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EFFECTS OF ACUTE AND CHRONIC ETHANOL INTAKE ON SYNAPTOSOMAL GLUTAMATE BINDING ACTIVITY*

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Abstract—The effects of acute and chronic ethanol administration *in vivo*, on brain synaptosomal glutamate binding activity were explored. In the L-glutamic acid concentration range in which high affinity stereospecific binding to synaptic membranes takes place, both acute and chronic ethanol administration led to progressively greater glutamate binding depending on the chronicity of exposure to ethanol (2 hr to 16 days). These changes appeared to be primarily due to an increase in the maximum binding capacity of these membrane binding sites rather than to changes in their affinity towards the ligand. The withdrawal from ethanol following 16 days of continuous exposure brought about a slow reversal of the increased glutamate-binding activity over a period of 6 days. A brief (2 hr) exposure to ethanol *in vitro*, produced a small decrease in glutamate binding, whereas prolonged exposure to the alcohol during equilibrium dialysis had a biphasic effect on ligand binding to synaptosomal membranes. These results are suggestive of a possible role for L-glutamic acid in the nervous system during ethanolism and the post-withdrawal reaction.

The mechanisms by which physical dependence on alcohol develops as a result of chronic ethanol intake are still unknown, as are the relationships of the physiological adaptation of ethanol dependence to the withdrawal signs seen upon discontinuation of alcohol intake. It has been proposed that the primary, and probably most important, site of ethanol activity might be on the cell membranes, expecially the neuronal cell membranes [1, 2]. Some of the membrane-related effects of ethanol include its interaction with hydrophobic regions of the membrane proteins that may alter the activity of membrane-attached enzymes such as Na^+–K^+–ATPase and the neuronal adenylate cyclases [2–6]. Additionally, the penetration of ethanol into the membrane matrix probably affects the conformation or activity of proteins or lipoproteins that control ionic translocations across the membrane or the uptake of some neurotransmitters [7, 5]. Such ethanol-induced protein changes may be directly or indirectly related to changes in neuronal excitability and the capacity of stimulated neurons to release sufficient amounts of neurotransmitters as has been demonstrated for a variety of transmitter substances in both *in vivo* and *in vitro* preparations [8–14].

The possibility that one of these neurotransmitter systems may be involved in the hyperexcitable state associated with ethanol withdrawal was examined by Goldstein [15] who analyzed pharmacologically the role that some neurotransmitters play in the ethanol withdrawal syndrome in mice. She concluded that during withdrawal γ-aminobutyric acid (GABA) and the catecholamines can act to inhibit the "overactivity of an endogenous brain excitatory system of unknown nature, apparently not cholinergic". We [16] have recently used the same paradigm as Goldstein to gather pharmacologic evidence which points to a probable hyperactivity of the glutamate excitatory system in the central nervous system (CNS) during chronic ethanol intoxication. This hyperactivity appears to have some characteristics of a glutamate-receptor supersensitivity. In order to explore this possibility further, we have examined the effects of *in vitro* and *in vivo* ethanol administration, both acute and chronic, on the capacity of brain crude synaptosomal membrane preparations to bind glutamate. We have previously shown that such membrane preparations contain Na^+-independent glutamate binding sites that have the expected characteristics of the physiologic glutamate receptor [17, 18]. The present study reveals that ethanol intake causes a time-dependent increase in glutamate binding interactions with the synaptosomal membranes in chronically ethanol-exposed animals.

MATERIALS AND METHODS

Animals and diets. Adult male Sprague–Dawley rats were fed laboratory chow until the start of the experiment when they reached a body wt of 250–300 g. They were then pair-fed a diet consisting of 71 per cent commercial Slender, 6% (w/v) ethanol for the experimental animal or an isocaloric amount of sucrose for the control animal of the pair, and different volumes of distilled water to bring the diets to their final volume. This diet is similar to that which has previously been employed to induce chronic ethanolism in rodents [6, 19]. Average daily consumption of this diet by each animal was 70–90 ml, which corresponds to a daily ethanol intake of 4.2–5.4 g. Animals employed for the determination of changes following ethanol withdrawal were maintained on the ethanol or control diet for 16 days and

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then were changed to a laboratory chow and *ad libitum* water intake for 24, 72, or 144 hr prior to sacrifice. The rats used for the acute administration experiments were maintained on laboratory chow until the day they were pair-treated with either an intraperitoneal (i.p) injection of ethanol (4 g/kg) or an injection of isocaloric sucrose solution 2 hr prior to sacrifice.

Differential centrifugation and glutamate binding assays. Each rat was rapidly decapitated and the brain was extirpated, weighed, and placed in ice-cold 0.32 M sucrose solution (10 volumes per g brain wet wt). After homogenization of the minced brain tissue in a glass homogenizer with a Teflon pestle, the homogenates were centrifuged at 1000 *g* for 10 min to remove the nuclei and cell debris material, and the supernatant was centrifuged at 17,000 *g* for 10 min to obtain the crude mitochondrial fraction. The supernatant from this step was carefully decanted and the white membrane "halo" of the pellet resuspended in 3.0 ml of 50 mM Tris–Tricene* buffer, pH 7.5. Care was taken to avoid resuspending the brown mitochondrial center of the pellet. The membrane subfractions that precipitate in the "crude mitochondrial" fraction have previously been shown to include neuronal endings (synaptosomes, approximately 40 per cent of particulate), myelin fragments, and a mixed population of glial and neuronal plasma membranes [20, 21].

The glutamate binding assays were conducted as previously described employing equilibrium dialysis [17]. The resuspended "crude synaptosomal" membrane fraction was further diluted to a final protein concentration of 0.2 mg/ml with 50 mM Tris–Tricene buffer that contained D-glutamate (final D-glutamate concentration was 2 mM). The presence of D-glutamate has been found to diminish the non-specific interactions of L-glutamic acid [17]. Binding of L-[^{3}H]-glutamate (0.1 μCi per assay tube), isotopically diluted to the desired concentration with unlabeled L-glutamate, was measured by allowing overnight equilibration at 4°C of a 0.1 ml aliquot of the membrane suspension inside the dialysis sac against 5 ml of 50 mM Tris–Tricene buffer which contained the ligand. The time allowed for equilibration was based on preliminary determinations which showed that the amount bound did not change between 18–48 hr. In addition, there appeared to be no metabolic conversion of L-[^{3}H]-glutamate during the equilibration period [17]. When the *in vitro* effects of ethanol on glutamate binding were being investigated, the appropriate amount of ethanol was added into the buffer that was employed in the external solution of the dialysis system and equilibrium was allowed to develop in its presence. Protein was measured according to Lowry [22].

At the end of the equilibration period, 0.1 ml samples from inside and outside the dialysis sac were removed and the radioactivity was determined by liquid scintillation spectrometry in 10 ml of a Triton X-100–Toluene (1:2) scintillation fluid mixture. The efficiency of tritium counting for each sample was determined and the radioactivity was expressed as disintegrations per min. Average efficiency was 50 per cent.

Mathematical and statistical analysis. All data were computer analyzed to determine the amount of L-glutamate bound, the amount bound per mg protein, and the concentration of the free ligand. Results are presented as means ±S.E. of the mean. Significance levels were determined by the two-tailed Mann–Whitney U test. Values of P less than 0.05 were considered as significant.

Materials. L-[G-^{3}H]-glutamic acid (50 Ci/m-mole) was purchased from New England Nuclear Corp., Boston, MA. L-glutamate, D-glutamate, Tris, and Tricene were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals were of reagent grade.

RESULTS

Effect of acute ethanol administration on synaptosomal glutamate binding. The acute administration of ethanol (4 g/kg) produced some small changes in glutamate binding activity of the crude synaptosomes from the treated animals as compared to their paired controls. This brief 2 hr exposure to ethanol was sufficient to bring about small to moderate increases in glutamate binding in the range of glutamate concentrations that have been found to represent the high-affinity binding interaction of this putative neurotransmitter with the synaptic membranes [17, 18]. The increased binding activity in the ethanol-treated animals was significantly different only at a glutamate concentration of 0.4 μM. This rather limited increase in glutamate binding may represent either a direct effect of ethanol on the membranes if it is still associated with them at the time of the assay or it may represent the beginnings of a cellular response that is expressed as a change in membrane binding activity. The first possibility

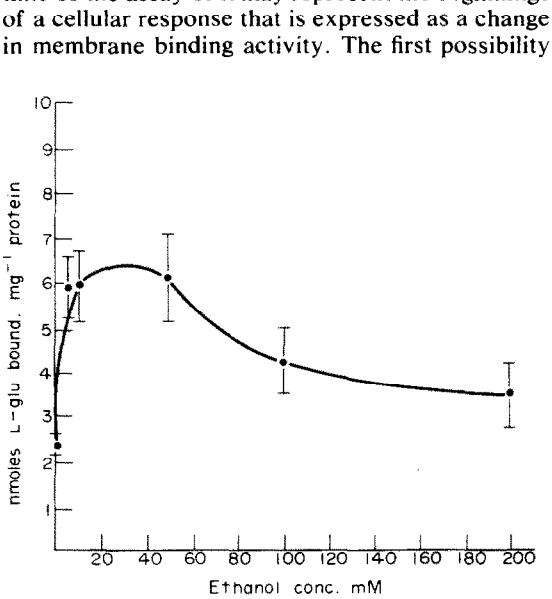

Fig. 1. Effects of ethanol added *in vitro* to the equilibrium dialysis medium on L-glutamate binding to the synaptosomal membranes. Ethanol was added at variable concentrations to the dialysis medium and glutamate binding was measured in its presence or absence as described in Methods. Membrane preparations used were from control, untreated animals [4]. Each point is the mean of 6–8 determinations (± S.E.).

* Tris = Tris-(hydroxymethyl) aminomethane; Tricene: (*N*-Tris [Hydroxymethyl] methyl Glycine).

was explored in studies designed to evaluate the *in vitro* effects of ethanol on synaptosomal glutamate binding.

In vitro *effects of ethanol on glutamate binding.* The presence of ethanol at variable concentrations in the buffer medium employed for equilibrium dialysis produced a biphasic change in glutamate binding activity of synaptosomes prepared from normal, untreated animals (Fig. 1). Ethanol concentrations of 5–50 mM caused a large increase in glutamic acid (0.4 μM) binding to the membranes, whereas ethanol concentrations of 50–200 mM resulted in a gradual decrease in L-glutamate binding activity towards the baseline levels.

On the basis of published rates of elimination and of equilibrium distribution of ethanol from blood into brain tissue, it can be calculated that the brain alcohol levels 2 hr after the acute injection of ethanol employed in methods would be approximately 55 mM. When ethanol at that concentration was added into the medium used in the equilibrium dialysis binding assay, it caused a substantial increase in glutamate binding activity (Fig. 1). However, these results may not be truly representative of the effects of an acute ethanol injection since the brain tissue from these animals has undergone a 1:10 dilution during homogenization, and the membrane pellet has been resuspended in an even larger volume of hypotonic buffer medium (1:30 dilution as an average), and no ethanol was added in the equilibrium dialysis medium.

In an effort to examine further the possibility that the mere exposure for 2 hr of brain neuronal membranes to high ethanol concentrations, even in an *in vitro* system, could lead to the same small increases in glutamate binding as those observed for the treated animals, a synaptosomal membrane preparation from a non-ethanol exposed animal was allowed to stand with 50 mM ethanol for ½ hr at room temperature and for 1-½ hr at 4°. After precipitation of these membranes and resuspension in 50 mM Tris–Tricene buffer, the binding activity of the ethanol-exposed membranes at 0.4 μM L-glutamate concentration was slightly less than that of a similarly processed membrane aliquot that had received only an equal volume of the Tris–Tricene buffer (glutamate binding activity: alcohol-exposed membranes 2.68 ± 0.69 nmoles · mg^{-1} protein; control membranes 3.56 ± 1.27 nmoles · mg^{-1} protein). These observations would suggest that exposure of synaptosomal membranes *in vitro* for a brief period rather than for the duration of the equilibrium dialysis assay does not lead to an increase in glutamate binding but rather to a small decrease in such binding interactions between glutamate and the synaptosomal membranes.

Chronic ethanol intake and glutamate binding activity. The small increases in glutamate binding observed after an acute injection of ethanol may represent a functional change in membrane interaction with this neuroexcitatory amino acid. This type of change might then become increasingly apparent under a more prolonged exposure to ethanol. Chronic ethanol intake for 8 and 16 days did lead to higher glutamate binding interaction in the ethanol-treated animals as compared to their pair-fed con-

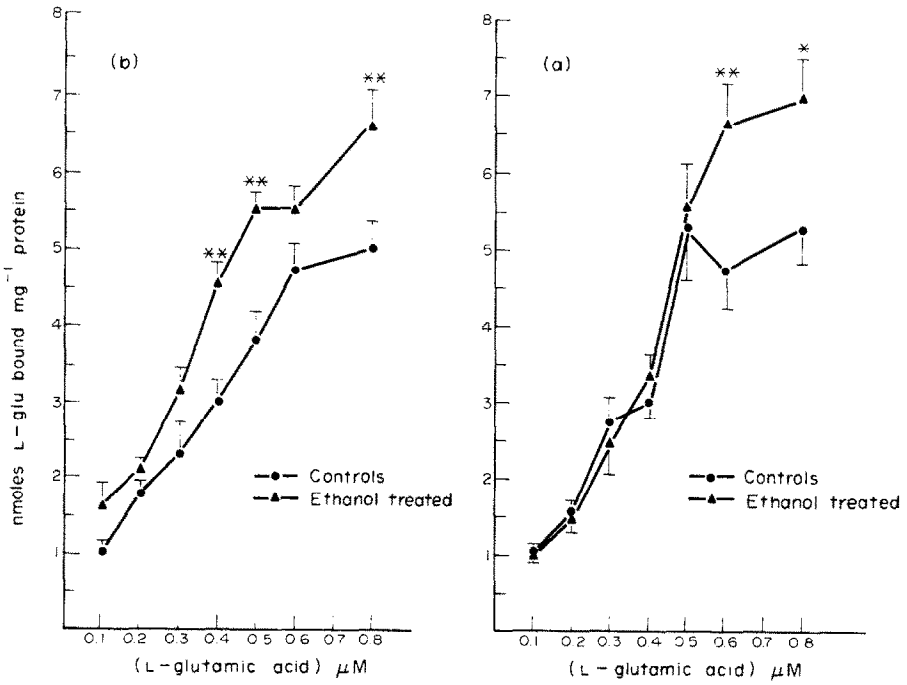

Fig. 2. Effects of chronic ethanol treatment on L-glutamate binding to brain synaptosomal membranes. Glutamate binding was measured in synaptosomal membranes from rats that had been ingesting ethanol for 8 days (panel a) and their pair-fed controls, or in animals that took ethanol for 16 days (panel b) and their pair-fed controls. Each point is the mean of 10–18 determinations for the 8 day treated and control animals and 8–12 determinations for the 16 day treated controls. * indicates significant differences at $P < 0.05$, a ** indicates significant differences at $P < 0.01$ level.

trols (Fig. 2). These changes in glutamate binding activity are more apparent in the L-glutamic acid concentration range of 0.5–0.8 μM and they are greater for the animals that have undergone 16 days of treatment with alcohol. For the latter group of animals the glutamate binding activity is enhanced at all concentrations of L-glutamate in the range of 0.1–0.8 μM. Because of the marked positive cooperativity of glutamate binding in the concentration range of 0.3–0.8 μM (Hill coefficients varied from 2.39–4.88 for the various groups), it was difficult to determine accurately the dissociation constants (K_d) for these binding interactions. From Scatchard plot analyses [23], the estimated K_d for the high affinity binding appeared to be quite similar for the treated and the control groups (0.23–0.38 μM). Thus, the differences seemed to be confined primarily to a higher maximum amount of bound glutamate for the set of high affinity binding sites in the synaptosomal membrane preparations. Chronic ethanol treatment also seemed to produce changes in the Hill coefficient of binding in the ligand concentration range of 0.3–0.8 μM, e.g. from a Hill coefficient of 2.39 for the 16 day exposure controls to a coefficient of 3.50 for the 16 day ethanol treated animals. There was no difference between the sensitivity of membranes from alcohol-treated rats and those from controls in the inhibition of L-glutamic acid binding produced by the physiologic glutamate antagonist glutamate diethyl ester [17, 18].

Effect of ethanol withdrawal on glutamate binding. Since chronic alcohol intake produced increases in glutamate binding it was of interest to determine how soon after alcohol withdrawal such changes reverted to a normal level of binding activity. Animals that consumed ethanol for 16 days continuously were allowed to undergo withdrawal for 3 days prior to being sacrificed. The differences in binding activity had been partially reversed in the ligand concentration range of 0.1–0.8 μM (Fig. 3), but at the higher L-glutamate concentrations (0.8–1.2 μM), the binding interaction in the animals undergoing withdrawal was considerably higher than that of their paired controls (Fig. 3).

The partial reversal of the increased L-glutamate binding activity of the synaptosomal membranes following 3 days of withdrawal from chronic ethanol intake was suggestive of a gradual process of return to a basal state of these membranes. This time-dependent membrane change was followed through an examination of the glutamate binding activity of synaptosomal membranes from animals that had undergone withdrawal from ethanol for 1 day and for 6 days (Fig. 4). As can be seen in Fig. 4 the pattern of glutamate binding interactions for the animals that were in the first day of withdrawal from ethanol is very similar to that seen with the rats treated for 16 days with ethanol and not allowed to undergo any withdrawal (Fig. 2). On the other hand, the synaptic membranes from animals that had undergone 6 days of withdrawal showed only small differences from the control membranes (Fig. 4). These findings are indicative of a gradual return of the high affinity glutamate binding activity of synaptic membranes toward a normal state over a period of 6 days. However, despite the partial reversal of the synaptic membrane binding capacity towards baseline levels after 3 days of withdrawal, the remaining increased interaction between the ligand and its membrane receptors appeared to be once again due to some intrinsic changes in the membrane binding activity of the treated animals. This was shown by measuring the changes in glutamate binding at 0.1–0.6 μM concentrations brought

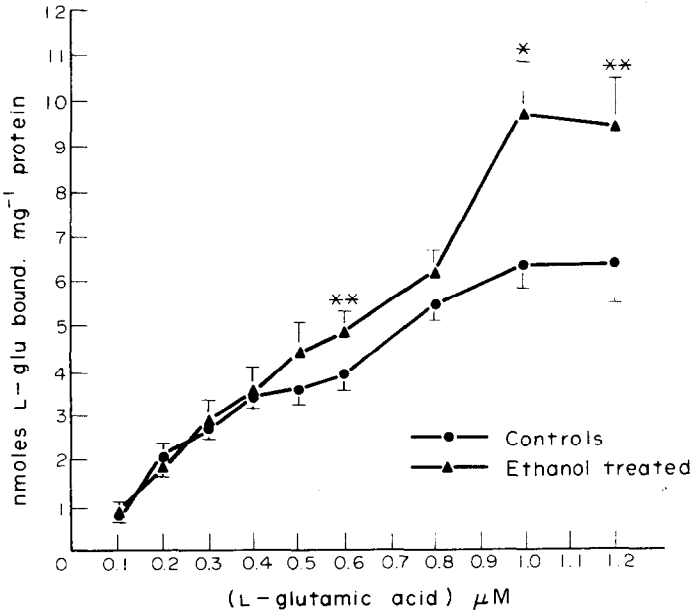

Fig. 3. Effects of 16 day ethanol exposure and 3 days of withdrawal from ethanol on the brain synaptosomal glutamate binding activity. The ethanol-exposed animals were allowed to undergo withdrawal from alcohol for 3 days prior to sacrifice as described in Methods (six animals in each group). Each point is the mean of 10–12 determinations. All other symbols are the same as in Fig. 2.

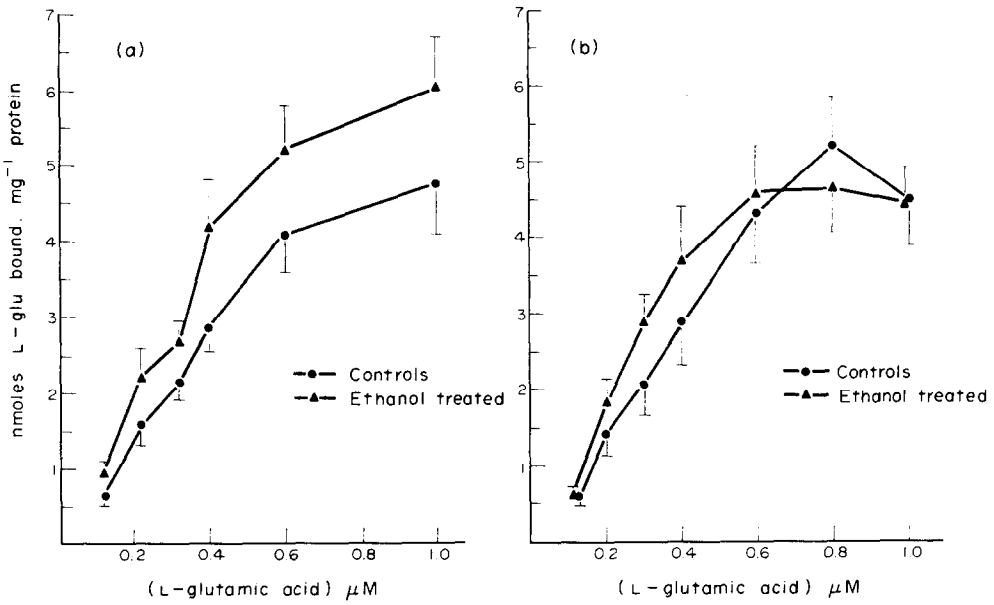

Fig. 4. Comparative effects of 1 day (panel a) and 6 days (panel b) of withdrawal following 16 days of continuous ethanol ingestion on brain synaptosomal glutamate binding activity. Each point is the mean (±S.E.) of 6–8 determinations from four animals in each group of ethanol-exposed animals or their paired controls.

about by the addition of 25 mM ethanol in the equilibrium dialysis medium of tissue from both the control and the alcohol-treated animals. As is shown in Fig. 5 the binding activity of the control tissues was increased by this *in vitro* addition of ethanol, but the ligand-interaction capacity of the synaptosomal membranes from animals in the post-ethanol withdrawal phase increased to an even higher level than that of the controls in the presence of 25 mM ethanol. This may be indicating an intrinsic shift in the activity of the glutamate binding sites as a result of the chronic ethanol exposure.

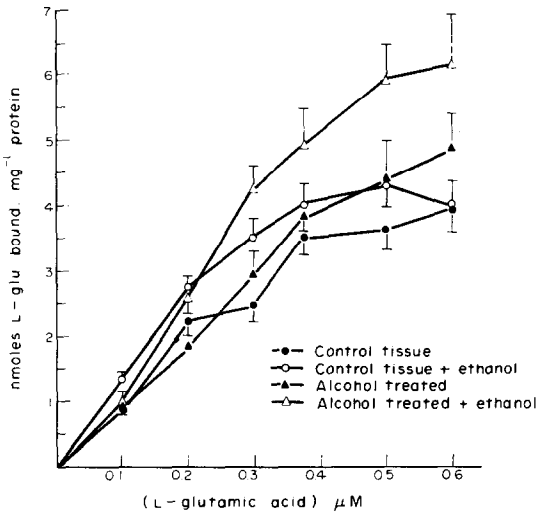

Fig. 5. Comparative effects of the *in vitro* exposure to ethanol of synaptosomal membranes from ethanol-treated and withdrawn animals and their controls. Tissues from four of the six animals of each type shown in Fig. 3 were used in these studies. Ethanol was added to the equilibrium dialysis medium at a final concentration of 25 mM. Each point for the binding of L-glutamate in the presence of ethanol in the medium is the mean of eight determinations. The values for the control tissues, in the absence of 25 mM ethanol and for the tissues from the ethanol-exposed and withdrawn animals in the absence of added ethanol are the same as those in Fig. 3.

DISCUSSION

The effects of ethanol on glutamate binding observed in these experiments once again are suggestive of the activity of ethanol on biological membranes. Our previous studies have demonstrated that the brain synaptic membranes are the subcellular fraction most enriched in the glutamate binding glycoprotein whose characteristics resemble those of the glutamate receptor [17, 18]. The activity of these neuronal membrane proteins is apparently affected by exposure to ethanol, especially under chronic conditions. The increase in glutamate binding as a result of chronic ethanol treatment shown in this study may represent a neuronal adaptation to the effects of ethanol on CNS excitability states. Since high ethanol concentrations produce generalized depression of CNS function, it is possible that the type of adaptation that the CNS neurons make in response to the depressant effects of ethanol is to increase their ability to respond to excitatory stimuli [24, 25]. This could be accomplished by an alteration in the degree of neurotransmitter–receptor interactions for a putative excitatory transmitter such as glutamate. Such a CNS adaptation could be the basis of the phenomenon of physical and behavioural tolerance and may help explain the post-withdrawal CNS hyperexcitability. The hypothesis of neurotransmitter receptor involvement in the development of physical tolerance and dependency was first advanced by Collier [26] and has recently received experi-

mental support through an analysis of the effects of chronic narcotic-analgesic administration [27–29].

The observation that the increased glutamate-binding activity of the neuronal membranes persisted for 3 days after withdrawal from chronic ethanol administration indicates that the membrane protein changes induced by chronic ethanol intake are stable in nature. This type of altered state of the membrane proteins could be the result of either the incorporation of more binding protein units into the membrane matrix under the influence of chronic ethanol exposure, or otherwise they might be the result of unmasking of receptor sites. The latter possibility is consistent with the observations that chronic ethanol intake causes increases in the exposure of 5,5′-dithiobis-(2-nitrobenzoic acid) fast reacting sulfhydryl groups of brain microsomes without inducing an increase in the total number of such sulfhydryl groups in these membranes [30]. Ethanol treatment could thus allow for the appearance of time-dependent and stable conformational changes of membrane proteins, one of which may lead to increased glutamate binding to the synaptosomal.

The *in vitro* effects of ethanol on glutamate binding to the synaptosomal preparations resemble the biphasic effects of Ca ion concentration changes on glutamate binding activity that we have observed [17]. Ethanol at concentrations of 20 mM and greater enhances Ca binding to erythrocyte ghost membranes [31]. Ross and colleagues [32, 33] have recently shown that the acute administration of small amounts of ethanol *in vivo* causes synaptosomal depletion of bound Ca, whereas chronic ethanol treatment brings about increases in membrane-bound Ca. Recent observations in our laboratory (unpublished observations) however, have shown that ethanol exposure of the synaptic membranes *in vitro* brings about increases in Ca binding as measured by increased fluorescence of the divalent cation chelator probe chlortetracycline [34]. These ethanol-induced increases in Ca binding were observed with 5 mM ethanol as the half maximal effective concentration of the alcohol. Thus, it is conceivable that some of ethanol's *in vitro* effects on the glutamate binding could be the result of Ca ion mobilization in the synaptic membranes.

Finally, it is of interest to note that chronic exposure *in vivo* of the rat brain tissue to ethanol in a manner that is known to produce a withdrawal reaction such as was employed in this study, leads to an enhancement of synaptosomal glutamate binding activity, whereas a similar type of treatment produces a significant decrease in synaptosomal glutamate transport activity [35, 5]. Thus, for the same putative neurotransmitter, in the same species, the uptake and "receptor" binding processes are differentially affected by ethanol treatment. No information exists at this point on another important aspect of glutamate activity, i.e. the *in vivo* effects of ethanol on the depolarization-induced release of glutamate.

On the basis of the findings in this study one could suggest that if the neuronal adaptation to the depressant effects of chronic ethanol exposure is that of enhanced receptor sensitivity to an excitatory agent such as glutamate, then it would seem unlikely that the sensitivity to an inhibitory neurotransmitter agent such as GABA should change in the same direction. On the other hand, during the withdrawal phase one could predict that there should be a compensatory increase in the neuronal responsiveness to an agent like GABA as an attempt to control the existing imbalance in excitatory activity. This would fit with the observations in the literature that the GABA receptor agonist di-*n*-propyl acetate can ameliorate post-ethanol withdrawal seizures, whereas GABA antagonists enhance such convulsive activity [36, 15]. We are currently examining the changes in GABA-receptor binding activity under conditions similar to those employed for the study of glutamate binding presented in this paper.

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