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Chronic Ethanol Administration Downregulates Neurotensin Receptors in Long- and Short-Sleep Mice

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CAMPBELL, A. D. AND V. G. ERWIN. *Chronic ethanol administration downregulates neurotensin receptors in long- and short-sleep mice.* PHARMACOL BIOCHEM BEHAV **45**(1) 95–106, 1993.—Neurotensin (NT) has been shown to differentially alter many of the physiologic responses to ethanol administration in long-sleep (LS) and short-sleep (SS) mice, which were selectively bred for differences in hypnotic sensitivity to ethanol. These mice have been shown to differ in NT receptor densities in cortical and mesolimbic brain regions and it has been suggested that ethanol actions may be mediated, in part, by neurotensinergic processes. The present study was conducted to further examine this hypothesis by determining the effects of acute and chronic ethanol administration on NT receptor systems in these mice. Scatchard analysis of [^{3}H]NT binding in brain membranes from mice chronically treated with ethanol yielded a one-site model, whereas binding in membranes from control mice were best described by a two-site model. Values for binding capacity (B_{max}) were significantly reduced in several brain regions, and binding site density for total, levocabastine-sensitive, and levocabastine-insensitive binding sites were also reduced. The maximum effect was seen after 2 weeks of chronic ethanol consumption. Three weeks after withdrawal from ethanol, K_d and B_{max} had returned to control values. Similarly, binding density in all regions for total, levocabastine-sensitive, and levocabastine-insensitive sites had returned to control values within 2 weeks. NT receptor characteristics measured 2 h post-3.0 g/kg ethanol revealed that ethanol caused a rapid downregulation of both subtypes of NT receptors. The finding that both acute and chronic ethanol significantly downregulate the neurotensin receptor systems further supports the hypothesis that ethanol's actions may be mediated in part by neurotensinergic systems.

Neurotensin Chronic ethanol LS mice SS mice Neurotensin receptors Acute ethanol Dopamine

THE 13 amino acid peptide, neurotensin (NT), first isolated from bovine hypothalamus (4) and later shown to be widely distributed in the CNS (10,27), satisfies many of the criteria for a neurotransmitter. The neuropeptide is concentrated in the synaptosomal fractions (50), is rapidly degraded by specific peptidases (6), is released in a calcium-dependent manner (26), binds to specific high-affinity sites (31,37,49), and activates second messenger systems coupled to its receptors (23). Pharmacological studies have shown that central administration of NT produces a variety of responses similar to those produced by ethanol administration, including hypothermia (2), analgesia (7), and altered locomotor activity (30). However, the physiological function of endogenous NT in the CNS remains unclear.

Two binding sites for NT in mouse and rat brain have been characterized. The high-affinity receptor subtype (NT_H, K_d = 0.3 nM) has been hypothesized to be the physiologically relevant receptor, and the low-affinity receptor (NT_L, K_d = 3 nM) to be solely an "acceptor site" (32,46). However, uneven distribution (32), saturability (3), specificity (44), and high affinity support a role for both receptor subtypes in NT action. Previous studies have shown that NT binding in long-sleep (LS) and short-sleep (SS) mice, which were selectively bred for differences in ethanol sensitivity (39), is best described by two independent binding isotherms (3). Binding to NT_L is selectively inhibited by the nonpeptide histamine (H_1) antagonist, levocabastine, in rats and mice (3,32,46), providing a powerful tool to discriminate between NT_H and NT_L.

Central administration of NT enhanced ethanol-induced anesthesia and hypothermia in mice (13,35), and a genotype-dependent NT enhancement of ethanol sensitivity has been reported with SS mice more sensitive than LS mice (16,19). Similarly, administration of NT into the ventral tegmental area (VTA) produced a greater dose-dependent increase in locomotor activity in SS than in LS mice (13). These results are consistent with SS mice possessing higher densities of receptors in the VTA (3). Indeed, membranes from the ventral midbrain (MB) from SS mice possess significantly higher total (NT_{total}), as well as NT_H, and NT_L receptor densities and it has been suggested that the differences in ethanol sensitivity in these mouse lines may be due, in part, to differences in NT receptor densities (3).

Whereas administration of NT into the VTA causes locomotor activation that correlates with an increase in dopamine

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(DA) turnover in the mesolimbic terminal fields in the nucleus accumbens (NA) (28,29), administration of NT into the NA produces opposite behavioral responses. Administration of NT into the NA has been shown to cause a decrease in locomotor activity produced by administration of DA (17) or amphetamine (11), and no changes in DA metabolites were observed. It is interesting how NT can both mimic and inhibit psychomotor stimulant actions on the same neuronal pathway. Similar to NT administration into the VTA, drugs of abuse including ethanol have been shown to preferentially increase DA turnover in the NA (9), suggesting a role for this peptide in addictive behaviors.

The aim of the present study was to examine the hypothesis that the differences in ethanol sensitivity in LS and SS mice are due to differences in NT receptor-mediated processes by determining the affect of acute and chronic ethanol administration on NT receptor systems in LS and SS mice. Using a method of chronic ethanol administration that produced marked functional tolerance to acute ethanol administration (18), we determined the effects of chronic ethanol on NT receptor subtypes in membranes from nine brain regions. Acute ethanol effects were also investigated. Because neurotensinergic systems are closely associated, and in some cases colocalized (1,42), with dopaminergic pathways, the D_1 and D_1 receptor subtypes were also investigated in membranes from control and acute and chronic ethanol-treated animals.

METHOD

Animals

Male LS/Ibg and SS/Ibg mice were obtained from the Institute for Behavioral Genetics, University of Colorado, Boulder, CO. All experiments were conducted with mice 60–80 days of age, which were maintained in an environment of constant temperature (22°C), humidity (20%), and light (12 L : 12 D cycle).

Materials

Reagent and chemicals of the highest purity commercially available were obtained as follows: [3,11-Tyrosyl-3,5-^{3}H(N)] neurotensin$_{1-13}$ (105 Ci/mmol in ethanol) and *N*-methyl-[^{3}H]SCH23390 (87 Ci/mmol in ethanol) were obtained from New England Nuclear (Boston, MA); [^{125}I]epidepride (2000 Ci/mmol in ethanol) was a generous gift from Dr. Chet Mathis, Lawrence Berkeley Laboratory (Berkeley, CA); neurotensin$_{1-13}$, neurotensin$_{1-11}$, neurotensin$_{1-8}$, neurotensin$_{8-13}$, neuromedin N, Tris-HCl, bovine serum albumin [BSA; radioimmunoassay (RIA) grade], 1,10 phenanthroline, and polyethylenimine (50% aqueous) were obtained from Sigma Chemical Co. (St. Louis, MO); EDTA and Scintiverse II were obtained from Fisher Scientific (Fair Lawn, NJ); bacitracin (60,000–70,000 U/g) was obtained from P-L Biochemicals, Inc. (Milwaukee, WI); (+)-butaclamol HCl was obtained from Research Biochemicals, Inc. (Natick, MA); levocabastine was a generous gift from Janssen Pharmaceutica (Beerse, Belgium); and the Whatman GF/B filters were obtained from Millipore Corp. (Bedford, MA).

Acute Ethanol Administration

Ethanol (3.0 g/kg) was administered IP to male LS and SS mice, and animals were sacrificed 2 h postinjection. Control animals received an equal volume of sterile saline vehicle and were also sacrificed 2 h postinjection. Tissue preparation and binding assays were performed as described below, and membranes from the MB and entorhinal cortex (RC) were investigated. Additionally, the effects of this treatment on the sensitivity of these membranes to levocabastine was studied.

Chronic Ethanol Administration

A simple method of chronic ethanol administration that produced marked functional tolerance was performed essentially as previously described (18). Mice were housed five per cage and provided lab chow ad lib. Animals were required to drink ethanol solutions in water (% v/v) in the following regimen: 10% for 4 days and 15% for the remainder of the chronic treatment. Blood ethanol concentrations varied from 10–200 mg/dl over a 24-h period of ethanol consumption (18). Control animals and animals withdrawn from ethanol

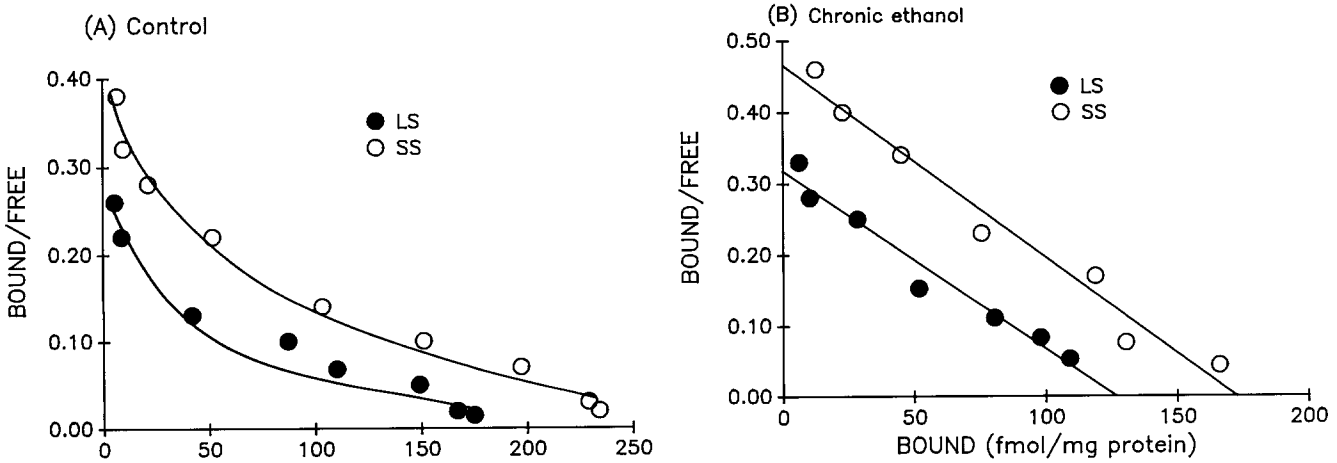

FIG. 1. Saturation binding (Scatchard analysis) of [^{3}H]neurotensin (NT) in ventral midbrain (MB) membranes from control (A) and chronic ethanol-treated (B) long-sleep (LS) and short-sleep (SS) mice. Membranes from the ventral midbrain were prepared as described in the text and incubated for 20 min with concentrations of [^{3}H]NT from 0.02–20 nM. Chronic ethanol-treated animals received ethanol in their drinking water (v/v, 10% for 4 days and 15% for 10 days). Typical experiments shown. Data were analyzed by the linear/nonlinear least squares regression analysis program LIGAND.

TABLE 1

EFFECTS OF CHRONIC ETHANOL ON NT RECEPTOR BINDING CHARACTERISTICS IN VENTRAL MIDBRAIN (MB) MEMBRANES OF LS AND SS MICE

Line	Brain Region	Treatment	Levocabastine	Receptor Subtype	K_d (nM)	B_{max} (fmol/mg protein)
LS	MB	None	−	NT_H	0.31 ± 0.02	38.80 ± 7.01*†
LS	MB	None	−	NT_L	2.98 ± 0.56	178.52 ± 16.50
LS	MB	None	+	NT_H‡	0.36 ± 0.04	51.26 ± 2.53*§
LS	MB	Ethanol	−	¶	1.66 ± 0.33	137.70 ± 13.40§#
LS	MB	Ethanol	+	NT_H‡	0.49 ± 0.05	80.18 ± 9.38§**
SS	MB	None	−	NT_H	0.29 ± 0.06	82.38 ± 5.54
SS	MB	None	−	NT_L	2.52 ± 0.70	219.11 ± 11.90
SS	MB	None	+	NT_H‡	0.35 ± 0.02	70.48 ± 3.82
SS	MB	Ethanol	−	¶	1.58 ± 0.31	173.10 ± 15.30†#
SS	MB	Ethanol	+	NT_H‡	0.40 ± 0.06	104.82 ± 12.23**†

Values were determined by Scatchard analysis as described in the Method section, and represent the mean ± SEM of 4–10 experiments. All data were analyzed by the linear/nonlinear least squares regression analysis program EBDA/LIGAND to determine K_d and B_{max} values. Comparisons were made by ANOVA to assess between- and within-subjects differences.

*Compared to corresponding SS value.

†$p < 0.01$, by one-way ANOVA.

‡ Represents levocabastine – insensitive receptors (putatively NT_H).

§$p < 0.05$, by one-way ANOVA.

#Compared to total density in control membranes (NT_H + NT_L B_{max} values in control membranes).

¶Receptor subtypes were not distinguishable by Scatchard analysis.

**Compared to control NT_H values in the presence of levocabastine (no treatment).

for the indicated periods were provided water and lab chow ad lib.

Tissue Preparation

Animals were sacrificed by cervical dislocation and decapitated and the brains were removed and for NT binding rapidly chilled in cold (4°C) 50 mM Tris, pH 7.4, containing 40 mg/l bacitracin and 1 mM EDTA (buffer 1). A typical experiment involved the use of 5–10 mice from each line. Brain regions were quickly dissected using mouse brain atlas guidelines (49) and a modification of a previously described method (22) and homogenized in 10 vol buffer 1. The homogenate was centrifuged at 500×g for 10 min and the resulting pellet was resuspended by homogenizing in the original volume of buffer and centrifuged for 10 min at 500×g. The pellet was discarded

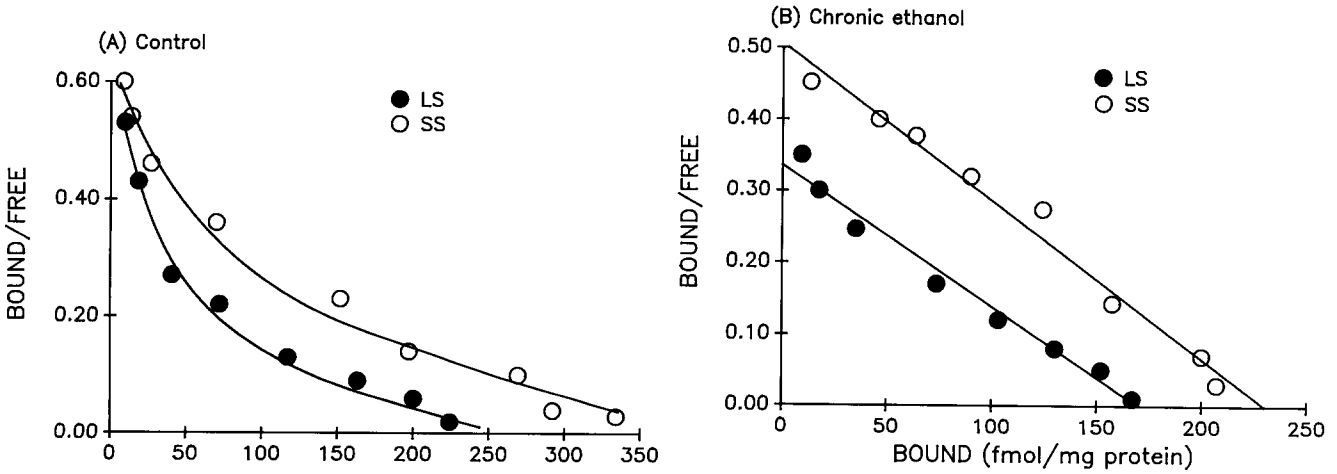

FIG. 2. Saturation binding (Scatchard analysis) of [^{3}H]neurotensin (NT) in entorhinal cortex (RC) membranes from control (A) and chronic ethanol-treated (B) long-sleep (LS) and short-sleep (SS) mice. Membranes from the entorhinal cortex were prepared as described in the text and incubated for 20 min with concentrations of [^{3}H]NT from 0.02–20 nM. Chronic ethanol-treated animals received ethanol in their drinking water (v/v, 10% for 4 days and 15% for 10 days). Typical experiments shown. Data were analyzed by the linear/nonlinear least squares regression analysis program LIGAND.

TABLE 2

EFFECTS OF CHRONIC ETHANOL ON NT RECEPTOR BINDING CHARACTERISTICS IN ENTORHINAL CORTEX (RC) MEMBRANES OF LS AND SS MICE

Line	Brain Region	Treatment	Levocabastine	Receptor Subtype	K_d (nM)	B_{max} (fmol/mg protein)
LS	RC	None	−	NT_H	0.30 ± 0.16	86.72 ± 6.30*†
LS	RC	None	−	NT_L	2.38 ± 0.52	210.01 ± 12.90*†
LS	RC	None	+	NT_H‡	0.47 ± 0.05	112.77 ± 4.28
LS	RC	Ethanol	−	§	0.76 ± 0.08	176.70 ± 12.76†¶
LS	RC	Ethanol	+	NT_H‡	0.50 ± 0.04	179.91 ± 17.34#**
SS	RC	None	−	NT_H	0.29 ± 0.10	131.42 ± 11.04
SS	RC	None	−	NT_L	3.41 ± 1.25	266.36 ± 17.80
SS	RC	None	+	NT_H‡	0.49 ± 0.08	131.52 ± 5.89
SS	RC	Ethanol	−	§	0.78 ± 0.05	212.30 ± 14.50†¶
SS	RC	Ethanol	+	NT_H‡	0.46 ± 0.07	150.47 ± 9.21††

Values were determined by Scatchard analysis as described in the Method section, and represent the mean ± SEM of 4–10 experiments. All data were analyzed by the linear/nonlinear least squares regression analysis program EBDA/LIGAND to determine K_d and B_{max} values. Comparisons were made by ANOVA to assess between- and within-subjects differences.

*Compared to corresponding SS value.

†$p < 0.01$, by one-way ANOVA.

‡Represents levocabastine – insensitive receptors (putatively NT_H).

§Receptor subtypes were not distinguishable by Scatchard analysis.

#Compared to control NT_H values in the presence of levocabastine (no treatment).

¶Compared to total density in control membranes (NT_H + NT_L B_{max} values in control membranes).

††Compared to value in the absence of levocabastine.

**$p < 0.05$, by one-way ANOVA.

and the combined supernatant liquids were centrifuged at 100,000 × *g* for 30 min. The resultant membrane pellet was washed by rehomogenization in 5 ml 50 mM Tris buffer, pH 7.4, containing 40 mg/l bacitracin with no EDTA (buffer 2). This procedure was repeated twice, and the final pellet was resuspended in a volume of buffer 2 to yield a protein concentration of 5 mg/ml [as determined by the method of Lowry (34); each assay tube contained 250 *v*g membrane protein].

Preparation of membranes for binding of the dopamine D_1 receptor antagonist [^{3}H]SCH23390 and the dopamine D_1 antagonist [^{125}I]epidepride were as above using 50 mM Tris buffer with 120 mM NaCl (buffer 4) used throughout the preparation (43).

Binding Assays

Neurotensin binding assays were performed essentially as described previously (31). Binding mixtures containing 250 μg membrane protein, 0.5 mM 1,10-phenanthroline, 50 mM Tris buffer, pH 7.4, containing 0.2% BSA (buffer 3) and various concentrations of levocabastine or NT fragments as indicated in the figure legends, in a final volume of 0.1 ml, were incubated at 25°C for 3 min. [^{3}H]NT was added to each tube and incubated at room temperature for 20 min. After incubation, 0.9 ml cold (4°C) buffer 3 was quickly added and the suspension was rapidly filtered under reduced pressure on Whatman GF/B glass fiber filters, which were presoaked for 3 h in 0.2% polyethylenimine to minimize nonspecific binding of labeled NT. The filters were washed two times with 2 ml each of cold (4°C) buffer 3 and were placed in vials containing 4 ml liquid scintillation cocktail. Radioactivity was determined by a Beckman L3-3133P scintillation counter. Nonspecific binding was determined as the radioactivity bound in the presence of 1 μM unlabeled NT_{1-13}, and specific binding was determined by subtracting nonspecific from the total bound radioactivity. Scatchard analyses were performed as above with increasing concentrations of [^{3}H]NT (0.02–20 nM) with and without 50 μM levocabastine, as indicated.

Saturation binding experiments of [^{3}H]SCH23390 were performed essentially as described elsewhere (44). Binding mixtures containing 250 μg membrane protein, buffer 4, and various concentrations of [^{3}H]SCH23390 (10 pM–10 nM), in a final volume of 100 μl, were incubated for 120 min at 22°C and rapidly filtered and washed as above. Nonspecific binding was determined by binding in the presence of 1 μM (+)-butaclamol.

[^{125}I]Epidepride binding parameters were obtained as described above for [^{3}H]SCH23390. Concentrations of [^{125}I]epidepride ranged from 3 pM–1 nM, and the final volume was 100 ml. Nonspecific binding was determined by binding in the presence of 1 μM (+)-butaclamol.

Data for all saturation binding experiments were analyzed by use of the linear/nonlinear regression analysis program EBDA/LIGAND (41).

Competition curves were obtained with 20 nM [^{3}H]NT incubated with concentrations of levocabastine from 1 nM–100 mM included in the assay tubes. Competition with [^{3}H]NT binding by various fragments and analogs of NT (NT_{1-11}, NT_{1-8}, NT_{8-13}, NT_{1-13}, or Neuromedin N) were performed similarly with concentrations of fragments from 1 nM–10 mM as indicated in the legends.

RESULTS

Equilibrium Binding of [^{3}H]NT in Membranes From Control vs. Chronic Ethanol-treated LS and SS Mice

Equilibrium binding (Scatchard plot) of [^{3}H]NT binding in MB membranes from LS and SS mice (Fig. 1A) reveals that

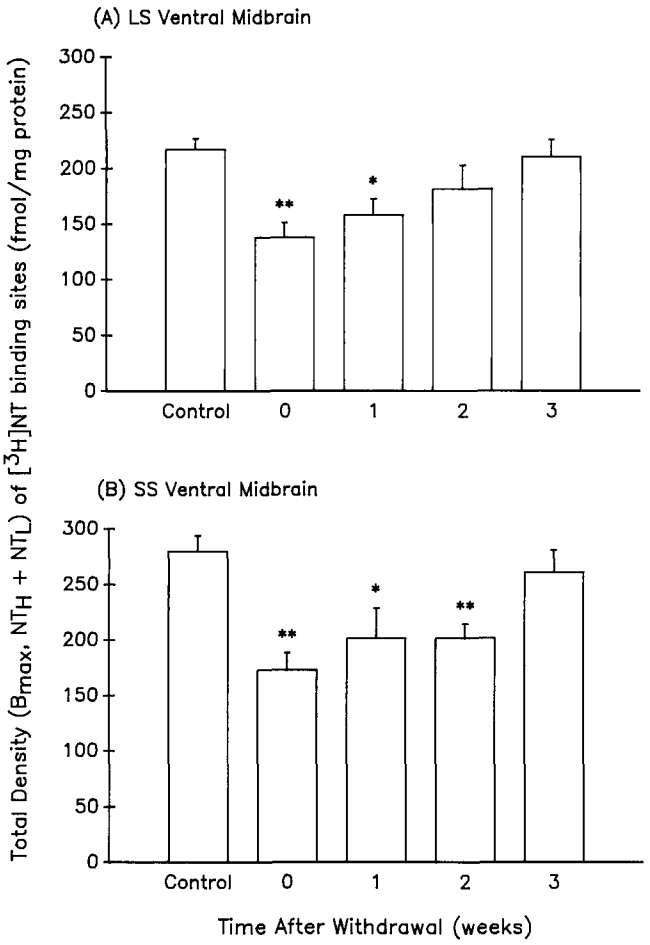

FIG. 3. Time course of the effects of chronic ethanol treatment and withdrawal on the total density of receptors in the ventral midbrain (MB) from long-sleep (LS) (A) and short-sleep (SS) (B) mice. Animals received ethanol in their drinking water for 2 weeks (0 weeks withdrawn) and were withdrawn for the indicated times. The total density of receptors was determined by Scatchard analysis as described in Figs. 1 and 2. Values represent the mean ± SEM of 4–10 experiments.

the binding is best described by a two-site model, with high- (NT_H) and low (NT_L)-affinity components. The K_d and B_{max} values can be seen in Table 1. The two lines differed in B_{max} values for NT_H, $F(1, 8) = 23.8$, $p < 0.01$, as reported previously (3). There were no differences between lines in K_d or B_{max} values for NT_L in the MB.

Figure 2A displays a Scatchard plot of [^{3}H]NT binding in RC membranes from LS and SS mice. Again, the binding isotherms were best described by two separate sites, with no differences in K_d values (Table 2) for NT_H or NT_L between the two lines. In the RC, B_{max} values were different between LS and SS mice for both NT_H, $F(1, 8) = 10.65$, $p < 0.05$, and NT_L, $F(1, 8) = 5.97$, $p < 0.05$.

Chronic ethanol treatment changed markedly the characteristics of equilibrium [^{3}H]NT binding. The binding in membranes from chronic ethanol-treated animals (Figs. 1B and 2B) was best described by a linear model (one site) for both LS and SS in the MB and RC. In the MB and RC, LS and SS (chronic ethanol treated) did not differ significantly in K_d or B_{max} values for the one identifiable binding site (Tables 1 and 2). The total density of receptors were significantly lower in membranes from chronically treated animals as compared to controls (NT_H + NT_L). For LS membranes, both the MB [217.3 ± 17.18 vs. 137.7 ± 13.43 fmol/mg pro, $F(1, 12) = 11.67$, $p < 0.01$] and RC [296.7 ± 16.55 vs. 176.7 ± 12.76 fmol/mg pro, $F(1, 9) = 35.55$, $p < 0.001$] showed a reduction in B_{max} values for total binding site density. Similarly, SS membranes revealed a highly significant reduction in binding site density [MB: 301.48 ± 17.05 vs. 173.1 ± 15.33 fmol/mg pro, $F(1, 11) = 26.38$, $p < 0.001$; RC: 398.8 ± 15.95 vs. 212.3 ± 14.47 fmol/mg pro, $F(1, 9) = 74.91$, $p < 0.0001$] after 2 weeks of chronic ethanol treatment.

The time course for effects of chronic ethanol on NT receptor systems in the MB can be seen in Fig. 3 (although not shown, the RC gave similar results). The total density of binding sites was significantly reduced after 2 weeks of chronic ethanol treatment in both lines of mice. The conversion of [^{3}H]NT binding from a nonlinear, two-site, model to a linear, one-site, model started to appear after 1 week (not shown) and had reached a maximum after 2 weeks (0 weeks withdrawn in Fig. 3). Binding characteristics at 3 and 4 weeks of chronic ethanol treatment were not different from 2 weeks (results not shown). At 1 and 2 weeks after withdrawal from ethanol, Scatchard analysis of equilibrium binding remained linear; however, after 3 weeks the binding parameters had returned to control values (data not shown). As can be seen in Fig. 3, the binding site density returned to control values within 3 weeks of withdrawal.

The competition curve shown in Fig. 4 reveals that the specificity for binding to frontal cortex membranes from chronically treated animals does not change (control not shown). The values obtained from these experiments are in good agreement with those previously published for untreated animals (3). NT_{8-13} and NT_{1-13} were equipotent in competing for binding with 20 nM [^{3}H]NT. NT_{1-11} and NT_{1-8} (not shown) were ineffective at competing for binding. Levocabastine inhibited approximately 50% of the binding at 1 μM, and neuromedin N was significantly less potent than NT_{1-13} or NT_{8-13}.

Effects of Levocabastine on [^{3}H]NT Binding

Binding in the presence of levocabastine has been shown to selectively inhibit NT binding to the NT_L form of the receptor (3,47). To determine which receptor subtype(s) might be changing upon chronic ethanol administration, Scatchard analysis in the presence of 50 μM levocabastine was performed on membranes from chronic ethanol-treated animals (Table 1). The K_d values were not different from those obtained for NT_H in control membranes; however, the B_{max} values were different. Levocabastine sensitivity remained in membranes from treated animals; thus, it was of interest to utilize this compound to characterize the effects of chronic ethanol treatment on levocabastine-sensitive and -insensitive binding sites in various regions.

Characterization of Chronic Ethanol Effects On Regional [^{3}H]NT Binding to Receptor Subtypes

Because NT_L (putative) receptor binding remained sensitive to levocabastine, binding in the presence of 50 μM levocabastine (20 nM [^{3}H]NT) reveals levocabastine-insensitive or NT_H (putative) binding sites (Fig. 5). In LS membranes, significant reductions in the caudate putamen (CP), hippocampus (HC), hypothalamus (HT), nucleus accumbens (NA), and RC were seen after 2 weeks of chronic ethanol treatment. Significance

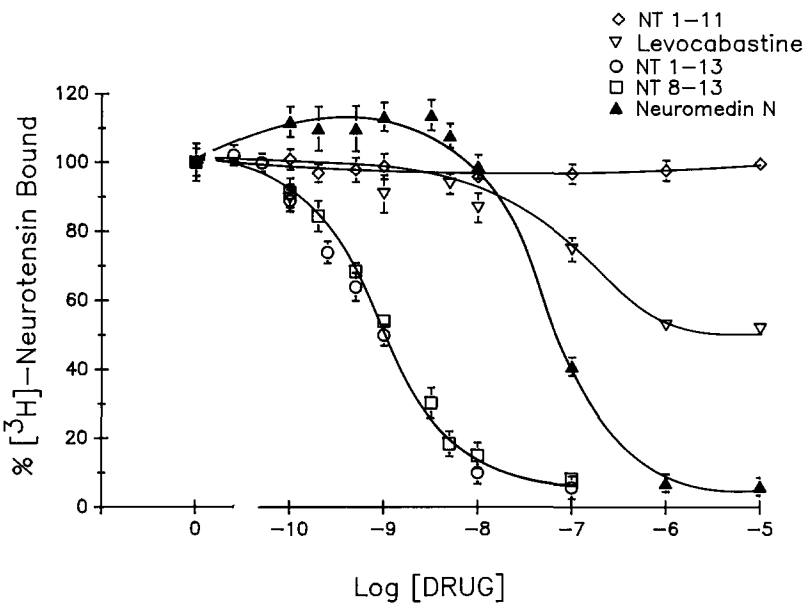

FIG. 4. Competition of various ligands and analogs of neurotensin (NT) for [^{3}H]NT binding in long-sleep mice (LS) frontal cortex membranes from chronic ethanol-treated animals. Animals were treated with ethanol as described in Fig. 1 (2 weeks) and equilibrium binding was performed with 20 nM [^{3}H]NT, as described in the text. Values represent the mean ± SEM of four separate experiments.

was determined by ANOVA followed by the Dunn (Bonferroni) multiple comparison test ($p < 0.01$). Chronic ethanol treatment tended to cause a reduction of levocabastine-insensitive binding sites in all regions of LS brain membranes, with the NA most affected: Binding in the NA was reduced by 68%. Similar reductions in NT binding were found in SS mice, with a decrease of 59% in the NA.

By subtracting binding in the presence of levocabastine from total binding (in the presence of 20 nM [^{3}H]NT, not shown), levocabastine-sensitive or NT_L (putative) sites can be determined (Fig. 6). In the MB of LS mice, a significant reduction was observed. Interestingly, the NA showed an increase in these binding sites after chronic ethanol. The results show NT_L sites in SS membranes to be reduced in the CP and MB.

Figure 7 shows the time course of the changes in levocabastine-insensitive (putative NT_H) binding sites in the NA of both LS and SS mice. One to 2 weeks after withdrawal from ethanol, the receptor density returned to control values. Similar time courses for return to control levels were observed for total, levocabastine-sensitive, and levocabastine-insensitive binding sites in membranes from all affected brain regions for both lines (data not shown).

Acute Ethanol Effects on NT Binding Sites

Table 3 reveals that no changes were produced by acute ethanol treatment except a significant decrease in NT_H receptor sites in the RC was observed in LS membranes. There was, however, a general decrease in the density of both receptor sites in both lines for both the MB and RC.

Equilibrium Binding of [^{3}H]SCH23390 and [^{125}I]Epidepride in Membranes from Control vs. Chronic Ethanol-treated LS and SS Mice

As Table 4 shows, there were no effects of chronic ethanol treatment on binding of the D_1 receptor antagonist, SCH23390, in MB and RC membranes. Data in the table are from naive, control mice, and mice chronically treated with ethanol for 2 weeks. The binding characteristics of the high-affinity D_2 antagonist [^{125}I]epidepride were also unchanged after chronic ethanol treatment. There were also no differences between LS and SS brain membranes in K_d or B_{max} values for either dopamine receptor subtype.

DISCUSSION

The finding that LS and SS brain membranes contain two forms of the NT receptor replicates previous observations (3) and is consistent with results in other species, including the rat (30,44), guinea pig (44), and bovine (40). Similarly, the saturability, uneven distribution, and high affinity support a physiological role for both receptor subtypes. Previous studies in this laboratory have shown that SS MB membranes contain higher densities of NT_{total}, NT_H, and NT_L receptors than LS membranes (3). It was suggested that this difference may account for the difference in locomotor activity observed upon administration of NT into the ventral tegmental area, where SS showed a greater increase in locomotor activity than LS (13). Likewise, acute low doses of ethanol produce a greater increase in locomotor activity in SS than in LS, and it has been proposed that the differences in sensitivity to ethanol may be due, in part, to differences in NT receptors (16). Therefore, it was of interest to investigate the effects of acute and chronic ethanol administration on MB NT receptors, and because the RC has been shown to contain high densities of receptors (3,33) and contain terminal projections of MB DA neurons (20) this region was included in the present study.

Previous studies have shown that the paradigm of chronic ethanol consumption used in the present study produced marked tolerance to ethanol-induced locomotor inhibition in LS and SS mice (18). The results reported in the present study show that chronic ethanol treatment changes brain NT recep-

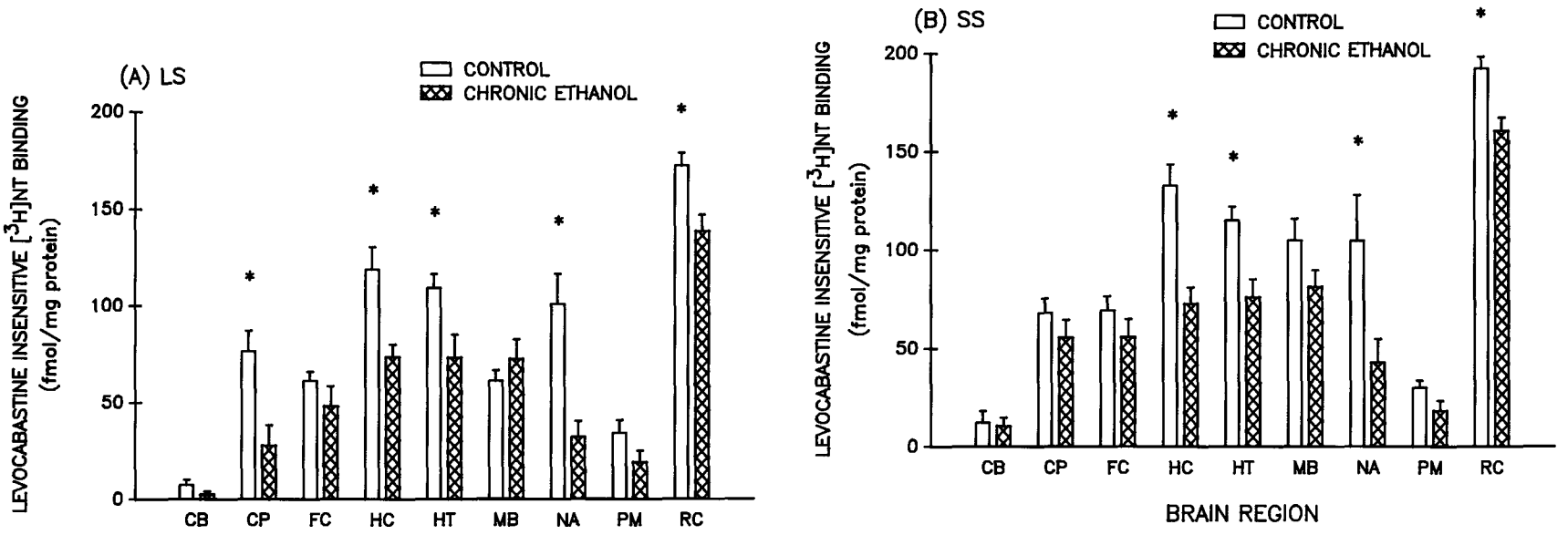

FIG. 5. Levocabastine-insensitive [^{3}H]neurotensin (NT) binding sites in membranes from nine brain regions of control and chronic ethanol-treated animals in long-sleep (LS) (A) and short-sleep (SS) (B) mice. Membranes were incubated with 20 nM [^{3}H]NT for 20 min at room temperature in the presence of 50 mM levocabastine. Brain regions were dissected as described in the Method section, and each value represents the mean ± SEM of six to eight experiments. Each experiment pooled tissue from five animals (each line). Significance was determined by ANOVA followed by a Dunn (Bonferroni) multiple comparison test. *$p < 0.01$.

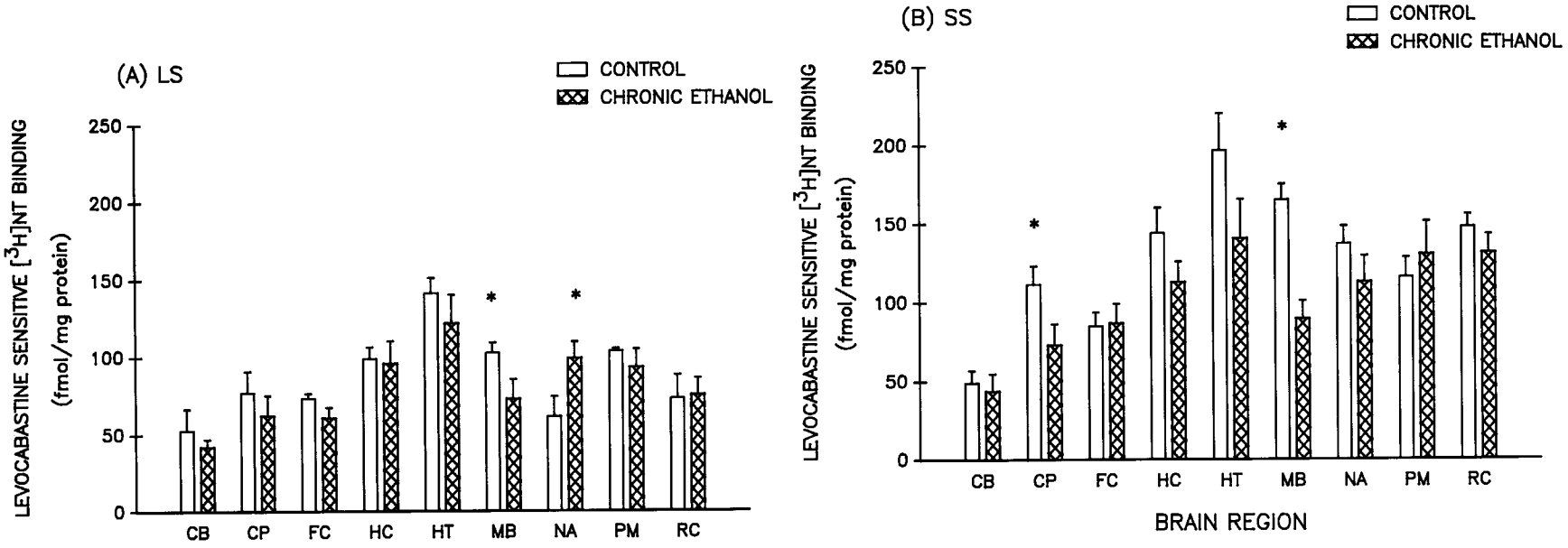

FIG. 6. Levocabastine-sensitive [^{3}H]neurotensin (NT) binding sites in membranes from nine brain regions of control and chronic ethanol-treated animals in long-sleep (LS) (A) and short-sleep (SS) (B) mice. Membranes were incubated with 20 nM [^{3}H]NT for 20 min at room temperature in the presence of 50 mM levocabastine. Values were determined by subtracting the amount bound in the presence or absence (total) of levocabastine from the total amount bound. Brain regions were dissected as described in the Method section, and each value represents the mean ± SEM of six to eight experiments. Each experiment pooled tissue from five animals (each line). Significance was determined by ANOVA followed by a Dunn (Bonferroni) multiple comparison test. *$p < 0.01$.

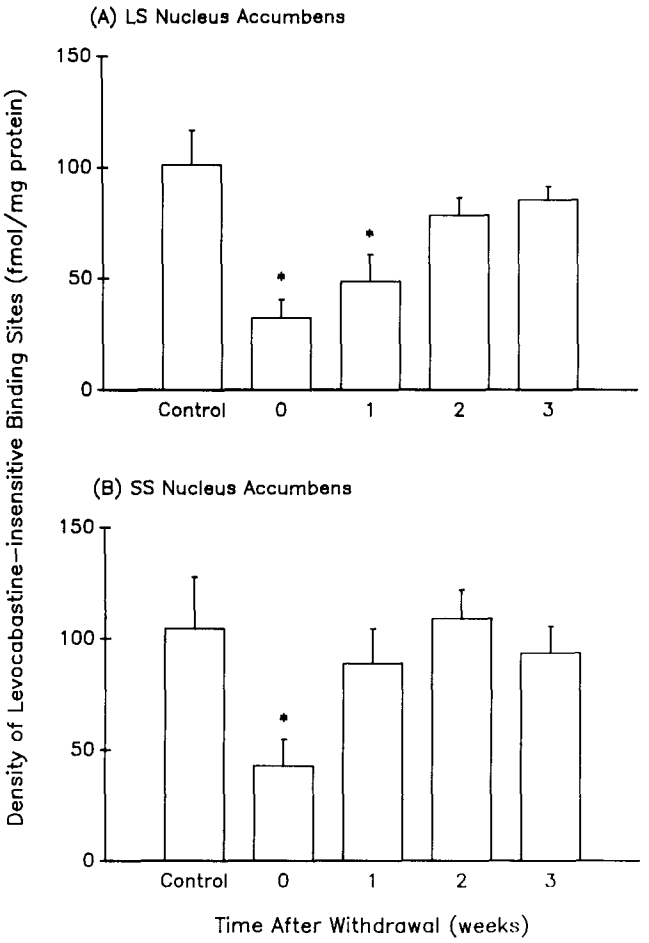

FIG. 7. Time course of the effects of chronic ethanol treatment and withdrawal on the density of levocabastine-insensitive (putatively NT_H) receptors in the nucleus accumbens (NA) from long-sleep (LS) (A) and short-sleep (SS) (B) mice. Animals received ethanol in their drinking water for 2 weeks (0 weeks withdrawn) and were withdrawn for the indicated times. The total density of receptors was determined by binding of 20 nM [^{3}H]neurotensin (NT) in the presence of 50 mM levocabastine as described in Fig. 5. Values represent the mean ± SEM of 4–10 experiments.

tor binding such that only one receptor subtype of intermediate affinity and density is detectable by Scatchard analysis. Whether NT_H, NT_L, or both subtypes change in response to chronic ethanol administration cannot be determined from these saturation studies. However, results show that membranes from chronically treated animals retained sensitivity to levocabastine inhibition of [^{3}H]NT binding, suggesting that both NT_H and NT_L receptor subtypes may be affected. It appears that the MB and RC differ in the degree to which chronic ethanol treatment affects the levocabastine-sensitive and -insensitive receptor subtypes (Tables 1 and 2). [^{3}H]NT binding to both MB and RC membranes from chronic ethanol-treated animals in the presence of levocabastine revealed K_d values that are not different from the K_d values for NT_H calculated from LIGAND analysis of the two-site model in control membranes. However, the B_{max} values obtained from MB membranes (chronic) in the presence of levocabastine suggest that both receptor subtypes are changed in this region after 2 weeks of chronic ethanol treatment. The treatment affected RC membranes differently, as it appears that the levocabastine-sensitive (putatively NT_L) receptor is primarily affected in this region. The reason for this difference remains obscure.

The time course for changes in NT binding characteristics closely parallels the development of behavioral tolerance to acute ethanol (18) with maximum tolerance and NT receptor changes being observed after 2 weeks of chronic ethanol administration. Similarly, the time to return of control values for NT binding parameters (ca. 2–3 weeks, Fig. 3) is in agreement with the return to control behavioral responses to acute ethanol administration (18). Alterations in NT binding was not genotype dependent in that LS and SS were similarly affected. It is of interest that the effects of chronic ethanol consumption in these lines of mice produced similar alterations in behavioral responses to challenge doses of ethanol.

It was important to determine whether chronic ethanol administration produced alterations in NT receptor selectivity for NT fragments and analogs, whether the changes in NT receptors generalized to other receptors, and whether NT receptor downregulation was evident in various brain regions. The data in Fig. 4 indicate that chronic ethanol consumption did not alter selectivity to various ligands and analogs. The requirement of the C-terminus for binding and biological activity has been well established (5,24), and the results show that relatively high affinity was maintained for NT_{8-13} and neuromedin N but not for NT_{1-8}. The unique and selective downregulation of NT receptors was demonstrated by showing that neither D_1 nor D_1 receptor binding characteristics were altered by chronic ethanol administration. These receptors were investigated because of the well-known association between neurotensinergic processes and dopaminergic systems (1,42).

Because it was not practical to perform saturation binding and Scatchard analyses on a large number of brain regions, the relative binding densities of NT receptors were measured at 20 nM [^{3}H]NT in the absence and presence of levocabastine to estimate NT_L and NT_H as described in the Method section. As shown in the Results section, it is of interest that chronic ethanol administration produced significant reductions in NT_L and NT_H in selected brain regions with NT_H altered in a larger number of regions than NT_L in both LS and SS mice. In general, it appears that chronic ethanol administration produced a downregulation of NT receptors in the hypothalamus and in mesolimbic, striatal, and cortical regions that are thought to be associated with motor activities, reinforcing behaviors, and thermoregulation induced by ethanol and NT administration (8).

Acute ethanol has been shown to produce a rapid decrease in NT levels in hypothalamus and mesolimbic brain regions of LS and SS mice (14). It was suggested that these in vivo effects of ethanol may be mediated by enhanced release and degradation. The present findings that acute ethanol causes a general decrease in NT receptor sites is consistent with a downregulation of NT receptors after occupancy by NT. The mechanism of this receptor downregulation is unknown; possibilities include internalization, second messenger uncoupling, or some other desensitization mechanism. Indeed, several groups (21,37) have recently shown that NT is rapidly internalized following occupation of the NT receptor in cell cultures. The finding that low doses, but not high doses, of ethanol cause changes in NT levels (14) is consistent with enhanced release of NT being the mechanism of chronic ethanol-induced changes in NT receptor density. The paradigm of

TABLE 3

THE EFFECTS OF ACUTE ETHANOL TREATMENT ON NT RECEPTOR CHARACTERISTICS

Line	Brain Region	Treatment	K_d High	B_{max} High	K_d Low	B_{max} Low
LS	MB	Saline	0.12 ± 0.01	22.28 ± 4.60	2.27 ± 0.24	204.67 ± 25.17
	MB	Ethanol	0.11 ± 0.03	24.25 ± 5.86	2.41 ± 0.37	181.40 ± 12.44
	RC	Saline	0.20 ± 0.02	89.87 ± 6.56	1.68 ± 0.31	222.32 ± 16.95
	RC	Ethanol	0.14 ± 0.02	63.15 ± 8.12 *	2.36 ± 0.49	204.29 ± 27.24
SS	MB	Saline	0.23 ± 0.08	61.32 ± 16.95	5.01 ± 1.82	216.38 ± 10.13
	MB	Ethanol	0.16 ± 0.06	42.16 ± 12.56	3.78 ± 1.23	192.41 ± 14.91
	RC	Saline	0.25 ± 0.06	106.75 ± 10.10	2.34 ± 0.39	249.02 ± 36.96
	RC	Ethanol	0.15 ± 0.04	74.37 ± 20.38	4.07 ± 1.69	252.32 ± 21.34

LS and SS mice were injected with ethanol, 3.0 g/kg, IP, and 2 h later prepared for receptor binding as described in the Method section. All data were analyzed by the linear/nonlinear least squares regression analysis program, EBDA/LIGAND to determine K_d and B_{max} values. K_d values are in nM and B_{max} values are in fmol/mg protein.

*Significantly different from saline treatment, as determined by one-way ANOVA ($p < 0.05$).

chronic ethanol administration used in the present study involves consumption of low doses of ethanol over a long period of time (18), and this is hypothesized to account for the changes seen in NT receptors. Studies ongoing in our laboratory have shown that chronic ethanol causes an increase in NT in all regions studied (12). Whether these increases are due to chronic increases in NT release and turnover or to inhibition of degradation is unknown. However, if ethanol increases NT release then chronically elevated concentrations of NT in the extracellular spaces might lead to a downregulation of NT receptors. The present study supports this hypothesis.

Another possible mechanism by which chronic ethanol might alter NT binding characteristics involves the well-known membrane fluidizing effects of ethanol (25,36). Chronic ethanol has been shown to alter neuronal membranes by making them less readily "fluidized" by temperature or ethanol (36); and, it has been suggested that such an effect might account for chronic tolerance to ethanol. Chronic ethanol administration has been shown to alter neuronal membrane protein phosphorylation in the presence of calcium and calmodulin (47). At present, it is unknown whether NT receptors are phosphorylated and whether such phosphorylation might alter NT bind-

TABLE 4

EFFECTS OF CHRONIC ETHANOL ON DOPAMINE D_1 AND D_2 RECEPTOR SUBTYPE BINDING IN MEMBRANES FROM LS AND SS MICE

Line	Brain Region	Treatment	Receptor	K_d (pM)	B_{max} (fmol/mg protein)
LS	MB	None	D_1	209 ± 16	149.44 ± 11.55
LS	MB	Chronic ethanol		244 ± 18	142.43 ± 9.21
LS	MB	None	D_2	69.68 ± 19.23	47.03 ± 13.17
LS	MB	Chronic ethanol		50.08 ± 11.45	74.43 ± 14.75
LS	RC	None	D_1	218 ± 21	86.19 ± 9.25
LS	RC	Chronic ethanol		275 ± 28	91.95 ± 3.98
LS	RC	None	D_2	19.31 ± 5.73	5.91 ± 2.25
LS	RC	Chronic ethanol		20.85 ± 2.34	11.79 ± 3.23
SS	MB	None	D_1	245 ± 60	124.18 ± 7.61
SS	MB	Chronic ethanol		229 ± 19	141.97 ± 2.13
SS	MB	None	D_2	62.26 ± 17.13	42.06 ± 10.51
SS	MB	Chronic ethanol		48.33 ± 5.14	74.05 ± 10.65
SS	RC	None	D_1	358 ± 51	86.29 ± 10.94
SS	RC	Chronic ethanol		273 ± 20	90.42 ± 5.6
SS	RC	None	D_2	34.52 ± 17.36	8.58 ± 3.89
SS	RC	Chronic Ethanol		16.00 ± 3.13	11.76 ± 0.70

Ventral midbrain (MB) or entorhinal cortex (RC) membranes from control or 2-week chronic ethanol-treated animals were prepared and binding of [^{3}H]SCH23390 (D_1) or [^{125}I]epidepride (D_2) was performed as described in the Method section. K_d and B_{max} values represent mean ± SEM of four separate experiments. All data were analyzed by the linear/nonlinear least squares regression analysis program, EBDA/LIGAND to determine K_d and B_{max} values. Comparisons between lines and treatments was performed by one-way ANOVA, and no significant differences were found

ing characteristics. Because either of these possible mechanisms would be expected to cause heterologous receptor effects, and as indicated in Table 4 and as noted above the changes in NT receptors are selective, it is possible that the NT receptor system fits into the "membrane domain" hypothesis (51), in which selective portions of the neuronal membrane are sensitive to the fluidizing properties of ethanol.

Although LS and SS mice may not be ideal models for the study of ethanol tolerance, the finding that chronic ethanol markedly affects [^{3}H]NT binding in LS and SS mice and that these effects closely parallel the rates of acquisition and decay of behavioral tolerance support the hypothesis that some of the effects of ethanol may be mediated by neurotensinergic processes. For example, if ethanol-induced hypothermia or locomotor inhibition were mediated, in part, by actions via NT receptors then downregulation of NT receptors would be expected to attenuate these behavioral effects of ethanol. Additionally, because acute ethanol was presently shown to cause general decreases in NT receptor subtype density and this same treatment has been shown to cause rapid decreases in NT levels consistent with enhanced peptide release, it is possible that the mechanism of chronic ethanol-induced changes in NT receptor density is due to continuous enhanced release of NT.

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