Effects of Chronic Alcohol Consumption on Hippocampal Anatomy and Associated Behaviors in Three Inbred Strains of Mice

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Abstract: The goal of this study was to investigate the influence of 6 months of chronic alcohol consumption on hippocampal neuroanatomy, notably the sizes of the intra- and infrapyramidal mossy fiber (IIPMF) terminal fields, and several behaviors, such as radial-maze learning, intermale aggression and anxiety-like behavior, in three inbred strains of mice (NZB, CBA/H, C57BL/6). Based upon several reports highlighting the toxicity of chronic alcohol exposure on the hippocampus, we expected a general diminution of cognitive abilities, with reduced spatial learning skills, increased aggression and anxiety; and concomitantly, a reduction in the sizes of the IIPMF. Contrary to our hypothesis, we did not find an effect of chronic alcohol exposure, neither an effect per se or in interaction with the genotype. Possible explanations for this unexpected finding include age effects and species differences between rats and mice.

Keywords: Alcohol, inbred mouse strains, spatial learning, hippocampus, aggression.

INTRODUCTION

Ethanol has a wide-range of physiological and behavioral effects, but remains one of the least understood psychoactive drugs. The main reason is that alcohol does not touch upon a simple interaction between ligand and receptor, but acts as a multipotential pharmacologically active substance. As evidenced by a current of publications, pinpointing a site of action or single mechanism underlying alcohol effects is difficult because the drug affects virtually all neurochemical and endocrine systems. Consequently, many studies have been carried out, each trying to understand specific pathways affected by alcohol. In this paper, we focus on the genetic susceptibility to the effects of chronic exposure to alcohol, a less frequently investigated part as most studies in this field have concentrated on the effects of chronic exposure to alcohol per se.

Long-term use of alcohol leads to multiple and durable changes in the central nervous system. Alcoholics exhibit brain lesions [1], such as reductions of the size of the forebrain and hippocampus [2], as well as neurodegenerative changes in the cholinergic basal forebrain [3]. In animal models, several groups have reported specific neuronal loss in the dentate gyrus, increased arborizations of the dendritic spines of the granule cells [4, 5], reduction of the number of spines of the CA3-pyramidal cells [6], and a reorganization of synaptic formations [7]. Chronic exposure to alcohol also induces long-term physiological changes, such as a decrease of specific neurotrophic factors [8, 9], and more generally, changes in patterns of neurotransmission [10]; for a review see [11]. Concomitantly, behavioral modifications are observed. Whereas the acute effect of alcohol is stimulatory and results in a behavioral "disinhibition" (e.g. exultation, euphoria, or desolation), long-term use provokes other patterns of behavior, subtle and variable according to age, sex, time of exposure, and models used [10, 12-14]. Not surprisingly given the plasticity of the brain, some of these toxic effects lessen or even disappear after withdrawal from alcohol [7, 15, 16], although this may take several months [5]. However, most brain damage often remains irreversible [17]; for instance, alcoholics who had been consuming alcohol for a long time revealed damages and shrinkage in several brain regions [1, 2]. In addition, the behavior of former alcoholics often does not recover completely [18].

Many reports have demonstrated that the effects of chronic exposure to alcohol on brain and behavior are variable from one person to another. Genetic factors, in interaction with environmental ones are likely to underlie these inter-individual differences (for review, see [19]). The aim of this study was to investigate the genetic susceptibility to chronic exposure of alcohol in a mouse model. To this end, mice from three inbred strains were exposed to an alcohol-containing solution as their only source of fluid for 6 months. Necessary control groups (pair-feds, standard) were
included. After an obligatory period of withdrawal in which all groups were housed under standard laboratory conditions, all animals were run through a behavioral test battery, including radial-maze learning, aggressive behavior (resident-intruder and neutral-cage paradigms), and anxiety-like behavior. Next, animals were sacrificed and the sizes of the hippocampal intra- and infrapyramidal mossy fiber (IIPMF) terminal fields were determined.

Bearing in mind that (i) long-term alcohol intake results in a loss of hippocampal pyramidal cells [6, 7, 20], (ii) dendritic spines of these pyramidal cells induce growth of the IIPMF terminal fields [21], (iii) newly born cells in the dentate gyrus are highly demanding on neurotrophic factors [22, 23], and (iv) long-term alcohol exposure decreases the concentration of such factors [8], we hypothesized that chronic alcohol exposure would diminish the sizes of the IIPMF terminal fields. In consequence, we would expect animals exposed chronically to alcohol to perform poorly in the radial maze, because the extent of the IIPMF terminal fields is positively correlated with spatial navigation skills [24, 25]. We also anticipated an increase in aggression as this behavior is negatively correlated with the IIPMF sizes [26-29]. Finally, if aggressive behavior is indeed increased, we would expect more anxiety-like behavior in the Light/Dark box, given the correlation between these two behaviors (Guilhot and Chapouthier, 1996).

The three strains used in this study, NZB/B1NJ (NZB), CBA/H (CBA) and C57BL/6J (B6), have distinct genotypes and therefore each strain might be differentially protected against or be vulnerable to chronic alcohol exposure. In addition, these three strains differ in voluntary alcohol consumption (Fuller, 1978) as well as their sizes of the IIPMF terminal fields. Hence, instead of or in addition to a general effect of chronic alcohol exposure, we might expect a strain-dependent, differential response to chronic alcohol consumption, i.e., a gene-environment interaction.

MATERIALS AND METHODS

Animals

All experimental animals were born and raised in our former animal facility at the Université René Descartes in Paris, France, which was approved by the French Ministry of Agriculture. Animals were kept under the following conditions: temperature: 23 ± 0.5°C; light/dark schedule: 12:12, lights on at 8:00 AM; Food (IM UAR) and tap water ad libitum; dust-free sawdust bedding; weaning at approximately 4 weeks; housing in plastic cages (42 x 27 x 17) with littermates (not more than four per cage) until alcoholization commenced. Three inbred strains were used in this study: NZB/B1NJ, CBA/H, and C57BL/6J. All strains had been maintained in our animal facility for several years. Only males were used. All experiments were performed in accordance with the applicable European Union and French regulations. WEC holds a valid French permit for animal experimentation (nr 3306013, Préfecture de la Gironde).

Experimental Design

At two months of age, animals from each strain were subdivided into three groups: (1) an alcohol group, which had its beverage replaced with a 15% ethanol solution (v/v); (2) a pair-fed group, which was provided with an isocaloric solution of dextrimaltose and served as a control for the alcohol group; (3) a non-treated control group which was supplied with regular tap water (standard laboratory procedure). Hence, nine groups (3 strains x 3 treatments) were created. This procedure was followed for six months. Liquid consumption and weight gain were similar across groups. After the period of alcohol consumption, mice were put back on a regular tap water regime for five consecutive weeks, as we wanted to avoid testing animals under influence or suffering from withdrawal effects. Animals were first tested in the LD box followed by the first aggression test (neutral-cage paradigm). Subsequently, their spatial learning was determined in the radial maze. Finally, animals were tested in the second aggression test (resident-intruder paradigm). All tests were separated in time by one week. At the beginning of the testing animals were 40 weeks of age.

Light-Dark Box Test

Anxiety was tested in the Light-Dark (LD) box, also known as the Black and White Box or two-compartment activity box. The LD box was first proposed by Crawley and Goodwin [30] and further developed and validated by Costall and colleagues [31, 32] and Misslin and colleagues [33]. The version used in the present experiments was described previously by Guillot et al. [34]. Briefly, it consists of two darkened Plexiglas boxes of the same size (23 x 15 x 15) cm. The light box has a transparent cover and is illuminated by a 100 W desk lamp. Animals can cross from one box into the other through a small hole in the wall. Each mouse was placed in the illuminated box and observed for 5 minutes after the first entry in the dark box. A mouse whose four paws were in the next box was considered as having changed boxes. Behavioral variables were latency to the dark box, percentage of time spent in the light compartment, overall number of transitions between the light and dark box and defecation.

Aggression Test

Aggressive behavior was measured in two tests: the neutral-cage and the resident-intruder paradigm. In both tests a DBA/2J male (supplied by IFFA-CREDO, Lyon, France) of the same age was used as a standard opponent. This strain was used for its low propensity to attack as an intruder [35].

The neutral-cage paradigm has been portrayed at length by Roubertoux et al. [36]. The test took place in a transparent Makrolon cage (42 x 26 x 18 cm) with a transparent lid. The floor was covered with a mix of sawdust from cages of different strains including the tested and DBA/2 animals. This procedure is known to accelerate the appearance of the first attack without affecting the proportion of males exhibiting at least one attack [37]. The experimental animal was placed in the test cage for a 2 minutes habituation period, after which a standard opponent was carefully put in the corner. Recording of the variables started when the experimental animal sniffed the opponent and lasted 6 minutes maximum. The experiment was stopped 2 minutes after the first attack of the experimental animal. The following behavioral variables were measured: latency to the first attack, number of attacks, number of tail rattlings, and number of attacking males.

The rationale of the second aggression test used in this study, the resident-intruder paradigm, has been explained...
MF, and IIPMF) was performed on a Macintosh computer. The central part of the radial maze measured 20 cm in diameter. Its arms (25 cm long, 6 cm high, 6 cm wide) were closed and made of transparent Plexiglas. At the end of each arm was a perforated partition behind which fresh food pellets were deposited. In this way, the presence or absence of a reward could not be smelled by the animals. All arms were reinforced by placing a small food pellet (~10 mg) behind a low barrier preventing the animal from seeing whether a specific arm was still baited or not. The maze was always oriented in space in the same way. Several extra-maze cues were provided close to the arms. A confinement procedure was used to disrupt chaining responses and kinesthetic strategies [38]. The radial maze was placed directly on the floor to avoid possible elevation-induced anxiety.

Animals were habituated for 1 day and subsequently trained for 5 days. The habituation consisted of a 15-minutes exploration trial with free access to all arms but without a food reward. Immediately afterwards they were deprived of food. During the training sessions, animals were kept at 80-90% of their original weight. On the first two days, trials were terminated after the animal had eaten all rewards. From day 3 up to 5, the time limit was set at 30 minutes. The situation of animals not eating all rewards occurred frequently on the first two days, but never on days 3 to 5. For this reason, data from days 1 and 2 were not included in the analysis. Two variables representing learning performances were sampled: the first variable is the number of errors. An error is noted if an animal enters an arm previously visited or does not eat the reward. The second variable is the number of new entries, the number of different arms visited during the first eight arm-visits. The maximum number of new entries is eight. A random choice of entries of the first eight arms leads to an expected mean of 5.3 [39].

Hippocampal Morphometry

Within a week following the radial maze test animals were sacrificed in order to measure the sizes of the hippocampal mossy fiber terminal fields. For a detailed description the reader is referred to [38, 40]. Briefly, animals were deeply anesthetized and perfused intracardially with sodium sulfide and glutaraldehyde. Brains were removed and post-fixed 24 hours in 3 % glutaraldehyde with 20 % sucrose and subsequently cut horizontally in 40 μm cryostat sections after which Timm's silver sulfide staining was applied [41].

Methods used for visualization and measurement of the hippocampal terminal fields were similar to those described previously [38, 42]. Sampling started directly below the most ventral extension of the septal pole of the fascia dentata. Five defined horizontal sections per animal were pseudo-randomly sampled, alternating between the left and right hippocampus, and taking every other section. The analysis of the mossy fiber (MF) terminal fields (CA4, suprapyramidal MF, and IIPMF) was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Macros developed especially for such types of assessments allowed objective and standardized measurements. Sizes of the three mossy fiber terminal fields were expressed as percentages of the total mossy fiber size, limiting possible variations in cutting plane or tissue shrinkage. Results are expressed as percentages of total mossy fibers.

Statistical Analyses

The data of the LD box, aggression tests, and hippocampal morphometry were analyzed using a two-way ANOVA with genotype (or strain) (three levels: B6, CBA and NZB) and treatment (also three levels: alcohol, pair-fed, and control) as between-subject factors. Radial maze data were analyzed using a two-way repeated measures ANOVA, with training days 3 up to 5 as the within-subject factor and genotype and treatment as between-subject factors (both having the same levels as above). The numbers of new entries were tested against chance level (5.3) using a t-test. Least Square Means (LSM) were calculated for all variables.

RESULTS

Light-Dark Box Test

The results are presented in Table 1. Treatment did not affect any of the anxiety-related variables, neither per se nor in interaction with the genetic background. The following measures varied among strains: latency to enter the dark compartment: F2,59=5.21; p<0.01 (B6=NSB=CBA); time spent in the light compartment: F2,59=7.47, p<0.01 (NZB=B6<CBA); crosses: F2,59=8.15, p<0.01 (CBA<NZB=B6).

Aggression Test

Results are presented in Table 2. In both tests strain differences were observed for all variables.

Neutral cage: attack latency: F2,67=5.01; p<0.01 (CBA=NSB<B6); attacks: F2,67=6.60, p<0.01 (B6<NSB=CBA); tail rattles: F2,67=4.00, p<0.05 (B6=CBA, CBA=NSB, B6<NSB).

Resident intruder paradigm: attack latency: F2,67=19.46; p<0.001 (CBA<NSB<B6); attacks: F2,67=9.97 p<0.001 (B6=NSB<CBA); tail rattles: F2,67=8.17, p<0.01 (B6=NSB<CBA). Neither a treatment effect per se nor an interaction between treatment and strain were detected.

Radial-Maze Test

The results of the radial maze test are presented in Fig. (1). Treatment did not affect radial maze learning, neither alone nor in interaction with days or strain. By contrast, strain differences as well as day effects were found for both the number of errors and the new entries (errors: STRAIN: F2,58=3.22, p<0.05, B6>CBA, B6=NSB, CBA=NSB; DAY: F2,116=12.49 p<0.001; new entries: STRAIN: F2,58=11.94, p<0.001, B6=NSB<CBA; DAY: F2,128=9.30, p<0.001). No interactions between day and strain were detected. More detailed analyses showed that on day 4 and 5, CBA made less errors than B6 (both days p<0.01). As for new entries, none of the groups performed above chance level on any given day.
Table 1. Results of the Light-Dark Box Test

<table>
<thead>
<tr>
<th></th>
<th>Latency (Sec ± SEM)</th>
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<td></td>
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<td>n</td>
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<td>C57BL/6J</td>
<td>8</td>
<td>73 ± 18</td>
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<td>70 ± 16</td>
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<td>CBA/H</td>
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<td>8</td>
<td>213 ± 74</td>
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<tr>
<td>NZB/B1NJ</td>
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<td>179 ± 65</td>
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<td>78 ± 29</td>
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**Time in Lit Side (Sec ± SEM)**

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<td>8</td>
<td>28 ± 5</td>
<td>8</td>
<td>16 ± 4</td>
<td>9</td>
<td>36 ± 5</td>
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<tr>
<td>CBA/H</td>
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<td>28 ± 6</td>
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<td>26 ± 6</td>
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<td>17 ± 5</td>
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**Crossings (Total ± SEM)**

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<td>C57BL/6J</td>
<td>8</td>
<td>10 ± 2</td>
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<td>7 ± 1</td>
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<td>9 ± 1</td>
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<td>CBA/H</td>
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<td>5 ± 1</td>
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<tr>
<td>NZB/B1NJ</td>
<td>8</td>
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<td>8</td>
<td>9 ± 2</td>
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<td>6 ± 1</td>
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**Boli (Total ± SEM)**

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<td>0 ± 0</td>
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<td>0 ± 0</td>
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<tr>
<td>CBA/H</td>
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<td>4 ± 1</td>
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Note: Results are expressed as means ± SEM.

Table 2. Results of the Intermale Aggression Tests

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<th></th>
<th>% Attacking Males</th>
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<th>Attack Latency (Sec ± SEM)</th>
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<th>% Attacking Males</th>
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<td>n</td>
<td>Alcohol</td>
<td>n</td>
<td>Control</td>
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<td>360 ± 0</td>
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<td>360 ± 0</td>
<td>9</td>
<td>360 ± 0</td>
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</tr>
<tr>
<td>CBA/H</td>
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<td>10</td>
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<tr>
<td>NZB/B1NJ</td>
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<td>334 ± 17</td>
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<td>312 ± 38</td>
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% Attacking Males

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<tr>
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<td>0</td>
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<td>0</td>
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<td>0</td>
<td>8</td>
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<tr>
<td>CBA/H</td>
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<td>63</td>
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</tr>
</tbody>
</table>

Note: Latencies are expressed in seconds (means ± SEM).
Hippocampal Morphometry

The sizes of the IIPMF terminal fields are shown in Fig. 2 while the other hippocampal variables are displayed in Table 3. Strain differences were observed for all variables: IIPMF: $F_{2,51}=45.34, p<0.001$, B6>CBA>NZB; suprapyramidal MF: $F_{2,51}=12.67, p<0.001$, B6<NZB=CBA; CA4: $F_{2,51}=9.94, p<0.001$, CBA=B6<NZB. No treatment effects were detected.

DISCUSSION

The goal of this study was to investigate the genetic susceptibility to chronic exposure of alcohol on specific neurobehavioral variables in three inbred strains of mice. Unexpectedly, chronic alcohol administration appeared not to influence the sizes of the IIPMF terminal fields nor did it appear to affect radial maze learning, aggression, and anxiety-like behavior. Interactions with the genetic background were not observed either.
Table 3. Sizes of the Suprapyramidal and CA4 Mossy Fiber Terminal Fields

<table>
<thead>
<tr>
<th></th>
<th>Suprapyramidal Layer (% of Total Mossy Fibers Surface)</th>
<th>CA4 (% of Total Mossy Fibers Surface)</th>
</tr>
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<tbody>
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<td></td>
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<td>Control</td>
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<td>C57BL/6J</td>
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<td>37 ± 1</td>
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<td>CBA/H</td>
<td>7</td>
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<td>NZB/B1NJ</td>
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<td>C57BL/6J</td>
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<td>CBA/H</td>
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<tr>
<td>NZB/B1NJ</td>
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<td>55 ± 2</td>
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</table>

Note: Results are expressed as mean percentages of total mossy fibers ± SEM.

The absence of effects of chronic alcohol intake is unlikely to be explained by the design of the experiment. The duration of the alcohol exposure (6 months) as well as the concentration of the alcohol solution used here have been demonstrated to affect behavior as well as brain anatomy in several independent studies [16, 20, 43-48], although some reports suggest that a longer exposure is necessary to have an effect on specific anatomical features of the hippocampus [6]. In addition, it is improbable that the 5-week withdrawal period resulted in recovery of initially present alcohol effects, as previous studies have shown that the loss of pyramidal and granule cells is long-lasting [17, 20]. In fact, withdrawal may worsen the effects of chronic alcohol exposure rather than lead to recovery [5].

One obvious possible explanation for the absence of effects is that the blood ethanol levels in our animals may have been too low to have an effect. However, this is unlikely as mice from our alcohol group were exposed to relatively high alcohol concentrations during a prolonged period of time as their only source of liquid and were drinking similar fluid amounts as both control groups. An alternative explanation for the surprising absence of ethanol effects might be a difference in the species used. Differences between rat and mouse models have been reported before [44, 49] and most studies that reported negative effects of protracted alcohol exposure, such as general learning disabilities and neuronal loss [3, 10, 14, 45-48], were conducted in rats. Mice have been used only rarely, which makes it difficult to generalize and compare. For instance, rats metabolize ethanol almost twice as slow as mice [50], although this does not necessarily lead to higher blood ethanol concentrations [51]. In addition, Béракочéa and collaborators showed that the memory deficits observed in mice after chronic alcohol exposure, are rather specific [52]. As for possible hippocampal damage in mice, Béракочéa himself later stated that "no major changes were observed in the hippocampus" of chronic alcohol consumption exposed mice and that chronic alcohol-induced amnesia "is not due to a dysfunction of the neural networks underlying memory storage processes" [56]. In addition, it is perhaps worth noting that in a previous study [57], we did not find the effects of prenatal exposure to ethanol reported in rats [58]. Hence, together with our current findings, this suggests that, in order to mimic the effects of chronic alcohol exposure in humans, mouse models are either less suitable than rat models (where both neuronal and behavioral effects are observed) or should be approached at a different level [16, 59].

The lack of treatment effects might also be related to the age of testing. At the time they were sacrificed, the animals were 10-11 months of age. Bearing in mind that aging effects are generally observed from 9 months on, possible negative influences of alcohol consumption may have been buffered, or even masked, by aging effects. Indeed, the size of the mossy fiber terminal fields has been shown to diminish over age [60], so a floor effect may have masked any effect of the alcohol treatment. The radial maze data support this explanation, as the absence of treatment effects here is most probably due to a floor effect. Even control animals showed only weak learning: the numbers of errors and new entries changed significantly over training and significant strain differences were observed, but in the end no single group performed significantly better than chance. Comparing our results with previously obtained data from younger animals of these strains, it is evident that learning performances in our controls were drastically lower than in untreated 3-months-old animals [35, 61].

CONCLUSIONS

Summarizing, despite a constant exposure to a 15% alcohol-containing solution as their only beverage for 6 months, male mice from the C57BL/6J, CBA/H and NZB inbred strains did not differ from pair-fed and control groups with respect to the sizes of the hippocampal intra- and infrapyramidal mossy fiber (IIPMF) terminal fields, radial maze learning, aggression, and anxiety-like behavior. Accordingly, our data indicate that, under these experimental conditions and within the limits discussed above, chronic alcohol exposure has no effect on the observed neurobehavioral variables.

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