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Cross-Tolerance Between Ethanol and Neurotensin in Mice Selectively Bred for Ethanol Sensitivity

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ERWIN, V. G., A. D. CAMPBELL, R. MYERS AND D. E. WOMER. *Cross-tolerance between ethanol and neurotensin in mice selectively bred for ethanol sensitivity.* PHARMACOL BIOCHEM BEHAV **51**(4) 891-899, 1995. – Neurotensin (NT), a tridecapeptide that satisfies criteria as a neurotransmitter, mimics many actions of ethanol, and evidence indicates that some of the acute effects of ethanol are mediated in part by NT. Recent studies have shown that chronic ethanol treatment produced a downregulation of NT receptors in mesolimbic brain regions of long sleep (LS) mice and that reduced NT binding capacity was associated with acquisition and decay of tolerance to ethanol-induced locomotor inhibition and hypothermia in these mice. The present study was undertaken to determine whether cross-tolerance develops between NT and ethanol and whether chronic NT infusion produces NT receptor downregulation. Animals chronically treated with ethanol were tolerant to NT-mediated locomotor inhibition at a dose of 1.8 pmol NT, ICV, and were tolerant to NT-induced hypothermia at 1.8 and 6.0 pmol NT. Following repeated injections or continuous infusion of NT ICV, LS mice showed tolerance to both NT- and ethanol-induced hypothermia and locomotor inhibition. Indeed, ethanol doses that are hypnotic in control mice (2.8 g/kg) were not effective in abolishing locomotor activity following chronic NT administration. Results with chronic saline infusion ICV indicate that stress alters sensitivity to ethanol-induced hypothermia. Chronic infusion of NT ICV produced a region-specific downregulation of high-affinity NT receptors in the striatum. The results demonstrate that cross-tolerance develops between NT and ethanol, and further support a role for neurotensinergic systems in the actions of ethanol.

Neurotensin Ethanol LS mice Cross-tolerance Locomotor activity Hypothermia

IDENTIFICATION of mechanisms mediating genetic-based differences in ethanol sensitivity has been the goal of much research conducted with the long-sleep (LS) and short-sleep (SS) mice. These lines of mice were selectively bred from a genetically heterogeneous stock (HS/Ibg) of mice for their differences in duration of loss of the righting response following acute ethanol administration (19,30). Current generations of these lines differ not only in hypnotic sensitivity to ethanol, but also in ethanol-induced hypothermia and low-dose effects of ethanol (8,17). For example, these lines differ markedly in locomotor responses to subhypnotic doses (1.0-2.5 g/kg) of ethanol (i.e., LS are more sensitive than SS to locomotor inhibition but SS are more sensitive to locomotor activation than LS). Quantitative genetic studies indicate that differences in ethanol sensitivity are mediated by multiple genes with additive effects, and genetic correlations among ethanol-related behaviors in LS × SS recombinant inbred (RI) strains show some overlap in those genes mediating differences in hypnotic and locomotor actions of ethanol, respectively (11,14). These results are consistent with reported differences in several neurotransmitter systems (5), including neurotensin (NT), in brain regions from LS and SS mice (2).

Challenge doses of ethanol produce widely divergent responses in LS and SS mice following chronic ethanol administration (16). For example, it has been shown that chronic ethanol consumption for 2 weeks produces tolerance to ethanol-induced (2.5 g/kg) locomotor inhibition in LS mice and an enhanced locomotor activation (sensitization) at this dose

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in SS mice. These results are consistent with the studies of others indicating that acute, subhypnotic doses of ethanol produce a genotype-dependent locomotor activation (e.g., in DBA/2J mice) or inhibition (e.g., in C57BL/6J mice), and that ethanol administration produces tolerance (in C57BL) or sensitization (in DBA) to ethanol in a genotype-dependent manner (4).

Neurotensin, a tridecapeptide, *p*-Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu (3), is widely distributed in mammalian brain; the studies of Frye et al. (18) and Luttinger et al. (28) first implicated NT in the actions of ethanol. These authors showed that NT potentiated ethanol-induced sedation, hypothermia, and impairment of the aerial righting reflex. These findings were extended by Erwin et al. (15,17), who showed that NT selectively increased ethanol sensitivity in SS and LS lines of mice. The SS mice were more sensitive than LS to NT potentiation of hypnotic, locomotor, and hypothermic effects of ethanol. These observations suggested differences in neurotensinergic processes in LS and SS mice.

Previous studies (12) have shown that NT levels are higher in specific brain regions from LS than from SS mice and that ethanol in vivo produced a rapid, dose-dependent decrease in NT levels in hypothalamus and striatum of LS mice. In addition, specific brain regions from LS and SS differed in NT receptor densities (2). Equilibrium binding and Scatchard analysis revealed two specific, saturable, and reversible ^{3}H-NT binding sites [high-affinity (NTH) and low-affinity (NTL)] in brain regions of LS and SS mice (2). Membranes from frontal cortex and ventral midbrain of SS mice contained higher densities of both receptor subtypes than these regions from LS mice (1,2). This difference in NT receptor density might account for the finding (13) that SS mice displayed greater locomotor activation than LS mice following microinjections of NT into the ventral tegmental area (VTA). Moreover, genetic correlations, in a panel of 24 LS × SS recombinant inbred strains, showed significant associations among NT receptor densities in frontal and entorhinal cortex and hypnotic and locomotor effects of ethanol (14).

Administration of NT into the nucleus accumbens (NA) or the VTA has been shown to modulate dopamine function in these brain regions (21,22). For example, administration of NT into the VTA produces locomotor activation concomitant with increased dopamine (DA) release in the NA, and NT injections into the NA, as well as into the lateral ventricles, produces locomotor inhibition (17). Thus, differential levels of NT or densities of NT receptors in these brain regions might mediate differential effects of ethanol on locomotor inhibition or activation.

Chronic ethanol administration has been shown to alter ^{3}H-NT binding characteristics, primarily by downregulation of high-affinity, levocabastine-insensitive, NT receptors (1). The time course for the reversible decreases in NT receptor densities closely paralleled the acquisition and decline of behavioral tolerance to chronic ethanol (10,16). If ethanol-induced hypothermia or locomotor inhibition/activation were mediated in part by actions via NT receptors (14), then downregulation of these receptors might be expected to attenuate the behavioral effects of ethanol. The present studies were conducted to determine whether NT and ethanol produced a cross-tolerance to locomotor inhibition and hypothermia and whether, like chronic ethanol, chronic NT administration causes a downregulation of NT receptors. The LS mice were used in these studies because of their high sensitivity to ethanol-induced locomotor inhibition and hypothermia, and because these mice readily develop tolerance to ethanol.

METHOD

Animals

Male LS/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 (12L : 12D), and were housed five mice per cage.

Materials

Reagents and chemicals of the highest purity commercially available were obtained as follows: neurotensin$_{1-13}$,Tris hydrochloride, bovine serum albumin (RIA grade), 1,10 phenanthroline, and polyethylenimine (50% aqueous) from Sigma Chemical Company (St. Louis, MO); radiolabeled [3,11-Tyrosyl-3,5-^{3}H(N)] neurotensin$_{1-13}$ (83 Ci/mmol in ethanol) was obtained from New England Nuclear (Boston, MA); EDTA and Scintiverse II were from Fisher Scientific (Fairlawn, NJ); bacitracin (60,000–70,000 U/g) were from P-L Biochemicals, Inc. (Milwaukee, WI); levocabastine was a generous gift from Janssen Pharmaceutica (Beerse, Belgium); the Whatman GF/B filters were from Millipore Corp. (Bedford, MA); and Durelon carboxylate cement was from ESPE (Seefeld, Germany).

Chronic Ethanol Administration

A simple method of chronic ethanol administration, which produced functional tolerance to ethanol-induced locomotor inhibition, was performed as described by Erwin et al. (16). Mice were housed five per cage and were 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. As previously reported (16), the mice consumed approximately 15 g ethanol/kg per 24 h. During the light phase, blood ethanol concentrations (BECs) were as low as 20 mg/dl, and during the dark phase (when mice eat and drink), BECs were as high as 200 mg/dl. Control animals were provided water and laboratory chow ad lib; the previous study (16) showed no difference between water-chow control animals and those receiving chow and equivalent volumes of sucrose in water, made isocaloric with ethanol consumed by the chronically treated mice.

Animal Surgery

Chronic ethanol-treated animals were withdrawn from ethanol for 2 h before surgery. Control (naive) and 2-week chronic ethanol-treated mice were anesthetized with sodium pentobarbital (80 mg/kg) and chloral hydrate (120 mg/kg), IP. After the animal was secured in a Kopf stereotaxic apparatus, holes were drilled in the exposed skull and stainless-steel (26-ga) injection cannula guides were lowered into the lateral ventricles. Animals were fitted with cannula guides unilaterally, and 33-ga obturators were placed in the guides to prevent occlusion. Coordinates for cannula placement were approximately 1 mm lateral to bregma at a depth of 3 mm as per the anatomical guidelines of Slotnick and Leonard (33). The cannula guides were glued to the scalp and secured with dental cement (Durelon carboxylate cement; ESPE). Animals were placed in an incubator set at 32°C until recovery (ca. 2 h) from the anesthesia. Animals were allowed to recover, with food and water available ad lib, for 24 h before use in experiments.

In experiments designed to infuse NT ICV continuously over a 3-day period, mice were fitted with cannula guides

(Brain Infusion Kit; Alza Corp, Palo Alto, CA) into the lateral cerebral ventricle, ICV. Alzet mini-osmotic pumps were filled with either sterile saline vehicle or NT in saline (10 mg/ml) under sterile conditions. The pumps were weighed before and after they were filled to assure proper filling procedures. After inserting a flow moderator into the pump, the complete infusion assembly was incubated in sterile saline at 37°C for 4 h before implantation. The pump flow rate was 1 μl/h. The osmotic pump was then carefully inserted into an SC pocket on the back of the neck of anesthetized animals and the cannulae glued to the skull; the assembly was covered with dental cement and a small loop of tubing was left exposed so that it could easily be cut to terminate NT infusion. A group of animals fitted only with the cannula guides and no pump were also included in this study as sham controls. In some experiments, mice fitted with the pump assembly and cannula guides in the left lateral ventricle were implanted with standard cannula guides in the right ventricle to administer challenge doses of NT. Following surgery the animals were placed in a 32°C incubator until recovery from anesthesia. In experiments with continuous NT infusions, the polyethylene tubes to the flow moderator were cut 2 h before challenge doses of NT or ethanol were administered.

Rectal Temperature and Locomotor Activity

Rectal temperatures were taken with a telethermometer (Bailey Instruments, Saddle Brook, NJ) immediately before saline, NT, or ethanol administration and behavioral testing. Initial temperature was used as an indicator of the animals' health, and mice with temperatures below 36°C were excluded from the experiment. Animals were injected ICV with either 0.9% sterile saline or NT in saline, or IP with saline or ethanol (15% v/v) in saline at the doses indicated in the tables and figures. Injections, 5.0 μl ICV, were made with 33-ga injection needles over a 30-s period.

After initial temperatures were taken, and following ICV or IP injections, animals were immediately placed in Omnitech Activity Monitors (Omnitech Electronics, Inc., Columbus, OH) to measure spontaneous locomotor activity as distance (centimeters) travelled following drug administrations. The activity monitors were enclosed in ventilated boxes, and activity was monitored under reduced lighting at 5-min intervals for 30 min by means of an MS-DOS computer. Rectal temperatures were taken immediately after the 30-min activity measurement. Ambient temperature was maintained at 22–23°C. Activity and temperature measurements were repeated at times indicated in the figures.

Brain Membrane Preparation and NT Receptor Binding

Tissue preparation. Animals were sacrificed by cervical dislocation and decapitated, and the brains were removed and rapidly chilled in cold (4°C) 50 mM Tris, pH 7.4, containing 40 mg/l bacitracin and 1 mM EDTA (buffer 1). Brain regions were quickly dissected using mouse brain atlas guidelines (33) and homogenized in 10–20 vol of 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 and the combined supernatant liquids were centrifuged 100,000 × g for 30 min. The resultant membrane pellet was washed by rehomogenization in 5 ml of 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 et al. (27)]; each assay tube contained 250 μg membrane protein.

Binding assays. Neurotensin binding assays were performed essentially as described by Kitabgi et al. (24). Binding mixtures containing 250 μg membrane protein, 0.5 mM 1,10-phenanthroline, 50 mM Tris buffer, pH 7.4, 0.2% bovine serum albumin (BSA) (buffer 3), and 10 μM levocabastine, a selective inhibitor of low-affinity NT receptors, as indicated in the figure legends, in a final volume of 0.1 ml, were incubated at 25°C for 3 min. ^{3}H-Neurotensin (20 nM) was added to each tube and incubated at room temperature for 20 min, a time found to be sufficient to allow the reaction mixture to reach equilibrium (13). In the presence of 1,10-phenanthroline, NT binding has been shown to reach a maximum after approximately 10 min, and remain constant for 20 min (13). 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 NT1-13, and specific binding was determined by subtracting nonspecific from the total bound radioactivity.

Data Analysis

Statistical analyses were performed with a CRUNCH 4.0 software program. Data from each experiment were analyzed by appropriate analysis of variance (ANOVA) to assess effects of between- and within-subject variables (ethanol dose, NT dose, or treatment condition). followed by Scheffé or *t*-test post hoc tests.

RESULTS

Effects of Chronic Ethanol Treatment on NT-Induced Hypothermia and Locomotor Inhibition

The effects of chronic ethanol treatment on the hypothermic response to ICV NT administration are shown in Fig. 1a. There was a significant ($p < 0.0001$) NT dose effect in both control and chronic ethanol-treated animals [$F(4, 41) = 76.8$ and $F(4, 50) = 27.4$, respectively]. A dose of 1.8 pmol NT produced a significant decrease in rectal temperature in control animals, and chronic ethanol-treated mice were clearly tolerant to NT-induced hypothermia at doses of 1.8 and 6.0 pmol, as indicated by an apparent shift to the right of the log dose–response curve. The dose–response curve for NT-induced hypothermia in chronic ethanol treated animals was biphasic, indicating sensitization to hypothermia at 0.6 pmol NT and tolerance at 1.8 and 6.0 pmol NT.

Neurotensin produced a dose-dependent inhibition ($p < 0.0001$) in locomotor activity (centimeters traveled in 10 min) in both control [$F(4, 38) = 57.9$] and chronic ethanol [$F(4, 51) = 42.8$] groups of LS mice (Fig. 1a). The magnitude of NT-mediated locomotor inhibition, following 0.6–1.8 pmol, ICV, in control mice differed significantly from inhibition in chronic ethanol-treated mice. Chronic ethanol-treated mice displayed a biphasic dose–response curve suggesting sensitization to locomotor inhibition at 0.6 pmol NT and tolerance to locomotor inhibition, at 1.8 pmol NT.

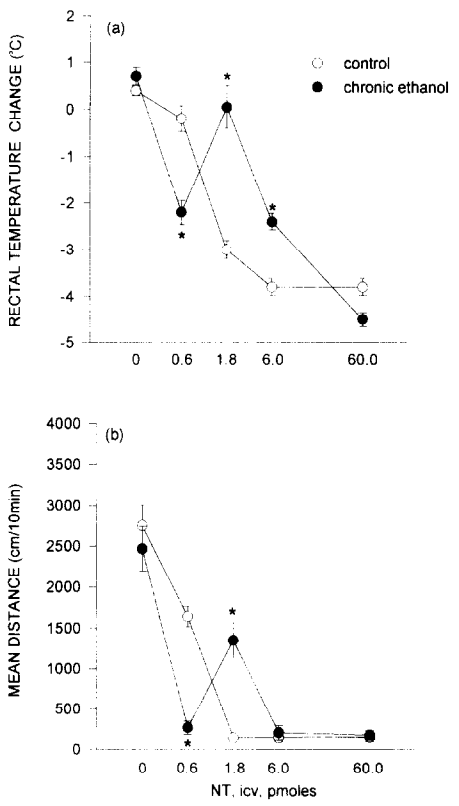

FIG. 1. Dose-response curves for NT-induced hypothermia and inhibition of locomotor activity in control and chronic ethanol-treated LS mice. Male LS mice were required to consume ethanol (ca. 15 g/kg per 24 h) in their drinking water for 2 weeks, as described in Method. Control and chronic ethanol-treated animals were implanted with cannula guides ICV, as described in Method, and allowed to recover for 24 h before testing. Animals received either 5 μl sterile saline (0 NT dose) or NT at doses of 0.6, 1.8, 6.0, or 60.0 pmoles in 5 μl saline. (a) Rectal temperatures were taken immediately before injections (basal values were 37.4 ± 0.4°C) of NT or saline ICV and were taken at 30 min after injections; rectal temperature change was plotted as a function of NT dose. Values represent means with SEM for 6–8 mice per group per NT dose. ANOVA showed significant effects of NT dose in both control and chronic ethanol groups [$F(4, 41) = 76.8$, $p < 0.0001$ and $F(4, 50) = 27.4$, $p < 0.0001$] respectively. *ANOVA (*t*-test) showed significant ($p < 0.01$) differences between control and chronic groups at 0.6, 1.8, and 6.0 pmol NT [$F(1, 14) = 25.5$, $F(1, 19) = 17.3$, and $F(1, 22) = 39.9$, respectively]. (b) Mice were the same as those used in (a) and locomotor activity was measured 5 min postinjection for 10 min, and plotted as a function of NT dose. ANOVA showed significant effects of NT dose in both control and chronic ethanol groups [$F(4, 38) = 57.9$, $p < 0.0001$ and $F(4, 51) = 42.8$, $p < 0.0001$, respectively]. *ANOVA (*t*-test) showed significant [$p < 0.01$] differences between control and chronic groups at 0.6 and 1.8 pmol NT [$F(1, 17) = 37.6$ and $F(1, 22) = 78.3$, respectively].

Effects of Repeated ICV NT Injections

The data in Fig. 2 show effects of repeated ICV injections of saline or NT (3 nmol/5 μl) on thermoregulation in LS mice. Repeated injections of saline had no effect on rectal temperature over the day 1 and day 2 periods, but, mice receiving 2 days of saline injections were as responsive as naive animals to the hypothermic effects of a challenge dose (3 nmol, ICV) of NT (data not shown). ANOVA for repeated measures showed a significant [$p < 0.0002$] main effect of NT at 60 min after the six injections [$F(5, 20) = 10.7$]. The initial dose of 3 nmol NT produced a marked decrease in rectal temperature (ca. 5°C). A Scheffé post hoc test showed that subsequent injections of NT on day 1 produced progressively less [$p < 0.01$] hypothermia, with the second and third injections producing approximately 3 and 2°C decreases in rectal temperature, respectively. On day 2 the initial injection of NT produced significantly less [$p < 0.01$] hypothermia than on day 1, and repeated injections during day 2 again produced progressively smaller [$p < 0.01$] decreases in rectal temperature.

As shown in Fig. 3, initial NT injections produced a marked decrease in locomotor activity, with subsequent injections producing progressively less inhibition. There was no main effect for saline injections, but ANOVA for repeated measures showed a significant main effect for NT injections [$F(5, 35) = 3.7$, $p < 0.01$] and a significant [$p < 0.01$] interaction between saline and NT injection groups. On day 2 locomotor activities were similar following either saline or NT injections at times 5 and 6, indicating the development of tolerance to locomotor inhibition.

A separate experiment was conducted to determine whether the tolerance to NT-induced locomotor inhibition was the result of acclimation to the locomotor activity arena [i.e., an environmentally cued tolerance (25,31)]. In this experiment, mice received six repeated NT injections ICV on the same regimen as in Fig. 2 and 3, but the mice were kept naive to the activity monitor by returning them to their home cage after each of the first five injections. After receiving five repeated saline injections, mice showed the same locomotor activity

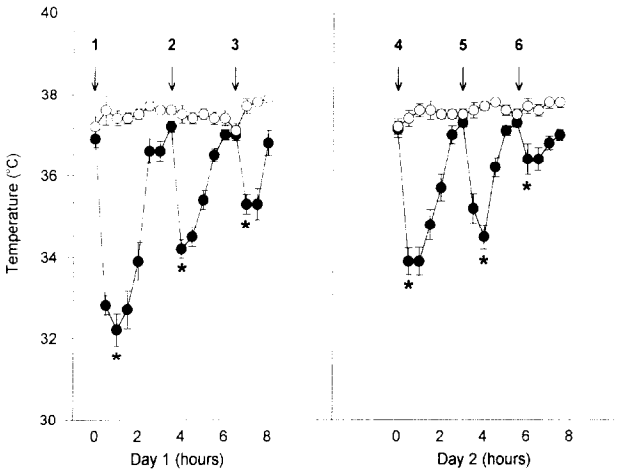

FIG. 2. Acquisition of tolerance to NT-induced hypothermia following repeated NT injections ICV. At the times indicated by the arrows (↓) saline, (○), or NT (3 nmoles/5 μl) in saline (●) was administered ICV via indwelling cannula in the lateral ventricles. Following initial injections on days 1 and day 2, rectal temperatures were measured every 30 min. When temperatures returned to control values mice received a subsequent injection of NT or saline as noted. Values represent means with SEM for six to eight mice per group (saline or NT ICV). There were obvious differences between saline and NT groups. ANOVA for repeated measures showed a significant main effect of NT at 60 min (*) following injections [$F(5, 20) = 10.7$, $p < 0.0002$]. Scheffé post hoc test showed significant [$p < 0.01$] rectal temperature differences following NT at times indicated (*): time 1 < time 2 < time 3 on day 1 and time 4 < time 5 < time 6 on day 2. Similarly, rectal temperatures were significantly ($p < 0.01$) lower at time 1 than at time 4.

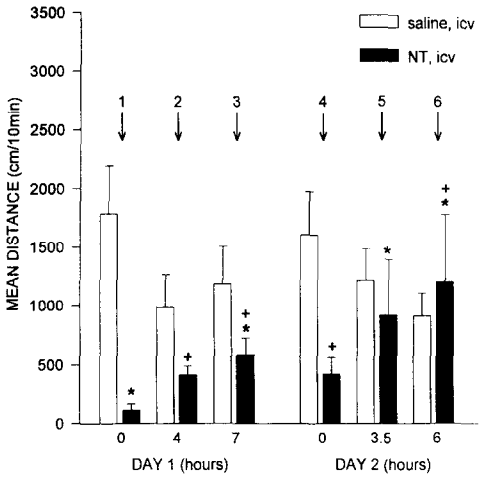

FIG. 3. Adaptation to locomotor inhibitory effects of NT following repeated NT injections. The mice in this experiment were the same as those described in Fig. 3. Five minutes after each ICV NT or saline injection animals were placed in Omnitech Activity Monitors and total distance traveled was measured for 10 min as described in Method. Arrows (↓) represent the times of saline or NT injection on days 1 and 2. There was no overall effect of saline but ANOVA for repeated measures showed a significant main effect of injection time for NT [$F(5, 35) = 3.7$, $p < 0.01$] and a significant ($p < 0.01$) interaction between saline and NT groups. Across all injections, values for NT differed significantly from those after saline [$F(1, 26) = 24$, $p < 0.0002$]. Values for the NT group at injection times 5 and 6 did not significantly differ from the corresponding saline group. As noted, post hoc t-tests showed the following significant ($p < 0.01$) differences in locomotor activity after NT: (*) times 3, 5, and 6 > time 1 and (+) time 6 > times 2, 3, and 4.

when tested after the sixth injection of saline as did mice tested on the first injection—that is, there was no change in locomotor response to saline. Mice receiving five injections of NT and tested for locomotor activity on the sixth NT injection showed no locomotor inhibition, indicating that tolerance to NT-induced locomotor inhibition, shown in Fig. 3, was not due to environmental cuing.

Because repeated NT injections for 2 days produced tolerance to NT, it was interesting to determine whether these animals were tolerant to the effects of ethanol, IP. As shown in Fig. 4a, ethanol (2.5 g/kg) produced significant hypothermia in control mice and in those receiving repeated ICV injections of saline. There was a significant [$F(2, 24) = 33.0$, $p < 0.0001$] difference among the groups (noninjected, saline, and NT injected) following ethanol administration IP, but not following saline injection IP. Animals receiving repeated injections of NT (NT-tolerant animals) were less sensitive to ethanol-induced hypothermia, as indicated by no change or small increases rather than decreases in rectal temperature.

The results in Fig. 4b confirm previous observations (9,16) that 2.5 g/kg ethanol produces locomotor inhibition in LS mice and show that repeated ICV injections of NT produce tolerance to locomotor inhibitory effects of ethanol. ANOVA for between-subject measures showed no significant treatment effect in locomotor activity among the noninjected, saline, and NT-injected groups following saline, IP. However, following IP ethanol there was a significant treatment effect among these groups [$F(2, 24) = 10.6$, $p < 0.01$]. Locomotor activities following ethanol in the saline- vs. NT-injected mice were significantly different [$F(1, 20) = 23.1$, $p < 0.0001$], indicating a tolerance to ethanol-induced locomotor inhibition.

Effects of 3-Day Continuous ICV NT Administration on Ethanol Sensitivity

Because tolerance following repeated injections of NT may be learned (25) (environmentally cued) as well as pharmacodynamic (31), experiments were conducted to minimize environmental cueing by determining whether continuous infusion of NT ICV, via an osmotic pump, produced behavioral tolerance to acute NT ICV or ethanol IP administration. The time course for effects of constant NT (6 nmol/h) or saline infusion

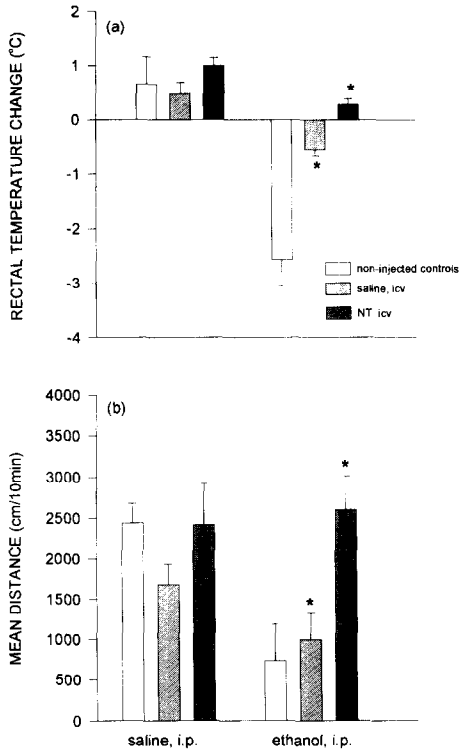

FIG. 4. Tolerance to ethanol-induced hypothermia and locomotor inhibition following repeated NT injections, ICV. Mice were administered NT or saline ICV, as described in Figs. 2 and 3. After rectal temperatures returned to control values (37.6 ± 0.65°C) following injection 6 on day 2, mice were given saline or ethanol (2.5 g/kg) in saline. (a) Hypothermia was determined by the difference in rectal temperatures taken before ethanol or saline and those obtained at 30 min after IP injections. Values represent means with SEM for four to six mice per group. ANOVA for between-subject measures showed no significant differences among the noninjected, saline-, and NT-injected groups following saline IP; but there was a significant difference among these groups following ethanol IP [$F(2, 24) = 33.0$, $p < 0.0001$]. Rectal temperatures following ethanol in the saline- vs. NT-injected groups (*) were significantly different [$F(1, 19) = 9.7$, $p = 0.005$]. (b) Locomotor activity was determined for 10 min beginning at 5 min after IP injections, as described in Method. Animals were the same as those used in (a) ANOVA for between-subject measures showed no significant differences among the noninjected, saline-, and NT-injected groups following saline IP; but there was a significant difference among these groups following ethanol IP [$F(2, 24) = 10.6$, $p < 0.01$]. Locomotor activities following ethanol in the saline- vs. NT-injected groups (*) were significantly different [$F(1, 20) = 23.1$, $p < 0.0001$].

ICV on rectal temperature can be seen in Fig. 5. Animals receiving saline via the osmotic pump showed no signs of hypothermia; however, NT-treated animals developed hypothermia within 6 h of recovery from surgery and initiation of infusion. The animals developed tolerance to the hypothermic effects of NT, as shown by a return to control temperatures in approximately 24 h.

Figure 6a and b shows that mice receiving 72 h of continuous infusions of saline were as sensitive as the no-pump control mice to hypothermia or locomotor inhibition produced by challenge doses of NT. Decreases in rectal temperature or locomotor activity were observed at 1.8 and 600 pmol NT in both no-pump and saline pump groups of mice. There were significant ($p < 0.001$) NT dose effects on hypothermia in the no-pump [$F(2, 19) = 34.9$] and saline pump [$F(2, 12) = 22.0$] groups. Also, there were significant ($p < 0.01$) NT dose effects on locomotor activity in the no-pump [$F(2, 19 = 7.1$] and saline pump [$F(2, 12) = 5.5$] groups. However, animals receiving 6 nmol/h NT ICV were completely tolerant to both the hypothermic and locomotor inhibitory effects of NT (i.e., there were no significant effects of NT dose in the chronic NT group).

Separate groups of mice were tested after 72 h infusion of NT or saline for sensitivity to challenge doses of ethanol (Fig. 7a and b). Animals receiving no osmotic pump and saline pump treatments showed significant effects of ethanol on rectal temperature and locomotor activity. There was a significant effect of pump groups for hypothermia following ethanol IP [$F(2, 27) = 19.6, p < 0.001$]. Scheffé post hoc test showed significant ($p < 0.001$) differences between no-pump vs. saline pump and no-pump vs. NT pump groups. However, there was no significant difference in hypothermia between the saline and NT pump groups following ethanol. Results indicated

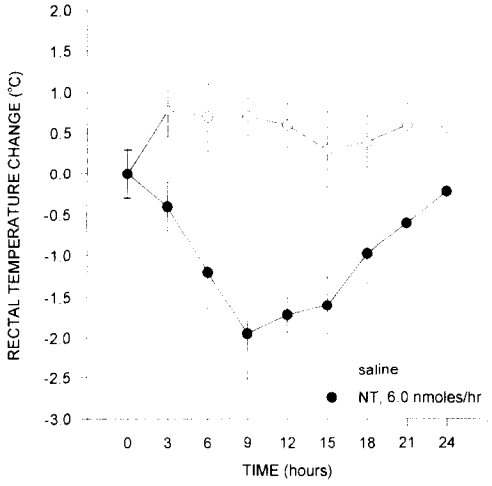

FIG. 5. Time course for development of tolerance to hypothermia following continuous infusion of NT into the cerebral ventricle. LS mice were fitted with cannulae into the cerebral ventricle and were continuously infused with saline or NT (6 nmol/h) in saline through an osmotic pump. Cannulae and pumps were inserted as described in Method. Rectal temperatures were taken every 3 h for 24 h following recovery from surgery, and the change was plotted as a function of time. Shown are the mean changes ± SEM ($n = 4$ for saline) and ($n = 6$ for NT). Only the first 24 h of this experiment are shown because temperatures in the NT-infused mice returned to saline control values (36.9 ± 0.36°C) within 30 h and remained at that level up to 72 h.

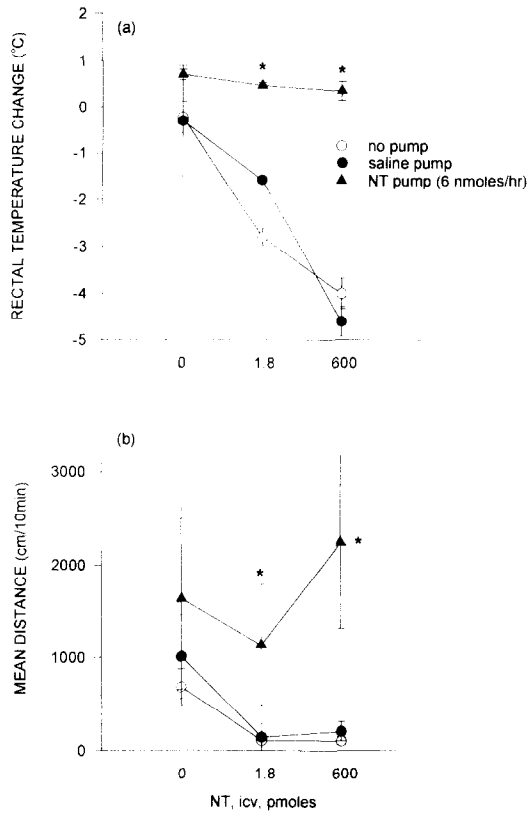

FIG. 6. Tolerance to NT-induced hypothermia and locomotor inhibition following continuous ICV infusion of NT for 72 h. LS mice were fitted with cannulae, ICV, and saline or NT in saline (6 nmol/h) was continuously infused for 3 days via an osmotic pump. Each animal was fitted with an osmotic pump and cannula into the left lateral ventricle and with a separate cannula guide into the right lateral ventricle; 72 h after infusion was initiated, animals were tested for sensitivity to acute ICV NT injection (given via the right cannula) by measuring rectal temperature change (basal values for rectal tempertures were 37.2 ± 0.4, 36.8 ± 0.32, and 37.5 ± 0.37°C for the no-pump, saline, and NT pump groups, respectively) and locomotor activity. (a) Hypothermia (decrease in rectal temperature at 30 min after ICV injection) values represent means with SEM for four to eight subjects per treatment group for each NT dose. Data were analyzed by ANOVA for between-subject differences, and ANOVA followed by a post hoc t-test to determine between-group effects of NT at each dose, 0, 1.8, and 600 pmol. There were significant overall dose effects on rectal temperature for the no-pump [$F(2, 19) = 34.9$, $p < 0.0001$] and saline pump [$F(2, 12) = 22.0, p < 0.001$] groups, but not for the NT pump group. There were no significant differences among pump groups after saline (0 NT dose); but, as noted (*), there were significant ($p < 0.001$) differences between NT pump and saline pump groups at 1.8 and 600 pmol NT ICV [$F(1, 11) = 19.6$, and $F(1, 10) = 29.4$, respectively]. (b) Locomotor activity was measured as described in Method using the same animals as in (a). There were significant overall dose effects on locomotor activity for the no-pump [$F(2, 19) = 7.1$, $p < 0.005$] and saline pump [$F(2, 12) = 5.5$, $p < 0.01$] groups, but not for the NT infusion group. There were no significant differences among pump groups after saline (0 NT dose); but, as noted (*), there were significant ($p < 0.01$) differences between NT pump and saline pump groups at 1.8 and 600 pmol NT ICV [$F(1, 10) = 6.0$, and $F(1, 12) = 7.2$, respectively]. There were no significant differences between no-pump and saline pump controls.

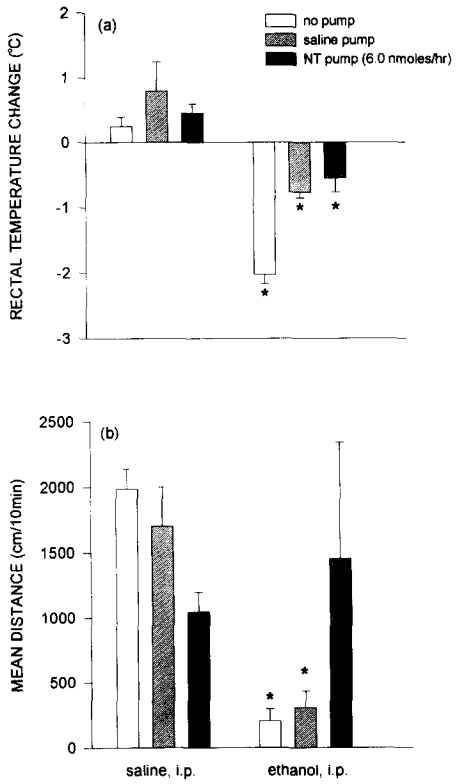

FIG. 7. Ethanol-induced changes in rectal temperature and locomotor activity in LS mice following continuous infusion of NT or saline, ICV. As in Fig. 6, LS mice were fitted with cannulae ICV, and saline or NT in saline (6 nmol/h) was continuously infused for 3 days via osmotic pumps. Two hours after infusion was terminated, animals were tested for sensitivity to acute ethanol injection, 2.5 g/kg, IP, by measuring rectal temperature change (basal values weere similar to those in Fig. 6) and locomotor activity. Values represent means and SEM for three to 10 animals per pump infusion group. Data were analyzed by ANOVA for between-subject differences, and ANOVA followed by a post hoc Scheffé or *t*-test to determine between-group effects of saline or ethanol, IP. (a) Hypothermia. There was no significant effect of pump group following saline IP; however, there was a significant [$F(2, 27) = 19.6$, $p < 0.0001$] effect of pump group following ethanol, IP. Post hoc Scheffé test showed significant ($p < 0.001$) differences between no-pump and saline pump and between no-pump and NT pump groups, but not between saline and NT pump groups following ethanol. As noted (*), values for hypothermia following ethanol were significantly ($p < 0.001$) lower than values after saline in the no-pump and saline pump groups [$F(1, 8) = 19.3$ and $F(1, 9) = 10.6$, respectively]. (b) Locomotor activity. There was a significant pump group effect after saline IP [$F(2, 19) = 13.0$, $p < 0.001$] and after ethanol, IP [$F(2, 27) = 4.5$, $p < 0.01$]. The mean values after saline, IP, for the saline pump and NT pump groups are shown and the standard errors are too small to be indicated on the graph (i.e., 35 and 55 cm, respectively). Unfortunately, $n = 3$ for each of these values. As noted (*), activity values in the no-pump [$F(1, 11) = 24.9$, $p < 0.001$] and saline pump [$F(1, 6) = 7.7$, $p < 0.05$] groups were significantly reduced by ethanol administration. Ethanol did not significantly change locomotor activity in the NT pump group.

a significant pump group effect for locomotor activity following saline and ethanol IP [$F(2, 19) = 13.0$, $p < 0.001$, and $F(2, 27) = 4.5$, $p < 0.02$, respectively]. The 2.5-g/kg dose of ethanol produced significant locomotor inhibition in both no-pump and saline pump groups, but not in NT pump group, indicating that chronic NT produces tolerance to ethanol-induced locomotor inhibition.

Effects of Chronic NT Administration on NT Receptors

Previous studies have shown that chronic ethanol administration, which produces tolerance to ethanol effects (16) and to NT-mediated hypothermia and locomotor inhibition (Figs. 1a and b), causes a downregulation of NTH receptors in brain regions of LS mice (1). Thus, it was interesting to determine whether 72 h chronic NT infusion ICV altered NT receptors. The data in Table 1 show binding capacities (fmol ^{3}H-NT/mg membrane protein) for NT in brain regions following 72 h infusions of saline or 6 nmol/h NT in saline. As shown, chronic NT but not saline infusion produced a significant decrease in levocabastine-insensitive [NTH receptor (23)] ^{3}H-NT binding in the striatum (combined nucleus accumbens and caudate putamen). Binding to NTH receptors in ventral midbrain or entorhinal cortex was not altered by chronic NT infusion. The levocabastine-sensitive, NTL receptors were not altered by chronic NT treatment.

DISCUSSION

Several studies were performed to determine whether cross-tolerance develops between NT and ethanol. It was shown that chronic ethanol administration produced tolerance to hypothermia and locomotor inhibition mediated by 1.8–6.0 pmoles ICV but not higher doses of NT (Fig. 1a and b). As shown in previous studies (16), the procedure used to administer ethanol chronically does not produce dependence as measured by handling-induced seizures following withdrawal, nor does the

TABLE 1

EFFECTS OF CHRONIC NT ADMINISTRATION ON NT RECEPTOR LEVELS IN LS BRAIN MEMBRANES

Brain Region*	Group†	*n*	NT_H (fmol/mg protein)	NT_L (fmol/mg protein)
EC	c	3	100 ± 7	78 ± 19
	s	4	79 ± 21	85 ± 14
	nt	8	88 ± 8	91 ± 6
STR	c	3	64 ± 4	64 ± 12
	s	4	51 ± 14	72 ± 11
	nt	8	29 ± 6‡	58 ± 7
VMB	c	3	55 ± 5	89 ± 3
	s	4	56 ± 4	83 ± 7
	nt	7	64 ± 2	82 ± 18

Binding capacities of ^{3}H-NT in membranes from brain regions of LS mice were measured as described in Method. High (NT_H) and low (NT_L) affinity receptors were identified by measuring binding capacities in the presence or absence of 10 μm levocabastine. Three groups of mice were compared with (*n*) representing the number of experiments using brain regions from two mice per experiment. Saline or NT (6 nmol/h) were continuously infused ICV as described in Method.

*EC = entorhinal cortex and hippocampus; STR = caudate nucleus and nucleus accumbens; VMB = ventral midbrain.

†c = control; s = chronic saline; nt = chronic neurotensin.

‡ANOVA showed that there was a significant [$p < 0.01$, $F(1, 10) = 15.2$] effect of NT on NT receptors.

method produce significant tolerance to hypnotic effects of ethanol (16). However, those studies demonstrated that this method of ethanol administration produced tolerance to locomotor inhibition and hypothermia elicited by subhypnotic doses of ethanol. Chronic administration of ethanol at higher doses than those used in the present studies produces tolerance to hypnotic as well as hypothermic effects of ethanol (25,31). It is possible that chronic exposure to higher ethanol doses might render mice tolerant to higher doses of NT.

Because the mice were required to drink ethanol in water solutions, they only received a challenge injection of ethanol once; thus, it may be assumed that the observed tolerance to ethanol is a functional and not environment-cued tolerance (25,31). Whether this procedure causes tolerance to locomotor inhibition and hypothermia produced by other hypnotic agents (e.g., barbiturates) has not been examined. Nevertheless, the observation that chronic ethanol consumption produces tolerance to NT-induced hypothermia suggests that NT and ethanol may share similar mechanisms of hypothermia induction.

Neurotensin produces locomotor inhibition or activation when injected into the NA or VTA, respectively (21,22). Thus, evidence indicates that locomotor activity is regulated by a balance of NT effects on dopamine function in these brain regions. As shown in the present studies, NT ICV produces a dose-dependent decrease in activity, indicating that inhibitory actions at the level of the NA dominate VTA effects after ICV administration. Because chronic ethanol administration decreases high-affinity NT receptors in the NA as well as in the VTA, the observed tolerance to NT-induced inhibition at 1.8 pmol would be expected assuming there were no spare receptors for NT in these brain regions. Paradoxically, sensitivity to 0.6 pmol NT was enhanced in chronic ethanol-treated mice. This result might be attributed to differential effects of chronic ethanol administration on NT receptor subtypes and on NT-immunoreactivity in different brain regions.

The second set of experiments showed that chronic administration of NT produced tolerance not only to challenge doses of NT, but also to challenge doses of ethanol (Figs. 4 and 7). Two methods were used chronically to administer NT ICV; the first was by repeated injections of 3 nmol/5 μl doses with three doses on each day for 2 consecutive days; the second was by continuous infusion (6 nmol/h) for 3 days. Both methods produced tolerance to NT- or ethanol-mediated hypothermia and locomotor inhibition. The results of experiments with the repeated injection method (Figs. 2–4) clearly show an environmental-cued tolerance (31); thus, it was important to compare results with a continuous infusion method that requires only one challenge dose of either NT or ethanol and minimizes cued responses.

Three days of constant ICV infusion of NT (6 nmol/h) caused tolerance to the locomotor inhibitory and hypothermic effects of NT in LS mice. Other investigators have recently reported a rapid development of tolerance to NT-induced hypothermia using a stable NT analogue, [D-Trp^{11}] NT, in rats (7). In those studies no tolerance developed to [D-Trp^{11}] NT-mediated analgesia; in the present study, chronic NT administration produced tolerance to NT-induced locomotor inhibition. These observations indicate that acquisition of tolerance to NT is behavior-specific. Such results might be explained by changes in NT receptors in some brain regions, but not in others.

Evidence for some selectivity in NT effects on tolerance is provided by the finding that chronic NT infusions produced a region-specific downregulation of high-affinity NT receptors in the striatum. Because injection of NT into the nucleus accumbens or caudate putamen produces locomotor inhibition (17,22), downregulation of NT receptors in these brain regions would be expected to decrease sensitivity to NT-mediated inhibitory effects. Further studies are in progress to determine the time course for NT receptor downregulation following continuous administration.

The mechanisms mediating region-specific downregulation of NT receptors are unclear; however, it is possible that neurons within different brain regions are exposed to different concentrations of NT during ICV infusion. For example, NT is rapidly degraded in brain by endopeptidases (10) and the half-life for ^{3}H-NT administered ICV in LS mice has been estimated to be <5 min. (15). Differential rates of degradation within, and rates of diffusion to, various brain regions might expose those regions to different NT concentrations. Initial steps in downregulation of NT receptors might be NT binding to and internalization of the NT receptors. Indeed, several groups have shown that NT is rapidly internalized following receptor occupancy (8,29). Differential rates of desensitization and internalization of NT receptor subtypes in different brain regions could account for behavioral-specific tolerance to NT and ethanol.

Continuous administration of NT produced tolerance to ethanol-induced locomotor inhibition. Indeed, doses that are hypnotic in control LS mice (ca. 2.8 g/kg) were ineffective in inhibiting locomotor activity following chronic NT administration. It is clear that continuous infusion of saline for 72 h produces tolerance to ethanol-induced hypothermia (Fig. 7a) but not locomotor inhibition (Fig. 7b). Whether the tolerance to hypothermia in control animals was due to hypothermia experienced during surgical anesthesia is not known. Other investigators have shown cross-tolerance between ethanol and pentobarbital (23) and that acclimation to cold causes animals to be resistant to hypothermia produced by ethanol or NT (26,32).

The results of the present study suggest influences of stress associated with repeated injections or with continuous infusions of NT ICV on neuroadaptive processes mediating tolerance. The effect of stress on ethanol actions was shown previously by Jones et al. (20), who demonstrated that individually housed LS mice were more tolerant to the hypothermic and hypnotic effects of ethanol than group-housed animals. The future development of nonpeptide NT analogs, which readily cross the blood–brain barrier, will provide the tools necessary to distinguish nonspecific (stress)-related from NT-specific effects.

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