ELSEVIER

Alcohol, Vol. 19, No. 1, pp. 65–74, 1999

0741-8329/99/$–see front matter

PII S0741-8329(99)00026-9

GM1 and Piracetam Do Not Revert the Alcohol-Induced Depletion of Cholinergic Fibers in the Hippocampal Formation of the Rat

FERNANDO BRANDÃO*, ALFREDO RIBEIRO-DA-SILVA†, AND ANTÓNIO CADETE-LEITE*

*Department of Anatomy, Porto Medical School, Alameda do Prof. Hernâni Monteiro, 4202-451 Porto, Portugal
†Departments of Pharmacology and Therapeutics, and Anatomy and Cell Biology,
McGill University, Montreal, Quebec, Canada H3G 1Y6

Received 30 December, 1998; Accepted 15 March, 1999

BRANDÃO, F., A. RIBEIRO-DA-SILVA AND A. CADETE-LEITE. *GM1 and piracetam do not revert the alcohol-induced depletion of cholinergic fibers in the hippocampal formation of the rat.* ALCOHOL **19**(1) 65–74, 1999.—Chronic alcohol consumption causes a depletion of the cholinergic fiber network in the rat hippocampal formation, which is not ameliorated by alcohol withdrawal. Following withdrawal from alcohol, there is a further loss of intrinsic hippocampal cholinergic neurons. In this study, we investigated whether treatment with putative neuroprotective agents during the entire withdrawal period would have beneficial effects upon the hippocampal cholinergic innervation. Adult male rats were alcohol-fed for 6 months and subsequently withdrawn from alcohol for 6 months. Some animals were treated with either ganglioside GM1 (35 mg/kg body weight s.c.), vehicle (saline s.c.), or piracetam (800 mg/kg body weight p.o.) for the entire withdrawal period. Choline acetyltransferase (ChAT) immunoreactive (IR) fibers and neurons were analyzed quantitatively in all four animal groups. There were no significant differences in the density of the ChAT-IR hippocampal fiber network when the pure withdrawal and withdrawal + vehicle groups were compared to the withdrawal + GM1 or withdrawal + piracetam groups. In contrast, the number of ChAT-IR interneurons in the hippocampal formation was higher in the withdrawal + GM1 or withdrawal + piracetam groups than in the pure withdrawal and withdrawal + vehicle groups. These results indicate that, in the doses used, neither neuroprotective agent had an effect upon the extrinsic cholinergic innervation, but they had a beneficial effect upon the hippocampal intrinsic cholinergic system.

Ethanol Withdrawal Gangliosides Nootropic agents Morphometry Image analysis Acetylcholine

STUDIES in the rodent have shown that the hippocampal formation is particularly sensitive to the neurotoxic effects of prolonged alcohol ingestion [for reviews, see (41,49)]. Studies using morphometric techniques have shown that all main neuronal populations of the hippocampal formation (i.e., the dentate granular and hilar cells and pyramidal cells from the CA1 and CA3 fields) are affected following long-term alcohol intake (1,8,12,15,21,34,35,38,43,45,46,48). Recently, we have detected a reduction in the density of the choline acetyltransferase (ChAT)-IR fiber network and in the number of ChAT-IR neurons of the hippocampal formation after chronic alcohol treatment (14). These last results confirm and expand previous observations, using morphological and neurochemical approaches, on the effects of prolonged alcohol intake upon the basal forebrain cholinergic projection, including the septo-hippocampal system (2,3,5,6,9,29).

We have extended our studies to investigate the effects of withdrawal from alcohol after long periods of ingestion (12,15, 17–20,41). The studies in animals withdrawn from chronic alcohol intake allowed us to investigate the possible regenera-

Requests for reprints should be addressed to Dr. António Cadete-Leite, Department of Anatomy, Porto Medical School, Alameda do Prof. Hernâni Monteiro, 4202-451 Porto, Portugal; Tel: +351-2-5096808; Fax: +351-2-5505640; E-mail: acadete@med.up.pt

tive or remodelling capabilities of the brain during prolonged alcohol abstinence and, therefore, contribute to the clarification of some controversial issues regarding the reversibility of alcohol-induced changes (18,20,27,43,44). Withdrawal from alcohol does not impede the ongoing degenerative activity triggered by alcohol intake. In particular, the regressive changes in the hippocampal excitatory circuitry, as observed at neuritic and synaptic levels, are not prevented by alcohol withdrawal (12,19,20,41). We have also found that the decrease in the hippocampal cholinergic fiber network and in ChAT-IR neurons (17), as well as the reduction in the number of dentate GABA-immunostained cells, were all aggravated following withdrawal from alcohol (15).

The above results prompted us to assess the possible beneficial effects, during withdrawal from alcohol, of the grafting of immature hippocampal tissue, as well as of the administration of the neuroprotective agents piracetam and ganglioside GM1. These two drugs were chosen because both piracetam [for reviews see (32,37)] and GM1 [for reviews see (24,36)] have been considered as possessing a wide variety of CNS neuroprotective effects.

We have observed that piracetam (11,13), the ganglioside GM1 (16), and hippocampal grafts (40), all displayed to a variable degree protective effects upon neurons and synapses of the hippocampal trisynaptic circuitry. In addition, we have shown that intracerebral grafts of immature hippocampal tissue promote recovery of the hippocampal cholinergic innervation in rats withdrawn from alcohol (17). The above results confirm and expand previous observations by Arendt and collaborators in the cerebral cortex and hippocampal formation of alcohol-treated rats, which provided compelling evidence that the grafting of suspensions of basal forebrain neurons resulted in morphological and functional recovery (3,4). Also, we have provided evidence that piracetam has protective effects upon GABAergic neurons of the dentate gyrus in the same experimental paradigm (15).

The extrinsic cholinergic innervation of the hippocampal formation is considerably reduced following chronic alcohol consumption and withdrawal (14,17). Such innervation is provided mainly by the septo-hippocampal pathway, which is known to play an important role in memory (2–4,22,30). Because the preservation of memory function is important, we decided to investigate the effect of the neuroprotective drugs piracetam and ganglioside GM1 during the withdrawal phase.

Therefore, the specific aim of the present study was to investigate whether the administration of piracetam and GM1 during withdrawal from alcohol would have beneficial effects upon the hippocampal cholinergic fiber network and the intrinsic cholinergic neurons, which are both affected by prolonged alcohol treatment and withdrawal.

METHOD

Animals, Diets and Treatments

Male Sprague-Dawley rats from the colony of the Gulbenkian Institute of Science (Oeiras, Portugal), weighing 200 ± 20 g, were used in this study. All animals were maintained during the entire experimental period under standard laboratory conditions (light/dark cycle of 12/12 h and a room temperature of 20–22° C). Solid standard food and water were available ad libitum. At 8 weeks of age, the animals were individually housed and separated into four groups of four rats each and treated for 12 months as follows. (a) Withdrawal group: Animals from this group had unrestricted access to a 20% (v/v) aqueous ethanol solution as the only available liquid source and to standard laboratory pellet food (Letica, Barcelona, Spain) for 6 months. Ethanol was introduced gradually, beginning with a 5% (v/v) alcohol solution on the first day and increasing the concentration by 1% per day until the final concentration of 20% was attained. After these 6 months of alcohol feeding the animals were switched to standard laboratory chow and water ad libitum for another period of the 6 months. The fluid intake was measured weekly and the average volume ingested per day calculated. (b) Withdrawal + GM1: Rats were treated as in (a) and subcutaneously injected, every two days, with a 0.9% saline solution containing 35 mg, per kg of body weight, of monosialoganglioside GM1 during the entire withdrawal period (16). (c) Withdrawal + Vehicle: Animals were treated as in the previous group but received saline instead of GM1 (15). (d) Withdrawal + Piracetam: Animals were treated as in (a), but during the entire withdrawal period, piracetam was added to the drinking water at a dose of 800 mg per kg body weight per day (13). The concentration of piracetam in the drinking water was adjusted, taking into account the fluid daily consumption by the animals and the body weight, to obtain an average dose of 800 mg/kg/day.

In the different withdrawal groups, the shift from alcohol feeding towards water intake was performed gradually during a 2-week period by a progressive reduction in the concentration of ethanol in the drinking water. This graded withdrawal was performed to avoid symptoms such as seizures and consequent lesions that might occur following acute abstinence from alcohol (48). Therefore, rats were allowed to progressively adapt to the new metabolic conditions. In the different animal groups, the consumed fluids were supplemented with vitamins and minerals as previously described (14).

Blood ethanol concentrations were assessed in an additional set of four alcohol-treated rats. After 6 months of alcohol treatment, 100 μl blood samples were collected in the morning (8:00 A.M.) and in the evening (6:00 P.M.) from the tail vein and were analyzed for ethanol using an enzymatic assay kit (Boehringer-Mannheim).

During the first month of the experimental period, the animals were weighed every three days and thereafter every two weeks.

Tissue Fixation

Tissue fixation was performed as previously described (14,23). At the end of the experimental periods, animals were anaesthetised with diethyl ether and injected intracardially with 0.1 ml of a heparin solution (containing 10 units USP per ml), followed by 1 ml of 1% sodium nitrite in saline. Then, the rats were perfused transcardially with 15–20 ml of 0.1 M phosphate buffer (PB) (pH 7.4) for vascular rinse, followed by a mixture of 3% (w/v) paraformaldehyde, 0.1% (v/v) glutaraldehyde and 15% (v/v) saturated picric acid in PB at room temperature. This was followed by a perfusion with 0.5% hydrogen peroxide in phosphate-buffered saline (PBS) and, lastly, by 10 % sucrose in PB, as previously described (23). The brains were then removed in toto, weighed, and coded to allow for analysis by investigators blinded to the treatment groups. Fifty-μm-thick coronal sections were cut on a Vibratome and collected as free-floating.

Immunostaining

Immunocytochemistry was performed as previously described (14,31). A rat monoclonal antibody against ChAT (Boehringer-Mannheim) was used. This antibody has been well characterised and displays immunological characteristics

identical to those of the antibody reported by Eckenstein and Thoenen (28).

Eight Vibratome sections per animal, each containing both the right and left hippocampal formations, were selected for immunostaining. Of those sections, two were from the septal part, four from the midseptotemporal part and two from the temporal part. PBS with 0.2% Triton X-100 was used to dilute all the antibodies and for the washes. The free-floating sections were incubated for 16 h in the primary antibody at 4°C. All the following steps were carried out at room temperature. After two washes, the sections were incubated for 1 h in a rabbit anti-rat IgG antiserum (Sigma) and washed twice. The tissue was then incubated for 2 h in a rat monoclonal anti-peroxidase antibody (Medicorp, Canada) (26). After three washes, the sections were processed for the demonstration of peroxidase activity using diaminobenzidine (DAB) and H_2O_2. Subsequently, the sections were rinsed three times, mounted on gelatine-subbed glass slides, air-dried overnight, dehydrated in ascending alcohol concentrations, and cover slipped with Permount. Particular care was taken to ensure that the DAB reaction was carried out for the same length of time and at the same temperature for all sections.

Control of the specificity of the immunostaining was performed in two sections from each animal, which were processed as described above, except for the replacement of the primary antibody by normal rat serum. These sections included the hippocampal formation and were selected at random. No immunostaining was observed.

Measurements of the Cholinergic Fiber Network

ChAT-IR fibers in the hippocampal formation were quantified as described in previous publications (14,17), using a Polyvar (Reichert) microscope equipped with a × 40 plan apochromatic objective and a × 12.5 projection lens. A video camera was used to capture the image into an image analysis system (Quantimet 920, Cambridge Instruments). Two randomly selected sections from the midseptotemporal part of the hippocampal formation were used per animal and the measurements were carried out bilaterally. A total of 15 rectangular adjacent fields (105 μm × 135 μm) were used to sample the regio superior and dentate gyrus at the midseptotemporal level as shown in Figure 1. To ensure that equivalent hippocampal areas were quantified in all samples, measurements were always carried out along a vertical line crossing the hilus just medial to the inner limit of CA3 pyramidal cell layer. Labeled fibers were detected by the image analysis system with the help of a fiber analysis program involving skeletonisation (39). With this method, detected immunostained fibers are extracted from background and reduced to one-pixel in width (skeletonisation). Therefore, the total number of pixels measured per area represents total fiber length. Results are expressed as total length/unit area and, therefore, represent fiber density.

Neuronal Quantification

For neuronal measurements, eight sections from each animal were used and measurements were carried out bilaterally as previously described (14,17). In each section, the boundaries of the different regions and layers of the hippocampal formation were drawn with the aid of a camera lucida at a final magnification of × 43 (Figure 1). In the dentate gyrus, the hilus and the suprapyramidal and infrapyramidal limbs of the granular and molecular layers were drawn separately. In the hippocampus proper, the CA1 (regio superior) and CA2-3

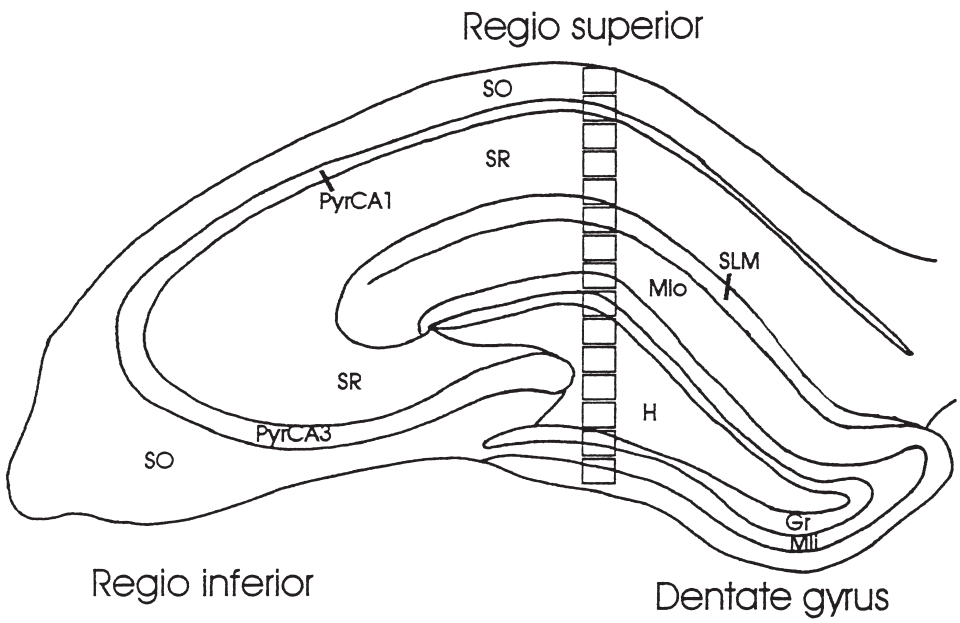

FIG. 1. Example of one of the camera lucida drawings that were used to plot the ChAT-IR neurons for quantitative analysis. The subdivisions of the hippocampal formation are shown. Note also the 15 rectangles that represent the fields where the fibers were measured with the help of the image analysis system. All fiber measurements were performed at the midseptotemporal level. GR, granular cell layer; H, hilus; Mli, inner molecular layer; Mlo, outer molecular layer; Pyr CA1, CA1 pyramidal cell layer; Pyr CA3, CA3 pyramidal cell layer; SLM, stratum lacunosum moleculare; SO, stratum oriens; SR, stratum radiatum.

(regio inferior) fields were also separately delineated; in each of these fields, the limits of different strata were also drawn. The cross-sectional area of each of the above zones was measured using a Videoplan image analysis system.

ChAT-IR cell bodies with intact perikaryal contours were identified in each of the above layers, in each section, using a $\times 40$ plan objective (final magnification $= \times 600$). Their locations were plotted in each of the previously drawn layers. The number of ChAT-positive neurons/unit surface area (areal density) was calculated from the number of plotted cell bodies in each layer and the area of the cross-sectional area of the respective layer.

Statistical Analysis

Due to the normality and homogeneity of variance of the sampled populations, and because there are not differences in the frequency of ChAT-IR neurons along the septotemporal axis of the hippocampal formation in the animals from the experimental groups, a two-way ANOVA was applied to discern main effects. Treatment and location (region or layer) were used as independent variables and animals as replicates. The remainder mean square was utilized as the error term. Taking into account the small size of the sample and that the SD was known, a z-test was applied to evaluate whether group means differed significantly from each other. For more accuracy when applying z-test, the z score of the more unfavorable element of each group relative to the comparison group was chosen. A Multistage-Bonferroni test was used to control for unacceptable levels of type I error, thus providing stronger protection against false rejections of the null hypothesis. Results for which the null hypothesis would be rejected according to the uncorrected procedure are regarded as borderline.

Throughout this study, values are expressed as means and standard deviations. A p value < 0.05 was considered as statistically significant.

RESULTS

Animals, Diets and Treatments

During the first 3 months of alcohol treatment, the average intake was approximately 7.5 g/kg body weight/day, whereas

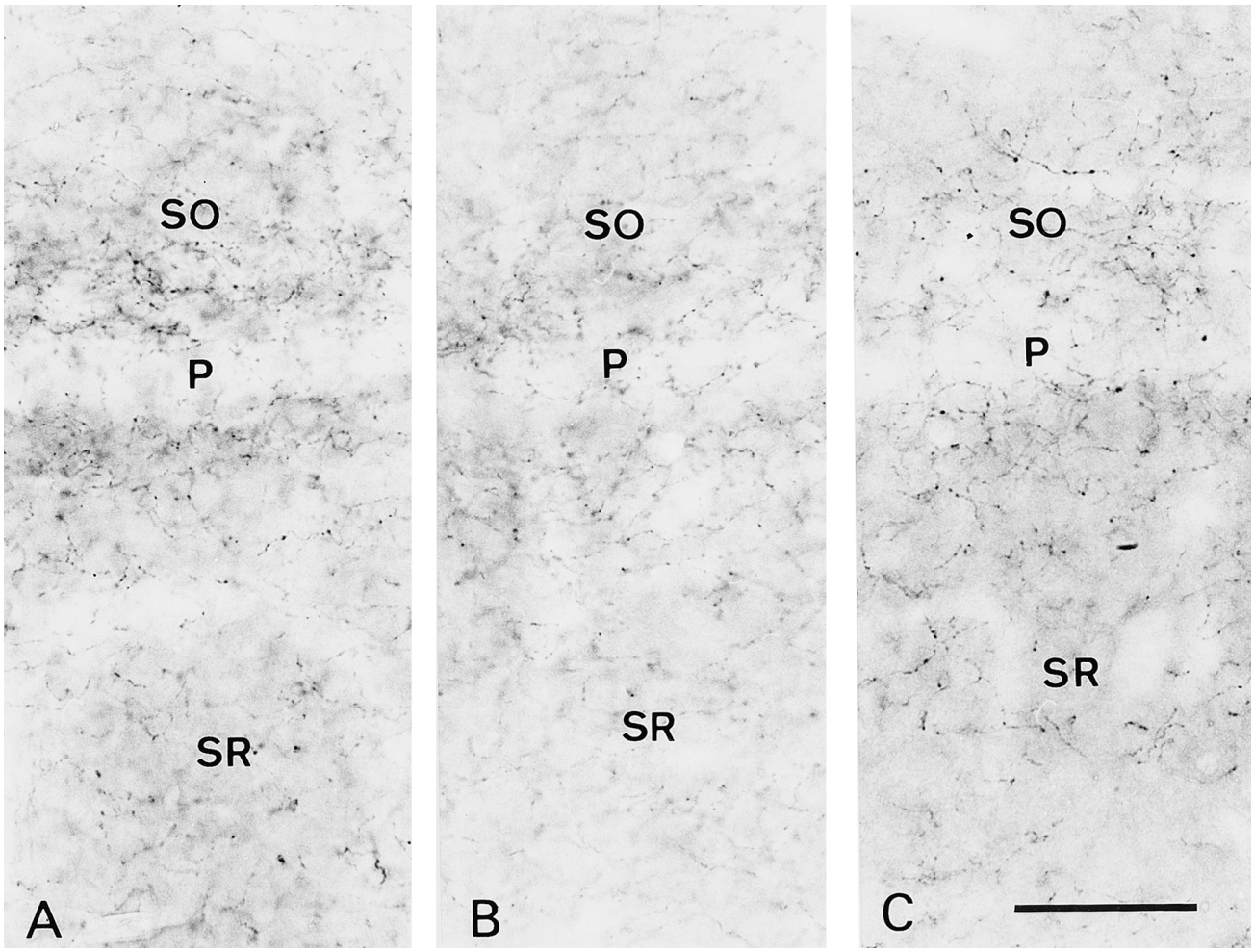

FIG. 2. Micrographs of part of the regio superior at the midseptemporal level in pure withdrawn (A), withdrawn + GM1-treated (B) and withdrawn + piracetam-treated (C) animals. ChAT immunostaining. Note that there is no apparent difference in the density of ChAT-IR fibers when B and C are compared to A. SO, stratum oriens; P, pyramidal cell layer of CA1; SR, stratum radiatum. Scale bar = 50 μm.

at 6 months, alcohol consumption attained a mean of 12.2 g/kg/day. The mean alcohol intake throughout the entire period of alcohol consumption (6 months) was approximately 9.0 g/kg body weight /day and corresponded to a mean value of 18 ml of daily alcohol ingestion (2.8 g).

The mean blood ethanol concentration was 102.4 ± 13.9 mg/dl. Values ranged from 83 to 122 mg/dl and the highest concentrations were always observed in blood samples collected in the morning.

Piracetam-treated rats had a fluid consumption of 43.0 ± 2.5 ml, a value that did not differ from the animals in the other withdrawal groups. The concentration of piracetam in the drinking water for the animals used in this study was adjusted according to the body weight and fluid consumption, to obtain the average dose of 800 mg/kg/day. Such concentration was on average 0.47% during the first two months, and increased to an average value of 0.60% during the last month. During the entire withdrawal period, each animal ingested on average 229.2 ± 20.8 mg/day of piracetam.

During the first month of alcohol treatment, the body weight of the animals remained unchanged, whereas in the subsequent 5 months average body weight increase was 10.9 ± 4.2 g per month. After the removal of alcohol the mean body weight increase in animals from the withdrawal group was 9.3 ± 3.1 g/month. In a previous study, in which we also analyzed the cholinergic system of the hippocampal formation and used the same duration of alcohol consumption and withdrawal from alcohol, the average body weight of the age-matched controls was of 12.2 ± 5.3 g during the first month. Subsequently, the increase was of 14.4 ± 2.3 g/month (17). At the end of the experimental period, the mean body weight of animals from all experimental groups in the present study did not differ significantly with the group. Furthermore, the values of mean body weight were very similar to those observed in the previous study in the alcohol-fed and withdrawal group and were lower but not significantly different from the control group in the same study (17).

No significant differences were found among the mean brain weights of pure withdrawn and withdrawn-treated rats.

Cholinergic Fiber Network

Qualitatively, no obvious differences were detected in the density of the hippocampal cholinergic fiber network when the withdrawal-treated groups were compared to the withdrawal-untreated group (Figs. 2 and 3).

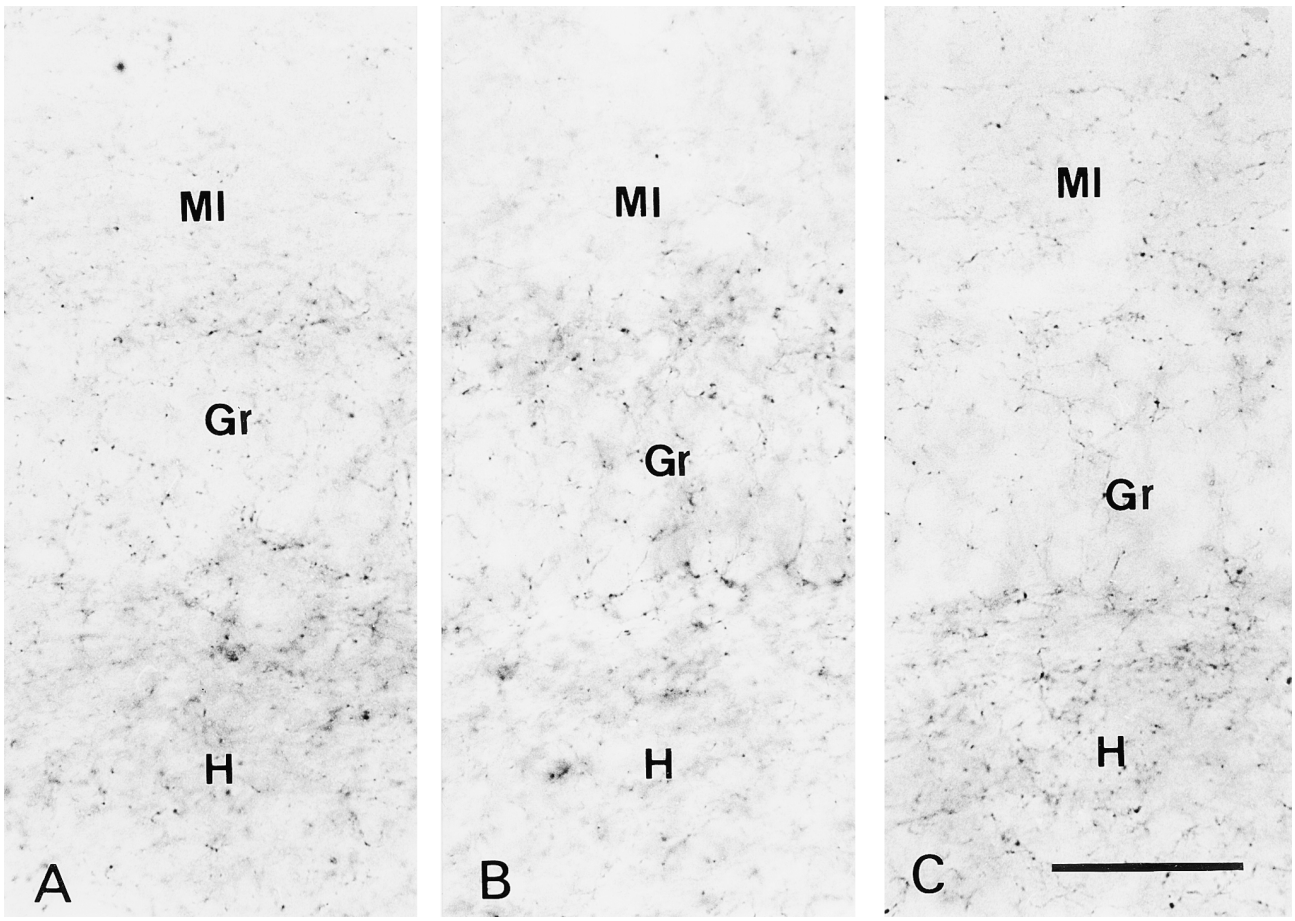

FIG. 3. Micrographs of part of the suprapyramidal limb of the dentate gyrus at the midseptemporal level in pure withdrawn (A), withdrawn + GM1-treated (B) and withdrawn + piracetam-treated (C) animals. ChAT immunostaining. Note that there is no apparent difference in the density of ChAT-IR fibers when B and C are compared to A. Ml, molecular layer; Gr, granular cell layer; H, hilus. Scale bar = 50 μm.

TABLE 1

HIPPOCAMPAL CHOLINE-ACETYLTRANSFERASE IMMUNOREACTIVE FIBER LENGTH (μm) OF WITHDRAWAL AND WITHDRAWAL-TREATED GROUPS OF ANIMALS

	Withdrawal	Withdrawal + Vehicle	Withdrawal + GM1	Withdrawal + Piracetam
Hippocampal formation	14528.72 ± 4724.23	15040.82 ± 689.29	13139.29 ± 3591.43	13932.25 ± 3628.26
Dentate gyrus	16158.22 ± 5925.53	16905.48 ± 290.72	14502.20 ± 3695.17	14998.75 ± 4394.79
Regio superior	12218.82 ± 3255.82	12438.41 ± 1541.61	11438.23 ± 3543.95	13064.46 ± 2794.65

Numbers represent mean fiber length (μm) ± SD per field (a rectangle measuring 105 μm × 135 μm).

Table 1 shows the results of the measurements of the cholinergic fiber network in the hippocampal formation as a whole, in the regio superior and in dentate gyrus. Analysis of these data revealed that there are no significant differences in the total fiber length per unit surface area (fiber density) when GM1-treated and piracetam-treated groups were compared to pure withdrawal and vehicle-treated groups.

Neuronal Number and Distribution

The total number of identified ChAT-immunostained neurons in 224 sections was 1610 of which 353 were from pure withdrawal group, 375 from the vehicle-treated group, 446 from the GM1-treated group, and 436 from the piracetam-treated group. The average number of neurons per section was 6.3 ± 2.9 in pure withdrawn animals, 6.6 ± 4.1 in vehicle-treated rats, 8.0 ± 3.4 in GM1-treated rats, and 7.8 ± 3.7 in piracetam-treated animals.

The number of ChAT-immunoreactive neurons per unit surface area (areal density − N_A) in the different regions and layers of the hippocampal formation in animals of all the groups studied is shown in Table 2. The number of ChAT-immunoreactive neurons per unit surface area from vehicle-treated animals did not differ from the pure withdrawn animals. However, a significant increase in the N_A of the ChAT-immunoreactive neurons was detected in the hippocampal formation as a whole (24% and 22%) and in regio superior (30% and 32%) and its stratum lacunosum moleculare (40% and 42%) when the GM1-treated and piracetam-treated groups were compared to the pure withdrawal and vehicle-treated groups. No significant differences were detected when the GM1-treated group was compared to the piracetam-treated group.

ANOVA tests indicated that treatment and region, as well as the interaction between these two variables, were all significantly related to the N_A of ChAT-immunoreactive neurons.

TABLE 2

AREAL DENSITY (N_A) OF CHOLINE ACETYLTRANSFERASE-IMMUNOREACTIVE NEURONS IN THE HIPPOCAMPAL FORMATION OF WITHDRAWAL AND WITHDRAWAL-TREATED GROUPS OF ANIMALS

	Withdrawal	Withdrawal + Vehicle	Withdrawal + GM1	Withdrawal + Piracetam
Hippocampal formation	11.3 ± 1.0	11.8 ± 1.3	14.0 ± 1.0*†	13.8 ± 1.3*††
Dentate gyrus	10.3 ± 2.1	10.9 ± 0.4	11.2 ± 1.8	11.0 ± 1.7
Infrapyramidal limb	6.3 ± 2.6	5.7 ± 0.6	6.3 ± 1.2	6.4 ± 2.3
Molecular layer	5.6 ± 3.3	5.1 ± 1.1	5.7 ± 1.0	5.9 ± 1.8
Granular layer	9.0 ± 2.2	8.2 ± 2.7	7.7 ± 2.6	7.8 ± 3.8
Hilus	6.9 ± 3.2	5.1 ± 3.4	6.0 ± 0.9	6.0 ± 1.3
Suprapyramidal limb	16.6 ± 4.4	16.9 ± 2.6	16.2 ± 3.1	16.2 ± 1.4
Granular layer	17.4 ± 8.9	17.8 ± 12.0	17.1 ± 1.5	16.2 ± 4.8
Molecular layer	16.6 ± 5.8	16.7 ± 1.0	16.0 ± 3.8	16.3 ± 2.4
Regio superior	19.9 ± 1.5	20.2 ± 2.0	25.8 ± 3.0*†	26.2 ± 3.0*†
St. Lacunosum moleculare	63.0 ± 5.1	64.3 ± 1.8	88.1 ± 6.2*†	89.2 ± 8.7*†
St. Radiatum	11.7 ± 3.8	12.4 ± 3.2	12.3 ± 1.4	11.8 ± 2.1
St. Pyramidale	8.9 ± 5.8	10.7 ± 6.5	10.8 ± 2.7	10.8 ± 7.7
St. Oriens	5.7 ± 2.0	5.2 ± 3.6	6.5 ± 2.6	6.2 ± 2.6
Regio inferior	3.8 ± 0.2	4.1 ± 1.8	3.9 ± 0.7	3.8 ± 0.6
St. Lacunosum moleculare	8.2 ± 4.9	9.8 ± 5.2	8.0 ± 4.9	8.4 ± 2.7
St. Radiatum	4.5 ± 0.7	4.1 ± 1.8	4.7 ± 0.7	4.5 ± 0.9
St. Pyramidale	4.5 ± 1.0	4.1 ± 4.5	4.7 ± 2.1	4.1 ± 1.6
St. Oriens	1.7 ± 0.8	1.7 ± 1.5	1.7 ± 0.8	1.9 ± 0.6

Values represent means ± SD; N_A (N/10 mm^2).
Withdrawal vs. Withdrawal + GM1 and Withdrawal + Piracetam:* $p < 0.0005$;
Withdrawal + Vehicle vs. Withdrawal + GM1 and Withdrawal + Piracetam: †$p < 0.0005$; ††$p < 0.005$.
ANOVA, *z*-test, and Bonferroni test were used for statistical evaluation.

Cross-Sectional Area of the Hippocampal Formation

No significant differences in the cross-sectional area of the hippocampal formation were found when all groups were compared. The mean cross-sectional area of the hippocampal formation as a whole was 78.3 ± 3.1 mm^2 in pure withdrawn animals, 79.5 ± 1.5 mm^2 in vehicle-treated rats, 79.6 ± 1.5 mm^2 in GM1-treated animals, and 79.0 ± 0.3 mm^2 in piracetam-treated rats ($F = 0.22$, $p = 0.88$). In the dentate gyrus, values were 21.5 ± 2.1 mm^2, 24.7 ± 1.0 mm^2, 22.7 ± 0.2 mm^2, and 22.2 ± 2.0 mm^2, respectively. In the regio superior, values were 28.1 ± 1.8 mm^2, 28.1 ± 0.4 mm^2, 29.4 ± 2.3 mm^2, and 28.4 ± 1.5 mm^2, respectively. In the regio inferior, values were of 28.7 ± 1.4 mm^2, 26.7 ± 2.1 mm^2, 27.6 ± 1.8 mm^2, and 28.5 ± 1.0 mm^2, for pure withdrawn, vehicle-treated, GM1-treated, and piracetam-treated animals, respectively.

ANOVA tests did not show any significant effect of treatment upon the cross-sectional area of the three regions of the hippocampal formation ($F = 2.42$, $p = 0.12$ for dentate gyrus; $F = 0.40$, $p = 0.75$ for regio superior; and $F = 0.98$, $p = 0.43$ for regio inferior).

DISCUSSION

This study is part of a series of investigations aimed at determining whether drug treatments can improve recovery following withdrawal from alcohol. The approaches used included the performance of intracerebral grafting and the administration of drugs claimed to possess neuroprotective properties (11,13,15–17,40). In this investigation, we addressed the issue of a possible morphological recovery following treatment by measuring the cholinergic fiber density in the hippocampal formation from animals withdrawn from prolonged alcohol consumption, in the presence or absence of treatment with GM1 and piracetam. We also calculated the number of cholinergic neurons in different regions and layers of the hippocampal formation in the above groups.

As in previous studies (14,15), the measurements of the cross-sectional area of the hippocampal formation did not reveal any significant differences among groups. Taken together, these results indicate an absence of alcohol and/or drug-induced tissue shrinkage.

In previous morphological studies on the cholinergic innervation of the hippocampal formation of the rat, we reported an extensive depletion of the cholinergic fiber network, as well as a reduction in the number of cholinergic cell bodies, after 6 and 12 months of alcohol consumption (14,17). After 6 months of alcohol consumption (14, present study), we have detected a reduction of 40% in the density of the hippocampal cholinergic fiber network and a 37% decrease in the number of ChAT-IR neurons per unit surface area. Compared to controls, animals that were alcohol-fed for 12 months (17) had a 40% reduction in cholinergic fiber density and a 32% decrease in the areal density of ChAT immunopositive neurons. Also, the inclusion of a withdrawal group in our experiments provided evidence that 6 months of withdrawal, following 6 months of ethanol intake, did not alter the previously reduced hippocampal cholinergic fiber density but further decreased the number of hippocampal cholinergic cell bodies (17). Indeed, compared to controls (17), withdrawn animals displayed a 63% reduction in fiber density and a 45% reduction in the number of ChAT-IR neuron per unit surface area. Compared to alcohol-fed animals (17), those animals that were withdrawn from alcohol showed a reduction of 20% in the N_A of cholinergic neurons. Therefore, our observations provide no evidence of neuronal regeneration following withdrawal. On the contrary, our studies provide further support to the view that, in this CNS area, neuronal degeneration observed following alcohol intake is not reduced but rather augmented following ethanol withdrawal (1,15,18–20).

Following the administration of ganglioside GM1 to animals withdrawn from long periods of alcohol intake, we have detected an absence of recovery in the number of CA3 pyramidal and dentate granular, hilar and GABAergic cells as well as in the number of synapses from mossy fibers on CA3 pyramidal cells (10,15,16). In contrast, GM1 administration had a protective effect upon CA1 pyramidal cells (10).

The administration of piracetam to withdrawn animals protected them from additional cell loss of granular, hilar and GABAergic neurons in the dentate gyrus, as well as of hippocampal CA1 pyramidal cells (13,15). Also, we observed that the number of synapses between mossy fibers and CA3 pyramidal cells was higher in piracetam-treated than in alcohol-fed and withdrawn rats (11).

Effects of Ganglioside GM1

In previous investigations (10,15,16), we have applied to animals withdrawn from prolonged alcohol intake the same dose of GM1 used in this study. We have observed an absence of recovery in the number of CA3 pyramidal and dentate granular, hilar and GABAergic cells. Furthermore, we did not detect any recovery in the number of synapses from mossy fibers on CA3 pyramidal cells. In contrast, GM1 administration had a protective effect upon CA1 pyramidal neurons (10).

In the present study, in the dose used, the subcutaneous application of GM1 did not lead to a recovery of the cholinergic fiber network following withdrawal from chronic alcohol consumption. In contrast, it should be stressed that GM1 did protect hippocampal cholinergic neurons in withdrawn animals. The lack of regrowth of the cholinergic fiber network following GM1 treatment that we detected in the present study represents evidence that this ganglioside did not have a neurotrophic effect at the level of the terminal arborization of the cholinergic septo-hippocampal axons. This may have resulted from a lack of sufficient endogenous levels of neurotrophic agents for GM1 to be able to potentiate their effects in our experimental conditions. In agreement with this hypothesis, recent investigations (47) demonstrated that chronic exposure to alcohol is accompanied by a decrease in neurotrophic factor activity in the hippocampus.

However, in our material the number of hippocampal ChAT-immunoreactive neurons per unit surface area in animals withdrawn from alcohol and treated with GM1 was higher than those observed either in pure withdrawn or in withdrawn-vehicle-treated animals. This positive effect was obtained in a neuronal population that is intrinsic to the hippocampus and may not result from a neurotrophic effect but rather from neuroprotection. We can speculate that the administration of GM1 during the withdrawal period reduces cell death due to excitotoxicity because of the putative neuroprotective effects described above. Furthermore, chronic ethanol treatment is known to alter the physicochemical characteristics of cellular membranes, possibly destabilising its structure. As a role of GM1 in restoration of cell membrane structure and function has been proposed (33), it is likely that part of the neuroprotective role of GM1 in our model resulted from effects at the cell membrane level. Among other hippocampal neuronal populations, GM1 also had a neuroprotective effect upon CA1 pyramidal cells (10). However, dentate GABAer-

gic (15), granular and hilar cells, and CA3 neurons were not protected following GM1 administration (10,16). The reason why this neuroprotective effect is restricted to some hippocampal neuronal populations is at present unclear.

The dose of GM1 that we applied is comparable, or even higher, to the one given in other studies when using a systemic route. However, the very limited effect of GM1 could originate from an insufficient concentration of the drug in the CNS. In fact, GM1 is more efficacious when administered intracerebroventricularly, as it never reaches very high concentrations in the brain (25). Unfortunately, the intracerebroventricular route could not be used because of the prolonged period of administration of the drug. Also, we preferred the subcutaneous to the intraperitoneal route to avoid complications of the long-term administration of the drug in the peritoneal cavity. Our decision is justified, as all the systemic routes seem to lead to acceptable levels of the drug in the brain (7).

Effects of Piracetam

The results of the present study allow us to suggest that the end result of piracetam administration upon the extrinsic and intrinsic cholinergic innervation of the hippocampal formation of withdrawn animals is similar to that observed following the administration of GM1. Indeed, after the administration of either piracetam or GM1 there was no improvement in the density of the hippocampal cholinergic fiber network. The number of hippocampal intrinsic cholinergic interneurons was increased following either piracetam or GM1 treatment. This protective effect of piracetam upon an intrinsic hippocampal neuronal system concurs with a previous study in which we had found that this nootropic drug impeded additional cell loss in dentate granular and hilar neurons and in CA1 pyramidal cells in animals withdrawn from alcohol (13).

In our study, we used an oral administration of the drug. Although different studies have used different routes of administration, most studies use an oral administration because the drug reaches good concentrations in the blood and penetrates the blood brain barrier, although slowly (32). The dose utilized by us (800 mg/kg/day) was quite high. Furthermore, is thought that the efficacy is proportional to the duration of the treatment (32). As we have utilized one concentration of piracetam, we cannot rule out the possible greater efficacy of higher doses. However, this is not likely, based on what is said above.

CONCLUSION

Our study shows that neither ganglioside GM1 nor piracetam lead to a recovery of the cholinergic fiber network in the hippocampal formation of animals withdrawn from chronic alcohol consumption. In contrast, however, both agents showed a significant protective effect upon the intrinsic hippocampal ChAT immunoreactive neurons.

The significant increase in the number of ChAT immunoreactive neurons which we detected in the hippocampal formation of the withdrawn rats following treatment with either the ganglioside GM1 or the piracetam shows that there is an apparent recovery in the number of cholinergic neurons. Whether such recovery represents a real increase in the number of cholinergic neurons, as a result of prevention of cell death, or rather an increase in the expression of ChAT, is an issue that cannot be answered with the data that we possess. In fact, it cannot be ruled out that ChAT would be down regulated in withdrawn animals and the levels restored following treatment, leading to detection of previously "silent" cells.

It is difficult in a morphological study like ours to infer the functional relevance of the GM1 and piracetam-induced protective effects upon the intrinsic hippocampal cholinergic neurons in animals withdrawn from alcohol. Neurochemical and behavioral studies carried out in parallel would be required. Although the functional role of the hippocampal intrinsic cholinergic neurons has yet to be determined, it is universally accepted that the cholinergic innervation provided by these neurons is not particularly relevant when compared to that provided by the septo-hippocampal system. However, it should be pointed out that it has been proposed that these small neurons may play a specific role in the fine modulation of the excitability of other hippocampal cells (42). We can speculate that the sparing of certain intrinsic hippocampal neuronal circuits may avoid further anomalies of hippocampal function following withdrawal from chronic alcohol consumption.

ACKNOWLEDGEMENTS

The authors thank M. M. Paula-Barbosa for helpful criticisms. We are also grateful to J. Paulo Andrade for help with immunocytochemistry, A. Pereira for technical assistance, and Alan Forster (McGill University) for photographic expertise. We also thank Fidia Research Laboratories (Italy) and UCB (Portugal) for providing, respectively, the GM1 and piracetam used in this study. This work was supported by Fundação para a Ciência e a Tecnologia (project PECS/C/SAU/93/95 and Unit 121/94—Centro de Morfologia Experimental da Faculdade de Medicina da Universidade do Porto), Program PRAXIS XXI, and NATO Grant CRG 971176.

REFERENCES

1. Andrade, J. P.; Fernando, P. M.; Madeira, M. D.; Paula-Barbosa, M. M.; Cadete-Leite, A.; Zimmer, J.: Effects of chronic alcohol consumption and withdrawal on the somatostatin-immunoreactive neurons of the rat hippocampal dentate hilus. Hippocampus 2:65–72; 1992.
2. Arendt, T.: The cholinergic deafferentation of the cerebral cortex induced by chronic consumption of alcohol: reversal by cholinergic drugs and transplantation. In: Hunt, W. A.; Nixon, S. J., eds. Alcohol-induced brain damage. Rockville, MD: U.S. Department of Health and Human Services; 1993:431–460.
3. Arendt, T.; Allen, Y.; Marchbanks, R. M.; Schugens, M. M.; Sinden, J.; Lantos, P. L.; Gray, J. A.: Cholinergic system and memory in the rat: effects of chronic ethanol, embryonic basal forebrain brain transplants and excitotoxic lesions of cholinergic basal forebrain projection system. Neurosci. 33:435–462; 1989.
4. Arendt, T.; Allen, Y.; Sinden, J.; Schugens, M. M.; Marchbanks, R. M.; Lantos, P. L.; Gray, J. A.: Cholinergic-rich brain transplants reverse alcohol-induced memory deficits. Nature 332:448–450; 1988.
5. Arendt, T.; Brückner, M. K.; Pagliusi, S.; Krell, T.: Degeneration of rat cholinergic basal forebrain neurons and reactive changes in nerve growth factor expression after chronic neurotoxic injury. I. Degeneration and plastic response of basal forebrain neurons. Neurosci. 65:633–645; 1995.
6. Arendt, T.; Hennig, D.; Gray, J. A.: Marchbanks, R. M.: Loss of neurons in the rat basal forebrain cholinergic projection system after prolonged intake of ethanol. Brain Res. Bull. 21:563–570; 1988.
7. Bellato, P.; Milan, F.; Toffano, G.: Disposition of exogenous tritium-labelled GM1 monosialoganglioside in the rat. Clin. Tri. J. 26:39–48; 1989.

8. Bengoechea, O.; Gonzalo, L. M.: Effects of alcoholization on the rat hippocampus. Neurosci. Lett. 123:112–114; 1991.
9. Beracochea, D.; Durkin, T. P.; Jaffard, R.: On the involvement of the central cholinergic system in memory deficits induced by long term ethanol consumption in mice. Pharmacol. Biochem. Behav. 24:519–524; 1986.
10. Brandão, F.: Acção de agentes neuroprotectores na formação do hipocampo de ratos em abstinência após longos períodos de consumo de álcool. Ph.D. Thesis. Porto, Portugal: Faculdade de Medicina do Porto; 1996.
11. Brandão, F.; Cadete-Leite, A.; Andrade, J. P.; Madeira, M. D.; Paula-Barbosa, M. M.: Piracetam promotes mossy fiber synaptic reorganization in rats withdrawn from alcohol. Alcohol 13:239–249; 1996.
12. Brandão, F.; Cadete-Leite, A.; Paula-Barbosa, M. M.: Morphological brain changes induced by alcohol and aging. Effects of piracetam during withdrawal following long-term alcohol consumption. In: Racagni, G.; Mendlewicz, J., eds. Treatment of age-related cognitive dysfunction: pharmacological and clinical evaluation. International Academy of Biomedical Drug Research. Vol. 2. Basel: Karger; 1992:63–68.
13. Brandão, F.; Paula-Barbosa, M. M.; Cadete-Leite, A.: Piracetam impedes hippocampal neuronal loss during withdrawal after chronic alcohol intake. Alcohol 12:279–288; 1995.
14. Cadete-Leite, A.; Andrade, J. P.; Sousa, N.; Ma, W.; Ribeiro-da-Silva, A.: Effects of chronic alcohol consumption on the cholinergic innervation of the rat hippocampal formation as revealed by choline acetyltransferase immunocytochemistry. Neurosci. 64: 357–374; 1995.
15. Cadete-Leite, A.; Brandão, F.; Andrade, J. P.; Ribeiro-da-Silva, A.; Paula-Barbosa, M. M.: The GABAergic system of the dentate gyrus after withdrawal from chronic alcohol consumption. Effects of intracerebral grafting and putative neuroprotective agents. Alcohol Alcohol. 32:471–484; 1997.
16. Cadete-Leite, A.; Brandão, F.; Madeira, M. D.; Paula-Barbosa, M. M.: Effects of GM1 ganglioside upon neuronal degeneration during withdrawal from alcohol. Alcohol 8:417–423; 1991.
17. Cadete-Leite, A.; Brandão, F.; Tajrine, D.; Antunes, S.; Ribeiro-da-Silva, A.; Andrade, J. P.: Intracerebral grafts promote recovery of the cholinergic innervation of the hippocampal formation in rats withdrawn from chronic alcohol intake. An immunocytochemical study. Neurosci. 79:383–397; 1997.
18. Cadete-Leite, A.; Tavares, M. A.; Alves, M. C.; Uylings, H. B.; Paula-Barbosa, M. M.: Metric analysis of hippocampal granule cell dendritic trees after alcohol withdrawal in rats. Alcohol. Clin. Exp. Res. 13:837–840; 1989.
19. Cadete-Leite, A.; Tavares, M. A.; Pacheco, M. M.; Volk, B.; Paula-Barbosa, M. M.: Hippocampal mossy fiber-CA3 synapses after chronic alcohol consumption and withdrawal. Alcohol 6:303–310; 1989.
20. Cadete-Leite, A.; Tavares, M. A.; Paula-Barbosa, M. M.: Alcohol withdrawal does not impede hippocampal granule cell progressive loss in chronic alcohol-fed rats. Neurosci. Lett. 86:45–50; 1988.
21. Cadete-Leite, A.; Tavares, M. A.; Uylings, H. B. M.; Paula-Barbosa, M. M.: Granule cell loss and dendritic regrowth in the hippocampal dentate gyrus of the rat after chronic alcohol consumption. Brain Res. 473:1–14; 1988.
22. Chrobak, J. J.; Napier, T. C.: Intraseptal administration of bicuculline produces working memory impairments in the rat. Behav. Neural Biol. 55:247–254; 1991.
23. Côté, S.; Ribeiro-da-Silva, A.; Cuello, A.C.: Current protocols for light microscopy immunocytochemistry. In: Cuello, A. C., ed. Immunohistochemistry II. Chichester: John Wiley & Sons; 1993:147–168.
24. Cuello, A. C.: Glycosphingolipids that can regulate nerve growth and repair. Adv. Pharmacol. 21:1–50; 1990.
25. Cuello, A. C.; Garofalo, L.; Kenigsberg, R. L.; Maysinger, D.: Gangliosides potentiate *in vivo* and *in vitro* effects of nerve growth factor on central cholinergic neurons. Proc. Natl. Acad. Sci. 86:2056–2060; 1989.
26. Cuello, A. C.; Milstein, C.; Wright, B.; Bramwell, S.; Priestley, J. V.; Jarvis, J.: Development and application of a monoclonal rat peroxidase-antiperoxidase (PAP) immunocytochemical reagent. Histochem. 80:257–261; 1984.
27. Durand, D.; Saint-Cyr, J. A.; Gurevich, N.; Carlen, P. L.: Ethanol-induced dendritic alterations in hippocampal granule cells. Brain Res. 477:373–377; 1989.
28. Eckenstein, F.; Thoenen, H.: Production of specific antisera and monoclonal antibodies to choline acetyltransferase: characterization and use for identification of cholinergic neurons. EMBO J. 1:363–368; 1982.
29. Floyd, E. A.; Young-Seigler, A. C.; Ford, B. D.; Reasor, J. D.; Moore, E. L.; Townsel, J. G.; Rucker, H. K.: Chronic ethanol ingestion produces cholinergic hypofunction in rat brain. Alcohol 14:93–98; 1997.
30. Gage, F. H.; Björklund, A.; Stenevi, U.; Dunnett, S. B.; Kelly, P. A. T.: Intrahippocampal septal grafts ameliorate learning impairments in aged rats. Science 225:533–536; 1984.
31. Garofalo, L.; Ribeiro-da-Silva, A.; Cuello, A. C.: Potentiation of nerve growth factor-induced alterations in cholinergic fiber length and presynaptic terminal size in cortex of lesioned rats by the monosialoganglioside GM1. Neurosci. 57:21–40; 1993.
32. Gouliaev, A. H.; Senning, A.: Piracetam and other structurally related nootropics. Brain Res. Rev. 19:180–222; 1994.
33. Heaton, M. B.; Paiva, M.; Swanson, D. J.; Walker, D. W.: Ethanol neurotoxicity in vitro: effects of GM1 ganglioside and protein synthesis inhibition. Brain Res. 654:336–342; 1994.
34. Lescaudron, L.; Seguela, P.; Geffard, M.; Verna, A.: Effects of long-term ethanol consumption on GABAergic neurons in the mouse hippocampus: a quantitative immunocytochemical study. Drug Alcohol Depend. 18:377–384; 1986.
35. Lescaudron, L.; Verna, A.: Effects of chronic ethanol consumption on pyramidal neurons of the mouse dorsal and ventral hippocampus: a quantitative histological analysis. Exp. Brain Res. 58:362–367; 1985.
36. Mahadik, S. P.: Gangliosides: new generation of neuroprotective agents. In: Marangos, P.; Lal, H., eds. Emerging strategies in neuroprotection. Boston: Burkhauser Press; 1992:187–223.
37. Martin, P. R.; Nimmerrichter, A. A.: Pharmacological treatment of alcohol-induced brain damage. In: Hunt, W. A.; Nixon, S. J., eds. Alcohol-induced brain damage. Rockville, MD: U. S. Department of Health and Human Services; 1993:461–477.
38. McMullen, P. A.; Saint-Cyr, J. A.; Carlen, P. L.: Morphological alterations in rat CA1 hippocampal pyramidal cell dendrites resulting from chronic ethanol consumption and withdrawal. J. Comp. Neurol. 225:111–118; 1984.
39. Mize, R. R.; Holdefer, R. N.; Nabors, L. B.: Quantitative immunocytochemistry using an image analyzer. I. Hardware evaluation, image processing and data analysis. J. Neurosci. Methods 26:1–24; 1988.
40. Paula-Barbosa, M. M.; Brandão, F.; Andrade, J. P.; Madeira, M. D.; Zimmer, J.; Cadete-Leite, A.: Intracerebral grafting impedes hippocampal cell loss during withdrawal after long-term alcohol consumption in rats. Alcohol Alcohol. 26:177–190; 1991.
41. Paula-Barbosa, M. M.; Brandão, F.; Madeira, M. D.; Cadete-Leite, A.: Structural changes in the hippocampal formation after long-term alcohol consumption and withdrawal in the rat. Addiction 88:237–247; 1993.
42. Phelps, P. E.; Houser, C. R.; Vaughn, J. E.: Small cholinergic neurons within fields of cholinergic axons characterize olfactory-related regions of rat telencephalon. Neurosci. 48:121–136; 1992.
43. Phillips, S. C.: Qualitative and quantitative changes of mouse cerebellar synapses after chronic alcohol consumption and withdrawal. Exp. Neurol. 88:748–756; 1985.
44. Phillips, S. C.; Cragg, B. G.: Chronic consumption of alcohol by adult mice: effect on hippocampal cells and synapses. Exp. Neurol. 80:218–226; 1983.
45. Riley, J. N.; Walker, D. W.: Morphological alterations in hippocampus after long-term alcohol consumption in mice. Science 201:646–648; 1978.
46. Walker, D. W.; Barnes, D. E.; Zornetzer, S. F.; Hunter, B. E.; Kubanis, P.: Neuronal loss in hippocampus induced by prolonged ethanol consumption in rats. Science 209:711–713; 1980.

47. Walker, D. W.; Heaton, M. B.; Lee, N.; King, M. A.; Hunter, B. E.: Effect of chronic ethanol on the septohippocampal system: a role for neurotrophic factors? Alcohol. Clin. Exp. Res. 17:12–18; 1993.
48. Walker, D. W.; Hunter, B. E.; Abraham, W. C.: Neuroanatomical and functional deficits subsequent to chronic ethanol administration in animals. Alcohol. Clin. Exp. Res. 5:267–282; 1981.
49. Walker, D. W.; King, M. A.; Hunter, B. E.: Alterations in the structure of the hippocampus after long-term ethanol administration. In: Hunt, W. A.; Nixon, S. J., eds. Alcohol-induced brain damage. Rockville, MD: U. S. Department of Health and Human Services; 1993:231–247.