THE GABAERGIC SYSTEM OF THE DENTATE GYRUS AFTER WITHDRAWAL FROM CHRONIC ALCOHOL CONSUMPTION: EFFECTS OF INTRACEREBRAL GRAFTING AND PUTATIVE NEUROPROTECTIVE AGENTS

A. CADETE-LEITE*, F. BRANDÃO, J. P. ANDRADE, A. RIBEIRO-DA-SILVA¹ and M. M. PAULA-BARBOSA

Department of Anatomy, Porto Medical School, Portugal and ¹Department of Pharmacology & Therapeutics, McGill University, Montréal, Québec, Canada

(Received 28 October 1996; in revised form 17 January 1997; accepted 26 February 1997)

Abstract — We have demonstrated that, in the rat hippocampal formation, withdrawal from chronic alcohol consumption aggravates the ethanol-induced loss of pyramidal neurons and dentate granule cells. We have also shown that intracerebral grafting and piracetam could have a protective effect in these conditions. In this study we utilized immunocytochemical methods to investigate whether y-aminobutyric acid (GABA)ergic dentate gyrus cells, which are known to be inhibitory, were also affected by withdrawal from alcohol and, if so, whether putative neuroprotective agents could ameliorate the alterations found. Rats were alcohol-fed for 6 months and further divided into several groups: (1) alcohol-fed for an extra 6 months; (2) withdrawn from alcohol for 6 months; (3) withdrawn and grafted with newborn rat hippocampal tissue; (4) withdrawn and orally treated with piracetam for 6 months; (5) withdrawn and treated systemically with monosialoganglioside GM1 for 6 months; (6) withdrawn and treated with the vehicle used to dissolve the GM1. Control animals were pair-fed. All animals were killed 12 months after the beginning of the experiment and processed for GABA immunocytochemistry. GABA-immunoreactive (IR) neurons in the dentate gyrus were quantified and we found that alcohol-fed animals had a significant reduction in the numerical profile density of GABA-IR neurons in the dentate gyrus as a whole and in the hilus and in the granular layer of the suprapyramidal limb. Withdrawal from alcohol aggravated the GABAergic neuronal loss. Of the treatments used, only piracetam had a striking beneficial effect. Data gathered from the present work and from our previous studies indicate that the neuronal loss following chronic alcohol consumption and withdrawal affects both excitatory and inhibitory neurons in the dentate gyrus and that piracetam may have a useful protective role in this condition.

INTRODUCTION

There is considerable evidence that the brain is strongly affected by chronic ethanol consumption. Degenerative changes have been observed in many brain areas, and have revealed striking regional variations (Tavares and Paula-Barbosa, 1982, 1984; Beracoechea et al., 1987; Cadete-Leite et al., 1988b, 1990; Madeira et al., 1993; Pentney, 1993; Sousa et al., 1994). Studies from different laboratories have demonstrated that, in rodents submitted to long-term ethanol intake, the hippocampal formation is one of the areas most vulnerable to this neurotoxic agent (for review, see Paula-Barbosa et al., 1993; Walker et al., 1993). This finding is important, because the hippocampus, which plays an important role in learning and memory (Squire, 1986; Shen et al., 1994), is known to be affected by alcohol intake. Using unbiased stereological techniques, we have observed that the main neuronal populations which form the excitatory hippocampal trisynaptic circuit (Cadete-Leite et al., 1988b, 1989; Brandão et al., 1992) were severely damaged. Furthermore, we have shown that the choline acetyltransferase (ChAT)-immunoreactive (IR) fibre network and neurons of the hippocampal formation (Cadete-Leite et al., 1995), as well as cells of the dentate

*Author to whom correspondence should be addressed at: Department of Anatomy, Porto Medical School, Alameda Prof Hernani Monteiro, 4200 Porto, Portugal.
hilus and its somatostatinergic subpopulation (Andrade et al., 1992), were also affected by chronic ethanol consumption. In addition, we have also found a global reduction in the number of γ-aminobutyric acid (GABA)-IR neurons in the dentate gyrus from ethanol-treated rats (Cadete-Leite et al., 1993).

We have extended our investigations to animals withdrawn from alcohol after long periods of consumption to evaluate whether the removal of ethanol would reverse the ethanol-induced degenerative changes. In these studies, we found that withdrawal from ethanol ingestion did not reduce hippocampal degeneration, but rather augmented it leading to a further loss of dentate granule and CA1 pyramidal cells (Cadete-Leite et al., 1988a; Brandão et al., 1992) as well as of ChAT-IR neurons (Cadete-Leite et al., 1997). This somewhat unexpected finding might be of the utmost importance if a homology between rodents and humans is established. We decided, therefore, to evaluate whether agents known to display trophic or protective effects upon the damaged CNS have the capability to arrest the degenerative activity observed during ethanol withdrawal. For this purpose, we have used intra-hippocampal grafts of immature hippocampal tissue performed at the beginning of the withdrawal as well as neuroprotective drugs during the entire abstinence period. The above studies revealed that, whereas grafting and piracetam displayed unequivocal protective effects upon the main hippocampal neuronal populations (Paula-Barbosa et al., 1991; Brandão et al., 1995), the administration of monosialoganglioside GM1 during the withdrawal period had a protective effect which seemed restricted to the CA1 pyramidal cells (Cadete-Leite et al., 1991; Brandão, 1996).

Therefore we found it worthwhile to quantify the GABAergic neurons of the dentate gyrus using the model that we developed for evaluating the effects of long-term ethanol consumption and withdrawal in rodents (Cadete-Leite et al., 1988a, 1989).

Thus, the purpose of the present study was to investigate whether withdrawal from alcohol interferes with the reduction in the number of dentate GABAergic neurons observed after alcohol intake as well as whether the intracerebral grafting of immature hippocampal tissue and the administration of piracetam and GM1 ganglioside during withdrawal would have beneficial effects upon the GABAergic intrinsic innervation of the fascia dentata.

MATERIALS AND METHODS

Animals and diets

Male Sprague–Dawley rats from the colony of the Gulbenkian Institute of Science (Oeiras, Portugal) 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 until the rats were eight weeks old. Then rats were individually housed and randomly assigned to one of seven experimental groups. Twelve months after the beginning of the treatment (i.e. at 14 months of age) groups of four animals each were formed by sampling animals at random. Rats were treated as follows:

(1) Alcohol-fed. Rats had unrestricted access to a 20% (v/v) aqueous ethanol solution as the only available liquid source. At the beginning of the experiment, the administration of ethanol was gradual, starting with a 5% (v/v) solution and increasing 1% per day to a final concentration of 20% 2 weeks later. Alcohol feeding was continued until the animals were killed, to prevent the occurrence of withdrawal symptoms. Rats had free access to standard laboratory pellet food (Letica, Barcelona, Spain). The amounts of food and ethanol solution consumed were measured every other day.

(2) Pair-fed controls. Animals were given the same amount of food consumed by alcohol-treated rats. Sucrose was added to the drinking water to replace the caloric intake of ethanol. The percen-
DENTATE GYRUS AFTER CHRONIC ALCOHOLISM IN THE RAT

473

tagge of sucrose in the drinking water of the pairfed controls was calculated by taking into account the caloric values of both alcohol and sucrose, as well as the volume of ethanol consumed by alcohol-fed rats (Cadete-Leite et al., 1995).

(3) Withdrawal. Animals were alcohol-fed for 6 months and then switched to a diet in which standard chow and water ad libitum were provided for 6 months.

(4) Withdrawal + grafts. Rats were alcohol-treated as in (1) and then grafted at the beginning of the withdrawal period with immature hippocampal tissue (Paula-Barbosa et al., 1991).

(5) Withdrawal + piracetam. Rats were treated as in (3). In addition, during the entire withdrawal period, piracetam was added to the drinking water to allow an average daily intake of 800 mg/kg body weight (Brandão et al., 1995).

(6) Withdrawal + GM1. Rats were treated as in (3) and subcutaneously injected, every 2 days, with a 0.9% saline solution containing 35 mg/kg body weight of monosialoganglioside GM1 during the entire withdrawal period (Cadete-Leite et al., 1991).

(7) Withdrawal + vehicle. Animals were treated as in the previous group, but received saline instead of the GM1.

In the different withdrawal groups, the shift from alcohol treatment to water was performed gradually through a progressive reduction of the concentration of the ethanol in the drinking solution over a period of 2 weeks. This graded withdrawal was performed to avoid the symptoms and lesions that might occur following acute abstinence from alcohol (Walker et al., 1981; Maier and Pohorecky, 1989). With this approach, rats are allowed to adjust to the new metabolic conditions (Eckardt et al., 1986), and no seizures were observed. The diets were supplemented with vitamins and minerals as previously described (Cadete-Leite et al., 1995).

During the first month of the experimental period, the animals were weighed every 3 days and thereafter every 2 weeks.

Blood ethanol analysis

Blood ethanol concentrations were assessed in an additional set of four alcohol-treated rats. Blood samples (100μl) were collected in the morning (08:00) and in the evening (18:00) from the tail vein after 6 and 12 months of treatment, and were analysed for alcohol using an enzymatic assay kit (Boehringer-Manheim).

Grafting technique

Graftings of hippocampal tissue blocks were performed as previously described (Paula-Barbosa et al., 1991), using newborn rats as donors. In brief, the donor animals were decapitated and brains removed in toto. After exposing the hippocampal region, the septotemporal middle two-thirds of the hippocampal formation were dissected out, immersed in Ringer solution and then sucked into a glass capillary cannula connected to a 50μl Hamilton syringe. Blocks were trimmed in order to contain both the dentate gyrus and the hippocampus proper. Simultaneously with the preparation of the donor tissue, the recipient animals were anaesthetized using a mixture of pentobarbital (8.85 mg/ml) and chloral hydrate (42.5 mg/ml) in a dose of 4 ml/kg body weight i.p., and prepared to serve as recipients by exposing and trepanating the skull. Using a stereotaxic technique, the grafting cannula was lowered into the right dorsal hippocampus and the graft expelled. The cannula was retracted 1 min after.

Tissue fixation

At the end of the respective experimental periods, animals were anaesthetized 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. Next, the animals were perfused transcardially with 15–20 ml of phosphate buffer pH 7.4 (PB) for vascular rinse, followed by a 20–30 min fixation with a mixture of 3% (w/v) paraformaldehyde, 0.1% glutaraldehyde and 15% (v/v) saturated picric acid in PB at room temperature. The perfusion mixture was then switched to a 0.5% hydrogen peroxide solution in phosphate-buffered saline (PBS) for 30 min, followed by a 10% sucrose/PB rinse for a period of 30 min (for details, see Côté et al., 1993). The brains were then removed in toto, weighed, and coded to allow blind analysis of the material. Fifty-μm-thick coronal sections were cut on a Vibratome and collected as free-floating. On the right hemisphere of the grafted animals, the tract of the grafting cannula was followed from section to section in order to detect the presence of grafted material.
Sections from each grafted animal were collected into gelatin-subbed slides and stained with the cresyl violet method, to allow the evaluation of the success of the grafts. Animals in which grafted material had been reabsorbed were used as sham-operated.

**Immunostaining**

Six Vibratome sections per animal, each containing both the left and right hippocampal formations, were selected for immunostaining. Of those sections, two were from the septal part, two from the temporal part, and two from the midseptotemporal part. In grafted animals, 12 sections were immunostained, four from each of the three parts of the hippocampal formation. PBS was used for the washes and PBS with 0.2% Triton X-100 (PBS + T) was used to dilute all the antibodies. Following a pretreatment in 10% normal goat serum, the sections were incubated as free-floating at 4°C for 16 h in rabbit anti-GABA antibody (Sigma). All subsequent steps were carried out at room temperature. After three washes, the sections were incubated in biotinylated goat antirabbit IgG followed by an avidin-biotin complex (Vector Laboratories). The peroxidase reaction was developed with 3,3'-diaminobenzidine (DAB) and H$_2$O$_2$. Subsequently, the sections were rinsed three times, mounted on gelatin-subbed glass slides, air-dried overnight, dehydrated through ascending alcohols, and coverslipped 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.

**Cell measurements**

For neuronal quantification, 12 hippocampal sections from each animal were used and measurements were carried out bilaterally. In each section, the boundaries of the different layers of the dentate gyrus — the hilus and the suprapyramidal and infrapyramidal limbs of the granular and molecular layers — were schematically drawn with the aid of a camera lucida at a final magnification of ×43. The cross-sectional area of each of these layers was measured using a Videoplan image analysis system.

GABA-IR cell bodies with intact perikaryal contours were identified in each section using a ×40 plan objective (final magnification of ×600) and plotted in each of the previously drawn layers. The number of GABA-IR neurons per unit surface area (numerical profile density, $N_A$) was calculated from the number of plotted cell bodies in each layer and the area of the cross-sectional area of the respective layer.

For measurements of the perikaryal area, the contours of all GABA-IR neurons referred to above were individually drawn, using a camera lucida at a final magnification of ×600. In those layers in which the number of plotted GABA-IR cells was >15, only the contours of the first 15 neurons visualized were drawn. Measurements were made with the aid of the Videoplan and the values were averaged within layers in each dentate gyrus.

Because in a previous study (Cadete-Leite et al., 1993) we have verified that the number of GABA-IR neurons from the dentate gyrus did not differ along the septotemporal axis of the hippocampal formation, data from the septal, midseptotemporal, and temporal parts were pooled within each animal.

**Statistical analysis**

Normal quantile plots were used as a tool for assessing the normal distribution of the data. This assessment was important in this study, because normality was not obvious in all cases and there was incomplete variance homogeneity. Due to the normality and homogeneity of variance of the sampled populations and because there are no differences in the frequency of dentate GABA-IR neurons along the septotemporal axis of the hippocampal formation from animals of the experimental groups, a two-way ANOVA was applied to discern main effects. Treatment and location of neurons in each layer of the dentate gyrus were used as independent variables and animals as replicates. The remainder mean square was utilized as the error term.

Taking into account the size of the sample and given 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 unfavourable 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 rejec-
Table 1. Morphometric data from control and experimental groups of animals

<table>
<thead>
<tr>
<th>Group</th>
<th>Total no. of neurons per group</th>
<th>No. of neurons per section (mean ± SD)</th>
<th>No. of neurons in a single section</th>
<th>Cross-sectional area (mean ± SD) of dentate gyrus (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3293</td>
<td>68.6 ± 3.9</td>
<td>52–119</td>
<td>1.49 ± 0.03</td>
</tr>
<tr>
<td>Alcohol</td>
<td>2808</td>
<td>58.6 ± 5.5*</td>
<td>28–85</td>
<td>1.51 ± 0.02</td>
</tr>
<tr>
<td>Withdrawal</td>
<td>2499</td>
<td>52.2 ± 4.5*†</td>
<td>36–80</td>
<td>1.48 ± 0.03</td>
</tr>
<tr>
<td>Withdrawal + graft</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ipsilateral</td>
<td>2397</td>
<td>49.9 ± 2.7***</td>
<td>33–79</td>
<td>1.50 ± 0.03</td>
</tr>
<tr>
<td>Contralateral</td>
<td>2484</td>
<td>51.8 ± 2.9***</td>
<td>38–99</td>
<td>1.50 ± 0.04</td>
</tr>
<tr>
<td>Withdrawal + piracetam</td>
<td>2795</td>
<td>58.1 ± 5.2*</td>
<td>34–83</td>
<td>1.49 ± 0.01</td>
</tr>
<tr>
<td>Withdrawal + GM1</td>
<td>2582</td>
<td>53.8 ± 4.0***</td>
<td>38–93</td>
<td>1.49 ± 0.04</td>
</tr>
<tr>
<td>Withdrawal + vehicle</td>
<td>2545</td>
<td>53.0 ± 2.6***</td>
<td>40–91</td>
<td>1.48 ± 0.02</td>
</tr>
</tbody>
</table>

Twelve hippocampal sections from each animal were used for neuronal quantifications. Comparisons of mean number of neurons per section: control vs all experimental groups, *P < 0.0005; alcohol vs withdrawal and withdrawal-treated groups, †P < 0.05, ††P < 0.005; withdrawal + piracetam vs withdrawal, withdrawal + graft (ipsilateral and contralateral sides), withdrawal + vehicle, and withdrawal + GM1, ‡P < 0.05, ‡‡P < 0.005. ANOVA, z-test and Bonferroni test were used for statistical evaluation. ANOVA did not show any significant effect of treatment upon cross-sectional area of dentate gyrus (F<sub>7,24</sub> = 0.366, P = 0.913).

RESULTS

Animals and diets

The average ethanol consumption in alcohol-treated animals throughout the entire experimental period was ~9.0 g/kg body weight/day. During the first 3 months of alcohol treatment the average intake was <9.0 g/kg body weight/day, whereas at 12 months alcohol consumption attained an average of 13.0 g/kg body weight/day. In any case, the mean daily alcohol intake was lower than that reported in other experimental models (Walker et al., 1981; Lescaudron et al., 1986) and corresponded to a mean value of 18 ml of daily alcohol ingestion (2.8 g; 19.5 kcal). The average daily sucrose solution consumed by the pair-fed controls was ~41 ml, with a mean daily intake of 11.7% (w/v) of sucrose (4.8 g; 19.5 kcal).

During the entire experimental period, the mean blood ethanol concentration was 101.4 ± 12.7 mg/dl. Values ranged from 80 to 120 mg/dl and the highest concentrations were always observed in the blood samples collected in the morning. No significant differences were found in blood alcohol levels from samples collected after 6 and 12 months of alcohol treatment.

During the first month of the experiment, the body weights of alcohol-treated rats remained unchanged as opposed to a mean increase of 11.9 ± 4.8 g in pair-fed controls. However, this difference was not significant. Thereafter, the mean body weight increase of pair-fed controls was 13.8 ± 2.4 g/month, whereas that of alcohol-fed rats was 11.1 ± 3.0 g/month. In all withdrawal groups the mean body weight increase after the removal of alcohol was 9.3 ± 2.9 g/month. At the end of the experimental period, no significant differences were found between the mean body weights of alcohol-treated rats and pair-fed controls. The mean body weight of the withdrawn and withdrawn-treated animals did not differ significantly from those of alcohol-treated and pair-fed control animals.

No significant differences were found among the mean brain weights of pair-fed control and experimental rats.
Fig. 1. Micrographs of part of the suprapyramidal limb and hilus of the dentate gyrus. Micrographs are from control (A), alcohol-fed (B), withdrawn (C) and withdrawn + piracetam-treated (D) rats. Note the reduction in number of GABA-IR neurons in B–D compared to A, particularly noticeable in C. MI = molecular layer; Gr = granular cell layer; H = hilus. Scale bar = 50 μm.
Morphology of the GABA-IR neurons

Qualitatively, no obvious differences were detected in the morphological properties of GABA-IR neurons in the dentate gyrus when the experimental groups were compared to controls. The morphology of the cells was identical to that described in previous studies (see e.g. Woodson et al., 1989). Figure 1 gives typical examples of GABA-IR cells from the suprapyramidal limb of the granular cell layer in control and experimental groups, and shows an apparent reduction in the number of GABA-IR cells in the experimental groups relative to the control group.

Number and distribution of neurons

The total number of identified dentate GABA-IR neurons in the 384 sections studied was 21,403. The distribution of these neurons among animal groups is shown in Table 1, which also shows the average number and range of neurons per section, as well as the mean cross-sectional area of the dentate gyrus per section.

The analysis of the data presented in Table 1 revealed a reduction in the number of GABA-IR neurons in all experimental groups compared to the control group. When withdrawal groups were compared to the alcohol-fed group, significant reductions in the mean number of neurons per section were detected in all groups except the withdrawal + piracetam group. In this group, the values found for this parameter were higher than those of either the withdrawal group or the withdrawal + graft, withdrawal + GM1 and withdrawal + vehicle groups. ANOVA indicated that the mean number of GABA-IR neurons per section was dependent on treatment ($F_{7,120} = 6.49, \ P = 0.0002$). There were no significant differences between groups in the cross-sectional area of dentate gyrus.

Numerical profile density of neurons

When compared to controls, there was a significant reduction in the number of GABA-IR neurons per unit surface area (numerical profile density, $N_A$) in the dentate gyrus as a whole and in the suprapyramidal limb and its granular layer in all experimental groups (Table 2). In the hilus, the above $N_A$ reduction was also observed in all experimental groups with the exception of the withdrawal + piracetam group.

Withdrawal from alcohol resulted in a further reduction (compared to the alcohol-fed group) in the $N_A$ in the dentate gyrus as a whole and in the suprapyramidal limb and its granular layer (Table 2). The association of withdrawal with grafting and GM1 treatment did not result in any significant difference from the withdrawal group regarding the $N_A$ values (Table 2). However, the withdrawal + piracetam group displayed $N_A$ values that were not statistically different from those of the alcohol-fed group in the dentate gyrus as a whole as well as in the suprapyramidal limb and its granular layer, and in the hilus (Table 2).

ANOVA revealed that treatment ($F_{7,120} = 18.21, \ P < 0.0005$) and location ($F_{4,120} = 712.32, \ P < 0.0005$), as well as the interaction between these two variables ($F_{28,120} = 6.56, \ P < 0.0005$), were significantly related to the variations in the $N_A$ of GABA-IR neurons.

Neuronal perikaryal area

No significant differences from control were observed in the perikaryal area of GABA-IR neurons in the dentate gyrus as a whole or in any of its layers in the experimental groups (Table 3). ANOVA indicated a significant effect of location ($F_{4,120} = 17.91, \ P < 0.0005$) on the perikaryal area of the GABA-IR neurons, but not of the treatment ($F_{7,120} = 0.31, \ P = 0.95$) or the interaction between these two variables ($F_{28,120} = 0.25, \ P = 1.00$).

DISCUSSION

We are aware of the fact that absolute values, e.g. total neuronal numbers, instead of intermediate parameters such as the neuronal densities employed in this investigation, are essential to reach sound conclusions. Unfortunately, we could not use such estimators, because the technical procedures for brain grafting alter the anatomy of the hippocampal formation from the recipient animals, namely its boundaries, whose discreteness is pivotal to the estimation of total numbers. Thus, the numerical profile density of neurons was the estimator chosen because it allowed us to obtain data comparable among the groups under evaluation.

The present study reinforces the view that chronic alcohol consumption leads to a consider-
Table 2. Numerical profile density \( (N_A) \) of GABAergic neurons in the dentate gyrus of control and experimental animals

<table>
<thead>
<tr>
<th>Location</th>
<th>Control</th>
<th>Alcohol</th>
<th>Withdrawal</th>
<th>Ipsilateral</th>
<th>Contralateral</th>
<th>Withdrawal + piracetam</th>
<th>Withdrawal + GM1</th>
<th>Withdrawal + vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dentate gyrus</td>
<td>48.9 ± 2.7</td>
<td>42.1 ± 2.5***</td>
<td>36.7 ± 0.9***↑↑↑</td>
<td>37.1 ± 2.2***↑↑↑</td>
<td>38.5 ± 1.5***↑↑↑</td>
<td>41.3 ± 2.9***↑↑↑</td>
<td>38.1 ± 0.8***↑↑↑</td>
<td>36.2 ± 1.4***↑↑↑</td>
</tr>
<tr>
<td>Infra-pyramidal limb</td>
<td>31.6 ± 2.5</td>
<td>31.9 ± 4.9</td>
<td>31.1 ± 2.0</td>
<td>31.0 ± 2.2</td>
<td>29.8 ± 0.9</td>
<td>29.6 ± 1.4</td>
<td>33.0 ± 1.9</td>
<td>31.6 ± 1.7</td>
</tr>
<tr>
<td>Molecular layer</td>
<td>16.4 ± 1.0</td>
<td>16.8 ± 2.6</td>
<td>14.8 ± 2.4</td>
<td>15.7 ± 1.8</td>
<td>14.5 ± 1.5</td>
<td>14.9 ± 1.4</td>
<td>17.5 ± 1.3</td>
<td>16.3 ± 1.4</td>
</tr>
<tr>
<td>Granular layer</td>
<td>84.0 ± 12.0</td>
<td>77.4 ± 12.3</td>
<td>80.0 ± 8.3</td>
<td>77.3 ± 6.1</td>
<td>72.8 ± 6.0</td>
<td>73.2 ± 7.8</td>
<td>74.7 ± 6.4</td>
<td>76.3 ± 4.3</td>
</tr>
<tr>
<td>Hilus</td>
<td>87.7 ± 13.2</td>
<td>65.0 ± 15.8**</td>
<td>57.0 ± 4.7***</td>
<td>60.4 ± 7.7***</td>
<td>60.5 ± 5.8***</td>
<td>71.6 ± 14.2</td>
<td>60.0 ± 3.1***</td>
<td>58.6 ± 2.6***</td>
</tr>
<tr>
<td>Supra-pyramidal limb</td>
<td>45.7 ± 5.0</td>
<td>40.7 ± 2.0*</td>
<td>32.0 ± 3.1***↑↑↑</td>
<td>33.1 ± 2.0***↑↑↑</td>
<td>35.8 ± 1.5***↑↑↑</td>
<td>39.5 ± 2.4*</td>
<td>34.9 ± 0.9***↑↑↑</td>
<td>33.0 ± 1.9***↑↑↑</td>
</tr>
<tr>
<td>Granular layer</td>
<td>148.2 ± 13.2</td>
<td>113.7 ± 14.8***</td>
<td>87.4 ± 2.5***↑↑↑</td>
<td>87.3 ± 3.2***↑↑↑</td>
<td>87.9 ± 2.6***↑↑↑</td>
<td>113.1 ± 5.5*</td>
<td>84.1 ± 2.8***↑↑↑</td>
<td>84.2 ± 2.2***↑↑↑</td>
</tr>
<tr>
<td>Molecular layer</td>
<td>19.2 ± 2.7</td>
<td>21.8 ± 3.4</td>
<td>16.7 ± 2.5</td>
<td>17.9 ± 0.9</td>
<td>18.2 ± 1.6</td>
<td>20.8 ± 2.0</td>
<td>20.4 ± 0.9</td>
<td>19.1 ± 0.8</td>
</tr>
</tbody>
</table>

Values represent mean ± SD; \( N_A \) (N/mm\(^2\)). Control vs alcohol, withdrawal, withdrawal + graft (ipsilateral and contralateral sides), withdrawal + piracetam, withdrawal + GM1 and withdrawal + vehicle: *\( P < 0.05 \), **\( P < 0.005 \), ***\( P < 0.0005 \); alcohol vs withdrawal, withdrawal + graft (ipsilateral and contralateral sides), withdrawal + GM1 and withdrawal + vehicle: †\( P < 0.005 \), ††\( P < 0.0005 \); withdrawal + piracetam vs withdrawal, withdrawal + graft (ipsilateral and contralateral sides), withdrawal + GM1 and withdrawal + vehicle: ‡\( P < 0.005 \), ‡‡\( P < 0.0005 \). ANOVA, z-test and Bonferroni test were used for statistical evaluation.
Table 3. Perikaryal area (µm²) of GABAergic neurons in the dentate gyrus of control and experimental animals

<table>
<thead>
<tr>
<th>Location</th>
<th>Control</th>
<th>Alcohol</th>
<th>Withdrawal</th>
<th>Withdrawal + graft</th>
<th>Withdrawal + piracetam</th>
<th>Withdrawal + GM1</th>
<th>Withdrawal + vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dentate gyrus</td>
<td>97.8 ± 9.3</td>
<td>100.0 ± 9.7</td>
<td>100.7 ± 6.9</td>
<td>102.5 ± 8.9</td>
<td>104.4 ± 4.8</td>
<td>105.3 ± 5.4</td>
<td>100.2 ± 4.8</td>
</tr>
<tr>
<td>Infrapyramidal limb</td>
<td>105.1 ± 9.9</td>
<td>106.5 ± 12.1</td>
<td>107.9 ± 6.8</td>
<td>107.6 ± 12.8</td>
<td>107.5 ± 5.9</td>
<td>106.6 ± 8.7</td>
<td>104.9 ± 7.7</td>
</tr>
<tr>
<td>Molecular layer</td>
<td>99.8 ± 11.5</td>
<td>101.5 ± 12.6</td>
<td>101.1 ± 11.2</td>
<td>100.9 ± 13.6</td>
<td>104.3 ± 7.2</td>
<td>104.7 ± 7.3</td>
<td>100.1 ± 12.5</td>
</tr>
<tr>
<td>Granular layer</td>
<td>110.3 ± 8.7</td>
<td>111.6 ± 12.5</td>
<td>114.7 ± 6.1</td>
<td>114.2 ± 16.5</td>
<td>110.7 ± 5.5</td>
<td>115.4 ± 6.8</td>
<td>109.7 ± 6.4</td>
</tr>
<tr>
<td>Hilus</td>
<td>93.5 ± 8.5</td>
<td>96.8 ± 8.4</td>
<td>97.1 ± 8.1</td>
<td>101.7 ± 12.3</td>
<td>98.9 ± 9.0</td>
<td>101.3 ± 4.0</td>
<td>98.7 ± 6.5</td>
</tr>
<tr>
<td>Suprapyramidal limb</td>
<td>92.8 ± 9.9</td>
<td>95.2 ± 8.6</td>
<td>95.4 ± 8.2</td>
<td>97.7 ± 6.5</td>
<td>96.7 ± 8.2</td>
<td>98.7 ± 8.3</td>
<td>97.0 ± 5.3</td>
</tr>
<tr>
<td>Granular layer</td>
<td>99.6 ± 12.4</td>
<td>99.9 ± 7.9</td>
<td>104.0 ± 9.4</td>
<td>101.4 ± 12.5</td>
<td>99.2 ± 10.1</td>
<td>102.6 ± 8.0</td>
<td>103.8 ± 5.7</td>
</tr>
<tr>
<td>Molecular layer</td>
<td>85.9 ± 8.0</td>
<td>90.5 ± 9.3</td>
<td>86.8 ± 8.0</td>
<td>93.9 ± 10.1</td>
<td>94.1 ± 10.8</td>
<td>83.7 ± 13.3</td>
<td>90.2 ± 4.9</td>
</tr>
</tbody>
</table>

Values represent mean ± SD.
able reduction in the number of GABAergic cells in the dentate gyrus. This cell depletion was confined to the hilus and suprapyramidal limb of the granular layer. As far as we are aware, this is the first comprehensive study of the effects of chronic alcohol consumption upon the GABAergic cell population of the dentate gyrus using immunocytochemistry. Further, we have also expanded our data by investigating the effects of withdrawal from alcohol, a condition that aggravates the ethanol-induced cell loss. Finally, with respect to the action of protective agents during abstinence, grafting of hippocampal tissue from newborn animals and GM1 treatment were found to be ineffective. By contrast, the alcohol-fed and withdrawn animals treated with piracetam were indistinguishable from each other, suggesting that the above putative neuroprotective agent may be useful in preventing some of the brain damage following withdrawal from alcohol.

**GABAergic cells in the dentate gyrus**

Our study was restricted to the GABA-IR cells in the dentate gyrus. We decided to focus on these cells, as they are known to be the major source of inhibition of the dentate granular cells, which represent the first postsynaptic target of the hippocampal afferents arising from the entorhinal cortex (Amaral and Witter, 1989). Lescaudron et al. (1986) performed an immunocytochemical study in the mouse centred in the hippocampus proper, and found a loss of GABAergic cells confined to the ventral hippocampus and reaching 25% in the stratum radiatum of CA1. These data match the 24% reduction in GABAergic cells that we observed in the suprapyramidal granular layer of the dentate gyrus. In addition, although immunocytochemistry was not used and the morphological properties of the cells were not determined with certainty, a study in the ethanol-sensitive long-sleep mouse showed a depletion of 17% of dentate basket cells (Scheetz et al., 1987). These results are therefore also comparable to ours, as most of the dentate basket cells are known to be GABAergic (Woodson et al., 1989; Amaral and Witter, 1995).

Furthermore, it deserves to be emphasized that the reduction in the $N_A$ of GABAergic neurons induced by chronic alcohol consumption and withdrawal probably corresponds to parallel changes in the total number of neurons immunoreactive for GABA. Indeed, we found no differences in the cross-sectional area of the dentate gyrus among control and experimental groups. Likewise, we did not detect any difference among experimental groups and controls in the neuronal perikaryal area. This result indicates that treatments did not have any effect on cell size and that all GABA-IR cell populations were equally affected by the experimental conditions used.

The possibility that the neuronal loss observed may not result from neuronal death must be carefully considered. In fact, this finding might derive from the lack of expression of GABA leading to GABA levels below immunocytochemical detection. The likelihood of the latter hypothesis is small, because, in the experimental groups, the cells were as intensely immunostained as in controls and we did not find numerous lightly stained cells in alcohol-treated rats, as would be expected with lower GABA levels.

**GABAergic function after alcohol treatment**

Evidence from in vitro studies indicates that acute administration of ethanol induces an enhancement of the GABAergic inhibitory action in the CNS (for review see Little, 1991), which seems to be governed through an increase of chloride influx (Suzdak et al., 1986; Mehta and Ticku, 1988). However, tolerance to this effect develops quickly (for review see Buck and Harris, 1991). Thus, it is not surprising that, after chronic alcohol consumption a reduction of GABA$A$ receptor function was observed, albeit the underlying mechanism is at present unclear (Shanley and Wilce, 1993).

Regarding withdrawal from chronic alcohol consumption, there is evidence that it further increases the degenerative activity induced by alcohol (Phillips and Cragg, 1984; Cadete-Leite et al., 1988a), through a mechanism that seems to be mostly excitotoxic (Crews and Chandler, 1993). In previous studies in the hippocampal formation, we detected increased granular and CA1 pyramidal cell losses during withdrawal (Cadete-Leite et al., 1988a; Brandão et al., 1992), as well as increased loss of cholinergic neurons in the hippocampal regio superior (Cadete-Leite et al., 1997). Our present data show that the GABAergic cell population is also affected by withdrawal from alcohol, reaching a decrease attaining 41% of control levels. In other words, if a comparison is
made with alcohol-induced neuronal loss, the reduction observed in withdrawn rats is ~23%. The likelihood that the increased cell loss during withdrawal might have functional implications is high. In fact, a recent electrophysiological study in slices from the rat hippocampus showed a persistent reduction of GABA_A receptor inhibition after repeated episodes of withdrawal from alcohol (Kang et al., 1996).

Absence of protective effects of grafting and GM1 treatment

The absence of a beneficial effect of intracerebral grafting of hippocampal tissue from newborn rats was somewhat unexpected, as previous studies from our laboratory had shown a reduced loss of dentate granular and hilar cells as well as of pyramidal cells from CA1 and CA3 (Paula-Barbosa et al., 1991; Brandão et al., 1993), and a sprouting of hippocampal cholinergic fibres (Cadete-Leite et al., 1997). None the less, apparently, grafting did not have any effect upon the cholinergic cell bodies of the hippocampal formation (Cadete-Leite et al., 1997). Intracerebral grafts probably have their beneficial effect through the production and/or induction of the release of neurotrophic agents in the brain (for a discussion see Cadete-Leite et al., 1997). Should that be so, then it is likely that the absence of effect upon the GABAergic system might result from a lack of release by the hippocampal grafts of factors with trophic effects upon the dentate GABAergic cells.

GM1 treatment is known to have a neuroprotective effect in the CNS, both in vivo and in vitro, although the mechanism of action is not fully understood (for review see Cuello, 1990; Mahadik, 1992). For example, GM1 administered to rats bearing cortical infarcts (Elliott et al., 1989), medial septal lesions (Poplawsy and Isaacson, 1989), and electrolytic lesion of the substantia nigra (Jackson et al., 1989) has been shown to improve the performance in behavioural tests and promote functional recovery. In addition, there are numerous morphological and biochemical studies indicating the neuroprotective capabilities of this drug (Kojima et al., 1984; Wieraszko and Seifert, 1985; Tanaka et al., 1986; Schneider and Yuwiler, 1989; Fong et al., 1995). Furthermore, recent in vitro studies have demonstrated that GM1 treatment might protect against ethanol-induced glutamate neurotoxicity (Heaton et al., 1994; Hoffman et al., 1995). However, in the present study, GM1 applied subcutaneously did not protect the GABAergic neurons in the dentate gyrus during withdrawal from chronic alcohol consumption. This finding fits previous data from our laboratory, in which no ameliorative effects were observed in withdrawn animals treated with GM1 with respect to the number of CA3 pyramidal and dentate granule neurons as well as to the number of mossy fibre–CA3 pyramidal cell synapses (Cadete-Leite et al., 1991). However, recent observations revealed a protective effect upon CA1 pyramidal cells (Brandão, 1996). A possible cause for the absence of effect of the ganglioside is the route of administration. Indeed, GM1 has been shown to be more effective when applied intracerebroventricularly than systemically (Cuello et al., 1989), although it crosses the blood–brain barrier in small amounts (Bellato et al., 1989). It is therefore possible that the GABAergic hippocampal neurons require a higher concentration of GM1 than the other cell populations in which GM1 has been shown to have a beneficial effect when applied by a systemic route.

Effects of piracetam

The protection displayed by piracetam upon GABAergic neurons of the dentate gyrus from withdrawn animals is noteworthy. The results reported here fit previous non-immunocytochemical studies in which we had found that piracetam impeded additional cell loss in the granular and hilar cells of the dentate gyrus (Brandão et al., 1995). Likewise we observed in a different study that the number of synapses between mossy fibres and CA3 pyramidal cells was higher in piracetam-treated than in alcohol-fed and withdrawn animals (Brandão et al., 1996).

Piracetam is a cyclic derivative of GABA, but possesses a low affinity for GABA receptors (Giuergia, 1982). This agent has been considered as possessing a wide variety of neuronal protective actions among which a cognition-enhancing effect deserves to be emphasized (for review see Gouliaev and Senning, 1994). In addition, it has been claimed that piracetam and other nootropic drugs are capable of reversing certain types of amnesia, to protect against barbiturate-induced neuronal toxicity, and they seem effective, at the clinical level, in light or moderate dementia (for
GENERAL CONCLUSIONS

The results now reported, alongside those of our previous studies on hippocampal neurons known to be excitatory (Cadete-Leite et al., 1988a,b, 1989; Brandão et al., 1995), show that both excitatory and inhibitory systems of the hippocampal formation are affected by chronic alcohol consumption and withdrawal. As the normal balance between both systems is required for a normal hippocampal function, caution must be taken when measures are to be employed in order to prevent disruptions of hippocampal function in chronic alcoholism and withdrawal. In other words, any attempt to ameliorate such disruptions must interfere simultaneously with the inhibitory and excitatory systems. Even so, the apparent protective effects of piracetam in both inhibitory (this study) and excitatory systems of the hippocampal formation (Brandão et al., 1995, 1996) renders this drug suitable for use for this purpose.

REFERENCES


