Biochemical Pharmacology, Vol. 44, No. 7, pp. 1335–1339, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

CHRONIC ETHANOL CONSUMPTION AND WITHDRAWAL AFFECTS MITOCHONDRIAL BENZODIAZEPINE RECEPTORS IN RAT BRAIN AND PERIPHERAL ORGANS

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(*Received* 1 *May* 1992; *accepted* 6 *July* 1992)

Abstract—Ethanol administration to rats for 30 days resulted in a significant decrease (−28%; $P < 0.05$) in the density of mitochondrial benzodiazepine receptors (MBR) in the olfactory bulb. The reduction in [^{3}H]PK 11195 binding persisted 24 hr after cessation of alcohol and had returned to normal values when measured 4 days later. Alterations were confined to this brain region and were not detected in the cerebral cortex, cerebellum or hippocampus. [^{3}H]PK 11195 binding was elevated in the liver (100%; $P < 0.01$), heart (43%; $P < 0.01$) and testis (27%; $P < 0.05$) 30 days following ethanol consumption and this persisted for 1 and 4 days after abrupt withdrawal. A transitory decrease (−20%; $P < 0.05$) in MBR density was observed in the adrenal gland following 30 days of alcohol ingestion, but disappeared during withdrawal. The alterations in these receptors may be relevant to the cellular damage or dysfunction induced by chronic exposure to ethanol.

Chronic use of ethanol is associated with a variety of disorders throughout the body. Virtually every organ system in the body can be affected directly or indirectly by long-term alcohol consumption. Alcohol abuse causes neurological impairment and affects the liver as well as the cardiovascular, endocrine and reproductive systems.

The γ-aminobutyric acid (GABA‡)–benzodiazepine (BZ) receptor–chloride channel complex is activated by alcohol. Like barbiturates and BZs, alcohol stimulates $GABA_A$ receptor-mediated chloride ion uptake in rat brain synaptoneurosomes [1]. The ability to augment membrane chloride conduction has been correlated with the intoxication potencies of different types of alcohol in rats [2]. The partial cross-dependence between ethanol and BZs may indicate a change in BZ receptor sensitivity following repeated ethanol use. However, most studies have shown that chronic ethanol exposure does not affect the density or affinity of the central-type BZ receptor (CBR) [3–5].

Besides the CBR, a peripheral type of BZ receptor has been identified in peripheral tissues as well as in the brain [6–9]. These receptors are localized to the mitochondria and are involved in steroidogenesis in the adrenal gland and testis [10, 11]. Due to their subcellular localization, they have been designated mitochondrial BZ receptors (MBR). MBR are not coupled with the GABA–chloride ion channel and bind with high affinity the ligands [^{3}H]PK 11195 and [^{3}H]Ro 5-4864, but not clonazepam, which has a high affinity for the CBR [6]. In contrast to CBR, brain MBR have been reported to be up-regulated by chronic ethanol administration [3, 5, 12]. The increase in MBR is confined to the brain tissue and is not reflected in the kidney [5] or heart [13]. The relationship of the modulation of brain MBR during ethanol consumption to the development of physical dependence and tolerance is not clear. No changes in brain MBR were observed in rats following a single acute intoxicating dose of ethanol [5] or in mice that were tolerant to but not physically dependent on ethanol [12]. The possible modulatory effect of chronic ethanol ingestion on MBR in peripheral organs other than the heart and kidney has not yet been assessed.

The present study was designed to examine the effect of chronnic (30 days) ethanol ingestion and withdrawal (for 1 and 4 days) on MBR in the cerebral cortex, cerebellum, hippocampus and olfactory bulb, as well as in the heart, liver, testis and adrenal gland. The peripheral organs were chosen in order to reevaluate the effect of ethanol on cardiac MBR and to extend the study to other organs which are greatly affected by alcoholism.

MATERIALS AND METHODS

Materials. [^{3}H]PK 11195 (74.5 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Unlabeled Ro 5-4864 was kindly supplied by Drs H. Gutman and E. Kyburz (Hoffman-La Roche, Basel, Switzerland). Lumax was obtained from Lumac (Schaesberg, The Netherlands). Absolute ethanol was purchased from Riedel-De Haen (Seelze, Germany). All other compounds were purchased from commercial sources.

Procedure. Thirty male Sprague–Dawley rats (200–250 g) were housed in air-conditioned quarters

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‡ Abbreviations: GABA, γ-aminobutyric acid; BZ, benzodiazepine; CBR, central benzodiazepine receptor(s); MBR, mitochondrial benzodiazepine receptor(s).

with a 12-hr light/dark schedule. Standard rat pellets were provided *ad lib.*

Chronic ethanol ingestion was experimentally induced as described by Simler *et al.* [14]. Increasing concentrations of ethanol solution were administered to all the ethanol-treated rats (N = 21), beginning with 5% (v/v) ethanol for 3 days and followed by 10% (v/v) ethanol for the next 3 days, and maintained thereafter at 15% (v/v) ethanol for an additional 24 days. Liquids were supplied and measured daily. At the end of the spontaneous chronic ethanol consumption period, ordinary tap water was provided as the sole drinking source for 24 hr or 4 days in the groups subjected to abrupt ethanol withdrawal for 1 day (N = 7) or 4 days (N = 7), respectively. Water was provided to the control group (N = 9) *ad lib.* for 30 days and was measured daily. All rats were killed by decapitation. The kidneys, testes, liver, heart, adrenal gland, cerebral cortex, olfactory bulbs, cerebellum, hippocampus, hypophysis and hypothalamus were removed and stored at −70° until assayed. The wet weight of all the organs was similar in all groups.

Membrane preparation. Prior to binding assay, tissues were homogenized in 50 vol. of 50 mM Tris–HCl buffer, pH 7.4, at 4° using a Brinkmann Polytron (setting 10) for 15 sec and centrifuged at 49,000 *g* for 15 min. The pellet was homogenized in the same buffer to a final concentration of about 250–625 μg protein/mL and used for binding assay.

[^{3}H]*PK* 11195 *binding assay.* [^{3}H]PK 11195 binding was assayed in 50 mM Tris–HCl buffer, pH 7.4, in a final volume of 500 μL containing 400 μL membranes (100–240 μg protein) and 25 μL [^{3}H]PK 11195 (0.2–6 nM final concentration) in the absence (total binding) or presence (non-specific binding) of 75 μL unlabeled Ro 5-4864 (1 μM final concentration). After incubation for 60 min at 4°, samples were filtered under vacuum over Whatman GF/B filters and washed three times with 5 mL of 50 mM ice-cold Tris–HCl buffer. Filters were placed in vials containing 5 mL of xylene–Lumax (3 : 1) and counted for radioactivity after 12 hr. Protein content was determined according to the method of Lowry *et al.* [15], using bovine serum albumin as standard.

Statistical analysis. Equilibrium dissociation constant (K_d) and maximal number of binding sites (B_{max}) were determined by Scatchard analysis of saturation curves of [^{3}H]PK 11195, using the EBDA (Equilibrium Binding Data Analysis)/LIGAND program [16] (Packard Instrument Co., Meriden, CT, U.S.A.). The binding parameters were analysed for each animal individually. One-way variance (ANOVA) was used for intergroup comparisons. The results are expressed as means ± SEM.

RESULTS

The ethanol-treated rats drank smaller (25–42%) quantities of fluids than the control group. Increased water consumption was observed during the first day of withdrawal and returned to control values 4 days later. The weights of the peripheral organs and the brain regions examined did not differ among ethanol-treated, withdrawal (for 1 and 4 days) and control rats.

The effects of chronic ethanol administration and withdrawal (for 1 and 4 days) on MBR in peripheral organs and brain are depicted in Figs 1 and 2. Ethanol administration resulted in a significant increase in B_{max} values of [^{3}H]PK 11195 in the liver (100%; $P < 0.001$), heart (43%; $P < 0.01$) and testis (27%; $P < 0.05$), but significantly lowered MBR density in the adrenal gland (−20%; $P < 0.05$). The increase in B_{max} values observed in the liver, heart and testis persisted when measured 1 and 4 days following the abrupt withdrawal of ethanol administration. In the adrenal gland the decrease was transitory, and MBR returned to normal values 24 hr after cessation of ethanol ingestion (Fig. 1).

Diminished [^{3}H]PK 11195 binding was detected in the olfactory bulb of the ethanol-treated rats (−28%; $P < 0.05$), as well as 1 day after withdrawal (−29%; $P < 0.05$). The maximal binding capacity returned to normal values within 4 days of withdrawal (Fig. 2). The alterations in B_{max} values observed in the peripheral organs and brain were not accompanied by changes in K_d values (peripheral organs, 0.5–2.4 nM; brain, 0.5–1.4 nM). Ethanol consumption and withdrawal did not affect [^{3}H]PK 11195 binding values in the cerebral cortex, hippocampus or cerebellum.

DISCUSSION

The most striking finding in the present study was the sensitivity of MBR to chronic ethanol exposure in the peripheral organs examined. The elevation of MBR in the liver, heart and testis in ethanol-treated and withdrawn rats can be linked to the long-lasting effects of chronic ethanol exposure on the liver: fatty liver, hepatic fibrosis, alcoholic hepatitis and cirrhosis [17]; the heart: cardiomyopathy and reduced cardiac contractility [18, 19]; and the testis: gonadal dysfunction, testicular atrophy and reduced testosterone levels [20, 21]. It is noteworthy that chronic ethanol abuse damages the Leydig cells of the testis [21], which are especially rich in MBR [8, 13, 22]. The increase in testicular MBR can be related to the relatively short period of ethanol ingestion, or to a compensatory mechanism to preserve gonadal functionality. Guaza *et al.* [22] observed that acute alcohol administration to rats stimulates corticosterone release, while long-term alcohol administration does not alter corticosterone secretion. It seems that repeated ethanol treatment induces hyposensitivity of the adrenal gland to the stimulatory effect of alcohol on corticosterone secretion. This hyposensitivity may be reflected by the transitory down-regulation of adrenal MBR.

Another possibility is that alcohol administration is associated with stress. Acute ethanol ingestion leads to stimulation of the hypothalamic–pituitary–adrenocortical axis as well as of the sympathetic nervous system. The repeated stress associated with chronic alcohol ingestion may be responsible for the down-regulation of the adrenal MBR, as demonstrated previously in platelets of chronically anxious patients [23]. Alternatively, it is possible that stress from dehydration may be reflected by a transitory down-regulation of adrenal MBR. The

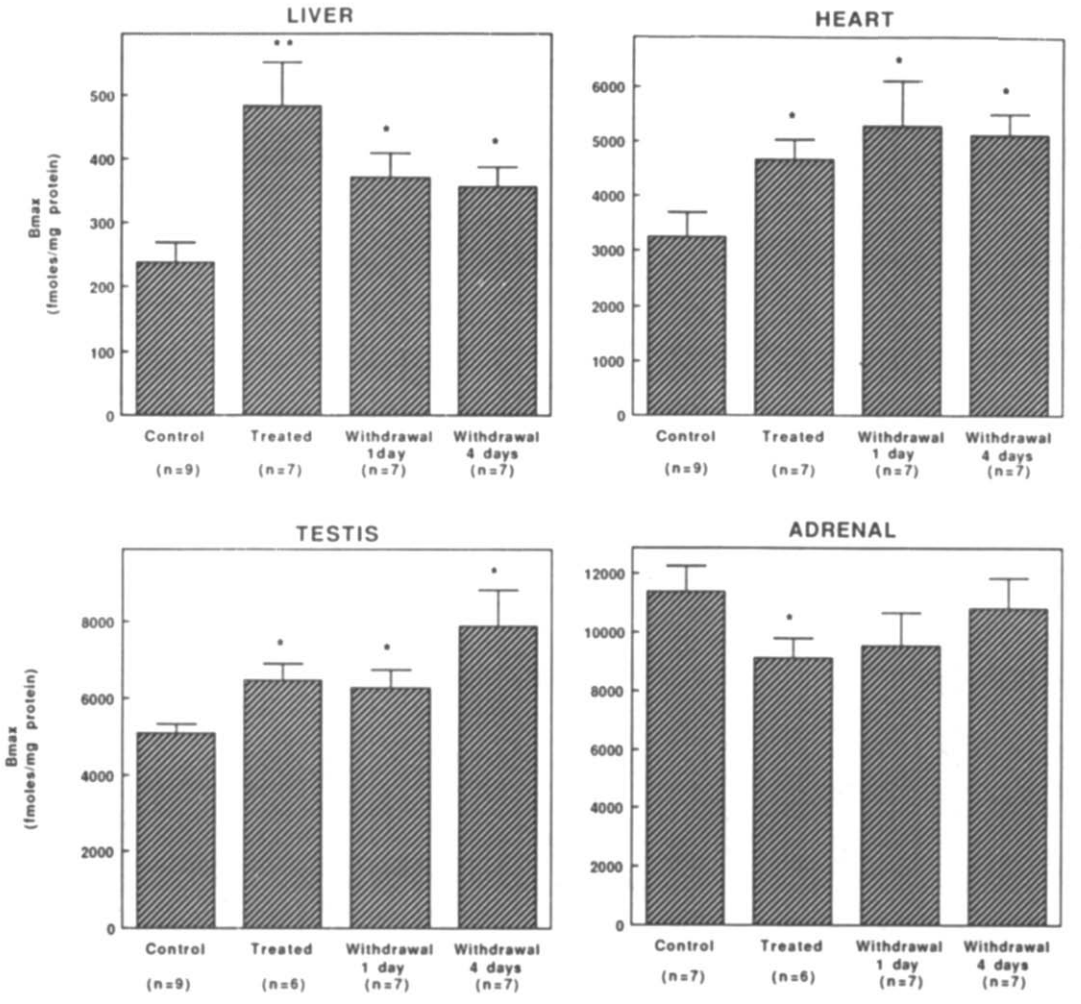

Fig. 1. Effect of chronic ethanol ingestion (30 days) and withdrawal (1 and 4 days) on the maximal binding capacity of MBR in the liver, heart, testis and adrenal gland of male Sprague–Dawley rats. Binding of [^{3}H]PK 11195 was assayed at six concentrations (0.2–6 nM final concentration) in the absence (total binding) or presence (non-specific binding) of unlabeled Ro 5-4864 (1 μM final concentration). Details are described in Materials and Methods. The results of each animal were analysed individually. Results are expressed as means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, when compared with control using one-way ANOVA.

role that water-deprivation stress plays in the ethanol-induced modulation of MBR in peripheral organs is not clear, since, in the present study, an opposite direction was detected in the adrenal gland (a decrease) in comparison to the other three organs (liver, heart and testis: an elevation).

Subcellular localization studies revealed the selective association of MBR with the mitochondrial outer membrane [24, 25]. Chronic ethanol consumption affects mitochondrial function in the heart [17, 26] and liver [27]. The mitochondrial dysfunction in the peripheral organs may be relevant to the modulation of MBR density.

Thirty days of continuous ethanol exposure induced a down-regulation of [^{3}H]PK 11195 binding sites in the olfactory bulb. The reduction persisted for 24 hr, but was not detectable after 4 days of withdrawal. The cerebral cortex, cerebellum and hippocampus were unaffected. Our results are in discordance with a previous study that reported an increase in MBR in the cerebral cortex, cerebellum and hippocampus, but not in the olfactory bulb, in rats following 4 days of ethanol administration [5]. This discrepancy may be attributable to the difference in the mode of ethanol administration, i.e. oral intubation of ethanol solution vs spontaneous oral ingestion in the present study. The difference in the duration of alcohol use (4 vs 30 days) may also explain the divergent results. It should be noted that a subsequent study demonstrated an up-regulatory effect of ethanol in whole brain as well as in the forebrain of mice, but not in the heart and kidney [12]. Alcohol had been adminstered by a liquid diet for 9 days. Again, the conflicting results may be due to differences in the manner and duration of ethanol administration and species differences (mice vs rats). Furthermore, we studied the effect of chronic exposure to ethanol *per se* on MBR

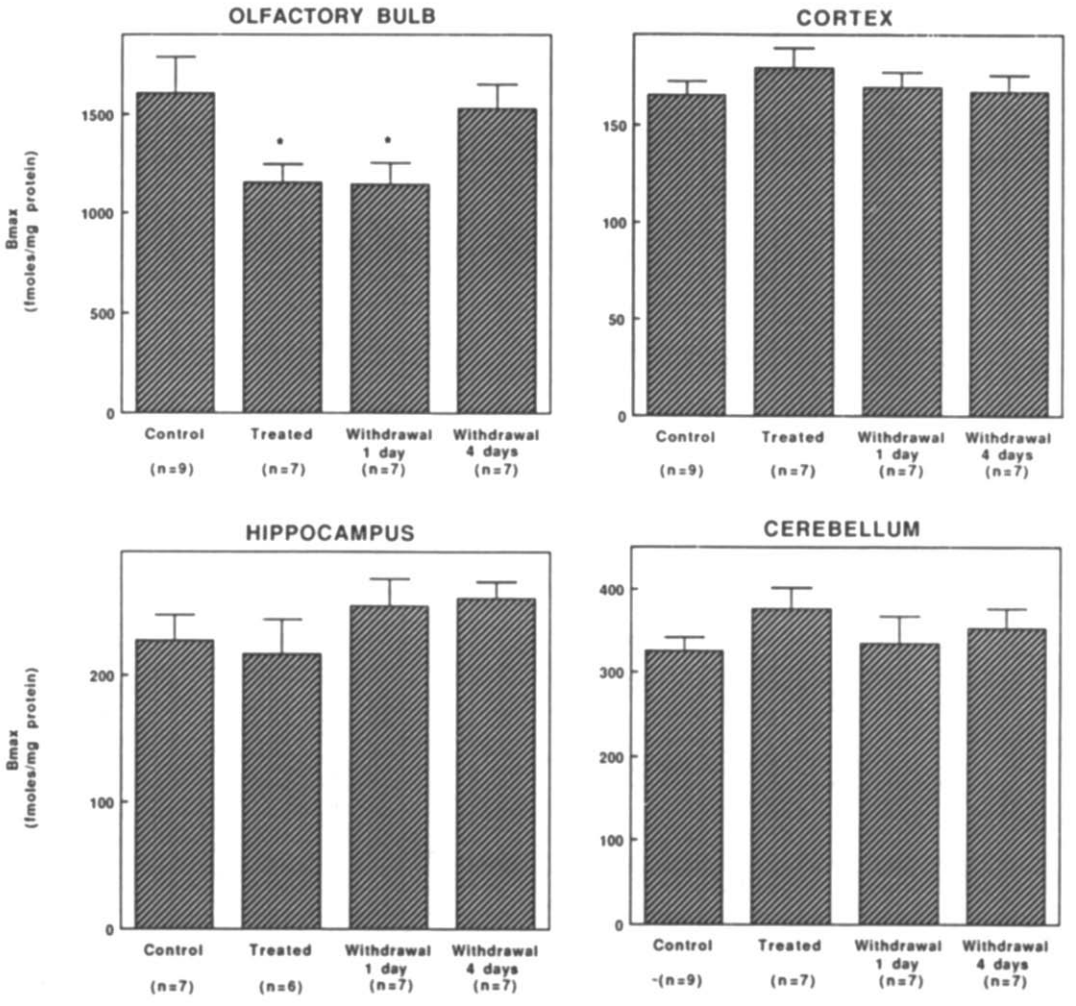

Fig. 2. Effect of chronic ethanol exposure (30 days) and withdrawal (1 and 4 days) on [^{3}H]PK 11195 maximal binding capacity in the olfactory bulb, cerebral cortex, hippocampus and cerebellum of male Sprague–Dawley rats. The binding of [^{3}H]PK 11195 was assayed as described in the legend to Fig. 1. The binding parameters for each animal were analysed individually. Results are expressed as means ± SEM. *$P < 0.05$ when compared with control using one-way ANOVA.

expression in various tissues, while the two previous studies investigated the effect of ethanol physical dependence [5, 12]. Unfortunately (because of technical problems), blood ethanol concentrations were not determined during the present study. The differences between our results and those of previous studies could also be explained by a large variation in ethanol intake between animals. It is noteworthy that the changes in MBR in brain were confined to the olfactory bulb, which is the brain region with the highest density of MBR; however, it is not clear why the sites in this brain region are more sensitive to alcohol exposure.

In summary, this study indicates that chronic ethanol ingestion is associated with alterations in MBR in the olfactory bulb as well as in the liver, heart, testis and adrenal gland. These results may be relevant to the pharmacological effects of chronic ethanol exposure. The time course and dose dependency of the effects of ethanol on MBR merit further investigation.

Acknowledgements—This work was supported in part by Grant 26-90 (to M.G.) from the Israel Institute for Psychobiology—the Charles E. Smith Family Foundation. We thank Ruth Singer for typing and editing the manuscript. This study is submitted in partial fulfillment of the requirements for the D.Sc. degree of M. Bidder at the Technion-Israel Institute of Technology.

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