside-NeuAc content in these investigated fractions were found. However, five months of diazepam treatment caused a significant decrease in most investigated fractions, except for the monosialoganglioside G_{M2}. Six months of treatment induced a generalized decrease in all the investigated fractions.

Following one-month recovery period the contents of gangliosides G_{T1b}, G_{D1a} and G_{M1} fractions remained decreased, while the contents of G_{Q1b} and G_{D1b} ganglioside fractions were significantly increased ($p<0.01$). The content of ganglioside-NeuAc in monosialoganglioside G_{M3} fraction was markedly increased ($p<0.001$) and almost reached its control value (Table 3).

Table 3. Ganglioside-NeuAc (mmol/g) in ganglioside fractions of rat cerebellum after prolonged duration of diazepam consumption (groups III, V, VI) and one month after diazepam exposure was discontinued (group V+1).

Group	III (n=6)		V (n=6)		VI (n=6)		V+1 (n=6)	
	C	DZP	C	DZP	C	DZP	C	I-REC
G_{Q1b}	0.127±0.022	0.124±0.009	0.146±0.017	0.111±0.018*	0.138±0.016	0.065±0.017***	0.138±0.016	0.136±0.006^{++}
G_{T1b}	0.498±0.008	0.488±0.016	0.425±0.016	0.384±0.015**	0.497±0.024	0.274±0.009**	0.497±0.024	0.330±0.011***$^{++}$
G_{D1b}	0.213±0.023	0.243±0.026	0.217±0.031	0.175±0.006**	0.218±0.007	0.172±0.009***	0.218±0.007	0.209±0.019^{++}
G_{D1a}	0.351±0.028	0.348±0.016	0.348±0.036	0.290±0.038**	0.368±0.021	0.264±0.024***	0.368±0.021	0.270±0.011***
G_{M1}	0.270±0.011	0.262±0.019	0.253±0.010	0.172±0.023**	0.266±0.035	0.144±0.004***	0.266±0.035	0.190±0.018***
G_{M2}	0.169±0.019	0.182±0.028	0.160±0.015	0.151±0.026	0.159±0.012	0.046±0.013***	0.159±0.012	0.143±0.018
G_{M3}	0.139±0.004	0.111±0.018	0.197±0.030	0.087±0.018**	0.190±0.029	0.053±0.016***	0.190±0.029	0.189±0.018^{+++}

Results are expressed as mean ± SD. The control groups did not differ from one another. Asterisks indicate experimental values that are significantly different ($p<0.05$, **$p<0.01$, ***$p<0.001$) from corresponding control values, whereas crosses denote significant differences between V-DZP and I-REC groups ($^{++}p<0.01$, $^{+++}p<0.001$).*

Discussion

The main aim of our investigation was to determine the influence of prolonged diazepam treatment on the ganglioside composition of the rat cerebellum as well as the possible ganglioside recovery after the withdrawal of prolonged diazepam treatment. The chronic treatment lasting 6 months is longer than that generally used in such studies and is more relevant to the clinical situation. The results reported here indicate that significant changes occur in the ganglioside profile but their physiological significance is still not clear.

After three months of diazepam consumption, no changes were observed in the total content of ganglioside-NeuAc or in investigated fractions. This is in agreement with the assumption that a three-month period is not sufficiently long to achieve biochemical alterations in the cerebellar ganglioside content. On the other hand, the above data indicate that diazepam treatment for five or six months causes significant changes in the content and distribution of cerebellar gangliosides. It is possible that our experimental regimen (10 mg DZP/kg/day) for 5 or 6 months may lead to the development of tolerance and physical dependence which was reflected by changes in major membrane ganglioside constituents such as G_{T1b}, G_{D1a} and monosialo-G_{M1}. Since these fractions did not recover during the withdrawal period, it can be proposed that the long-term presence of diazepam and its metabolite desmethyldiazepam in the brain, and especially in cerebellum, affect several membrane-bound enzyme systems involved in the synthesis and degradation of membrane gangliosides, possibly following transmitter-mediated activation of sialylase and sialyltransferase.

Furthermore, several lines of evidence suggest that the benzodiazepine receptor is part of a neuronal mechanism which serves as an amplifying system for GABAergic inhibition (Haefely 1980, Protic 1990). Benzodiazepines may also induce alterations in presynaptic mechanism involved in GABAergic neurotransmission. Presynaptic GABA receptors modulate Ca^{2+} entry into nerve terminals by reducing the phosphorylation of specific voltage-dependent Ca^{2+} channels (Guttman 1987). Moreover, the evidence concerning the specific neuronal function of gangliosides is not yet convincing but, because of their binding capacity for Ca^{2+} (Probst *et al.* 1982, Vaccarino *et al.* 1987) and neurotransmitters (Richardson *et al.* 1982), they may be involved in glutaminergic (Vaccarino *et al.* 1987) and GABAergic (Svennerholm 1980, Suzdak *et al.* 1986) neurotransmission. If gangliosides play a role of membrane-bound receptors in the CNS, or coreceptors for toxins, drugs, viruses, hormones, neurotransmitters, etc. (Svennerholm 1980, Schwarz and Futerman 1996), then changes in the ganglioside content during diazepam treatment and after its withdrawal suggest alterations in the ligand recognition properties of the pre- and postsynaptic cerebellar benzodiazepine/GABA receptor complex. This possibility will require further research. Certainly, some radioligand binding assays should be performed to assess the influence of the observed ganglioside changes on the ligand recognition properties of the cerebellar benzodiazepine/GABA receptor complex.

The period of recovery led to an increase of total cerebellar ganglioside content, but it did not reach its control values. The only fraction, which really recovered and reached its control value, was G_{M3}. In contrast, the monosialoganglioside G_{M2} was the only one that remained almost unchanged after recovery period. The most affected fraction after six months of diazepam treatment was G_{M3}, the content of which significantly increased after the cessation of diazepam exposure. It could be assumed that the simplest classes of gangliosides (monosialoganglioside G_{M3} is the precursor for ganglioside synthesis) are the least resistant to diazepam treatment, but that they are also prone to rapid recovery. However, it is still not clear whether the diazepam-induced decrease of G_{T1b}, G_{D1a} and G_{M1} gangliosides is permanent or whether they would ultimately recover to pre-treatment control levels.

Nevertheless, the changes in ganglioside composition might be important when considering the prolonged administration of this type of drugs in clinical medication, namely as a part of mechanisms responsible for diazepam addiction and withdrawal symptoms.

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