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Tolerance to and Cross Tolerance Between Ethanol and Nicotine

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COLLINS, A. C., J. B. BURCH, C. M. DE FIEBRE AND M. J. MARKS. *Tolerance to and cross tolerance between ethanol and nicotine*. PHARMACOL BIOCHEM BEHAV **29**(2) 365–373, 1988.—Female DBA mice were subjected to one of four treatments: ethanol-containing or control diets, nicotine (0.2, 1.0, 5.0 mg/kg/hr) infusion or saline infusion. After removal from the liquid diets or cessation of infusion, the animals were challenged with an acute dose of ethanol or nicotine. Chronic ethanol-fed mice were tolerant to the effects of ethanol on body temperature and open field activity and were cross tolerant to the effects of nicotine on body temperature and heart rate. Nicotine infused animals were tolerant to the effects of nicotine on body temperature and rotarod performance and were cross tolerant to the effects of ethanol on body temperature. Ethanol-induced sleep time was decreased in chronic ethanol- but not chronic nicotine-treated mice. Chronic drug treatment did not alter the elimination rate of either drug. Chronic ethanol treatment did not alter the number or affinity of brain nicotinic receptors whereas chronic nicotine treatment elicited an increase in the number of [^{3}H]-nicotine binding sites. Tolerance and cross tolerance between ethanol and nicotine is discussed in terms of potential effects on desensitization of brain nicotinic receptors.

Ethanol Nicotine Nicotinic receptors Tolerance Desensitization Cholinergic receptors
Ethanol metabolism Nicotine metabolism

ETHANOL and nicotine are consumed in alcoholic beverages and in tobacco, respectively, and are the most frequently used psychoactive substances. Furthermore, the simultaneous use of alcohol and tobacco is common. For example, several studies have demonstrated that a high correlation exists between alcohol and tobacco use; as the use of one substance increases so does the use of the other [4, 6, 8, 12, 15]. Consistent with this observation, Potthoff *et al.* [29] have reported that rats continuously treated with nicotine, via slow-release pellets, consume more of a 10% ethanol solution than do controls or animals treated chronically with caffeine, phencyclidine, secobarbital, mescaline or haloperidol.

There are many ways in which alcohol and tobacco could interact. For example, the continuous use of either agent has been shown to result in increased activity of hepatic drug metabolizing enzyme systems [3, 10, 34]. Alternatively, interactions within the nervous system are possible. Evidence is available which suggests that ethanol may alter nicotinic receptors. Ethanol and other aliphatic alcohols stabilize the nicotinic receptor from *Torpedo* in a non-functional (desensitized) state [9, 38, 39] and long-term ethanol treatment results in an increase in the number of nicotinic receptors in rat brain [37]. These findings suggest that ethanol may exert its behavioral actions, at least in part, by interacting with brain nicotinic receptors.

Several recent studies from our laboratory have examined the relationship between brain nicotinic receptors and the development of tolerance to selected behavioral and physiological effects elicited by a challenge dose of nicotine [19, 21–24]. In these studies, we infused nicotine intravenously into mice and measured brain nicotinic receptors using [^{3}H]-nicotine and α-[^{125}I]-bungarotoxin (BTX) as the ligands. The majority of these studies used DBA/2Ibg mice and, in this strain, we have observed that as the infusion dose of nicotine increases tolerance to nicotine increases. This is paralleled by an increase in the number of brain [^{3}H]-nicotine binding sites; at higher infusion doses the number of BTX binding sites also increases. Several other investigators have also demonstrated that chronic nicotine treatment elicits an increase in the number of brain nicotinic receptors [16, 27, 31, 32].

As noted above, one study has reported that chronic ethanol treatment results in an increase in the number of brain nicotinic receptors [37]. The ligand used to measure nicotinic receptors in this study was [^{3}H]-nicotine. Thus, it seems that both chronic nicotine and ethanol treatment may elicit an up-regulation of nicotinic receptors. Our studies

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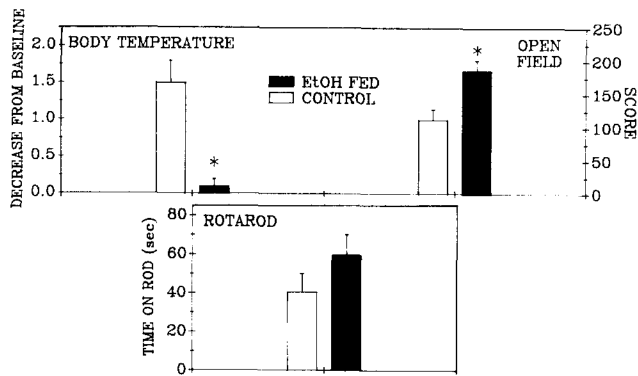

FIG. 1. Effect of chronic ethanol treatment on acute response to ethanol in mice. Mice fed with control or ethanol-containing (2.6% w/v) liquid diet for 10 days were injected with a 2.5 g/kg IP dose of ethanol. Results from control (open bars) or ethanol-treated (diagonal bars) mice represent the mean±S.E.M. for 12 animals. Results were analyzed by one-way analysis of variance. *Significantly different from corresponding controls ($p<0.05$).

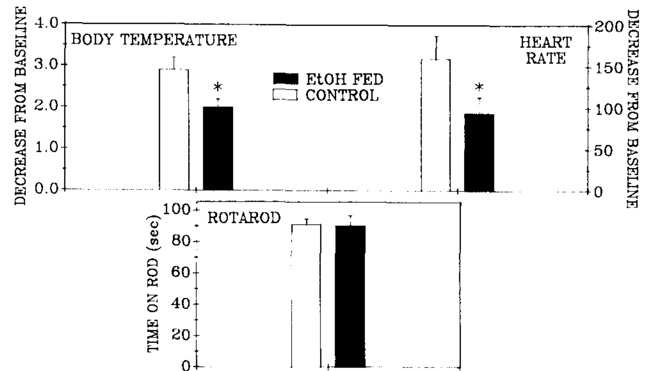

FIG. 2. Effect of chronic ethanol treatment on acute response to nicotine in mice. Mice fed with control or ethanol-containing (2.6% w/v) liquid diet for 10 days were injected with a 2.0 mg/kg IP dose of nicotine. Results from control (open bars) or ethanol-treated (diagonal bars) mice represent the mean±S.E.M. for 12 animals. Results were analyzed by one-way analysis of variance. *Significantly different from corresponding controls ($p<0.05$).

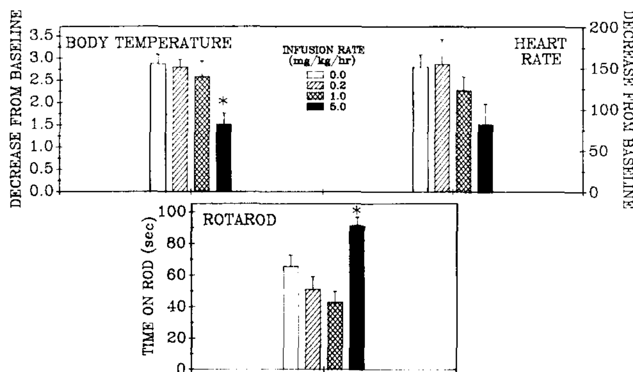

FIG. 3. Effect of chronic nicotine infusion on acute response to nicotine. Mice infused with the indicated doses of nicotine were injected with 2.0 mg/kg nicotine. IP. Results represent the mean± S.E.M. for 5 to 8 mice. The results were analyzed by analysis of variance. *Significantly different from the corresponding saline-infused control.

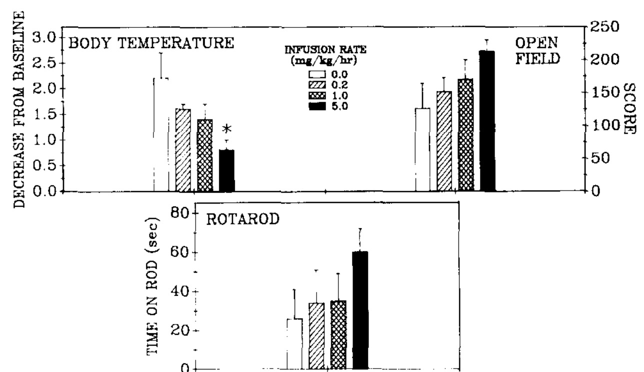

FIG. 4. Effect of chronic nicotine infusion on acute response to ethanol in mice. Mice infused with the doses of nicotine indicated were injected with 2.5 g/kg ethanol, IP. Results represent the mean±S.E.M. for 5 to 8 mice. The data were analyzed by one-way analysis of variance. *Significantly different from the corresponding saline-infused control ($p<0.05$).

with nicotine have demonstrated that an up-regulation of nicotinic receptors parallels the development of tolerance to nicotine. We have hypothesized that the up-regulation occurs because nicotine induces an initial activation and then a longer term desensitization of nicotinic receptors. That is, nicotine-tolerant animals have greater numbers of nicotinic receptors than do controls, but most of these receptors may be desensitized. Since ethanol may stabilize nicotinic receptors in the desensitized form [9, 38, 39], it may be that alcohol and tobacco are used together because they have similar effects on brain nicotinic receptors. It may be that tolerance to alcohol, which presumably promotes an increase in consumption, is due in part to altered nicotinic receptor number or function. If this is the case, it seems reasonable to anticipate that cross tolerance should exist between ethanol and nicotine. The data presented here demonstrate that cross tolerance does exist between ethanol and nicotine, but only for some measures.

METHOD

Materials

Poly-L-lysine, L-nicotine, α-bungarotoxin, 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, Tris (hydroxymethyl) aminomethane, Tris-HCl, alcohol dehydrogenase and sucrose were purchased from Sigma Chemical Co. (St. Louis, MO). Nicotine was redistilled before use. Toluene and isoamyl alcohol were purchased from J. T. Baker Chemical Co. (Phillysburg, NJ). 2,5 Diphenyloxazole was purchased from Fischer Chemical Co. (Fairlawn, NJ); Triton X-100 was obtained from Research Products International (Mount Prospect, IL). Nicotinamide adenine dinucleotide was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Viscarin was purchased from FMC Corporation (Springfield, NJ). Choline bitartrate, L-methionine, casein, dextrin, AIN vitamin mixture and AIN mineral mixture were obtained from ICN Pharmaceuticals (Cleveland,

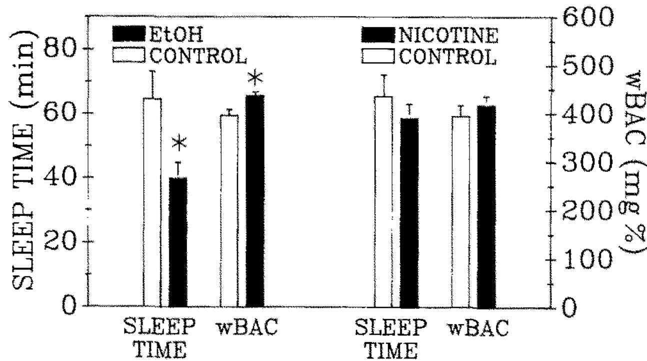

FIG. 5. Effect of chronic ethanol or nicotine treatment on sleep time and waking blood ethanol concentration. Mice were treated chronically with ethanol (2.6% w/v liquid diet) or nicotine (5.0 mg/kg/hr IV infusion) as described in the Method section. Control (open bars) or treated (diagonal bars) mice received an acute injection of ethanol (3.8 g/kg IP) and the time to regain loss of righting reflex (sleep time) and waking blood ethanol concentrations (wBAC) were determined as described in the Method section. Results are the mean±S.E.M. for 6 mice. Data were analyzed by one-way analysis of variance. *Significantly different from corresponding controls ($p<0.05$).

OH). DL-[^{3}H]-nicotine (N-methyl-^{3}H, specific activity 60.2 Ci/mmol and α-[^{125}I]-bungarotoxin (^{125}Tyrl, initial specific activity 23.0 μCi/μg) were obtained from New England Nuclear (Newton, MA).

Animals

The mice used in this study were females of the DBA/2Ibg strain bred in the mouse colony at the Institute for Behavioral Genetics. The mice were housed with two to five like-sex litter mates per cage and given free access to food (Wayne Lab Blox) and water. The animals were maintained on a 12-hr light-dark cycle (lights on 7:00 a.m. to 7:00 p.m.). All mice were 60–80 days old when used.

Chronic Ethanol Treatment

Mice were treated with ethanol by chronic feeding using the Shorey-AIN diet [13]. This is a nutritionally complete diet in liquid form which utilizes dextrin as a caloric replacement for ethanol in the control mixture. Singly housed mice received 20 ml of a 2.6% (w/v) formula (yielding 20% ethanol-derived calories) each day for 10 days. Control mice received equal volumes of control diet. This concentration of ethanol was chosen so as to facilitate the development of tolerance yet avoid, as much as possible, the development of severe ethanol dependence. Dependence is usually characterized by the appearance of withdrawal symptoms. Such symptoms could confound the behavioral and physiological parameters measured. The diet was presented 2–3 hours prior to the onset of the animal's dark cycle. Animal weight and ethanol consumption were monitored daily. To determine blood ethanol levels, a 10 μl blood sample was taken from the retro-orbital sinus approximately 5 hours after the diet had been presented to the mice. Blood samples were also taken from control mice to compensate for changes which might have occurred due to handling-induced stress in the treated mice.

Chronic Nicotine Treatment

Nicotine was administered via a constant intravenous infusion as reported previously [19]. This technique involves the surgical implantation of a silastic cannula in the right jugular vein of the mouse. Prior to surgery, animals were anesthetized with pentobarbital (45 mg/kg) and chloral hydrate (63 mg/kg). After 1–2 days convalescence, the mice were placed in individual cages (115×15×25 cm) and the cannulae were attached to thermoplastic tubing which was connected to a 1 ml syringe mounted on a Harvard Instruments infusion pump. The flow rate was 35 μl/hr. All mice were initially infused with saline for 1–2 days to allow for adaptation to the infusion apparatus. After this time, saline infusion was continued in the controls or nicotine infusion was initiated. Mice were treated with nicotine at an initial infusion rate of 0.2 or 1.0 mg/kg/hr. For some of the animals, the 1 mg/kg dose was increased by 1 mg/kg each day until a 5.0 mg/kg/hr dose was attained. All mice were maintained at their final infusion dose for 8–10 days.

Tests for Acute Ethanol Response

The ethanol test battery was chosen from a number of possible behavioral and physiological measures. Tests were selected which provide maximal information concerning acute ethanol response. The dose and time of testing after injection were derived from previously determined dose-response and time course data for each measure. Prior to testing, the diet was removed from the animal's cage for 6 hours to allow for metabolic clearance of the drug, i.e., blood alcohol levels were zero at the time of behavioral testing. Diets were removed at 7 a.m., shortly after the lights came on in the animal colony. The animals were tested for possible withdrawal symptoms by measuring performance on each of the tests. Subsequently, the animals received a 2.5 g/kg IP injection of ethanol (injection volume=0.02 ml/g body weight). The tests and time of testing after injection were: open field activity, 1–4 min; rotarod performance, 7–7.8 min; body temperature, 15 min. A separate group of animals was used for sleep time experiments.

Open field activity. The open field arena [7] is a device which quantifies locomotor activity. The arena consists of a Plexiglas box (91.4×91.4 cm) with equispaced photocells built into the sides. Thus, a light grid is generated across the floor of the arena. Mice were placed in the arena and activity was monitored by the interruption of the photocell light beams which was then electronically recorded. Mice were injected and placed in the corner in an upright clear plastic cylinder for 1 min. They were then released and activity was measured for 3 min.

Rotarod performance. The rotarod (Ugo Basile Co., Milan, Italy) is a device which tests motor coordination. The test animal must continually walk to stay on a rotating rod or fall a distance of 30 cm which inactivates a timer. Prior to testing, mice were trained to stay on the rotarod for at least two consecutive 100 sec tests. For the chronic ethanol-treated animals, this training was initiated 4 hr after the ethanol-containing liquid diets were withdrawn. The amount of time within the 100 sec period that the animal was able to stay on the rotarod following an acute injection was recorded. Rotation speed was 10 rpm and the rod diameter was 6.3 cm.

Body temperature. Body temperature was recorded by insertion of a Thermalet THS probe (Bailey Instrument, Saddlebrook, NJ) 2.5 cm into the rectal cavity.

TABLE 1

EFFECT OF CHRONIC ETHANOL OR NICOTINE TREATMENT ON ETHANOL OR NICOTINE ELIMINATION*

Treatment	Nicotine K_E (min^{-1})	Nicotine $T_{1/2}$ (min)	Ethanol K_E (mg%/min)	Ethanol $T_{1/2}$
Saline-infused	0.067 ± 0.010 (4)	12.7 ± 1.8 (4)	1.59 ± 0.04 (8)	—
Nicotine-infused (5.0 mg/kg/hr)	0.060 ± 0.010 (4)	11.7 ± 2.0 (4)	1.65 ± 0.08 (8)	—
Control Diet	0.071 ± 0.010 (4)	10.9 ± 2.3 (4)	1.32 ± 0.07 (6)	—
Ethanol fed (2.6% w/v diet)	0.070 ± 0.015 (4)	12.6 ± 3.5 (4)	1.55 ± 0.14 (6)	—

*The apparent elimination rate constants (K_E) are given for nicotine (min^{-1}) and ethanol (mg/100 ml/min). The half-life ($T_{1/2}$) for nicotine is given in minutes. Values represent the mean ± S.E.M. for the number of chronically treated mice in parentheses. Data were analyzed by one-way analysis of variance. There were no statistically significant differences between control and treated animals for either drug.

Sleep time. Sleep time, defined as the interval between the loss and the return of the righting reflex, was measured following the IP injection of a 3.8 g/kg (0.02 ml/g) dose of ethanol. The time between loss of and regaining the righting response was recorded. An animal was judged to be "awake" when it righted itself three times within a 30 sec time period. Ambient room temperature was maintained at 24.6±0.5°C. Upon waking, a 10 μl blood sample was taken from the retro-orbital sinus. Blood ethanol concentration was determined as described below.

Tests for Acute Nicotine Response

The nicotine test battery was derived in a similar fashion as that for ethanol (see [19] for dose-response and time course data). The tests and time of testing, respectively, for response to an acute dose of nicotine were: rotarod, 5–6.7 min; heart rate, 8–10 min; body temperature, 15 min. The challenge dose of nicotine was 2.0 mg/kg given IP (injection volume=0.01 mg/g body weight). Rotarod and body temperature were measured as described above.

Heart rate. Heart rate was recorded using an E & M Physiograph (Narco Bio-Systems, Houston, TX). The test animal was restrained in a Plexiglas half-cylinder with appropriate holes for entry, breathing, and electrode placement. Electrodes were placed immediately behind the left foreleg and immediately in front of the right hindleg. Heart rate was recorded for 6 sec.

Ethanol Elimination

Blood samples were taken at 15, 45, 90, 135, and 180 min after an acute ethanol injection. The apparent elimination rate constant (K_E) was calculated from these data. Blood ethanol concentration was determined using the method of Smolen *et al.* [33].

Nicotine Elimination

Nicotine elimination experiments were performed by taking a 40 μl retro-orbital blood sample at 3 min intervals for 15 min following a 2.0 mg/kg IP injection of nicotine containing 10 μCi of [^{3}H]-nicotine as a tracer. The procedure for nicotine extraction is a modification of the methods of Hatchell and Collins [12]. K_E and $t_{1/2}$ were calculated from nicotine concentrations vs. time data.

Tissue Preparation for Receptor Determination

Brain tissue was prepared essentially as described by Romano and Goldstein [30] except that three 5-min incubations at 37°C were added to allow dissociation of nicotine which may have been bound to the tissue [19]. After being tested for cross tolerance, the mice were sacrificed by cervical dislocation. Their brains were removed, chilled to 4°C, and dissected into seven brain regions: cortex, cerebellum, hindbrain (pons-medulla), hippocampus, hypothalamus, striatum, and midbrain. Each tissue was placed in 10 volumes of incubation buffer (NaCl, 118 mM; KCl, 4.8 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 20 mM; pH 7.5) and homogenized using a Teflon-glass homogenizer. Following homogenization, the samples were incubated for 5 min at 37°C and then centrifuged at 15,000×g for 20 min. The supernatant was discarded and the pellet was resuspended in 20 volumes of ice-cold water for 60 min. After this, the samples were incubated for 5 min at 37°C, then centrifuged at 15,000×g for 20 min. This pellet was resuspended in 10 volumes of homogenization buffer and used in the receptor assay.

The binding of [^{3}H]-nicotine and [^{125}I]-BTX was conducted at a single concentration in all of the brain regions. In addition, K_D and B_{max} were determined in cortex only by Scatchard analysis of saturation binding data for BTX. K_D and B_{max} for [^{3}H]-nicotine in cortex were estimated by displacement of DL-[^{3}H]-nicotine with L-nicotine. It was not possible to estimate K_D and B_{max} in every region because of the limited amount of tissue available. Thus, binding was conducted in all brain regions with radioligand concentrations at or near the B_{max} to provide an indicator of changes in the number of receptors.

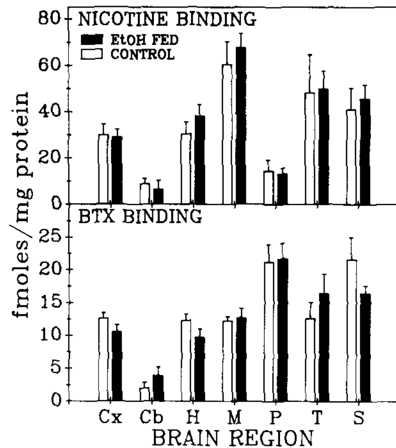

FIG. 6. Effect of chronic ethanol treatment on the binding of [^{3}H]-nicotine and [^{125}I]-BTX in seven brain regions. The brain regions are: cortex (Cx), cerebellum (Cb), hindbrain (H), midbrain (M), hippocampus (P), hypothalamus (T), and striatum (S). Membranes obtained from mice fed with control or ethanol-containing liquid diets for 10 days were assayed for [^{3}H]-nicotine and [^{125}I]-BTX binding using a single ligand concentration as described in the Method section. Each bar represents the mean±S.E.M. for 6 animals in each group for the nicotine binding assays and n=12 for the BTX assay. No significant effects of ethanol treatment were found.

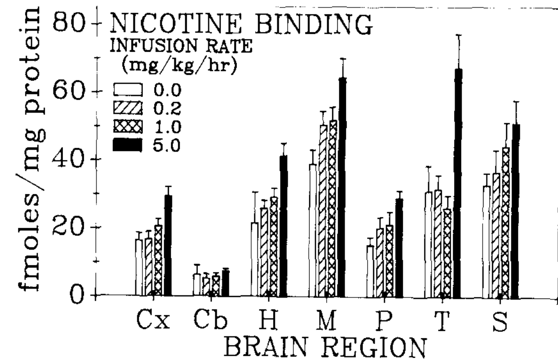

FIG. 7. Effect of chronic treatment on the binding of [^{3}H]-nicotine in seven brain regions. See legend to Fig. 6 for identity of brain regions. Membranes obtained from chronic saline- or nicotine-infused mice were assayed for [^{3}H]-nicotine binding using a single ligand concentration as described in the Method section. Each point represents the mean±S.E.M. of 6 to 16 animals at each nicotine dose.

DL-[^{3}H]-Nicotine Binding

The binding of DL-[^{3}H]-nicotine was determined using the method of Romano and Goldstein [30] as modified by Marks and Collins [20]. Final incubation volume was 250 μl and each sample contained 150 to 500 μg of tissue protein. Binding was initiated by the addition of [^{3}H]-nicotine (final concentration: 58.4 nM). Specific binding was determined as the difference between samples containing zero and 1×10^{-6} M unlabeled nicotine.

α-[^{125}I]-Bungarotoxin Binding

The binding of α-[^{125}I]-BTX was determined using the methods of Marks and Collins [21,25]. Incubations were conducted for 2.5 hr at 37°C in an incubation volume of 0.5 ml (final BTX concentration: 0.51±0.035 nM).

Scintillation Counting

After filtration and wash, filters were placed in 10 ml Nalgene filmware bags and 2.5 ml scintillation cocktail (toluene, 1.25 l; Triton X-100, 0.9 l; 2,5-diphenyloxazole, 10.6 g) was added. The bags were sealed and filters mechanically crushed. All samples were counted on a Beckman 7,000 liquid scintillation counter. Efficiency was 22% for ^{3}H and 80% for ^{125}I.

Protein

Protein was measured by the method of Lowry *et al.* [17] using bovine serum albumin as standard.

Statistical Analysis

The effect of chronic drug treatment on physiological or behavioral responses and on receptor levels was analyzed by analysis of variance. Tukey B *post hoc* tests were employed to determine which group means differed significantly. Significance levels for *post hoc* tests were set at $p=0.05$.

Drug elimination was analyzed by subjecting the drug concentration vs. time data to a mixed model analysis of variance (treatment group=between subjects variable, time=within subjects variable) and testing for a significant group by time interaction. In addition, K_E and $t_{1/2}$ were analyzed using one way analysis of variance.

RESULTS

The average ethanol consumption of animals chronically administered the ethanol-containing diet was 18.3±0.3 g/kg/day. Blood ethanol levels in these animals 5 hours after the diet was presented to them averaged 259.2±17.4 mg/100 ml. At 17 hours after the diet was presented (2 hours after the onset of the light cycle the following day) no detectable blood ethanol levels were found in these mice. The diet was generally completely consumed by this time. The average weight of control mice on the final day of treatment was 19.6±0.6 g; the average weight of ethanol-fed mice was 19.8±1.0 g. These values are not significantly different.

Chronic ethanol-treated and control diet-treated animals had virtually identical startle responses, heart rates, and body temperatures. No significant differences in startle response or heart rate (ethanol treated=692±11, control=708±7 beats/min) were noted, but a significant difference was observed in baseline body temperature between control- (38.7±0.1°C) and ethanol-treated (38.0±0.2°C) animals, $F(1,22)=13.5$, $p<0.01$.

The results of tests for the acute response to ethanol in control- and ethanol-fed mice are given in Fig. 1. A significantly higher open field score, $F(1,22)=10.4$, $p<0.01$, was seen in ethanol-fed mice following ethanol injection. Saline injected controls had an activity score of 182±38. Ethanol elicited a significant decrease in activity in the control diet animals, but was without effect on the chronic ethanol-fed mice. The ethanol-fed mice also showed less of a decrease in body temperature, $F(1,22)=16.5$, $p<0.001$, following challenge with ethanol. The chronic ethanol-treated and control animals did not differ in response to a challenge dose of ethanol for the rotarod test.

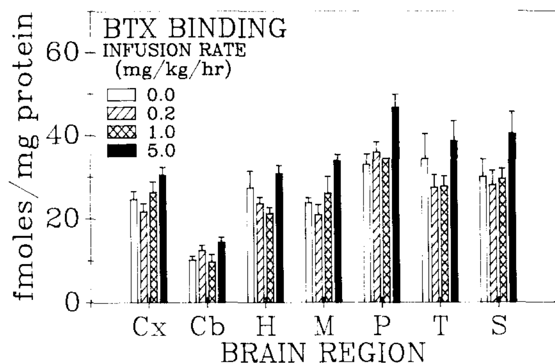

FIG. 8. Effect of chronic nicotine treatment on the binding of [^{125}I]-BTX in seven brain regions. See legend to Fig. 6 for identity of brain regions. Membranes obtained from chronic saline- or nicotine-infused mice were assayed for [^{125}I]-BTX binding using a single ligand concentration as described in the Method section. Each point represents the mean±S.E.M. of 6 to 16 animals at each nicotine dose.

The effect of chronic ethanol treatment on acute nicotine response is summarized in Fig. 2. The change in body temperature induced by nicotine was significantly less in ethanol-fed mice, $F(1,22)=4.8$, $p<0.05$. Following an acute dose of nicotine, control-fed mice had body temperatures of 35.2±0.3°C and ethanol-fed mice had body temperatures of 35.3±0.3°C, which were not significantly different. The apparent cross tolerance relates to the fact that ethanol- and control-fed animals had different baseline body temperatures (38.0±0.2 and 38.7±0.1°C, respectively). The nicotine challenge dose had virtually no effect on rotarod performance in either the chronic ethanol-fed or control groups. A reduced effect of nicotine on heart rate was seen in the ethanol-fed mice. Thus, the chronic ethanol-fed animals were cross tolerant to nicotine on two of the three tests used.

The baseline values for chronic saline- and nicotine-infused animals were also measured. The rotarod performances and body temperatures of the nicotine-infused animals were identical to those of chronic saline-infused animals. For example, the 5.0 mg/kg/hr nicotine-treated group had a rotarod score of 97.0±2.4 sec and body temperature was 38.2±0.2°C which are not different from the values obtained for the saline-infused controls (88.5±5.1 sec for rotarod and 38.3±0.1°C for body temperature). Saline-infused animals had a heart rate of 724±11 beats/min whereas the 1.0 and 5.0 mg/kg/hr nicotine-infused animals had baseline heart rates of 668±18 and 658±16 beats/min, respectively. The heart rates of the 1.0 and 5.0 mg/kg/hr treatment groups were less than control.

The responses of chronic saline- and nicotine-infused mice to a challenge dose of nicotine (2 mg/kg) are presented in Fig. 3. The challenge dose of nicotine elicited a decrease in rotarod performance, heart rate, and body temperature. Tolerance to nicotine's effects was detected for two of the three measures, one-way ANOVA: body temperature $F(3,43)=5.99$, $p<0.01$; heart rate $F(3,43)=2.48$, $p>0.05$; rotarod, $F(3,64)=9.50$, $p<0.01$. This tolerance appears to be dose-related; i.e., as the chronic nicotine infusion dose increased, the response to the challenge dose of nicotine progressively decreased.

The responses of the chronic saline- and nicotine-infused animals to a challenge dose of ethanol are presented in Fig. 4. Ethanol elicited decreases in open field activity, rotarod performance, and body temperature in the chronic saline-infused animals. These effects grew progressively less as the nicotine infusion dose was increased. The slope of the nicotine infusion dose-ethanol response curve was 14.74 (95%confidence limits 7.46–22.20) activity counts/(mg/kg/hr) for open field activity. The slope of the rotarod curve was 6.18 (95% CI=5.52–6.84) degrees/(mg/kg/hr) and the slope of the body temperature curve was −0.22 (95% CI=−0.061 to −0.38) degrees/(mg/kg/hr). These are all non-zero which indicates a trend towards reduced response to ethanol as the chronic nicotine infusion dose increased. In addition, the one-way ANOVA detected significant differences between nicotine-infused and control animals for the body temperature test (the 5.0 mg/kg/hr group). Thus, the chronic nicotine-treated animals that were clearly tolerant to nicotine were also clearly cross tolerant to alcohol's effects on body temperature. Suggestions of a reduced response to alcohol were also seen for the rotarod and open field activity tests.

The results of the sleep time experiments are summarized in Fig. 5. Ethanol-fed mice had shorter sleep times, $F(1,10)=6.1$, $p<0.025$ and higher waking blood ethanol concentrations (wBAC), $F(1,10)=6.9$, $p<0.05$, than did control mice. There was no statistically significant difference in sleep time between nicotine- (5.0 mg/kg/hr) and saline-infused mice nor were waking blood alcohol levels different. Thus, chronic ethanol-treated mice were tolerant to ethanol but chronic nicotine-treated animals were not cross tolerant for this test.

Table 1 presents the effects of chronic ethanol or nicotine treatment on ethanol and nicotine elimination. Ethanol and nicotine elimination studies were analyzed by a mixed measures analysis of variance. This analysis compared the plasma drug concentration in each group (control and treated) as a function of time. Any differences between groups in the plasma drug concentration vs. time curve would have been detected as a significant group-by-time interaction. No significant group-by-time interactions occurred for ethanol or nicotine elimination between nicotine- (5.0 mg/kg/hr) and saline-infused mice. In addition, the K_E and $t_{1/2}$ for nicotine were calculated for both treatment groups. No significant differences in K_E or $t_{1/2}$ were detected. Similarly, no significant differences in the K_E for ethanol were seen in either treatment group.

Figure 6 presents the effects of chronic ethanol treatment on the binding of [^{3}H]-nicotine (upper panel) and [^{125}I]-BTX (lower panel) in seven brain regions. These assays were carried out using a single ligand concentration, and should, as a result, reflect potential differences in the number of binding sites unless affinity (K_D) differences exist. No significant differences were detected between the control and ethanol-fed groups for either ligand in any brain region.

Figures 7 and 8 present the effects of chronic nicotine infusion on the binding of the two ligands in the seven brain regions as measured using a single ligand concentration. Chronic nicotine infusion resulted in statistically significant increases in [^{3}H]-nicotine binding in all of the regions, with the exception of striatum and cerebellum, one-way ANOVA: cortex, $F(3,53)=6.45$, $p<0.01$; cerebellum, $F(3,24)=0.77$, $p>0.05$; hindbrain, $F(3,50)=9.49$, $p<0.001$; midbrain, $F(3,50)=5.46$, $p<0.01$; hippocampus, $F(3,26)=6.60$, $p<0.01$; hypothalamus, $F(3,26)=6.60$, $p<0.01$; striatum, $F(3,24)=2.02$, $p>0.05$. These increases were dose-dependent. The binding of [^{125}I]-BTX was also affected by chronic nicotine treatment

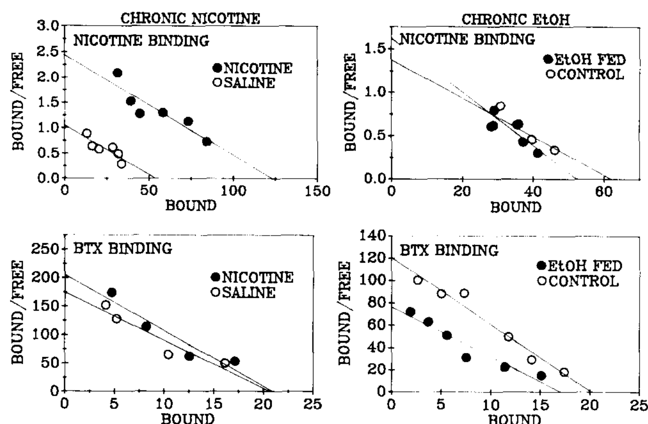

FIG. 9. Scatchard analysis of [^{3}H]-nicotine and [^{125}I]-BTX binding in membranes obtained from cortex of control and chronic nicotine- (left hand panels) or ethanol- (right hand panels) treated mice. Saturation analyses of ligand binding were carried out as described in the Method section. Each point represents the mean of 4 to 6 separate assays.

but significant changes were seen in only some brain regions, midbrain, $F(3,23)=3.77, p<0.05$; hippocampus, $F(3,23)=5.72$, $p<0.01$, due to changes in the 5.0 mg/kg/hr treatment group.

Figure 9 presents Scatchard plots for the binding of these two ligands to membranes prepared from cortex of nicotine- and saline-infused and for ethanol-fed animals and control-diet-fed. The K_D values for [^{3}H]-nicotine were 14±2 and 12±2 μM in the saline- and nicotine-infused groups and 24.3±2.3 μM for ethanol-fed and 30.9±4.1 μM for the control diet-fed animals. The K_D values for [^{125}I]-BTX were 0.16±0.03 μM and 0.1±0.02 μM, and 0.30±0.04 μM and 0.22±0.06 μM for the ethanol and control groups, respectively.

DISCUSSION

Chronic ethanol treatment clearly produced tolerance to a challenge dose of ethanol as is evidenced by the fact that the chronic ethanol-fed animals were not as susceptible to the open field activity and temperature depressing effects of ethanol as were the control diet-fed animals. The duration of ethanol-induced sleep time was also less in the chronic ethanol-treated animals. Because the rate of elimination of ethanol was not changed and because the animals regained the righting response at a higher blood alcohol level, we conclude that the tolerance that was seen is due to reduced CNS sensitivity to ethanol, not to alterations in elimination of ethanol.

We hoped to be able to measure tolerance, and cross tolerance, with minimal interference from a withdrawal syndrome. A recent report from our laboratory has demonstrated that ethanol-withdrawing mice exhibit decreases in heart rate and body temperature [11]. The baseline heart rates recorded from the chronic ethanol-fed animals were the same as control and body temperatures were only slightly reduced. This suggests that our goal was attained; i.e., a measurable tolerance was achieved without the development of severe ethanol dependence. Thus, it seems reasonable to conclude that the reduced response of the ethanol-fed animals to a challenge dose of nicotine for the heart rate and body temperature tests reflects cross tolerance between ethanol and nicotine rather than some confound related to ethanol withdrawal. Because chronic ethanol treatment did not result in an alteration in the pharmacokinetics of nicotine, it seems likely that the reduced response of the ethanol-fed animals to nicotine is due to a change in CNS sensitivity.

Adir *et al.* [1] have assessed the effects of chronic ethanol treatment on the rate of nicotine metabolism in rats. These investigators also failed to observe an effect of chronic ethanol treatment on the $t_{1/2}$ for nicotine. However, they did observe a modest overall decrease in blood nicotine levels in their ethanol-fed rats. This suggests that chronic ethanol treatment may alter nicotine metabolism, at least under some circumstances. Whether the difference between our study and Adir's study is due to species differences or to differences in the treatment method remains to be determined.

Chronic nicotine treatment resulted in a dose-related tolerance to nicotine. Statistically significant tolerance was not seen until the 5 mg/kg nicotine dose. Interestingly, statistically significant cross tolerance to ethanol was detected only in the 5 mg/kg/hr group and only for the body temperature test which was one of the most sensitive indicators of nicotine tolerance. Tolerance to nicotine and cross tolerance to ethanol paralleled one another in that at the lower infusion doses of nicotine a trend towards a reduced response to ethanol was seen. All of the dose-response (nicotine infusion dose-nicotine response and infusion dose-ethanol response) curves were non-zero. This finding is supportive of the contention that tolerance to nicotine and cross tolerance to ethanol were obtained in the nicotine-infused animals. The tolerance and cross tolerance are likely due to changes in CNS sensitivity because chronic nicotine treatment did not alter the metabolism of nicotine or ethanol.

Chronic nicotine treatment had no effect on ethanol-induced sleep time or waking blood ethanol concentration. This is consistent with the report that an acute dose of nicotine does not alter ethanol-induced sleep times [26]. Bhagat [2] has noted that daily injections of nicotine (1 mg/kg) for 4–6 weeks in mice significantly enhanced ethanol induced sleep time. This effect was not seen if treatment was extended for up to 9–10 weeks. Our study utilized a much higher dose (5.0 mg/kg/hr) of nicotine for a shorter duration (2 weeks). Perhaps the effect seen in the latter part of Bhagat's [2] study appeared much sooner in our study. Further experiments utilizing lower infusion doses or a shorter duration of treatment would be necessary to confirm this.

Chronic ethanol treatment had no effect on [^{3}H]-nicotine or [^{125}I]-BTX binding in the regions studied. This result is inconsistent with a previous report which indicated that chronic ethanol treatment increases [^{3}H]-nicotine binding [37]. In this earlier study, rats were treated with ethanol as 8% w/v or 16% w/v solutions in the drinking water for 5 months. This resulted in an increase in [^{3}H]-nicotine binding in hypothalamus (27%) and thalamus (50%) and a decrease in hippocampus (16%). Perhaps our failure to replicate this finding relates to the differences in treatment time. Alternatively, several investigators [26, 38, 46, 49, 50] have successfully altered the brain [^{3}H]-nicotine (^{3}H-ACh) binding site in rats using chronic injection techniques. We have not been successful in changing these same receptors in the mouse using chronic injection techniques. The more rigorous continuous infusion method was required to detect an effect. Thus, the rat and

mouse may differ in susceptibility to up-regulation of brain nicotinic receptors.

The chronic infusion of nicotine resulted in an increase in [^{3}H]-nicotine binding in most brain regions. This result is consistent with several other reports from our laboratory [19, 21–24] and other laboratories [16, 27, 31, 32]. Changes in the B_{max} but not in K_D occurred. Generally, nerve cells adapt to chronic stimulation with agonists by a decrease in number of receptor binding sites and increase the number of receptors in response to chronic antagonism (see Creese and Sibley [5] for a review). An increase in receptor number induced by nicotine, an agonist, is therefore unexpected but may not be inconsistent with the general interpretation of receptor changes described above. Nicotine has biphasic effects. Low doses elicit stimulatory responses whereas higher doses initially stimulate, then inhibit responses. The inhibition of response results from a conformational shift in the receptor to the desensitized form [35,36]. In this state, the receptor is functionally antagonized and no ion flux occurs. Given the high concentrations of nicotine obtained during chronic infusions in this study, [^{3}H]-nicotine binding sites were probably chronically antagonized and the adaptive response would therefore be an increase in receptor number. Thus, nicotine would be acting mechanistically as an agonist, but functionally as an antagonist, and the behavioral, physiological and biochemical effects noted in this study would be responses to the antagonist properties of nicotine.

Since tolerance to the acute effects of ethanol developed in the absence of any changes in nicotine receptors, a strict correlation between changes in nicotine receptors and the development of tolerance is not indicated. However, ethanol may interact with nicotinic receptors indirectly. Young *et al.* [38,39] and El-Fakahany *et al.* [9] have demonstrated that ethanol stabilizes the nicotinic receptor in the high affinity (desensitized) form. Thus, ethanol may interact with nicotine by disrupting nicotinic receptor function and alcohol-induced increases in smoking may be related to what amounts to an ethanol-induced functional blockade of nicotinic receptors. The observation that treatment with mecamylamine, a nicotinic receptor antagonist, increases smoking behavior in humans [28] is consistent with this argument. Thus, it may be that alcohol-induced stabilization of the nicotinic receptor in a desensitized form has the same effect on response to nicotine (a reduction) and tobacco consumption (an increase) as does mecamylamine because alcohol serves to inactivate nicotinic systems. Conversely, if alcohol acts, in part, by inactivating nicotinic receptors, nicotine-induced increases in receptors could result in an antagonism of alcohol effects. This, of course, could promote both tolerance to alcohol and consequently an increase in alcohol consumption.

In conclusion, chronic infusion of nicotine resulted in the development of tolerance to some of the effects of nicotine and chronic nicotine treatment also reduced some of the responses to ethanol. This suggests that the effects of nicotine and ethanol may be mediated through similar mechanisms. The observations that chronic ethanol treatment resulted in the development of tolerance to ethanol and cross tolerance to some of the effects of nicotine confirm this suggestion. This tolerance and cross-tolerance does not appear to be due to alterations in the rates of nicotine or ethanol metabolism or to changes in the number of [^{3}H]-nicotine binding sites. Since both drugs may desensitize nicotinic receptors, it may be that the cross tolerance develops because of changes in the functional status of brain nicotinic receptors. Clearly, further studies will be required to test this hypothesis.

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