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# Changes in Rat Brain Gangliosides Following Active Avoidance Conditioning

HELEN E. SAVAKI[1]

*Department of Biological Chemistry, University of Athens, School of Medicine Goudi, Athens 609, Greece*

AND

GABRIEL M. LEVIS

*Biochemical Research Laboratory, Department of Clinical Therapeutics, University of Athens School of Medicine, Alexandra Hospital, Vas. Sophias and K. Lourou Street, Athens 611, Greece*

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SAVAKI H. E. AND G. M. LEVIS. *Changes in rat brain gangliosides following active avoidance conditioning.* PHARMAC. BIOCHEM. BEHAV. 7(1) 7-12, 1977. – Brain gangliosides of rats trained in a conditioned avoidance Sidman task and undisturbed rats in their cages were studied. The ($^{14}$C) acetate was injected intracerebrally seven days before the starting of 30 days training. Thirty-seven days after injection all rats were killed and ganglioside fractions were isolated from neocortex, hippocampus, brain stem, cerebellum and residual cerebral tissue of each one brain. Trained rats had higher levels of ($^{14}$C)-labeled polysialogangliosides ($G_1$, $G_2$, $G_3$) in hippocampus and neocortex than the controls. Regarding the rest of the brain areas, a significant increase of $G_2$ in the residual cerebral tissue of the trained as compared with the controls was found. The results suggest that the sialic acid rich gangliosides of only certain parts of the brain are affected by the Sidman avoidance conditioning of the animals.

Gangliosides  Active avoidance conditioning  Sidman schedule  Learning

GANGLIOSIDES are concentrated at areas of high synaptic density in the nervous system [1, 4, 8, 11, 32]. They increase in amount as well as in complexity with ontogenetic and phylogenetic evolution [37, 41, 46, 47]. Cell contact-dependent ganglioside changes have been reported, supporting their role during the formation of synapses [17,49]. It has been suggested that gangliosides play an important role in the neuronal transmission [12,39], possibly by exerting an ion binding and releasing function [9,33] and that they may be involved in the information storage processes [15, 16, 26], or in the facilitation of the functional establishment of a synaptic pathway [5, 6, 7, 43]. Ganglioside metabolism in relation to sensory stimulation has recently been studied [10, 13, 36]. Small changes of ganglioside metabolism in the whole brain of the rats or mice have been found after short term behavioral stimulation [14,21]. It has been shown also that passive avoidance learning is inhibited by antiserum to brain gangliosides [28]. In view of these considerations we have studied the changes in ganglioside species, which have been identified in particular parts of the rat brain, after a long term active avoidance conditioning.

## METHOD

### *Behavioral Procedure*

Male Wistar rats were housed in a temperature controlled room with a constant photoperiod of 12 hr light/12 hr dark. At the 23rd day of their age each rat was given intracerebrally 50 μCi-l($^{14}$C)acetate (spec. radioactivity 55 mCi/mmole) in 50 μl of NaCl 0.9%, through the right parietal area into the center of the right cerebral hemisphere. Training of nine rats (conditioned group) according to Sidman schedule [45] started seven days later. The Sidman avoidance schedule operates as follows: The rat is placed in a Skinner box, the floor of which is an electrified grid; after 20 sec a light comes on for 10 sec and then a shock is delivered lasting 0.5 sec every 10 sec. The rat can avoid or terminate shock at any time by pressing a lever. This sets the schedule back to the beginning. The optimum time to press the lever to obtain maximum avoidance of shock with minimum lever presses is around 26-27 sec of the schedule. Using this schedule we can obtain one dependent and three independent measures. (a) Premature responses – Responses made before the conditioned stimu-

[1] Present Address: Laboratory of Cerebral Metabolism, National Institute of Mental Health, Bethesda, MD 20014.

lus (light) comes on. (b) Late responses – Responses made too late to avoid shock. (c) Reaction time – the time between the onset of the conditioned stimulus and the rat's response. (d) Efficient (effective) responses (the dependent variable) – those made while the conditioned stimulus is on and before shock. These will naturally increase in proportion that (a) and (b) increase. Effective, ineffective (premature plus late) responses and reaction time of each rat were tape recorded and analysed with the aid of a computer (ATAC 501–20 Nihon Kohden Kogyo, Japan). Another group of nine rats housed during the training period was used as control group.

*Biochemical Procedure*

At the 60th day of their age all rats were killed by decapitation and their brains dissected into hippocampus, neocortex, brain stem, cerebellum and residual cerebral tissue according to an atlas of the rat brain [29]. Until extraction of their lipids, the brains were stored at −20°C in sealed plastic vials. Gangliosides were isolated by the method of Holm and Svennerholm [18]. All assays were carried out in a blind fashion. The brain tissue was homogenized in an all glass homogenizer. The homogenate was extracted for 1 hr with 10 vol. of chloroform-methanol (C-M) 1:1 and the extract was passed through a glass filter. The tissue was re-extracted twice with 5 vol. of C-M 1:1; for the second extraction the solution was at boiling point. Total lipids recovered from the combined extracts were counted for radioactivity and used for the final correction of the radioactive data of individual brain areas. The combined extracts were evaporated and taken to dryness by addition of a small volume of toluene-ethanol 1:1 at the end of the evaporation. The extract was redissolved in 3 vol. of C-M 2:1 and the precipitate formed was removed by centrifugation. The supernatant fluid was evaporated and redissolved in a small volume of C-M 9:1. The total lipid extract was run on a glass column (2 cm inner diameter) packed with silica gel G (250–500 mg fresh brain tissue/g gel). Almost all lipids except gangliosides were eluted with 15 vol. of chloroform-methanol-water (C-M-W) 65:25:4, after which the gangliosides were eluted with 15 vol. of C-M-W 60:35:8. For further purification of the crude ganglioside fraction, the ganglioside from column chromatography was hydrolyzed in 5 ml of 0.1 M sodium methylate in methanol for 1 hr at room temperature. After neutralization with acetic acid, the hydrolysate was dialysed against distilled water at 4°C for 3 days. The dialysis residue was evaporated and redissolved in 10 ml of C-M 2:1. Aliquots of gangliosides of the same brain areas from control and trained rats were chromatographed on the same plate in one dimension, on silica gel G, with the solvent system C-M-2.5 N ammonia solution 60:40:9 [31,35]. A mixture of standard brain ganglioside from beef brain was chromatographed at the same time for identification. Radioactive spots were located by autoradiography, scraped out from the plate, powdered, suspended in 4% w/v Cab-O-Sil (Packard) in scintillation solution and counted in a Packard Liquid scintillation spectrometer. Sufficient counts were accumulated from each sample for a counting error of less than 3%. Results are expressed as dpm/200 mg of wet tissue/50 $\mu$Ci radioactivity of total lipid extracts. A standard curve of channel's ratio plotted against efficiency was used for estimation of dpm. Conditioned versus control values were tested by Student's test. Since the values might not be normally distributed we also recorded the non-parametric Wilcoxon test of the same hypothesis. Intramolecular distribution of radioactivity of gangliosides was measured following acid methanolysis [27] and separation of fatty acids and bases [34].

## RESULTS

*Behavioral*

The Sidman schedule was applied for 30 hr, one hr per day. Maximal performance ratio was achieved at 22 hr of training (Fig. 1). Statistical significance of the differences between means were determined using the paired $t$-test. The value of the 1st, 5th and 12th hr are significantly lower ($p < 0.05$) than those of the 22nd and 27th hr. Differences between the 22nd and 27th hr of training were not significant ($p > 0.05$). The number of responses during the period of conditioned stimulus (CS) increased with the training and remained at these accelerated levels, while the response rate during the 20-min segment preceeding the CS

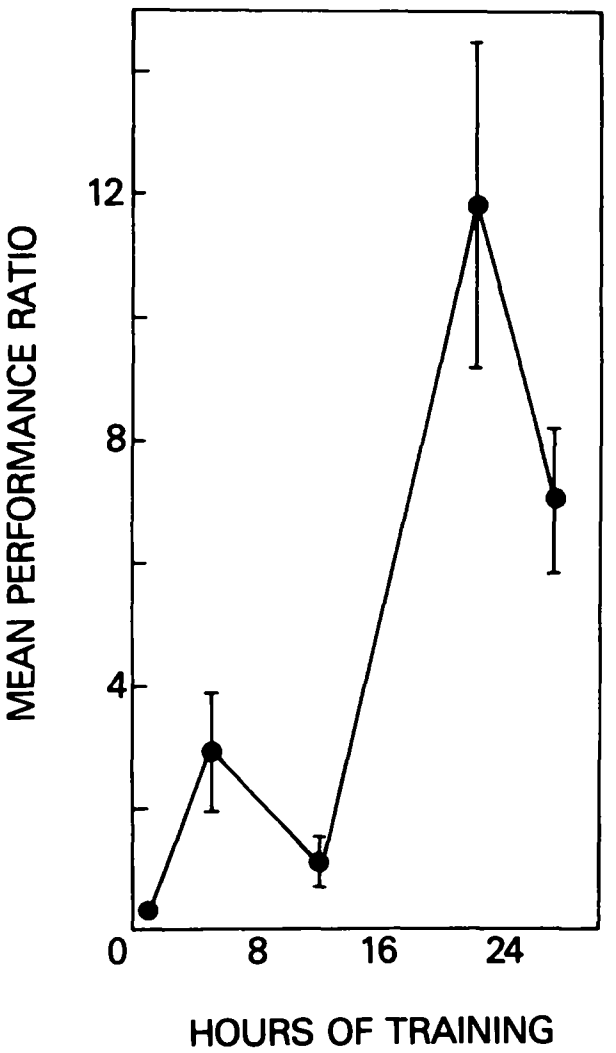


FIG. 1. Performance Ratio (effective/ineffective responses) during the training. Each point represents the mean ± standard error of 9 rats.

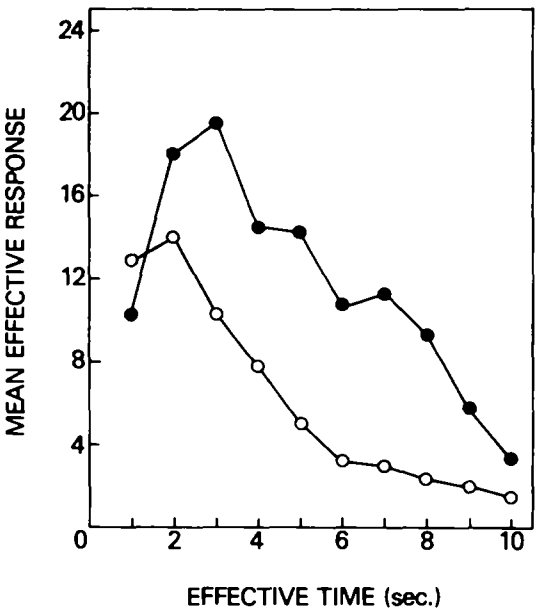


FIG. 2. Distribution of the mean reaction time of the 9 trained rats, at the 12th (○) and 27th (●) hr of training.

showed an initial increase that gradually disappeared as the animals adapted to the schedule. The proportion of premature and late responses both finally decreased and hence the proportion of effective responses increased. The animals also improved their reaction time (Fig. 2). At the 27th hr of training they acquired higher avoidance of shocks with fewer lever presses than at the 12th hr.

*Biochemical*

The total radioactivity of the C-M extract that included gangliosides did not differ significantly between the individual brain areas of the experimental and control groups. However, the final radioactivity data were normalized for radioactivity in the total lipid extract. Hippocampus and neocortex were the brain areas of the conditioned rats where most of the disialo ($G_2$, $G_3$) and trisialo ($G_1$) gangliosides contained significantly higher radioactivity as compared with the controls (Table 1). The disialoganglioside $G_{2A}$ was detectable only in hippocampus and cerebellum. The radioactivity of $G_{2A}$ fraction of hippocampus was also significantly higher than that of controls. In the residual cerebral tissue only the radioactivity of the disialoganglioside $G_2$ was higher in the conditioned rats, while no significant differences were found in any of the ganglioside fractions of the brain stem and the cerebellum. In the hippocampus and the neocortex the most marked change found in the gangliosides of the conditioned rats, as deduced by the significance of differences shown in Table 1, was the increase in the $G_2$ disialoganglioside. Maximal percent increase of radioactivity of trained over the control group (109%) was found in $G_2$ fraction of neocortex (Fig.

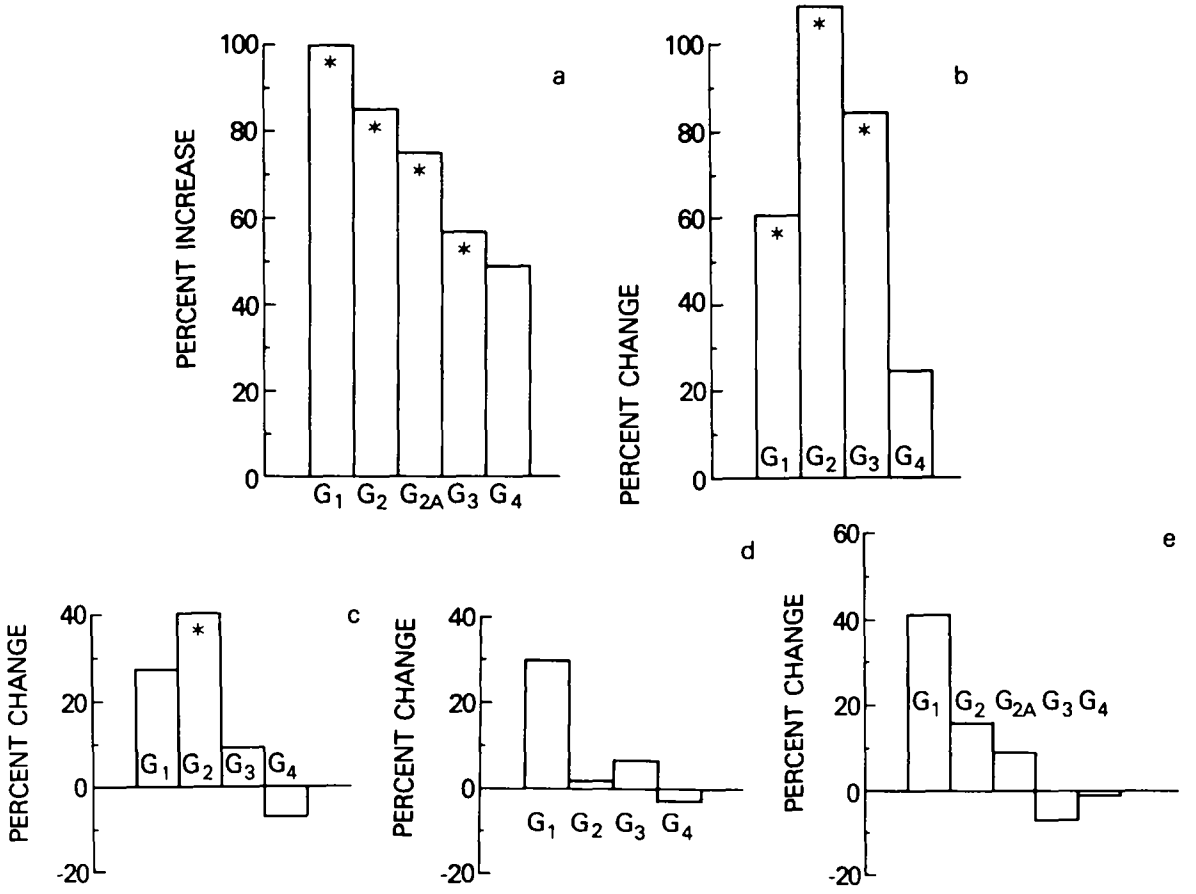


FIG. 3. Percent changes of labelled gangliosides (dpm/200 mg tissue/50 μCi pool) for trained rats relative to caged controls (baseline). *: significant differences; a: hippocampus; b: neocortex; c: residual cerebral tissue; d: brain stem; e: cerebellum.

TABLE 1

RADIOACTIVITY OF GANGLIOSIDES IN VARIOUS BRAIN PARTS OF CONTROL AND CONDITIONED RATS

| | dpm/200 mg wet tissue/50 μCi radioactivity of total lipid extract | | | |
|---|---|---|---|---|
| | Control | Conditioned | $P_t$ | $P_w$ |
| | | Hippocampus | | |
| $G_1$ | 124 ± 23 (6) | 249 ± 26 (9) | < 0.01 | < .01 |
| $G_2$ | 836 ± 184 (6) | 1549 ± 110 (9) | < 0.005 | < .02 |
| $G_{2A}$ | 159 ± 19 (4) | 279 ± 30 (7) | < 0.025 | < .01 |
| $G_3$ | 1115 ± 90 (6) | 1749 ± 181 (9) | < 0.02 | < .05 |
| $G_4$ | 879 ± 122 (6) | 1306 ± 197 (9) | > 0.1 | > .1 |
| | | Neocortex | | |
| $G_1$ | 86 ± 19 (8) | 139 ± 12 (9) | < 0.05 | .02 |
| $G_2$ | 513 ± 96 (8) | 1074 ± 117 (9) | < 0.005 | < .01 |
| $G_3$ | 779 ± 103 (8) | 1432 ± 213 (9) | < 0.025 | < .02 |
| $G_4$ | 653 ± 118 (8) | 813 ± 145 (8) | > 0.4 | > .1 |
| | | Residual Cerebral Tissue | | |
| $G_1$ | 67 ± 8 (7) | 86 ± 8 (7) | > 0.1 | > .1 |
| $G_2$ | 440 ± 57 (7) | 618 ± 49 (7) | < 0.025 | < .05 |
| $G_3$ | 514 ± 73 (7) | 561 ± 47 (7) | > 0.5 | > .1 |
| $G_4$ | 617 ± 233 (7) | 572 ± 136 (7) | > 0.7 | > .1 |
| | | Brain Stem | | |
| $G_1$ | 215 ± 43 (8) | 279 ± 30 (9) | > 0.2 | > .1 |
| $G_2$ | 1015 ± 267 (8) | 1032 ± 156 (9) | > 0.9 | > .1 |
| $G_3$ | 540 ± 205 (8) | 575 ± 148 (9) | > 0.8 | > .1 |
| $G_4$ | 2074 ± 743 (8) | 2011 ± 601 (9) | > 0.9 | > .1 |
| | | Cerebellum | | |
| $G_1$ | 307 ± 30 (8) | 433 ± 55 (9) | > 0.05 | .05 |
| $G_2$ | 1528 ± 124 (8) | 1780 ± 221 (9) | > 0.3 | > .1 |
| $G_{2A}$ | 362 ± 70 (8) | 393 ± 61 (9) | > 0.7 | > .1 |
| $G_3$ | 1385 ± 265 (8) | 1284 ± 160 (9) | > 0.7 | > .1 |
| $G_4$ | 1055 ± 160 (8) | 1043 ± 232 (9) | > 0.95 | > .1 |

Gangliosides of the five brain parts of conditioned and control animals isolated by column and thin layer chromatography as described in METHOD. Gangliosides nomenclature is that of Korey and Gonatas [30]. Results are mean values ± standard error of the mean for the number of samples shown in parentheses. Statistical significance of the differences between the means was determined using Student's *t*-test, as well as the distribution-free Wilcoxon test. Results from less than nine samples are presented in cases of unsuccessful dissection of the brain or loss of the sample.

3). The radioactivity of the acid methanolysis products of individual ganglioside species of the neocortex of the experimental and control animals are shown in Table 2. It can be seen that most of the radioactivity was found in the fatty acid and sphingosine residues, i.e., in the ceramide part, of the gangliosides. This finding also indicates that the gangliosides isolated under the present experimental conditions were not contaminated with radioactivity from nonlipid materials.

## DISCUSSION

These studies were planned to investigate possible changes occurring in the composition of gangliosides in five brain areas of the rat following long lasting avoidance conditioning. For this purpose and in view of the small quantity of samples which were available for analytical purposes it was thought necessary to produce gangliosides labelled in the ceramide moiety which is stable [18] so that changes of the ganglioside content and/or pattern relative to the controls could be identified by counting of radioactivity after a considerable long time period. Also, the labelling of brain gangliosides and training of the animals took place at an age period during which the content and pattern of the ganglioside of the whole brain do not change considerably [3,37] so that the effect of conditioning on ganglioside metabolism would not be obscured by changes due to brain development. Under these conditions pronounced differences between the control and the experimental groups of animals were identified in the radioactivity associated with the polysialosyl ganglioside fractions only of certain parts of the brain.

It is evident that under the present experimental conditions it is difficult to define the exact metabolic changes underlying the present findings as well as the exact point during training at which the changes occurred. However, the results show that in certain parts of the brain of the conditioned rats very notable changes in the metabolism of the sialic acid rich gangliosides have occurred.

It is known that acetate is quickly metabolized in the brain, and, therefore, at the beginning of the training (7 days after acetate injection), all acetate should be metabolized and stable ceramide residue of each ganglioside of both groups of animals equally labelled in proportion to their content. On this basis, at the end of the training, the

TABLE 2

INTRAMOLECULAR DISTRIBUTION OF THE RADIOACTIVITY IN THE GANGLIOSIDES OF THE NEOCORTEX

| | Control | | Conditioned | |
|---|---|---|---|---|
| | Percent Radioactivity | | | |
| Gangliosides | Fatty Acids | Sphingosine | Fatty Acids | Sphingosine |
| $G_1$ | 58 | 37 | 55 | 38 |
| $G_2$ | 54 | 43 | 56 | 40 |
| $G_3$ | 64 | 32 | 62 | 33 |
| $G_4$ | 57 | 36 | 59 | 35 |

Part of total gangliosides of the neocortex from three control and three conditioned animals not used for counting of radioactivity were pooled and separated into fractions by TLC. Each ganglioside was extracted from the silica gel G with three washings of chloroform-methanol-$H_2O$ 65:35:8 v/v. The extract was dried and subjected to acid methanolysis [27]. Fatty acids were extracted three times with petroleum ether, then the mixture was made alkaline pH 12 and sphingosine was extracted similarly [34]. Results are percentages of the radioactivity of the ganglioside fractions before methanolysis.

radio-activity of gangliosides per wet weight of the two groups can be compared. Also, the results showing increased radioactivity of the $G_1$, $G_2$, and $G_3$ species of the experimental group, as compared to the control group, strongly suggest an increased content of these gangliosides in the above-mentioned brain areas. The relative increase of the radioactivity incorporated into each of these ganglioside fractions, in the experimental as compared to the control group, is better illustrated when the results are expressed as percent change(Fig. 3).

The observed changes in these gangliosides during conditioning are well in accordance with the mechanisms proposed for the role postulated for these glycolipids in neurofunctions. These mechanisms depend on the content of the negatively charged sialic acid groups [33] and possibly as suggested by Holm and Svennerholm [18] on an increased sialyl transferase activity and on the affinity of the sialic acids for binding cationic compounds which is highest for disialosyl groups [48]. This hypothesis is also consistent with that of Irwin. Irwin and colleagues [21, 22, 23] using a swim-escape paradigm (short term duration experiment of conditioning) found a shift from disialo-to trisialogangliosides, which in the absence of changes in sialidase levels, has been attributed to an increase in sialyl transferase activity. Though their results concerning the small relative increase of $G_1$ is not at variance with our findings, the experimental conditions used by these investigators and especially the short term of training and the analysis of gangliosides from the brain as a whole do not allow further comparison of the results. It is possible that behavioral stimuli can produce specific changes in certain areas of the brain which can be masked by either a redistribution of local levels of functional and metabolic activity without significant change in the average of the brain as a whole, or the restriction of altered metabolic activity to regions too small to be detected in measurement of the brain as a whole. On the other hand, in short term experimental systems it is not possible to identify changes in ganglioside concentration due to the large half-life of ganglioside metabolism [18].

Our results are also in accordance with those of Dunn and Hogan [14] who observed increased incorporation of [1-$^3$H]glucosamine into gangliosides of the whole brain in response to an avoidance training similar to ours but of a short term duration. The increases were not specific for any ganglioside species and in this respect their results were different from ours most probably due to the facts that the brain was examined as a whole and they used a short term training.

It is for the first time that an emphasis has been given in the evaluation of the effect of the long term training on gangliosides of the various functionally different parts of the brain. In this respect the observed changes in particular parts of the brain become more important when correlated with the increased metabolic activity of macromolecules occurring in these particular brain parts of trained animals, like RNA and protein of the neocortex [19,24] and hippocampus [20, 25, 42, 44, 50]. It can therefore be suggested that the changes in ganglioside content may be relevant to the changes in RNA and/or the protein synthesis in the above mentioned brain parts and especially in the hippocampus, which is associated with the junction of arousal and reward system which consider to have significant role in the learning formation. Several other reports concerning electrophysiological activity [25,40], electrical resistance [2] and aspiration of certain brain areas [38] points to an implication of the hippocampus and the neocortex in the learning process.

The results presented in this study derive from the analysis of brain material obtained from conditioned rats with consolidated experience which was well documented. It was not possible to have appropriate functional controls undergo the same stimuli with the trained animals, because any manipulation with a supplementary control (functional control) on a fixed time with certain stimuli would have conditioning effect of another type. In order to use appropriate functional controls, we should know the analyzed components (sub-behaviors) of the Sidman schedule. The two-way shuttling behavior has been analyzed by Izquierdo, but this is a short-term training-avoidance conditioning. Consequently, we admit that the changes of ganglioside labelling cannot be correlated with any particular sub-behavior.

The consistency of our findings with those of Karpiak *et al.* [28], who reported a 95% inhibition of an avoidance learning in rats receiving the antiganglioside serum, compared with no inhibition of rats receiving absorbed antiserum, are strongly indicative of specific effect of conditioning on brain gangliosides. Furthermore, the increase of the content of certain ganglioside fractions in particular areas of the rat brain after long term training, suggested here, may result in more or less permanent functional changes (connections) of the neurons involved.

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