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μ-Opiate Receptor Binding and Function in HOT and COLD Selected Lines of Mice

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STUART, J. F., J. DOROW AND D. J. FELLER. *μ-Opiate receptor binding and function in HOT and COLD selected lines of mice.* PHARMACOL BIOCHEM BEHAV **46**(3) 519–526, 1993.—μ-Opiate receptor binding and function were examined in mice selectively bred for sensitivity (COLD) and resistance (HOT) to ethanol-induced hypothermia. These mice also have differential hypothermic sensitivity to μ-opiates. μ-Opiate receptor density was higher in the frontal cortex of HOT mice compared with COLD mice, but was the same in other brain areas. In addition, there were no line differences in K_d values. Basal adenylate cyclase (AC) activity was similar in both lines, as was the response to forskolin (FS) stimulation. Morphine was more effective at inhibiting FS-AC activity in the hypothalamus of HOT mice compared with COLD mice but was equally effective in the frontal and parietal cortex. There were no differences between lines in basal Ca^{2+}, Mg^{2+}, or Ca^{2+}/Mg^{2+}-ATPase activity. Further, 30 min after treatment ATPase activities were not altered in ethanol- or levorphanol-treated mice. These results suggests that μ-opiate biochemical pathways, but not ATPase enzyme systems, may be involved in mediating differential hypothermic sensitivity observed in HOT and COLD mice.

Selected lines HOT and COLD mice μ-Opiate Ethanol Hypothermia Adenylate cyclase ATPase

MICE have been selectively bred for sensitivity (COLD) or resistance (HOT) to ethanol (EtOH)-induced hypothermia (9,27). The study of correlated neurochemical responses to genetic selection could help our understanding of biochemical mechanisms underlying ethanol-induced hypothermia. One neurochemical system known to have important influences in thermoregulation is the opiate system (1,8,32). HOT and COLD selected mice also exhibit a differential hypothermic response to sedative drugs (12), μ-opiate agonists, and the κ-opiate agonist, U-50,488H (13). Opiates produce hypo- or hyperthermia depending upon the species and experimental conditions (1,4,8,32). κ-Opiate agonists produce hypothermia in mice while μ-opiate agonists produce either hypo- or hyperthermia depending upon the dose and ambient temperature (1,4).

Morphine and levorphanol, specific ligands for the μ_1-opiate receptor (6,14,24), are inhibited by the μ-opiate selective antagonist, naloxone. μ-Opiates inhibit adenylate cyclase (AC) activity through a pertussis toxin-sensitive G protein (7,16,36), while κ-agonists inhibit AC activity in some tissues (3,36) and not in others (15,31). Ethanol stimulates AC activity by interaction with Gs protein (17), but does not affect morphine inhibition of AC activity (16).

Pillai and Ross (29) suggest that ethanol-induced hypothermia in rats is mediated by opiate pathways ($\kappa > \mu$). In addition, they found that acute ethanol administration (29) increased Ca^{2+}/Mg^{2+}-ATPase activity in synaptic membranes in the hypothalamus but not in the cortex or brainstem. Ethanol's hypothermic effect and stimulation of ATPase activity were reduced by administration of naloxone. Similar to ethanol-induced hypothermia (29), Pillai and Ross also found that morphine-induced hyperthermia in rats was inhibited by naloxone but, in contrast with ethanol, morphine affected calcium stasis in the brain by decreasing Ca^{2+}/Mg^{2+}-ATPase activity (28).

This study has been designed to answer the following basic biochemical questions: a) Has genetic selection of HOT and COLD mice for ethanol-induced hypothermia altered the density or affinity of brain μ-opiate receptors? b) Has selection changed the coupling between μ-opiate receptors and AC or produced a direct effect on the catalytic subunit? c) Does ethanol and/or levorphanol affect the ATPase system in mice and, if so, is there a difference between HOT and COLD selected lines?

METHOD

Animals

Two pairs of HOT and COLD lines (HOT 1 and COLD 1, HOT 2 and COLD 2) were bred from a foundation stock by within-family selective breeding (9). The two COLD lines were bred for the maximum hypothermic response attained 30 or

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60 min after injection of 3 g/kg EtOH. The two HOT lines were bred for the minimum hypothermic (and sometimes hyperthermic) response to EtOH. They were housed four or five per cage on a 12 L : 12 D cycle with ad lib access to food and water.

HOT and COLD mice, both males and females, from selected generations 17–28 were used in these studies. There were at least three mice from each genetic replicate for an experiment. The age of the animals ranged from 7 to 14 weeks. Within any experiment, there was no more than a 3-week difference in age. Testing was always performed between 9:00 a.m. and 1:00 p.m.

Hypothermia Measurements

The approach used to test all drugs was similar to that previously described by Crabbe et al. (9). All experiments were at a room temperature of 21 ± 2°C. Body temperatures were taken rectally with a 0.5-mm probe (RET-3) coupled to a Sensortek TH-8 Digital Thermometer 5–15 s after gentle restraint and probe insertion. After taking baseline temperature, drug 1 was administered by IP injection and the mouse returned to its individual chamber. Drug 2 was administered by IP injection 2 or 15 min following the first injection. After the second injection, the mouse was returned to its individual chamber. Test temperatures of each mouse were taken at 15 and 30 min. After the last test temperature, mice were sacrificed and brains removed for biochemical studies.

Tissue Preparation

Mice were killed by cervical dislocation and decapitation. Brains were removed and dissected into the frontal cortex, parietal cortex, hypothalamus, and brainstem. Tissues were further prepared as required for each assay.

[^{3}H]DAGO Binding Assay

Standard receptor binding assay techniques were used to determine the B_{max} and K_d for the binding of [^{3}H]DAGO (specific activity = 56.26 Ci/mmol) (2). Tissue, pooled from five animals, was homogenized in 15 vol 50 mM Tris-HCl (pH 7.4 at 4°C) and centrifuged at 16,000 × *g* for 15 min. The pellet was washed once and resuspended in 100 vol (0.5–0.7 mg/ml protein) of the same buffer. Samples, 1 ml tissue in a final volume of 1.02 ml, were incubated with radioligand for 1 h at room temperature. Five concentrations of [^{3}H]DAGO (0.1–10.0 nM) were used to determine total binding and 10 μM morphine sulfate was used to determine nonspecific binding. Samples, collected on GF/B filters and washed twice with ice-cold 50 mM Tris-HCl (pH 7.4 at 4°C), were placed in vials containing 9 ml BioSafe II. Radioactivity was counted using Beckman LS 3801 (Beckman Instruments, Fullerton, CA) in a 0- to 400-nm window and corrected for 35% counting efficiency. The data were analyzed using the computer program LIGAND (21).

Adenylate Cyclase Assay

Homogenates were prepared according to a modification of the procedure described by De Vivo et al. (10). Tissue, collected from a single animal, was homogenized in 10 vol buffer containing 0.3 M sucrose, 5 mM EGTA, and 20 mM Tris-HCl (pH 7.4 at 4°C) and then diluted with 8 vol homogenizing buffer. A two-step centrifugation was used: First, homogenates were centrifuged for 10 min at 500 × *g*, followed by centrifugation of the supernatant for 10 min at 39,000 × *g*. AC activity was determined using a modification of the method of Salomon (33,34). Approximately 100–150 μg protein was incubated at 30°C in assay buffer containing 100 mM NaCl, 10 μM guanosine triphosphate (GTP), 2 mM $MgCl_2$, 0.2 mM adenosine triphosphate (ATP) (α-[^{32}P]), 1.0 mM cyclic adenosine monophosphate (cAMP), 4 mM theophyline, 5 mM creatine phosphate, 16 μg/ml creatine phosphokinase, and 80 mM Tris-HCl (pH 7.4 at 23°C). Basal AC activity was stimulated by addition of 3 μM of a water-oluble derivative of forskolin (FS), 7-β-deacetyl-7-β-[ν-(morpholin)] (20). The reaction was stopped by adding 30% trichloroacetic acid. Recovery was determined by addition of [^{3}H]cAMP. Counts were collected by a two-column separation method as described by Salomon (33,34). Nine milliliters of BioSafe II were added to each sample and the radioactivity was counted on a Beckman LS-3801 using a dual setting: 0–400 nm for [^{3}H] and 400–1,000 nm for [^{32}P].

ATPase Assay

Samples were prepared by homogenizing the tissue in 10 vol buffer containing 0.32 M sucrose, 10 mM HEPES (pH 7.4), and 1 mM EGTA. Homogenates, prepared from the tissue of a single animal, were centrifuged at 900 × *g* for 10 min and the pellets resuspended in 10 vol buffer. ATPase assays were performed according to a modification of the procedure outlined by Palayoor et al. (23). Mg^{2+}-independent Ca^{2+}-ATPase was measured in a buffer containing 25 mM HEPES (pH 7.4), 0.2 M sucrose with various concentrations of Ca^{2+} buffered by EGTA, and between 0.5–2.0 mM Na^+-ATP. Ca^{2+}/Mg^{2+}-ATPase activity was measured in a buffer containing 50 mM HEPES (pH 7.4), 2 mM $MgCl_2$, 100 mM KCl, 100 μM EGTA, 100 μM oubain, 0.5–2.0 mM Na^+-ATP, and 0.1–2.0 mM $CaCl_2$. Basal Mg^{2+}-ATPase was determined using Ca^{2+} free buffer. Assay buffer containing ≈15 μg protein was incubated at either 30 or 37°C for 5–15 min. The reactions were stopped by the addition of 2.5 N H_2SO_4. The amount of inorganic phosphate liberated was determined by the method of Baykov et al. (5) and reported as nmol Pi/mg protein/min.

Protein Assay

Proteins were determined by the Peterson modification of the Lowry assay (26).

Statistical Analyses

Statistical analyses were performed by analysis of variance (ANOVA) on the CRUNCH (version 4) analysis program, with posthoc analyses by simple main effects. If replicates within a selected line did not differ, data for each animal/sample from both replicates were averaged (collapsed across replicates) to simplify analysis and presentation. When the replicates differed, they were presented separately. There was no statistical interaction with age or gender in any of the experiments; therefore, the data were collapsed across these variables.

Drugs

Ethanol was purchased from Midwest Grain Products (Pekin, IL). [^{32}P]ATP, [^{3}H]cAMP, and [^{3}H]DAGO (specific activity = 56.26 Ci/mmol) were purchased from DuPont NEN (Wilmington, DE). Water-soluble forskolin was purchased from Research Biochemicals, Inc. (Natick, MA). BioSafe II was purchased from Research Products International Corp.

TABLE 1

DENSITY (fmol/mg PROTEIN) OF [^{3}H]DAGO RECEPTORS IN BRAIN AREAS FROM HOT AND COLD MICE

Brain Area	HOT	COLD
Frontal cortex	105.9 ± 8.7*†	59.0 ± 8.5
Parietal cortex	80.6 ± 4.7	71.7 ± 1.4
Hypothalamus	173.7 ± 24.0‡	179.7 ± 32.2
Brainstem	86.3 ± 8.9	84.5 ± 6.8

*Mean ± SEM, n = 7–8 mice/line, collapsed across replicate.
†HOT > COLD, $p < 0.003$.
‡n = 14–15/line.

(Mt. Prospect, IL). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Ethanol and levorphanol were diluted with 0.9% (w/v) saline before injection.

RESULTS

With the exception of the levorphanol hypothermia, experimental data from replicates within a selected line did not differ. Therefore, replicate results were pooled for most experiments to simplify statistical analyses and presentation. For the levorphanol hypothermia data, the individual replicate results were presented.

Our results show a significant line difference between HOT and COLD mice in frontal cortex μ-opiate receptor density, $F(1, 10) = 18.3$, $p < 0.003$. Table 1 shows the density was higher (2×) in HOT mice compared to COLD mice (selection generation 17–19). Selected lines did not differ in receptor density in other brain areas, and there was no line difference in K_d for [^{3}H]DAGO binding in any brain region tested (Table 2).

Basal and FS-stimulated and AC activity were the same in tissue from the frontal cortex, parietal cortex, and hypothalamus from HOT and COLD mice (Fig. 1). Morphine was equally effective in both lines at inhibiting FS-AC activity in the frontal and parietal cortex (Fig. 2). However, there was a statistically significant, $F(1, 80) = 16.7$, $p < 0.0002$, greater morphine inhibition of FS-AC in the hypothalamus from HOT mice compared with COLD mice. There was also a significant effect of morphine concentration, $F(1, 80) = 35.2$, $p < 0.0001$, and a significant replicate effect, $F(1, 80) = 14.3$, $p < 0.0004$. There was not a significant line × replicate interaction, $F(1, 80) = 1.8$, $p < 0.10$, nor line × drug concentration interaction, $F(1, 80) = .299$, $p < 0.90$. Using a different μ-opiate agonist, *l*-levorphanol, we confirmed our results presented in Fig. 2. Inhibition of hypothalamic FS-AC by levorphanol was significantly different between HOT and COLD selected lines, $F(1, 69) = 10.8$, $p < 0.002$. Hypothalamic homogenates from HOT mice were more sensitive to levorphanol inhibition than homogenates from COLD mice (Fig. 3). In contrast with the morphine data, the levorphanol data did not indicate an effect of replicate, $F(1, 69) = .9$, $p > 0.30$, or any interactions.

TABLE 2

AFFINITY (nM) OF [^{3}H]DAGO RECEPTORS IN BRAIN AREAS FROM HOT AND COLD MICE

Brain Area	HOT	COLD
Frontal cortex	1.07 ± 0.20*	0.75 ± 0.08
Parietal cortex	0.68 ± 0.05	0.79 ± 0.07
Hypothalamus	1.98 ± 0.34†	1.98 ± 0.29
Brainstem	0.79 ± 0.09	0.93 ± 0.11

*Mean ± SEM, n = 7–8 mice/line, collapsed across replicate.
†n = 14–15 mice/line, collapsed across replicate.

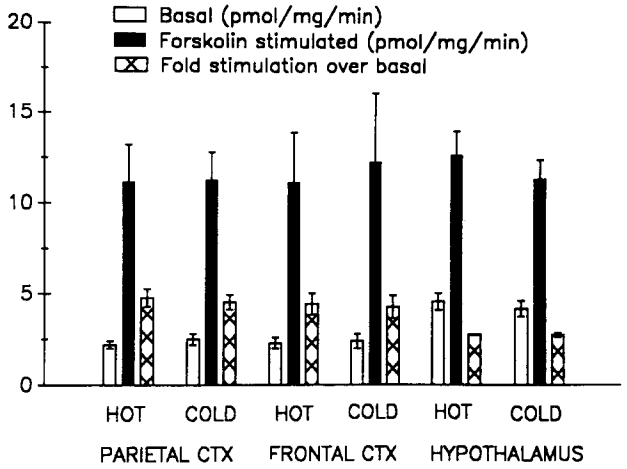

FIG. 1. Comparison of basal and forskolin-stimulated adenylate cyclase (FS-AC) activity in brain homogenates from naive mice. A water-soluble derivative of forskolin (3 μM) was used to stimulate basal activity, the assay was performed as described in the Method section, and the amount of stimulation determined by dividing FS-AC activity by basal activity. n = 12 mice/line, collapsed across replicate.

HOT and COLD mice did not differ in hypothermic response when injected with dexmedetomidine, an α_2-agonist (35) (unpublished results). It is known that AC activity is inhibited by receptor-bound α_2-agonists coupled to the Gi protein. Figure 4 shows there was no difference in dexmedetomidine inhibition of FS-AC activity between hypothalamic homogenates from HOT and COLD mice.

We also tested the effects of naloxone, a μ-opiate antagonist, on ethanol- and *l*-levorphanol-induced hypothermia and on brain Ca^{2+}/Mg^{2+} ATPase activity. Figure 5 shows, as previously reported (9), a significant difference between HOT and COLD selected lines in their hypothermic response to ethanol [COLD > HOT; $F(1, 64) = 165.4$, $p < 0.0001$]. There was no effect of naloxone on ethanol-induced hypothermia in either line, $F(1, 64) = 0.008$, $p < 0.9$. HOT and COLD mice had the same basal Ca^{2+}-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities in the hypothalamus, parietal cortex, and brainstem (Table 3). Further, there was no significant effect of ethanol or ethanol with naloxone (Fig. 6) on ATPase activity. Naloxone alone produced a slight, nonsignificant, decrease in Ca^{2+}/Mg^{2+}-ATPase activity.

We previously reported that both replicates of HOT and COLD mice from selection generation 18 differed in their hypothermic response to levorphanol (12). This result was not replicated when testing mice from selection generation 28 (Fig. 7). Using a paradigm that included pretreatment with either 1 mg/kg naloxone or saline 2 min before injection of 10 mg/kg levorphanol, we observed a significant line effect, $F(1, 96) = 31.5$, $p < 0.0001$, for hypothermia (COLD > HOT). There

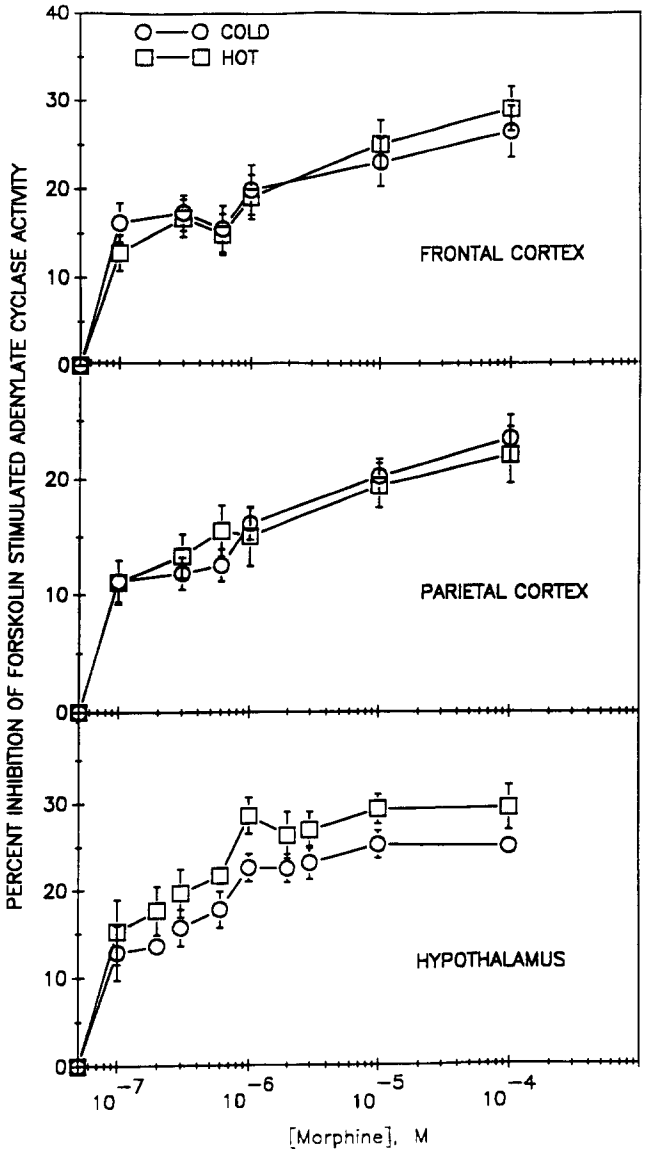

FIG. 2. Morphine inhibition of forskolin-stimulated adenylate cyclase (FS-AC) activity in brain homogenates from naive mice. Assays were performed as described in the Method section. Percent inhibition was calculated by the following equation:

$$\frac{(\text{FS-AC activity} - \text{morphine-inhibited FS-AC activity}) \times 100}{\text{FS-AC activity}}$$

There was a statistically significant line difference in the hypothalamus ($p < 0.001$, analysis of variance, $n = 12$ mice/line, collapsed across replicate).

was also a significant effect of replicate, $F(1, 96) = 7.2$, $p < 0.009$, and a significant effect of pretreatment, $F(1, 96) = 101.2$, $p < 0.0001$, and a selected line × replicate interaction, $F(1, 96) = 22.2$, $p < 0.0001$. Posthoc comparisons of individual replicates by simple main effects (18) indicated that replicate 1 mice did not differ ($p < 0.6379$) but replicate 2 mice did differ in their sensitivity to levorphanol ($p < 0.005$). Hypothermia in both selected lines was inhibited by pretreatment with naloxone. However, naloxone blocked only 75%

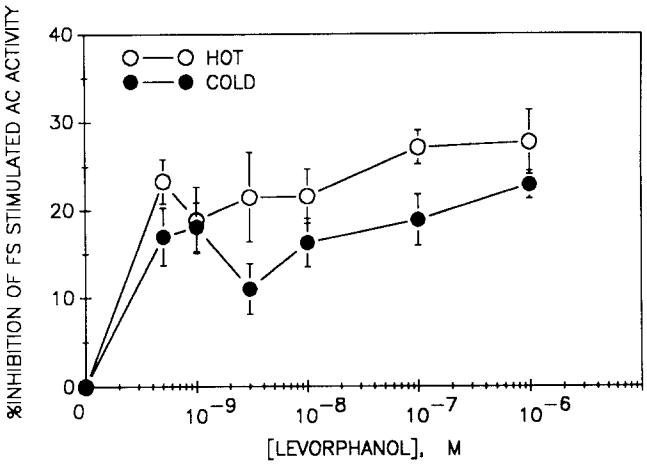

FIG. 3. Levorphanol inhibition of forskolin-stimulated adenylate cyclase (FS-AC) activity in hypothalamic homogenates from naive mice. Assays were performed as described in the Method section and percent inhibition was calculated as described in Fig. 2. There was a statistically significant line difference ($p < 0.0001$, analysis of variance, $n = 12$–16 mice/line, collapsed across replicate).

of the levorphanol-induced hypothermic response in COLD 2 mice while completely blocking hypothermia in HOT 1 and 2 mice and in COLD 1 mice.

Neither Ca^{2+}, Mg^{2+}, nor Ca^{2+}/Mg^{2+}-ATPase activities were different between HOT and COLD mice when they were treated with levorphanol or levorphanol with naloxone (see Fig. 8). The main effects were not statistically significant [line, $F(1, 47) = 0.249$, $p < 0.63$; pretreatment, $F(1, 47) = 0.01$, $p < 0.92$] and there were no significant interactions.

DISCUSSION

We found HOT mice to have a higher density of μ-opiate receptors in the frontal cortex than COLD mice, but not in

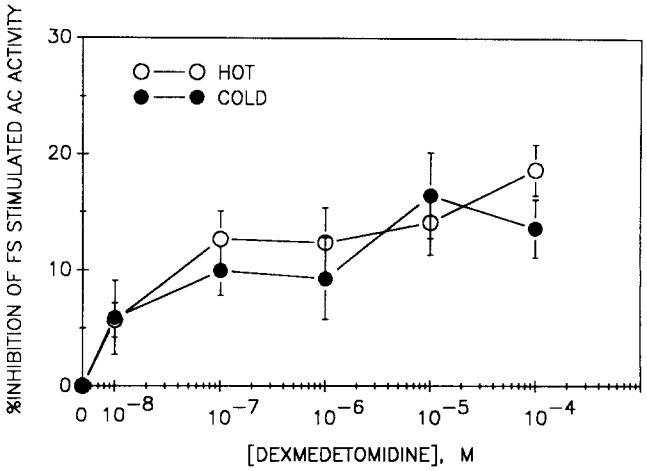

FIG. 4. Dexmedetomidine inhibition of forskolin-stimulated adenylate cyclase (FS-AC) activity in hypothalamic homogenates from naive mice. Assays were performed as described in the Method section and percent inhibition was calculated as described in Fig. 2. There was no statistically significant difference between the lines, $n = 7$–9 mice/line, collapsed across replicates.

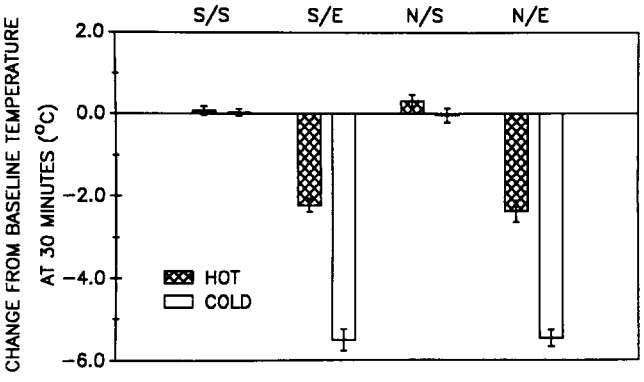

FIG. 5. Effect of naloxone on the hypothermic response to ethanol (EtOH). Either 10 mg/kg naloxone (N) or saline (S) was injected 15 min prior to EtOH (3 g/kg) or saline administration. A baseline temperature was taken before pretreatment and again before injection of EtOH. Body temperature was then taken at 15 and 30 min after injection of EtOH. The change from baseline temperature was calculated as the reading at 30 min minus the temperature reading taken before the pretreatment injection. There was a significant main effect of line ($p < 0.0001$, analysis of variance, n = 12–14 mice/line/treatment, collapsed across replicate).

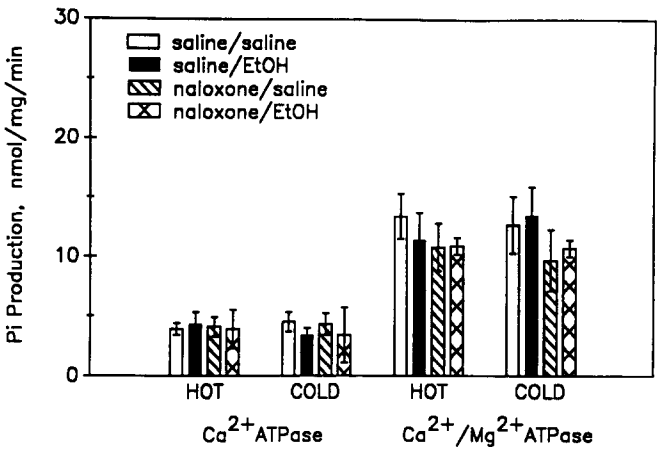

FIG. 6. Effect of ethanol (EtOH) and naloxone administration on Ca^{2+} and Ca^{2+}/Mg^{2+}-ATPase activity in hypothalamic homogenates. Mice were treated as described in Fig. 5 and after 30 min sacrificed by cervical dislocation, the brains removed, and the hypothalamus dissected out. Homogenates were prepared and assays performed as described in the Method section with the following modifications: The Ca^{2+}/Mg^{2+}-ATPase assay was performed in buffer containing 5 mM Mg^{2+}, 2 μM Ca^{2+}, and 2 mM ATP; the Ca^{2+}-ATPase assay was performed in Mg^{2+} free buffer. Samples were incubated for 5 min at 30°C. There were no significant line difference between any of the treatment conditions. n = 12–14 mice/line/treatment, collapsed across replicate.

the parietal cortex, brainstem, or hypothalamus. The K_d was the same in both lines in all brain areas. Muraki and Kato (22) found no direct correlation between [^{3}H]naloxone binding in seven brain areas, including the hypothalamus, and sensitivity to morphine-induced hypothermia in six strains of mice. Other investigators have reported a decrease in the density of μ-opiate receptors in the frontal cortex of C57BL mice following chronic ethanol ingestion (19). Although we found a lower μ-opiate receptor density in the frontal cortex of COLD mice compared with HOT mice, it is unclear if this is related to the differential hypothermic effect.

A major site of thermoregulation is in the preoptic area and anterior hypothalamus (POAH) (1,8,32). However, afferent pathways from other brain areas into the POAH may modulate POAH function. In addition, the hypothermic effect produced by μ-opiate agonists may be modulated by an interaction of the μ-opiate receptor system with other neuro-

TABLE 3

ATPase ACTIVITY (nmol P_i PRODUCED/mg PROTEIN/min) IN BRAIN HOMOGENATES FROM HOT AND COLD MICE

	HOT	COLD
Ca^{2+} ATPase		
Brainstem	82.8 ± 9.5*	78.4 ± 10.6
Frontal Cortex	102.6 ± 18.4	88.2 ± 12.1
Parietal Cortex	93.1 ± 10.9	102.7 ± 11.9
Hypothalamus	96.6 ± 7.8	75.0 ± 9.0
Ca^{2+}/Mg^{2+} ATPase		
Brainstem	92.3 ± 8.1	85.0 ± 11.5
Frontal Cortex	107.1 ± 15.8	120.5 ± 15.5
Parietal Cortex	147.0 ± 11.5	124.2 ± 13.1
Hypothalamus	74.4 ± 12.9	67.3 ± 13.1

Homogenates were prepared as described in the Method section. ATPase activity was assayed as described in the Method section with the following exceptions: Tthe Ca^{2+}/Mg^{2+}-ATPase assay was performed in buffer containing 5 mM Mg^{2+}, 2 mM Ca^{2+}, and 2 mM ATP; the Ca^{2+}-ATPase assay was performed in a Mg^{2+} free buffer. Samples were incubated for 15 min at 37°C.

*Mean + SEM, N = 9-19 mice/line, collapsed across replicates.

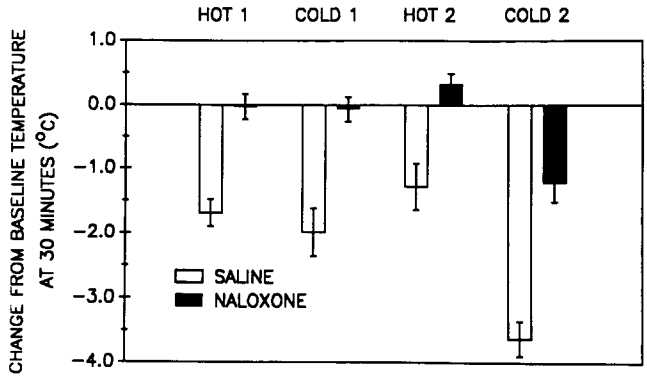

FIG. 7. Effect of naloxone on the hypothermic response to levorphanol. Either 1 mg/kg naloxone or saline was injected 2 min prior to levorphanol (10 mg/kg) or saline administration. A baseline temperature was taken before pretreatment. The change from baseline temperature was calculated as the reading at 30 min minus the temperature reading taken before the pretreatment injection. There was a significant main effect of line [$p < .0001$, analysis of variance (ANOVA)] and a significant effect of replicate ($p < 0.009$, ANOVA) and significant effect of pretreatment ($p < 0.0001$, ANOVA). There was a significant line × replicated interaction ($p < 0.0001$, ANOVA). Post-hoc analysis of simple main effect indicated that replicate 1 HOT and COLD mice did not differ but replicate 2 HOT and COLD mice did differ ($p < 0.003$) n = 7 mice/line/replicate/treatment.

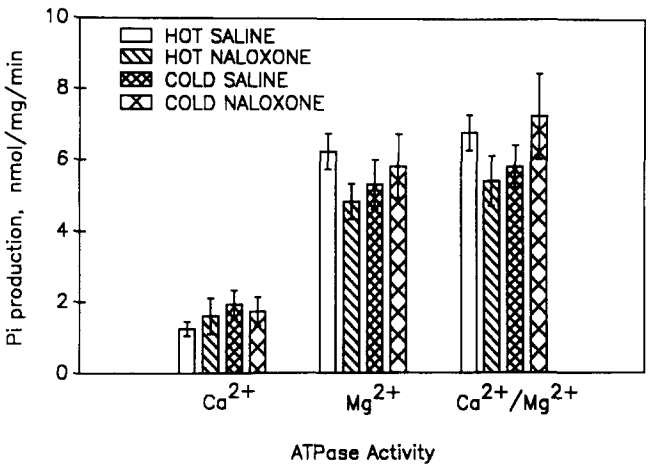

FIG. 8. Effect of levorphanol and naloxone on Ca^{2+}, Mg^{2+}, and Ca^{2+}/Mg^{2+}-ATPase activity in hypothalamic homogenates. Mice were treated as described in Fig. 7 and after 30 min sacrificed by cervical dislocation, the brains removed, and the hypothalamus dissected out. ATPase activities were performed as described in Fig. 6. Mg^{2+}-ATPase activity was performed in Ca^{2+} free buffer. There was no significant line difference between any of the treatment conditions. n = 14 mice/line/treatment, collapsed across replicate.

transmitters such as dopamine, norepinephrine, or serotonin. Nevertheless, this article focuses mainly on identifying direct, in vitro, μ-opiate effects in HOT and COLD mouse brains with specific emphasis on the hypothalamus.

Within the limits of our assay, we were unable to detect morphine inhibition of basal AC activity in HOT and COLD mice; therefore, we chose to stimulate basal activity with forskolin. Forskolin stimulates AC activity by acting on the G_s-adenylate cyclase complex. Valverius et al. (38) reported decreased forskolin binding after chronic ethanol ingestion in C57BL/6NCR mice. They observed this effect in the hypothalamus, cortex, and brainstem. Because HOT and COLD mice have been selected for their hypothermic response to ethanol, we might expect to see similar functional changes in their AC complex. However, basal AC and FS-AC activities were the same in the hypothalamus, cortex, and brainstem. Inhibition of hypothalamic FS-AC activity by morphine was greater in HOT than COLD mice, but morphine inhibition of FS-AC activity was equal in the cortex and brainstem. A change in the G_i protein or its receptor coupling may account for the differential morphine inhibition of AC activity in the hypothalamus. To test this possibility, we examined another receptor system known to couple to the G_i protein and inhibit AC activity. Dexmedetomidine, an α_2-agonist, was equally effective at inducing hypothermia and inhibiting hypothalamic AC activity in both lines. Together, these data suggest the coupling of the μ-opiate receptor to the AC system, not the G_i protein itself nor the G_s-AC complex, was responsible for the altered cyclase function.

It is also possible there are differences in endogenous μ-opiate agonists and/or their release in HOT and COLD mice. Endogenous ligands could be competing with exogenous ligands, such as morphine or levorphanol, for available receptors. If endogenous opiate agonists are unequal in HOT and COLD mice, different behavior might be observed with morphine or levorphanol. Decreases or increases in endogenous ligands could result in up- or downregulation of receptors, respectively. The difference in receptor density we see in the frontal cortex may result from a variation in endogenous μ-opiate ligand concentration.

Unequal endogenous ligands in HOT and COLD mice also might result in altered cyclase function. Van Vliet et al. (39) suggests that μ-opiate receptors inhibit AC activity stimulated by dopamine and noradrenaline. Patel and Pohorecky (25) suggest that, in rats, there is an interaction between endogenous μ-opiates, catecholamines, and ethanol in response to an acute dose of ethanol. We have not found differences in catecholamine turnover between HOT and COLD mice, nor, as of yet, have we measured endogenous μ-opiate ligands. However, we are pursuing this line of research.

The difference in morphine and levorphanol inhibition of FS-AC activity in HOT and COLD mice is a correlated neurochemical response to selection. This biochemical pathway may be involved in mediating the differential hypothermic sensitivity to morphine and levorphanol. Data presented here suggest that selection of mice for ethanol-induced hypothermia may have genetically altered the coupling of the μ-opiate receptor to the AC system, specifically to the G_i subunit. The magnitude of the difference between HOT and COLD mice in μ-opiate inhibition of FS-AC may not completely account for the difference in hypothermic response we have seen. Other neurochemical processes, such as ion channel or enzymatic functions, may have been altered by selection for HOT and COLD mice.

Most agonists increase intracellular Ca^{2+} (11); however, ethanol (37) and opiates (30) have been reported to lower intracellular Ca^{2+}. Agonist-induced changes in Ca^{2+} stasis can be accomplished either by the release of Ca^{2+} from internal stores, by opening/closing Ca^{2+} channels in the plasma membrane, or by activating uptake mechanisms in the internal storage systems. Therefore, mechanisms for the maintenance of Ca^{2+} stasis also may be altered by these agonists. We chose to examine Ca^{2+} and Ca^{2+}/Mg^{2+}-ATPase activities as an indicator of altered Ca^{2+} stasis.

We found no differences in basal Ca^{2+}-ATPase, Mg^{2+}-ATPase, or Ca^{2+}/Mg^{2+}-ATPase activity in the hypothalamus, parietal cortex, or brainstem of HOT and COLD mice. Further, and contrary to the findings of Pillai and Ross (28,29) in rats, ethanol stimulation of Ca^{2+}/Mg^{2+}-ATPase activity was not seen in the hypothalamus 30 min after treatment, nor did levorphanol have any effect on ATPase activity. The lack of a difference between Ca^{2+}-ATPase or Ca^{2+}/Mg^{2+}ATPase in naive or drug-treated HOT and COLD mice strongly suggests that these ATPase systems are not responsible for the differences in hypothermic sensitivity we see between the lines.

Pillai and Ross also reported that ethanol-induced hypothermia (29) and morphine-induced hyperthermia (30) in rats could be inhibited by a preinjection of naloxone, a μ-opiate antagonist. We did not observe an inhibition of ethanol-induced hypothermia when HOT and COLD mice were preinjected with naloxone. As expected, naloxone antagonized levorphanol-induced hypothermia. However, there was a component of levorphanol-induced hypothermia in COLD 2 mice that was not inhibited by naloxone. COLD 2 mice were more sensitive to μ-opiate-induced hypothermia than COLD 1 mice or either of the HOT lines.

There are several possible reasons for the inconsistency between our findings and those of Pillai and Ross. The effects of opiates on thermoregulation are species dependent and influenced by several external factors such as ambient temperature and restraint conditions (1,8,32). Pillai and Ross (28-30) used Sprague-Dawley rats for their studies. They report that

their rats were unrestrained while measuring temperature. Although mice used for our experiments were unrestrained most of the time, they were briefly restrained during temperature measurements. We injected all drugs IP, while Pillai and Ross (28–30) injected naloxone SC and ethanol or morphine IP. A higher dose of ethanol (3 vs. 2 g/kg) and lower dose of opiate (10 vs. 15–30 mg/kg) were used in our experiments. However, these doses of ethanol may not be relatively higher given the greater sensitivity of rats compared with mice. Preinjection of naloxone was 2 min (vs. 15 min) before administration of levorphanol and 15 min before ethanol in our experiments. We observed different biochemical results than Pillai and Ross (28,29) possibly because we used a single spin step to isolate our membranes while they prepared synaptosomes using Ficoll gradient followed by sucrose gradient centrifugation.

Based on the observation of a correlated response to selection between ethanol- and μ-opiate-induced hypothermia in the HOT and COLD selected lines, one might postulate that ethanol directly affects the μ-opiate pathway. If this postulate were true, ethanol-induced hypothermia should be antagonized by naloxone. Naloxone had no effect on ethanol-induced hypothermia in either HOT or COLD mice.

Another possible explanation for the correlation between ethanol- and μ-opiate-induced hypothermia in HOT and COLD mice is that ethanol and morphine were acting on parallel pathways feeding into a common mechanism that has yet to be identified or described. Additional evidence that μ-opiate-induced hypothermia is the result of activity in a different biochemical mechanism than ethanol-induced hypothermia in the selected lines is the recent observation that only replicate 2 HOT and COLD mice now differ in sensitivity to levorphanol while both replicate lines still differ in sensitivity to ethanol.

The binding of ligand to the μ-opiate receptor causes coupled and related biochemical responses in the brain. It is unlikely that the small differences we see in inhibition of AC activity in the hypothalamus of HOT and COLD mice is totally responsible for the differences in hypothermia. Some pharmacological effect of morphine/levorphanol other than its opiate receptor activity may mediate that portion of the hypothermic response in COLD 2 mice not inhibited by naloxone.

We are currently testing the hypothermic sensitivity of HOT and COLD mice to other neurotransmitters and mechanism-specific drugs. After identifying these drugs, we will test biochemical systems that respond to those drugs in tissue from HOT and COLD mice. These results will help us determine neurochemical mechanisms for ethanol-induced hypothermia.

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