ELSEVIER

Pharmacology Biochemistry and Behavior, Vol. 54, No. 2, pp. 525–532, 1996

0091-3057/96 $15.00 + .00

SSDI 0091-3057(95)02205-8

# Neurotensin Levels and Receptors in HAS and LAS Rat Brains: Effects of Ethanol

V. GENE ERWIN,[1] LAURA J. DRASKI AND RICHARD A. DEITRICH

*Alcohol Research Center, School of Pharmacy and Department of Pharmacology, University of Colorado Health Sciences Center, Denver, CO 80262 USA*

Received 3 May 1995; Revised 28 September 1995; Accepted 7 October 1995

ERWIN, V. G., L. J. DRASKI AND R. A. DEITRICH. *Neurotensin levels and receptors in HAS and LAS rat brains: Effects of ethanol.* PHARMACOL BIOCHEM BEHAV **54**(2) 525–532, 1996. – Previous studies of neurotensin (NT) levels and NT receptor densities in specific brain regions of mice selectively bred for differences in sensitivity to ethanol have shown that NTergic processes may mediate some actions of ethanol. In the present study, we have determined the levels of NT and NT receptor densities in specific brain regions of HAS and LAS rats that have been selectively bred for differences in sensitivity to ethanol-induced loss of righting response. Regional differences in NT levels were observed in brains from both HAS and LAS rats and values in hypothalamus, ventral midbrain, and nucleus accumbens from female rats were 25 to 75% higher than levels in corresponding regions from male rats. However, there were no significant line differences in NT-ir levels in corresponding regions from HAS and LAS animals. High-affinity binding (NTH $B_{max}$ values), measured by Scatchard analyses, were higher in ventral midbrain from HAS males than from LAS males. NTH receptor densities were higher in HAS males than in HAS females; sex differences were not observed in the LAS line. There were no significant line or sex differences between HAS and LAS in low-affinity ($NT_L$) $B_{max}$ values in any brain region. In HAS females, subhypnotic doses of ethanol produced a decrease in NT levels in nucleus accumbens, whereas, hypnotic doses caused an increase in NT levels. Likewise, hypnotic doses elicited increases in NT levels in hypothalamus of female HAS and LAS, but not in ventral midbrain or caudate putamen. These results are consistent with low dose activation of mesolimbic and nigrostriatal dopaminergic neurons in which NT is colocalized with dopamine and with high dose inhibition of these pathways.

Neurotensin Ethanol Brain HAS rats LAS rats

NEUROTENSIN (NT), an endogenous tridecapeptide, meets virtually all criteria for a neurotransmitter including wide distribution within perikarya, nerve fibers, and presynaptic terminals of nigrostriatal, mesolimbic, cortical, and hypothalamic neurons (8,19,23,28,34,44), $Ca^{2+}$-dependent release (27), specific high-affinity receptors (36,45), signal transduction processes (17,20,21) and rapid enzymatic degradation (4). Recent studies have described the gene for NT and neuromedin N, a peptide related to NT (30), and have utilized a cDNA clone to determine mRNA levels in several brain regions. The ratio of mRNA to NT immunoreactivity (NT-ir) levels are comparable for most brain regions except the cerebral cortex which had a higher than predicted mRNA level (30).

Specific, saturable, and reversible binding of NT to rat and mouse brain membranes show that the 8–13 *C*-terminal portion of the peptide is required for binding. Dissociation constants for NT binding were similar for membranes from selected brain regions from mice or rats (3,22,35). Studies using [$^{125}$I]-labeled NT have revealed biphasic binding isotherms best described by two independent, high-affinity ($NT_H$) and low-affinity ($NT_L$) binding sites (3,36); binding capacity is greater for $NT_L$ than for $NT_H$ in rat brain regions. Studies with recombinant inbred strains of mice indicate that the densities of $NT_H$ and $NT_L$ receptors are independently regulated (16). Kitabgi et al. (31) and Schotte et al. (41) have shown that levocabastine, an $H_1$ histamine antagonist, selectively inhibits binding to $NT_L$, providing a valuable tool to distinguish between the $NT_L$ and $NT_H$ receptors. Levocabastine has been used to determine the regional distribution of $NT_L$ and $NT_H$ binding in rat and mouse brain (3,31,41). Large differences were observed in NT receptor densities among brain regions with highest densities in cortical regions and lowest in the brain stem and cerebellum. Autoradiographic studies indicate NT receptors on dopamine cell bodies and dendrites in the

[1] Requests for reprints should be addressed to V. Gene Erwin, School of Pharmacy, Box C238, University of Colorado Health Sciences Center, 4200 E. Ninth Avenue, Denver, CO 80262.

substantia nigra and ventral tegmental area (VTA) and on cholinergic neurons in the basal forebrain (38,43,46).

Pharmacological studies have shown that administration of NT, ICV or into the nucleus accumbens (NA), elicits anxiolytic effects and locomotor inhibition (18,29,40) and injections into the VTA produces locomotor activation (29). It has been proposed that these effects are mediated by NT interactions with central dopaminergic processes (2,37,40). For example, microinjection of NT into the VTA enhances dopamine turnover in the (NA) and striatum (STR), but administration into the NA or ICV blocks dopamine-mediated motor activation (29,40). NT, administered centrally, elicits many effects similar to those produced by ethanol, including hypothermia (39), altered locomotor activity (18,29), and analgesia (5). Moreover, NT produces marked potentiation of ethanol-induced anesthesia and reversal of ethanol-mediated locomotor activation (15,18). These actions are genotype specific, as shown by differences in the LS/Ibg (LS) and SS/Ibg (SS) mice (15,18), which were selectively bred for differences in hypnotic sensitivity to ethanol.

Because LS and SS mice were selectively bred for differences in hypnotic sensitivity to ethanol and found to differ also in other ethanol actions (9,18), it was hypothesized that the genotype specific pharmacological interactions of NT with ethanol may be associated with genetically selected differences in NT levels or receptors. Campbell et al. (3) characterized the $NT_L$ and $NT_H$ receptors in LS and SS mice and found line differences in $NT_L$ receptor densities in ventral midbrain, nucleus accumbens, and entorhinal and frontal cortex, and in $NT_H$ in ventral midbrain. In these brain regions receptor densities were higher in SS than in LS mice. In recent studies, using recombinant inbred strains derived from LS and SS mice, Erwin et al. (11) found $NT_H$ densities were significantly correlated with ethanol-induced locomotor inhibition and hypnotic sensitivity.

Previous investigations (10,12) showed that endogenous levels of NT-ir were higher in LS than in SS hypothalamus and striatum. In those studies, acute ethanol administration produced a biphasic dose response; subhypnotic doses decreased while hypnotic doses increased NT-ir levels in specific brain regions (12). Because previous studies in mice indicate that neurotensinergic processes might mediate, in part, genetic differences in sensitivity to ethanol, the present study was undertaken to further test this hypothesis by comparing endogenous NT-ir levels, NT receptors, and the effects of ethanol on NT-ir levels in HAS and LAS rats that were selectively bred for differences in hypnotic sensitivity to ethanol (7).

## ABBREVIATIONS

HAS—high alcohol sensitive; LAS—low alcohol sensitive; NT—neurotensin; IP—intraperitoneal; $NT_L$ and $NT_H$—low-affinity and high-affinity neurotensin receptors, respectively.

## METHOD

### *Animals*

The high alcohol sensitive (HAS) and low alcohol sensitive (LAS) rats were selectively bred for differences in hypnotic sensitivity as measured by duration of loss of righting response and blood ethanol concentration (BEC) at regaining righting response (7). The selection was initiated from the genetically heterogeneous N/Nih stock and the lines used in these studies were from the 17th and 18th generations of selective breeding. The HAS and LAS lines differed in hypnotic sensitivity to ethanol to the extent that differential doses of ethanol, IP, were administered (2.45 g/kg for HAS and 3.6 g/kg for LAS) to achieve comparable duration of loss of righting response. HAS rats regain righting response at significantly lower BEC than LAS rats (see the Results section). The HAS and LAS rats are not compared for duration of loss of righting response (sleep time) because of different doses required to produce this effect. Rats were group housed at weaning (three per cage) in single-gender, Plexiglas cages and provided water and laboratory rat chow ad lib. The vivarium was maintained at 22 ± 1°C, with a 12 L : 12 D cycle (onset at 0600 h), with all testing occurring between 0800 and 1130 h. Rats were between 60–130 days of age at the time of testing, and no line differences in body mass at testing were observed for either males (HAS mean ± SEM = 313 g ± 8; LAS = 312 g ± 8) or females (HAS = 160 ± 3; LAS = 158 ± 3).

### *Materials*

Reagents and chemicals were obtained as follows: neurotensin$_{1-13}$ (NT), bovine serum albumin, 1,10 phenanthroline, Tris HCl, and polyethylenimine (50% aqueous) from Sigma Chemical Co., St. Louis, MO; Na[$^{125}$I] and [3,11-Tyrosyl-3,5-$^{3}$H(N)] neurotensin$_{1-13}$ (83 Ci/mmol in ethanol) from NEN Products, E. I. DuPont de Nemours & Co., Boston, MA; levocabastine was a generous gift from Janssen Pharmaceutica, Breese, Belgium; neurotensin antiserum, selective for the *C*-terminal region, was obtained through the courtesy of Dr. Marvin Brown, University of California at San Diego, San Diego, CA.

### *Handling Protocol*

Procedures were used to minimize possible effects of stress on neurotensin processes. All cages within a testing group were removed from their holding rack and individual rats were removed from their home cage one at a time, conventionally restrained using a leather-gloved hand, and an injection into their abdomen simulated with a capped syringe. After approximately 30 s the animal was replaced into its cage. When all rats had been handled and returned to their cage, they were transported to a separate experimental room where each cage was placed into a Plexiglas chamber used for $CO_2$ asphyxiation. Following a 1-min habituation session in the $CO_2$ chamber, all cages were returned to their holding rack in the colony room. Rats were handled as such for 4 days, with testing occurring on the fifth day. These handling procedures were conducted to minimize handling induced stress, because previous studies showed that these procedures minimized elevations in plasma corticosterone (7).

### *Testing Protocol*

Rats either were not injected (control) or were injected with either 0.5, 1.0, 2.0, or 3.5 g/kg ethanol (15% w/v) or a volume of saline equivalent to the highest dose (controls). Thirty minutes postinjection animals were anesthetized in a $CO_2$ chamber and sacrificed by decapitation. Brains were rapidly dissected for neurochemical analyses. Two 40 μl samples of trunk blood were obtained from the animals at this time for comparison of blood ethanol concentration (BEC) at the time of sacrifice.

### *Neurotensin Extraction and Radioimunoassay*

Brains were removed quickly (< 1 min), chilled in ice-cold saline for 30 s, sliced, and specific regions dissected according to anatomical guidelines similar to those used in previous studies with mice (42). Neurotensin was extracted and assayed as previously described (10,12). Tissues from the regions were

weighed, and homogenized in 10 to 20 vol of 0.01 N HCl, and placed in a boiling water bath for 5 min. Homogenates were centrifuged at 20,000 × *g* for 20 min, and the resulting supernatant extracts were lyophilized and stored at −70°C for less than 2 weeks prior to radioimmunoassay (RIA). Standard double antibody RIAs were used to measure NT concentrations (10,12).

NT was iodinated by the procedure of Hunter and Greenwood (25) by incubating 1 nmol NT with 20 g chloramine-T and 2 mCi Na[$^{125}$I] in 30 μl 0.5 M phosphate buffer, pH 7.4. The reaction was terminated by the addition of sodium metabisulfite (ca. 200 μg/50 μl), and labeled NT was purified by a Sep-Pac procedure followed by column chromatography on Sephadex G-50 fine. The ratio of bound/bound-plus-free [$^{125}$I]-NT was calculated for each level of NT in a standard curve. Displacement curves were obtained by plotting the ratio of [$^{125}$I]-NT tracer bound in the presence (B) and in the absence ($B_0$) of unlabeled NT standards against the $\log_{10}$ NT amount. Antiserum dilutions yielding approximately 30 to 40% binding of [$^{125}$I]-NT tracer (10,000 cpm) in the absence of NT standards were used. The antiserum was previously characterized (32) and shown to recognize the *C*-terminal region of NT. The concentrations of NT-ir in tissue extracts were calculated by regression analysis of standard curves. Aliquots of the reconstituted extracts were assayed in triplicate with at least two dilutions. Extraction efficiency, determined by addition of $NT_{1-13}$ to homogenates, was approximately 75 to 80%. Assay sensitivity was measured at 10 concentrations (each concentration in triplicate) between 1 to 120 pg NT with a mean $IC_{50}$ of 20 pg; this standard curve was run with each assay. Procedures described previously (10,12) were used for verification of NT-ir in brain extracts as authentic $NT_{1-13}$.

*Neurotensin binding assays.* Animals were sacrificed and brains dissected as described above; dissected and/or punched regions were rapidly chilled in cold (4°C) 50 mM Tris buffer, pH 7.4, containing 40 mg/ml bacitracin and 1 mM EDTA. Pooled regions from two to four brains were homogenized in 10 vol of buffer; the homogenates were centrifuged at 100,000 × *g* for 30 min. The resulting membrane pellet was rehomogenized and centrifuged; this wash procedure was repeated twice. The membrane pellets were resuspended in buffer to give a final protein concentration of 4 to 5 mg/ml (33); each binding tube contained 200 to 250 μg protein.

Binding assays were performed essentially as described by Kitabgi et al. (31) with membrane protein in 50 mM Tris buffer, pH 7.4, containing 0.5 mM 1,10-phenanthroline, 0.2% bovine serum albumin in a total volume of 0.1 ml. Assays were performed in the presence of nine concentrations of [$^3$H]-NT (0.01 to 20 nM). Scatchard analysis were performed with the LIGAND program for a two site model and iterated values for $K_d$ and $B_{max}$ were obtained for specific brain regions of HAS and LAS rats. Unlabeled $NT_{1-13}$ (10 μM) was used to determine nonspecific binding. After 20 min incubation at 22°C, a time shown to be sufficient to reach equilibrium, 0.9 ml cold binding buffer 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 twice with 2 ml each of cold buffer and were placed in vials containing 4 ml. Scintiverse II scintillation cocktail. Radioactivity was determined with a Beckman L3-3133P scintillation counter.

*Data Analysis*

Data from each experiment were analyzed by appropriate one-, two-, or three-way analysis of variance (ANOVA) to assess effects of between-subjects variables (rat line, sex, and ethanol dose) where needed. In each instance the number of between- and/or within-subjects variables and the Scheffe' post hoc test used is indicated. *F*-Ratios and corresponding *p* values are provided for values of $p < 0.05$.

## RESULTS

*Comparisons of Ethanol Sensitivities and Endogenous Neurotensin Levels*

The results in Table 1 show blood ethanol concentrations (BEC values) at regain of righting response in HAS and LAS rats. As expected, BEC values at regaining righting response were significantly higher for LAS than for HAS rats, verifying a greater sensitivity to hypnotic effects of ethanol in the HAS line. Values for HAS males (259 mg/dl) were significantly less than values for HAS females (296 mg/dl), but there was no significant difference between males and females in the LAS line. As noted in Table 1, there was a significant line by sex interaction. There were no significant differences between replicates (1 and 2) of HAS (253 and 266 mg/dl) or LAS (432 and 408 mg/dl) (data not shown in Table 1).

Data presented in Table 2 show levels of endogenous neurotensin immunoreactivity (NT-ir) obtained from brain regions of control (noninjected) HAS and LAS rats. Unfortunately, sufficient numbers of animals were not available to determine NT-ir levels in brain regions from both replicates of the HAS and LAS lines. Thus, data in Table 2 represents mean values of NT-ir for approximately equal numbers of replicates combined by line. As expected, brain regions differed markedly in NT-ir levels. Levels of NT-ir were similar in corresponding hypothalamus (HYP), ventral midbrain (VMB), or caudate putamen (CP) from HAS and LAS rats. It is of interest that in all brain regions examined, except the NA from LAS, NT-ir levels in females were significantly higher (28 to 75%) than in males of both rat lines.

*Effects of Ethanol on Neurotensin Levels*

Thirty minutes after saline or doses of ethanol (0.5 to 3.5 g/kg) were administered, rats were sacrificed and blood samples taken for BEC determinations and brains were dissected and extracted for NT-ir assays. Data in Table 3 show that BEC values were not significantly different for HAS and LAS rats at corresponding doses. Values for BEC in males and

TABLE 1

BLOOD ETHANOL CONCENTRATIONS IN HAS AND LAS RATS AT REGAINING RIGHTING RESPONSE

| | BEC (mg/dl) Mean ± SEM | |
|---|---|---|
| Line | Males (*n*) | Females (*n*) |
| HAS | 259 ± 9.6 (78) | 296 ± 11.0 (83) |
| LAS | 422 ± 4.6 (84) | 423 ± 5.9 (85) |

These values represent means of both replicates of the HAS and LAS lines. A three-way analyses of variance (line × sex × replicate) showed significant, $p < 0.001$, main effects for line, $F(1, 322) = 1111$, and for sex, $F(1, 322) = 19.8$, but not for replicate. There was a significant line by sex interaction, $F(1, 322) = 14.5$, but no significant line by sex by replicate interaction.

TABLE 2

COMPARISONS OF NEUROTENSIN LEVELS IN BRAIN REGIONS FROM HAS AND LAS RATS

| Line | Brain Region | (n) | Neurotensin Levels (ng/g Tissue) Mean ± SEM Males | (n) | Females |
|---|---|---|---|---|---|
| HAS | Hypothalamus | 5 | 69.3 ± 10.7 | 5 | 106.7 ± 13.6 |
| HAS | Vent. Midbrain | 4 | 30.7 ± 3.5 | 5 | 53.6 ± 8.0 |
| HAS | Nuc. Accumbens | 5 | 31.5 ± 2.3 | 5 | 40.3 ± 4.8 |
| HAS | Caud. Putamen | 4 | 7.9 ± 0.9 | 5 | 11.1 ± 1.0 |
| LAS | Hypothalamus | 5 | 74.5 ± 17.0 | 5 | 105.6 ± 11.9 |
| LAS | Vent. Midbrain | 4 | 29.6 ± 2.4 | 5 | 44.0 ± 4.7 |
| LAS | Nuc. Accumbens | 5 | 43.6 ± 13.1 | 5 | 33.9 ± 7.8 |
| LAS | Caud. Putamen | 5 | 8.6 ± 1.6 | 5 | 12.1 ± 3.2 |

ANOVA for within-subject variables for HAS and LAS (males and females) showed significant, $p < 0.001$, differences in NT levels by brain region, $F(3, 17) = 22.6$ and $F(3, 18) = 46.3$ for HAS, and $F(3, 18) = 22.0$ and $F(3, 19) = 82$ for LAS. There were no sex by strain or region by strain interactions.

ANOVA for between-subject variables showed significant, $p < 0.05$, effects for sex, $F(1, 36) = 6.0$ and $F(1, 37) = 6.9$ for HAS and LAS, respectively, across all brain regions. NT levels were lower in males than in females for all regions except in LAS nucleus accumbens and caudate putamen. Values, within region, in HAS were not significantly different from those in LAS.

females were not different at corresponding ethanol doses, data not shown. Thus, any between-line or sex effects of ethanol on NT-ir levels could not be accounted for by differences in BEC.

Administration of saline (0 ethanol dose) had no significant effect on the NT-ir levels of any brain region (Figs. 1-4 and Table 2). From the data in Fig. 1, ANOVA (line × sex × ethanol dose) indicated no significant main effect of line or ethanol dose, but a significant, $F(1, 59) = 27.9$, $p < 0.0001$, effect of sex on NT-ir levels in hypothalamus. There was a significant sex by treatment interaction, $F(2, 59) = 3.5$, $p < 0.05$, with post hoc tests indicating that in female rats there were significant ($p < 0.05$) effects of ethanol dose, $F(4, 24) = 3.5$, and $F(4, 24) = 2.7$, respectively. In the HAS, but not LAS females, values of NT-ir at 2.0 and 3.5 g/kg were significantly greater than values after saline (0 ethanol dose), 0.5, or 1.0 g/kg ethanol.

Effects of ethanol on ventral midbrain NT-ir levels are shown in Fig. 2. Following saline administration, NT-ir levels for both HAS and LAS females were significantly ($p < 0.05$) higher than in corresponding males. ANOVA showed no significant main effect of ethanol dose nor rat line, but a significant, $F(1, 55) = 27.6$, $p < 0.0001$, effect of sex was observed. Three-way ANOVA showed not significant interactions among line, sex, and ethanol dose.

Data presented in Fig. 3 show the effects of ethanol on NT-ir levels in nucleus accumbens from HAS and LAS rats. A three-way ANOVA (line × sex × dose) showed significant

TABLE 3

BLOOD ETHANOL CONCENTRATIONS IN HAS AND LAS RATS AT 30 MIN AFTER VARIOUS ETHANOL DOSES

| Ethanol Dose (g/kg) | BEC (mg/dl) Mean ± SEM HAS | LAS |
|---|---|---|
| 0.5 | 49 ± 3 | 43 ± 2 |
| 1.0 | 119 ± 6 | 116 ± 3 |
| 2.0 | 204 ± 3 | 200 ± 7 |
| 3.5 | 363 ± 11 | 357 ± 7 |

Two-way ANOVA (dose × line) showed a significant effect of dose, but no significant differences in BEC values between lines at corresponding doses.

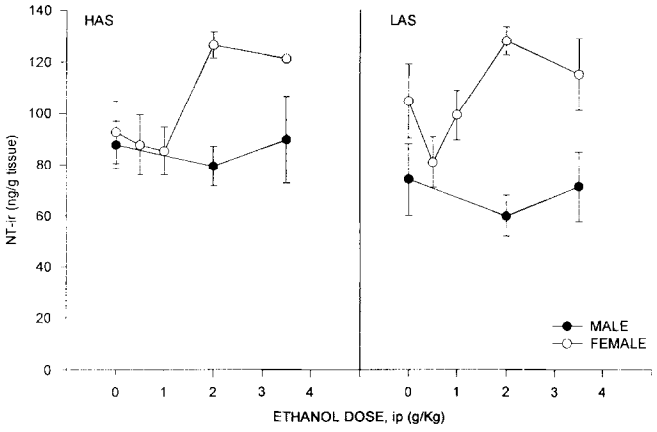


FIG. 1. Dose-response relationships for effects of ethanol on neurotensin levels in hypothalamus of HAS and LAS rats. HAS and LAS rats ($n = 4$ to 6 for males and females) were injected with saline or ethanol (20% v/v) in saline to give the doses indicated. Unfortunately, there were insufficient numbers of male HAS and LAS in generations 17 and 18 to include ethanol doses of 0.5 and 1.0 g/kg. Following $CO_2$ euthanasia and decapitation brains were rapidly removed, dissected and regions placed in 0.01 N HCl. Samples were homogenized and NT-ir extracted as reported previously. Data were analyzed by three-way ANOVA for between-subjects variables (line, sex, and ethanol dose). Scheffé test was used to evaluate comparisons between groups.

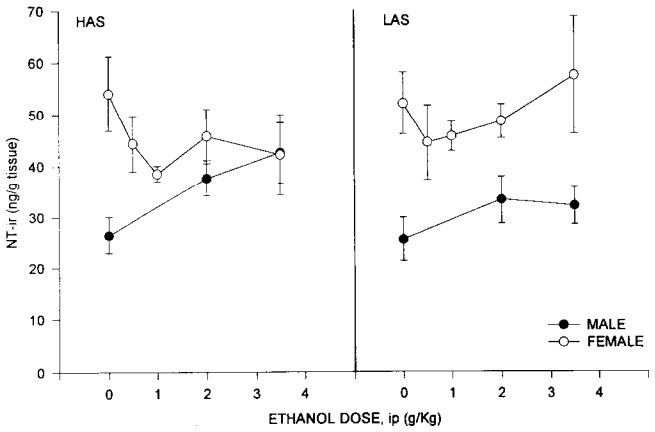


FIG. 2. Dose-response relationships for effects of ethanol on neurotensin levels in ventral midbrain of HAS and LAS rats. Experimental conditions and data analyses were as described in Fig. 1.

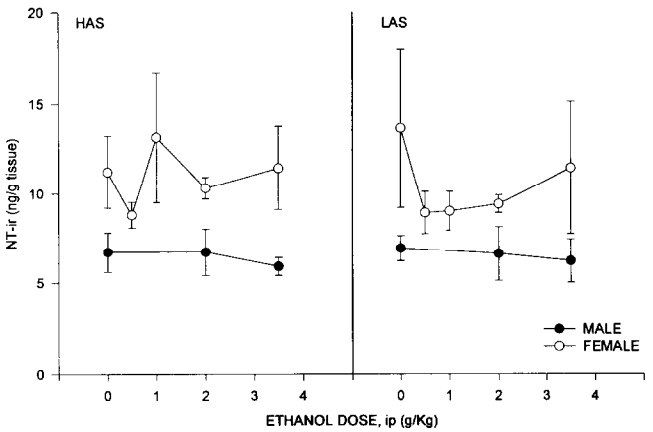


FIG. 4. Dose-response relationships for effects of ethanol on neurotensin levels in caudate putamen of HAS and LAS rats. Experimental conditions and data analyses were as described in Fig. 1.

main effects of sex, $F(1, 59) = 32.3$, $p < 0.0001$, line, $F(1, 59) = 9.1$, $p < 0.004$, and ethanol dose, $F(1, 59) = 14.4$, $p < 00001$. All interactions among these variables were significant ($p < 0.01$). Scheffé post hoc tests showed NT-ir levels at 3.5 g/kg ethanol to be significantly ($p < 0.05$) different from values after saline or 1.0 g/kg ethanol. In females, ANOVA showed a significant difference in NT-ir values between saline and 1.0 g/kg ethanol. These indicate that ethanol produces a biphasic dose response on NT-ir levels in the NA with subhypnotic doses causing a decrease and hypnotic doses eliciting a increase in levels.

As indicated in Fig. 4 and Table 2, NT-ir levels in the caudate putamen are quite low; thus, there is greater variability in the radioimmunoassays for NT in this brain region compared to other regions. Analysis of the data indicated no significant main effects of line, sex, or ethanol dose.

*Neurotensin Receptors*

Preliminary studies with the HAS and LAS rats indicated that these lines did not differ in either high-affinity ($NT_H$) or low-affinity ($NT_L$) NT receptor subtype in frontal cortex, striatum, or ventral midbrain, data not shown. These studies utilized the low-affinity NT receptor blocker, levocabastine (31), at one concentration (20 nM) of [$^3$H]-NT in the binding assay. The data presented in Tables 4 and 5 represent equilibrium binding assays and Scatchard analysis of NT binding at nine concentrations of [$^3$H]-NT ranging from 0.01 to 20 nM. Analysis of these binding data were performed with LIGAND for a two site model and iterated values for $K_d$ and $B_{max}$ were obtained for specific brain regions of both male and female HAS and LAS rats.

The results in Table 4 revealed that high-affinity NT ($NT_H$) receptors, putatively the pharmacologically relevant receptors, differed significantly by specific brain region in both HAS and LAS rats. However there were no significant differences in $K_d$ values for the high-affinity receptor in brain regions studies. Two-way ANOVA (line × sex) showed no significant line differences in $K_d$ or $B_{max}$ values for striatum (STR). Values for $B_{max}$ in STR from HAS, but not LAS, were significantly higher in males than in females, $F(1, 9) = 3.6$. Similar analyses showed no significant overall line or sex effects for VMB $B_{max}$ values; however, $B_{max}$ values in HAS males were significantly ($p < 0.05$) higher than values in LAS males, $F(1, 9) = 3.2$.

Binding parameters for the low-affinity NT receptor, $NT_L$, (levocabastine-sensitive receptor) are shown in Table 5. No between-line or sex differences were observed in $K_d$ or $B_{max}$ values for these receptors.

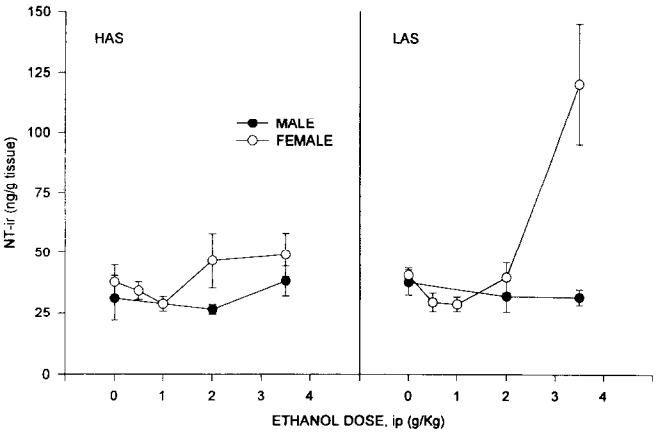


FIG. 3. Dose-response relationships for effects of ethanol on neurotensin levels in nucleus accumbens of HAS and LAS rats. Experimental conditions and data analyses were as described in Fig. 1.

## DISCUSSION

Previously, the LS and SS mice and recombinant inbred strains derived from these lines were shown to differ in neurotensin-immunoreactivity (NT-ir) and in NT receptor densities (14) in specific brain regions, and there were significant genetic correlations among these NT measures and hypnotic sensitivity to ethanol (11). Thus, we determined the levels of NT-ir and NT receptor densities in specific brain regions of HAS and LAS rats to determine whether hypnotic sensitivity to ethanol in rats might be associated with differences in NT-ergic processes. Data in Table 2 show significant differences in NT-ir levels by brain region in both HAS and LAS rats. ANOVA of the data showed that, within sex, NT-ir levels do

TABLE 4

HIGH-AFFINITY NEUROTENSIN RECEPTOR CHARACTERISTICS IN HAS AND LAS RAT BRAINS

| | | $NT_H$ Binding Parameters | | | |
|---|---|---|---|---|---|
| | | Males | | Females | |
| Rat Line | Brain Region | $K_d$ | $B_{max}$ | $K_d$ | $B_{max}$ |
| HAS | STR | 0.14 ± 0.05 | 29.7 ± 6.9 | 0.12 ± 0.04 | 17.3 ± 4.7 |
| HAS | VMB | 0.21 ± 0.04 | 39.0 ± 7.1 | 0.10 ± 0.02 | 24.8 ± 4.2 |
| LAS | STR | 0.10 ± 0.05 | 18.9 ± 4.0 | 0.23 ± 0.15 | 16.3 ± 5.3 |
| LAS | VMB | 0.10 ± 0.06 | 21.7 ± 5.3 | 0.06 ± 0.03 | 23.5 ± 4.3 |

All values are means ± SEM of $n = 4$ to 6 separate experiments using pooled brain regions from four to six animals of corresponding line and sex.

not differ by brain region between HAS and LAS rats. However, there were significant gender differences in NT-ir levels in virtually all brain regions in both rat lines with levels in females being approximately 25 to 75% higher than in males. The greatest gender differences were observed in the ventral midbrain (VMB) and the smallest differences were found in the nucleus accumbens (NA); indeed, no difference was observed in levels in NA from LAS males and females. Levels in the hypothalamus (HYP) and caudate putamen (CP) were about 40 to 50% higher in both HAS and LAS females than in males. These data are of interest in that female rats of the high alcohol sensitive, HAS, line are less sensitive than males to the hypnotic effects of ethanol. The results suggest that while NT-ir levels, per se, may not account for selectively bred differences in hypnotic sensitivity in rats, differences between males and females may be, in part, due to differences in NT-ir levels in specific brain regions.

Our previous studies showed that LS and SS mice differed in NT-ir levels in hypothalamus, but not in whole midbrain or striatum (caudate putamen plus nucleus accumbens). Those studies showed that high ethanol-sensitive, LS, had higher hypothalamic NT-ir levels than SS mice; whereas, in the present study, high alcohol-sensitive, HAS rats, have levels similar to the LAS rats. Comparisons of NT-ir levels in male and female mice from the LS and SS lines have not been made. The apparent discrepancy in hypothalamic NT-ir levels in LS vs. SS compared with HAS vs. LAS may reflect a species difference or might be accounted for in that differences in hypothalamic NT-ir levels may not be related to hypnotic sensitivity and may differ fortuitously in the selected mouse lines.

Previous studies with LS and SS mice have shown that ethanol administration produced a biphasic dose-response effect on NT-ir levels in hypothalamus, midbrain, and nucleus accumbens (12). Subhypnotic doses produced a decrease and hypnotic doses produced an increase in NT-ir levels. It is of interest that those observations are similar to the findings in the present studies in some brain regions, particularly the nucleus accumbens, from female HAS and LAS rats. We proposed that decreases in NT-ir levels, within 30 min after subhypnotic doses of ethanol, may result from enhanced NT release followed by rapid degradation by endopeptidases (4,12,15). Rapid increases in NT-ir levels following hypnotic doses of ethanol may be caused by inhibition of release with continued synthesis and/or by inhibition of degradation. These possibilities are consistent with the known biphasic effects of ethanol administration on dopaminergic processes. It has been shown that low doses of ethanol increases dopamine overflow in mesolimbic dopaminergic neurons as measured by increased dopamine release and turnover in rat nucleus accumbens (26). Inhibition of dopamine release has been reported for high doses of ethanol (24). Because dopamine and NT are colocalized in terminals of dopaminergic neurons in the frontal cortex and nucleus accumbens and are coreleased in the medial prefrontal cortex following medial forebrain bundle stimulation (1), comparable effects of ethanol on NT-ir levels and dopamine might be expected.

It has been well documented using LS × SS recombinant

TABLE 5

LOW-AFFINITY NEUROTENSIN RECEPTOR CHARACTERISTICS IN HAS AND LAS RAT BRAINS

| | | $NT_1$ Binding Parameters | | | |
|---|---|---|---|---|---|
| | | Males | | Females | |
| Rat Line | Brain Region | $K_d$ | $B_{max}$ | $K_d$ | $B_{max}$ |
| HAS | FC | 1.72 ± 0.18 | 213.7 ± 15.7 | 3.3 ± 0.9 | 210.8 ± 18.1 |
| HAS | STR | 2.16 ± 0.18 | 264.3 ± 24.7 | 3.0 ± 1.2 | 259.6 ± 16.6 |
| HAS | VMB | 2.14 ± 0.30 | 247.2 ± 23.3 | 1.3 ± 0.2 | 271.2 ± 17.0 |
| LAS | FC | 2.40 ± 0.17 | 217.0 ± 10.9 | 2.9 ± 0.67 | 176.0 ± 13.8 |
| LAS | STR | 2.90 ± 0.35 | 301.6 ± 43.4 | 1.7 ± 0.42 | 297.2 ± 19.1 |
| LAS | VMB | 2.24 ± 0.22 | 272.0 ± 33.6 | 1.6 ± 0.30 | 341.1 ± 22.4 |

All values are means obtained from the same experiment as described in Table 2. ANOVA showed no significant line or sex differences in any of the brain regions.

(RI) strains of mice that differences in hypnotic sensitivity and in locomotor effects at subhypnotic doses of ethanol are mediated by multiple genes with additive effects; estimates indicate a minimum of seven and four genetic loci, respectively (6,13). Thus, it is unlikely that in mouse or rat all of the genetic variance in ethanol sensitivity can be accounted for by one mechanism. In the LS × SS RI strains, genetic variability in NT-ir levels and NT receptor densities has been observed with evidence indicating polygenic influences on those levels (16). Significant genetic correlations between NT receptor densities (11) or NT-ir levels (unpublished data) and ethanol sensitivity have been shown. For example, $NT_H$ densities in striatal regions were significantly and positively correlated with hypnotic sensitivity; that is, the higher the receptor density the greater the sensitivity to ethanol. The present results, showing higher $NT_H$ density in striatum from HAS than from LAS males, are consistent with the genetic correlations in LS × SS RI strains. It is of interest that $NT_H$ densities in HAS males were higher than in females. These results show a reciprocal relationship between NT-ir levels and $NT_H$ receptor densities in the mesolimbic brain regions.

Results of the present study showing clear gender differences in NTergic processes indicate that differences in NT-ir levels and/or receptors may influence sex differences in ethanol sensitivity. Studies show that genetic differences in NTergic processes in specific brain regions may mediate, in part, differences in ethanol sensitivity in mice (11,12,14); however, this result was not confirmed in the HAS and LAS rats. The discrepancy between results with mice and rats might be explained by the absence of any significant genetic variation in NT receptor or NT-ir levels within the foundation stock from which the HAS and LAS were selected (7).

ACKNOWLEDGEMENTS

This research was supported, in part, by USPHS Grants AA 07330, AA 05868, and AA 03527.

## REFERENCES

1. Bean, A. J.; Roth, R. H. Extracellular dopamine and neurotensin in rat prefrontal cortex in vivo: Effects of median forebrain bundle stimulation frequency, stimulation pattern, and dopamine autoreceptors. J. Neurosci. 11:2694–2702; 1991.
2. Bissette, G.; Dauer, W. T.; Kilts, C. D.; O'Connor, L.; Nemeroff, C. B. The effect of the stereoisomers of butaclamol on neurotensin content in discrete regions of the rat brain. Neuropsychopharmacology 1:329–335; 1988.
3. Campbell, A. D.; Jones, B. C.; Erwin, V. G. Regional characterization of brain neurotensin receptor subtypes in LS and SS mice. Alcohol Clin. Exp Res. 15:1011–1017; 1991.
4. Checler, F.; Vincent, J. P.; Kitabgi, P. Degradation of neurotensin by rat brain synaptic membranes: Involvement of a thermolysin-like metalloendopeptidase (enkephalinase), angiotensin-converting enzyme, and other unidentified peptidases. J. Neurochem. 1:375–384; 1983.
5. Clineschmidt, B. V.; McGuffin, J. C.; Bunting, P. B. Neurotensin: Antinocisponsive action in rodents. Eur. J. Pharmacol. 54: 129–139; 1979.
6. DeFries, J. C.; Wilson, J. R.; Erwin, V. G.; Petersen, D. R. LSXSS recombinant inbred strains of mice: Initial characterization. Alcohol Clin. Exp. Res. 13:196–200; 1989.
7. Draski, L. J.; Spuhler, K. P.; Erwin, V. G.; Baker, R. C.; Deitrich, R. A. Selective breeding of rats differing in sensitivity to the effects of ethanol administration. Alcohol Clin. Exp. Res. 16: 48–54; 1992.
8. Emson, P. C.; Goedert, M.; Benton, H.; St.-Pierre, S.; Rioux, F. The regional distribution and chromatographic characterization of neurotensin-like immunoreactivity in the rat. Adv. Biochem. Psychopharmacol. 33:477–485; 1982.
9. Erwin, V. G.; Heston, W. D. W.; McClearn, G. E.; Deitrich, R. A. Effect of hypnotics on mice genetically selected for sensitivity to ethanol. Phamacol. Biochem. Behav. 4:679–683; 1976.
10. Erwin, V. G.; Jones, B. C. Comparison of neurotensin levels, receptors and actions in LS/Ibg and SS/Ibg mice. Peptides 10: 435–440; 1989.
11. Erwin, V. G.; Jones, B. C. Genetic correlations among ethanol-related behaviors and neurotensin receptors in long sleep (LS) × short sleep (SS) recombinant inbred strains of mice. Behav. Genet. 23:191–196; 1993.
12. Erwin, V. G.; Jones, B. C.; Radcliffe, R. Low doses of ethanol reduce neurotensin levels in discrete brain regions from LS/Ibg and SS/Ibg mice. Alcohol Clin. Exp. Res. 14:42–47; 1990.
13. Erwin, V. G.; Jones, B. C.; Radcliffe, R. A. Further characterization of LSXSS recombinant inbred strains of mice: Activating and hypothermic effects of ethanol. Alcohol Clin. Exp. Res. 14: 200–204; 1990.
14. Erwin, V. G.; Korte, A. Brain neurotensin receptors in mice bred for differences in sensitivity to ethanol. Alcohol 5:195–201; 1988.
15. Erwin, V. G.; Korte, A.; Marty, M. Neurotensin selectively alters ethanol-induced anesthesia in LS/Ibg and SS/Ibg lines of mice. Brain Res. 400:80–90; 1987.
16. Erwin, V. G.; Radcliffe, R.; Hinkle, B.; Jones, B. C. Genetic-based differences in neurotensin levels and receptors in brains of LL × SS mice. Peptides 14:821–828; 1993.
17. Erwin, V. G.; Radcliffe, R. A. Characterization of neurotensin-stimulated phosphoinositide hydrolysis in brain regions of long-sleep and short-sleep mice. Brain Res. 629:59–66; 1993.
18. Erwin, V. G.; Su, N. C. Neurotensin and ethanol interactions on hypothermia and locomotor activity in LS and SS mice. Alcohol Clin. Exp. Res. 13:91–94; 1989.
19. Frey, P.; Lis, M.; Coward, D. M. Neurotensin concentrations in rat striatum and nucleus accumbens: Further studies of their regulation. Neurochem. Int. 12:33–38; 1988.
20. Gilbert, J. A.; Richelson, E. Neurotensin stimulates formation of cyclic GMP in murine neuroblastoma clone N1E-115. Eur. J. Pharmacol. 99:245–246; 1984.
21. Goedert, M.; Pinnock, R. D.; Downes, C. P.; Mantyh, P. W.; Emson, P. C. Neurotensin stimulates inositol phospholipid hydrolysis in rat brain slices. Brain Res. 323:193–197; 1984.
22. Goedert, M.; Pittaway, K.; Williams, B. J.; Emson, P. C. Specific binding of tritiated neurotensin to rat brain membranes: Characterization and regional distribution. Brain Res. 304:71–81; 1984.
23. Hokfelt, T.; Everitt, B. J.; Theodorsson-Norheim, E.; Goldstein, M. Occurrence of neurotensin-like immunoreactivity in subpopulations of hypothalamic, mesencephalic, and medullary catecholamine neurons. J. Comp. Neurol. 222:543–549; 1984.
24. Hunt, W. A.; Majchrowicz, E. Alteration in turnover of brain norepinephrine and dopamine in alcohol-dependent rats. J. Neurochem. 23:549–552; 1974.
25. Hunter, W. M.; Greenwood, F. C. Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature 194:495–496; 1962.
26. Imperato, A.; Dichiara, G. Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. J. Pharmacol. Exp. Ther. 239:219–228; 1986.
27. Iversen, L. L.; Iversen, S. D.; Bloom, F.; Douglas, C.; Brown, M.; Vale, W. Calcium-dependent release of somatostatin and neurotensin from rat brain in vitro. Nature 273:161–163; 1978.
28. Jennes, L.; Stumpf, W. E.; Kalivas, P. W. Neurotensin: Topographical distribution in rat brain by immunohistochemistry. J. Comp. Neurol. 210:211–214; 1982.
29. Kalivas, P. W.; Nemeroff, C. B.; Prange, A. J., Jr. Neuroana-

tomical site specific modulation of spontaneous motor activity by neurotensin. Eur. J. Pharmacol. 78:471–474; 1982.
30. Kislauskis, E.; Bullock, B.; McNeil, S.; Dobner, P. R. The rat gene encoding neurotensin and neuromedin N. Structure, tissue-specific expression, and evolution of exon sequences. J. Biol. Chem. 263:4963–4968; 1988.
31. Kitabgi, P.; Rostene, W.; Dussaillant, M.; Schotte, A.; Laduron, P. M.; Vincent, J. P. Two populations of neurotensin binding sites in murine brain: Discrimination by the antihistamine levocabastine reveals markedly different radioautographic distribution. Eur. J. Pharmacol. 140:285–293; 1987.
32. Kobayashi, R. M.; Brown, M.; Vale, W. Regional distribution of neurotensin and somatostatin in rat brain. Brain Res. 126:584–588; 1977.
33. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275; 1951.
34. Makino, S.; Okamura, H.; Morimoto, N.; Abe, J.; Yanaihara, N.; Ibata, Y. Distribution of neurotensin-like immunoreactivity in the diencephalon of the Japanese monkey (*Macaca fuscata*). J. Comp. Neurol. 260:552–563; 1987.
35. Mazella, J.; Leonard, K.; Chabry, J.; Kitabgi, P.; Vincent, J. P.; Beaudet, A. Binding and internalization of iodinated neurotensin in neuronal cultures from embryonic mouse brain. Brain Res. 564:249–255; 1991.
36. Mazella, J.; Poustis, C.; Labbe, C.; Checler, F.; Kitabgi, P.; Granier, C.; Van Rietschoten, J.; Vincent, J. P. Monoiodo-[Trp11]-neurotensin, a highly radioactive ligand of neurotensin receptors. Preparation, biological activity, and binding properties to rat brain synaptic membranes. J. Biol. Chem. 258:3476–3481; 1983.
37. Merchant, K. M.; Letter, A. A.; Gibb, J. W.; Hanson, G. R. Changes in the limbic neurotensin systems induced by dopaminergic drugs. Eur. J. Pharmacol. 153:1–9; 1988.
38. Moyse, E.; Rostene, W.; Vial, M.; Leonard, K.; Mazella, J.; Kitabgi, P.; Vincent, J. P.; Beaudet, A. Distribution of neurotensin binding sites in rat brain: A light microscopic radioautographic study using monoiodo-[$^{125}$I]Tyr3-neurotensin. Neuroscience 22:525–536; 1987.
39. Nemeroff, C. B.; Bissette, G.; Prange, A. J., Jr.; Loosen, P. T.; Barlow, T. S.; Lipton, M. A. Neurotensin: Central nervous system effects of a hypothalamic peptide. Brain Res. 128:485–496; 1977.
40. Nemeroff, C. B.; Cain, S. T. Neurotensin-dopamine interactions in the CNS. Trends Pharmacol. Sci. 6:201–205; 1985.
41. Schotte, A.; Leysen, J. E. Further characterization of neurotensin binding in the rat brain: Levocabastine-displaceable neurotensin binding sites are not histamine-$H_1$ receptors. Biochem. Pharmacol. 38:3891–3893; 1989.
42. Slotnick, B. M.; Leonard, C. M. A Stereotaxic atlas of the albino mouse forebrain. DHEW Publication (ADN) 75-100; 1977.
43. Szigethy, E.; Beaudet, A. Selective association of neurotensin receptors with cholinergic neurons in the rat basal forebrain. Neurosci. Lett. 83:47–52; 1987.
44. Uhl, G. R. Distribution of neurotensin and its receptor in the central nervous system. Ann. NY Acad. Sci. 400:132–149; 1982.
45. Uhl, G. R.; Snyder, S. H. Neurotensin receptor binding, regional, and subcellular distribution favors transmitter role. Eur. J. Pharmacol. 41:89–91; 1977.
46. Young, W. S.; Kuhar, M. J. Neurotensin receptor localization by light microscopic autoradiography in rat brain. Brain Res. 206: 273–285; 1981.