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Sensitivity and tolerance to ethanol in mouse lines selected for ethanol-induced hypothermia

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Abstract

Within-family selective breeding techniques have been used to create two lines of mice to be insensitive (HOT) and two lines to be sensitive (COLD) to the hypothermic effects of an acute 3.0-g/kg ethanol (EtOH) injection. Previous studies have found HOT mice to be relatively resistant to the development of tolerance to this effect, whereas COLD mice readily develop tolerance. The breeding program is currently in selected Generation 52, and the HOT and COLD mice differ by about 10°C (average of both replicates) in their selected hypothermic response. Starting with selection Generation 20, separate lines of mice were inbred from the HOT-2 and COLD-2 selected lines, while selection continued for the original two replicate lines of HOT and COLD mice. To assess whether different dose treatments would produce differential tolerance development in the HOT and COLD selected lines, we administered different dose regimens across 5 days to HOT and COLD mice. The COLD mice developed tolerance while the HOT mice did not, regardless of total EtOH administered. In a separate study, we administered EtOH (3.0 g/kg) to mice for 3 days to assess a shorter tolerance paradigm. We also present here responses to the selection dose of 3.0-g/kg EtOH in the inbred HOT (IHOT-2) and COLD (ICOLD-2) mice tested after 41 generations of brother–sister mating. In addition, we report recent attempts to find doses of EtOH that would produce an equivalent initial hypothermic response in each of the six lines (HOT-1, COLD-1, HOT-2, COLD-2, ICOLD-2, and IHOT-2). When doses were selected to produce similar initial hypothermic sensitivity, tolerance was tested by giving three daily doses and examining the attenuation of the hypothermic response on the third day. All three COLD lines developed significant tolerance, while the HOT lines did not. The HOT and COLD mice provide a genetic model to study mechanisms mediating acute EtOH-induced hypothermia as well as tolerance development.

Keywords: Ethanol; Hypothermia; HOT mice; COLD mice; Selected lines; Inbred strains; Tolerance

1. Introduction

Acute administration of ethanol (EtOH) induces hypothermia in rodents maintained in a room-temperature environment. The thermoregulatory response of an animal to EtOH exhibits tolerance after repeated administration, and the degree of tolerance is inversely related to the degree of physical dependence and withdrawal [14]. Inbred mice differ in EtOH-induced hypothermia, indicating genetic influence on this response [2,5]. Sensitivity to the hypothermic effects of EtOH may also genetically correlate with sensitivity to other effects of EtOH including sedation and conditioned taste aversion [8,9]. EtOH-induced body temperature reduction has been shown to protect animals from the depression of the central nervous system resulting from an acute dose of EtOH [1]. Studying genetically distinct strains that differ in the hypothermic response will be helpful in elucidating possible mechanisms mediating EtOH-induced thermoregulation.

Mice insensitive (HOT) and sensitive (COLD) to EtOH-induced hypothermia have been selected using within-family selective breeding techniques. Mice maintained at room temperature were selected for maximal (COLD) or minimal (HOT) reduction from pre-injection rectal tempera-

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ture assessed 30 and 60 min after and EtOH injection. COLD mice show a pronounced hypothermic response, while HOT mice show little hypothermia, despite achieving similar blood EtOH concentrations [7]. The independently replicated pairs of lines (HOT-1 and COLD-1, HOT-2 and COLD-2) are currently in selected Generation 52. These lines originated from the heterogeneous mouse population HS/Ibg, the product of an eight-way cross of the inbred strains: A, AK, BALB/c, C3H, C57BL, DBA/2, Is/Bi, and RIII [17]. This population is the foundation population for a number of other selected mouse lines.

COLD mice develop tolerance to the hypothermic effect of EtOH with repeated administration, while HOT mice do not. Differences between the HOT and COLD lines in tolerance development persist even when initial hypothermia is equated by administering a higher EtOH dose to HOT mice [4]. When the dose dependence of both the peak and the duration of hypothermic response to EtOH were analyzed, tolerance magnitude was still greater in COLD than HOT mice [3], supporting the conclusion that hypothermic tolerance is a correlated response to selection. Under conditions of cold stress (produced by reducing ambient room temperatures to 4°C), HOT mice can develop some tolerance to EtOH-induced hypothermia, although still less than COLD mice [15].

At Generation 20 of selection, inbred strains from both replicates were initiated, resulting eventually in fully inbred HOT and COLD lines (ICOLD-1, IHOT-1, ICOLD-2, and IHOT-2, respectively). Fertility was a problem for the IHOT-1 line, which was lost after 39 generations of inbreeding, but the other inbred strains are viable. Therefore, of the inbred strains, only ICOLD-1 and -2 and IHOT-2 are currently available. The availability of inbred mice from selected Generation 20 to compare to mice that have continued under selection pressure for 52 generations provides a novel approach to the study of EtOH-induced hypothermia. In addition, these lines provide a useful tool in investigating correlated responses to selection. The main goal of this paper is to report the responses of the HOT and COLD mouse lines to additional generations of selection, and to investigate and compare sensitivity and tolerance in the inbred strains with the selected lines. We also present data from tolerance studies in the selected HOT and COLD mice. Because the degree of tolerance is dependent upon the degree of initial hypothermia [18] for the tolerance experiments, we attempted to ascertain genotypic specific doses that would induce equivalent initial hypothermia.

2. Materials and methods

2.1. Animals and drugs

HOT-1 and -2, COLD-1 and -2 selected lines of mice were produced using within-family selective breeding techniques [7]. HOT-1 and COLD-1 lines were derived from an initial population of nine breeding pairs of genetically heterogeneous mice (HS/Ibg) obtained from the Institute for Behavioral Genetics (Boulder, CO). HOT-2 and COLD-2 mice were derived from nine separate breeding pairs of HS/Ibg mice. Selection continued for 38 generations and then was relaxed for six generations; that is, for six generations, individuals were chosen for mating at random within each selected line. Selection resumed with the offspring of Generation 44 to produce filial Generation 45 ($S_{39}G_{45}$). At Generation 20, several brother–sister mating pairs chosen at random from each selected line were used to initiate inbreeding, resulting in the development of IHOT-2 and ICOLD-2 lines. The IHOT-2 and ICOLD-2 lines were developed by brother–sister matings of HOT-2 and COLD-2 mice for 41 generations, respectively. IHOT-1 and ICOLD-1 mice were also developed, but the IHOT-1 mice did not survive to the current generation; therefore, the ICOLD-1 mice were not tested in the present set of experiments.

All mice were born and reared in the Portland VA Veterinary Medical Unit. Mating pairs were housed on corncob bedding in clear polypropylene cages (33 × 16 × 13 cm) in a filtered Thoren rack system. Rodent chow and water was available ad libitum (except during behavioral testing) with an ambient colony room temperature of 22 ± 1°C with a 12-h light/dark cycle (lights on at 06:00 h). Pups were raised with their dam and weaned two to five per cage into same-sex litter groups at 21 ± 1 days of age. Mice were tested between 43 and 107 days of age.

EtOH was prepared (20% v/v) in 0.9% saline from 200-proof EtOH (Pharmco). Solutions were made fresh daily. All EtOH doses were injected intraperitoneally (ip) according to body weight. The experimental protocol was approved by an Institutional Animal Care and Use Committee, and procedures comply with the National Institutes of Health Guide for Care and Use of Laboratory Animals. All testing was performed between 2 and 6 h after lights on.

2.2. EtOH hypothermia

An hour before testing, mice were weighed and placed into well-ventilated individual clear Plexiglas chambers (8 × 19 × 8 cm). After a 1-h habituation period, each mouse was removed from its chamber and restrained lightly in a Plexiglas tube. A pretreatment (baseline) temperature was recorded with a Sensortek Th-8 Digital Thermometer attached to a 0.5-mm probe inserted 2.5 cm into the rectum for 5 s. Immediately following measurement of the baseline temperature, the mouse was injected with EtOH intraperitoneally and returned to its ventilated chamber. Mice were similarly handled for temperature assessments at 30 and 60 min following EtOH administration. For selection, mice were injected with 3.0-g/kg EtOH, while for the other experiments presented in this paper, doses varied according to genotype as noted.

2.3. Selection protocol

Methods pertaining to the selection of these lines have been reported previously [7]. After testing all mice from a given generation, the most extreme scoring male and female mice from each litter is entered into a rotational mating scheme to reduce the degree of inbreeding [7]. Selection is based on maximum (COLD mice) or minimum (HOT mice) reduction from pretreatment temperature at either 30 or 60 min after EtOH administration. HOT mice exhibiting hyperthermic responses were selected in preference to those exhibiting minimal or no hypothermia. Thus, selection was based on temperature sensitivity, which was the response at either 30 or 60 min that best fit the above criteria.

2.4. Ascending-dose hypothermic tolerance in selected lines

The purpose of this experiment was to assess whether the total cumulative dose of EtOH administered would differentially affect the development of tolerance between the HOT and COLD mice. That is, if a subthreshold dose of EtOH is administered (i.e., a dose that does not produce extreme hypothermia), will the COLD mice still show greater tolerance than the HOT mice or will this paradigm be insufficient to induce tolerance development in COLD mice. COLD-1 ($N=24$), COLD-2 ($N=23$), HOT-1 ($N=24$), and HOT-2 ($N=24$) male mice from Generation 41 were tested across 5 consecutive days. Mice were weighed and placed in individual holding chambers for hypothermia testing. These chambers were designed for hypothermia testing and have clear plastic walls, floors, and lids that are perforated with several 4-mm holes to provide ventilation. Rectal temperatures were taken at baseline and 45 min after injection (the time previously demonstrated to be peak hypothermia at the doses administered [3]). Immediately after baseline temperature was taken on Day 1, all mice were injected intraperitoneally with 3.0 g/kg EtOH (20% v/v in saline). Injections and testing were conducted daily for 5 days according to the following groups ($N=5–6$ HOT and 5–6 COLD mice for each group). Group denotes the dose of EtOH (g/kg) administered on Days 2–4, respectively of the experiment: Group 1: 1.0, 1.0, 1.0; Group 2: 3.0, 3.0, 3.0; Group 3: 1.0, 1.5, 2.0; and Group 4: 1.5, 2.0, 2.5. On Day 5, all mice were administered 3.0 g/kg EtOH.

2.5. 3-Day tolerance to selection dose in selected lines

The purpose of this experiment was to assess whether a 3-day tolerance paradigm was sufficient to induce tolerance in COLD mice. COLD-1 ($N=8$), COLD-2 ($N=8$), HOT-1 ($N=8$), and HOT-2 ($N=8$) female mice from Generation 49 were tested across 3 consecutive days. On Day 1, mice were brought into the testing room and placed into the hypothermia cages for a 1-h habituation period. Following habituation, a baseline measurement was taken from each animal, and mice were immediately injected with 3.0 g/kg EtOH. Temperatures were then taken at 30 and 60 min following injection. Following the 60-min temperature recording, mice were returned to their home cage and to the animal colony. On Day 2 of the experiment, mice were brought into the testing room and injected with 3.0 g/kg EtOH, but rectal temperatures of the mice were not measured on Day 2. On the third day, mice were treated as described for the first day of tolerance testing.

2.6. Characterization of inbred strains and selected lines

The purpose of this experiment was to characterize the inbred strains (IHOT-2 and ICOLD-2 from Generation 41) and selected lines (HOT-1, COLD-1, HOT-2, and COLD-2 from Generation 57) to observe the response to 3.0-g/kg EtOH as a result of further generations of selection. COLD-1 ($N=15$), COLD-2 ($N=14$), HOT-1 ($N=16$), HOT-2 ($N=13$), ICOLD-2 ($N=16$), and IHOT-2 ($N=14$) mice of both sexes were tested for their response to 3.0 g/kg EtOH (the selection dose) using the selection protocol described above. Immediately following the 60-min temperature measurement for each mouse, a 20-μl blood sample from the retro-orbital sinus was taken and immediately placed on ice. Samples and standards were analyzed for blood EtOH concentration using a gas chromatographic procedure described previously [19].

2.7. Initial sensitivity and tolerance to equi-effective doses of EtOH in HOT and COLD mice

COLD-1 ($N=15$), COLD-2 ($N=5$), HOT-1 ($N=12$), HOT-2 ($N=14$) mice from Generation 59 and ICOLD-2 ($N=9$), IHOT-2 ($N=11$) mice from Generation 43 of both sexes were tested in an attempt to identify equi-effective doses of EtOH. On Day 1 of testing, mice were transported to the testing room, weighed, and placed in the hypothermia chambers for a 1-h habituation period. Following habituation, a baseline measurement was taken from each animal as described. Immediately following baseline temperature measurements, mice were injected with the following doses of EtOH intraperitoneally: COLD-1 (1.5 g/kg), COLD-2 (1.25 g/kg), HOT-1 (5.41 g/kg), HOT-2 (5.55 g/kg), ICOLD-2 (1.5 g/kg), and IHOT-2 (5.41 g/kg). These doses were selected based on pilot testing. Temperatures were then taken at 30 and 60 min following injection, as described above. Following the 60-min temperature recording, mice were returned to their home cage and to the animal colony.

To investigate whether the genotypes would show differential tolerance development, tolerance was assessed using the 3-day paradigm described above.

2.8. Statistical analysis

Because analysis of raw temperature data could be complicated by differential changes in baseline temperatures, statistical analyses were performed on differences

between baseline temperature and temperatures taken after EtOH administration.

Statistical analyses utilized analysis of variance (ANOVA) testing of hypothermia at 30 and 60 min following injection of EtOH. The exception to this is the ascending-dose tolerance experiment where temperatures were instead collected at 45 min.

Preliminary data analyses were conducted, and in no instance were there significant interactions with sex, so data were collapsed across sex for analyses. Analyses were conducted for a main effect of Line (HOT or COLD) and Replicate (Replicate 1: HOT-1 and COLD-1; Replicate 2: HOT-2 and COLD-2; Replicate 3: IHOT-2 and ICOLD-2) with time (30 or 60 min) as a repeated measure. The exception to this is the ascending-dose tolerance experiment where analyses were conducted for a main effect of Line (HOT or COLD) and Group (designating different dose schedules). Significant interactions between factors were analyzed by performing one-way ANOVAs at the different levels of the between- or within-subjects factors. The criterion for significance for all analyses was set at $P < .05$.

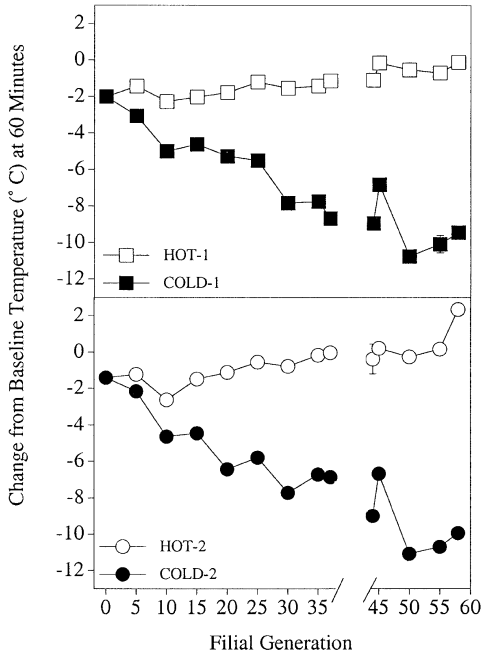

Fig. 1. Mean change from baseline temperature 60 min after intraperitoneal EtOH (20% v/v) of HOT and COLD mice approximately every five generations for 58 generations. Selection was relaxed after Generation 38 and resumed at Generation 45. Therefore, the last data point represents response to selection for mice from selection Generation 52 and filial Generation 58 ($S_{52}G_{58}$). Symbols are mean values of all animals tested for selection (approximately 70 per line per generation). The dark symbols represent the response of COLD mice, while the open symbols represent the response of HOT mice. The top panel depicts the response in Replicate 1, and the bottom panel illustrates the response of Replicate 2. Standard errors (S.E.) are smaller than symbol size.

3. Results

3.1. Response to further selection

Detailed analysis of selected Generations 0–14 has been published previously [17]. Mean hypothermic responses 60 min after injection of both replicate HOT and COLD lines across 52 generations of selection are presented in Fig. 1A and B. For the current selection generation ($S_{52}G_{58}$), the magnitude of response to 3.0 g/kg EtOH was a mean of -9.5°C in the COLD-1 mice and -10.0°C in the COLD-2 line. Mean HOT-1 and -2 temperatures were -0.1 and $+2.3$°C, respectively. It is evident from inspection of Fig. 1A and B that response to selection has been asymmetrical, with more response occurring in the COLD lines.

3.2. Ascending-dose hypothermic tolerance in selected lines

Results from this experiment are depicted in Fig. 2. There were no significant interactions with replicate, so for this experiment, replicate lines were combined. Mean ± S.E.M. baseline temperatures (°C) were 37.066 ± 0.124 for the HOT mice and 37.751 ± 0.122 for the COLD mice. The left panel shows the initial hypothermic response to 3.0 g/kg EtOH. There was a main effect of line [$F(1,86) = 334.32$, $P < .001$] but no significant effect of group and no interaction (P's > .7), indicating that the COLD mice showed greater hypothermia than the HOT mice with no group differences (n.b., group designated treatments to be administered Days 2–4). Following the first day of receiving 3.0 g/kg EtOH, mice were divided into the following four groups for different dose schedules (Days 2–4): Group 1: (1.0, 1.0, 1.0), Group 2: (3.0, 3.0, 3.0), Group 3: (1.0, 1.5, 2.0), and Group 4: (1.5, 2.0, 2.5). On Day 5, all mice were administered 3.0 g/kg EtOH.

The development of tolerance can be observed by creating a difference score for each animal. To do this, the response on Day 5 was subtracted from the response on Day 1, and these data are presented in the right panel in Fig. 2. A change in the negative direction (i.e., 0 or below on the y-axis) indicates tolerance, in that the response on Day 5 is greater (less negative) than the hypothermic response on Day 1. A change in the opposite direction (i.e., a positive score) indicates no tolerance development. While a change in the positive direction (e.g., in the HOT mice) might indicate sensitization, in the current experiments the magnitude of the response in this direction does not strongly support the development of sensitization. The lines differed significantly [$F(1,86) = 43.25$, $P < .001$], with HOT mice showing an increase in temperature (reflecting no tolerance) while the direction of change in the COLD mice indicates tolerance. There was no significant effect of group and no significant group by line interaction (P's > .7), indicating there was no significant effect of doses administered on the development of tolerance.

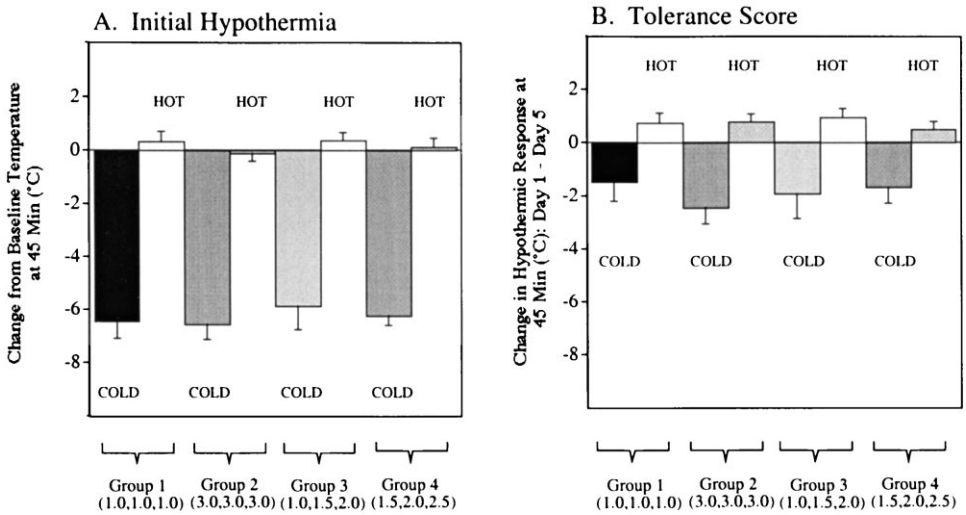

Fig. 2. Initial hypothermic response (Panel A) and development of tolerance (Panel B) to 3.0 g/kg EtOH as a function of different dose regimens of EtOH across a 5-day test schedule in HOT and COLD mice. Panel A: Change from baseline temperatures (mean ± S.E.M.) at 45 min following an injection of 3.0 g/kg EtOH on Day 1 to HOT (light bars) and COLD (dark bars) mice. Injections and testing were conducted daily for 5 days according to Group. Group denotes the dose of EtOH (g/kg) administered on Days 2–4 of the experiment, respectively. On Day 5, all mice were administered 3.0 g/kg EtOH, and tolerance was assessed as a change in response on Day 5 relative to Day 1. These data are presented in Panel B. Panel B: Mean ± S.E.M. tolerance scores (difference between Days 1 and 5 hypothermic response at 45 min in the same animal) for HOT (open bars) and COLD (dark bars) mice. Negative difference scores indicate tolerance, and positive scores no tolerance. For statistical analyses, see text.

3.3. 3-Day tolerance to selection dose in selected lines

Given previous evidence that tolerance in the HOT and COLD mice was not maintained after 3 days of testing [3], results from a 3-day tolerance paradigm are presented in Fig. 3. There were no significant interactions with replicate, so replicate lines were combined. Mean ± S.E.M. baseline temperatures (°C) were 37.463 ± 0.165 for the HOT mice and 37.494 ± 0.381 for the COLD mice. Initial sensitivity (hypothermic response on Day 1) is shown in panel A. There was a significant effect of line [$F(1,30) = 97.60$, $P < .001$], indicating that the COLD mice showed a hypothermic response while the HOT mice did not.

Tolerance is depicted in Panel B, as a tolerance score computed by subtracting the hypothermic response on Day 3 from that of the same mouse on Day 1. That is, if a mouse has less hypothermic response (less negative value) on Day 3 relative to its response on Day 1, the tolerance score will be negative (indicating tolerance development). A tolerance score in the opposite direction suggests no tolerance. An ANOVA computed on the data in Fig. 3B yielded a significant effect of line [$F(1,30) = 15.62$, $P < .001$], indica-

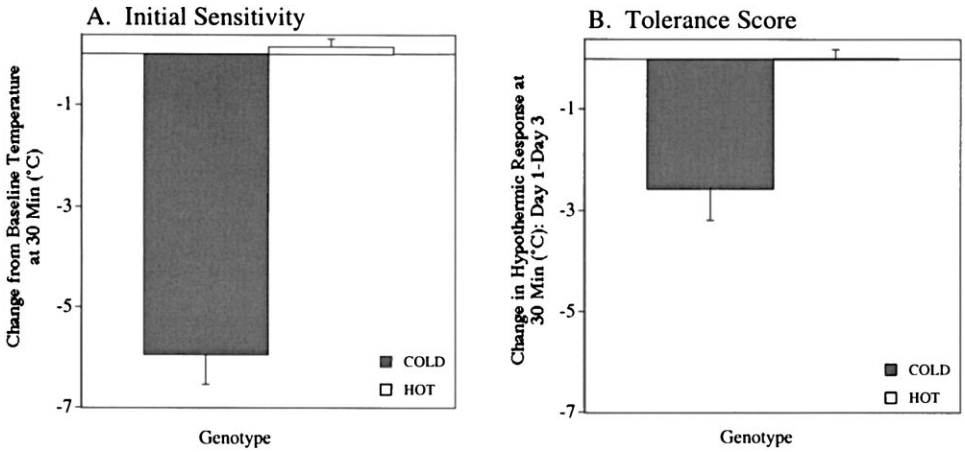

Fig. 3. Depicts the initial hypothermic response (Panel A) and development of tolerance (Panel B) to 3.0 g/kg EtOH in HOT and COLD mice using a 3-day tolerance paradigm. Panel A: Mean ± S.E.M. change from baseline temperature at 30 min following an acute injection of 3.0 g/kg EtOH. Mice were injected on Days 1 and 3 with 3.0 g/kg EtOH. Tolerance was assessed as a change in response on Day 3 relative to the response on Day 1. Panel B: Mean ± S.E.M. tolerance scores (difference between Days 1 and 5 hypothermic response at 30 min in the same animal) for HOT (open bars) and COLD (dark bars) mice. Negative difference scores indicate tolerance, and positive scores no tolerance. For statistical analyses, see text.

ting the development of tolerance in the COLD but not the HOT mice.

3.4. EtOH-induced hypothermia in response to 3.0 g/kg in the HOT and COLD lines

Fig. 4 illustrates the response of IHOT-2 and ICOLD-2 lines as well as HOT and COLD mice in response to the selection dose of 3.0 g/kg EtOH. Mean ± S.E.M. baseline temperatures (°C) were as follows: COLD1 (37.174 ± 0.310), COLD2 (37.901 ± 0.133), HOT1 (36.569 ± 0.304), HOT2 (36.953 ± 0.284), IHTSC2 (37.878 ± 0.151), and IHTSH2 (36.984 ± 0.162). A significant Line × Replicate × Time interaction was present [$F(2,82) = 14.84$, $P < .001$]; therefore, a repeated-measures ANOVA grouped on Line was performed for each HOT–COLD pair separately. In each comparison, COLD mice had greater hypothermia than HOT mice (P's < .001). When temperatures from the COLD mice were analyzed independently, there was a significant genotype (COLD-1 and -2, ICOLD-2) by time interaction [$F(2,42) = 22.89$, $P < .001$]. Follow-up ANOVAs indicate that the ICOLD-2 and COLD-2 mice differed [$F(1,28) = 55.03$, $P < .001$], and that there was a significant interaction with time [$F(1,28) = 34.65$, $P < .001$]. The COLD-1 and -2 mice also differed [main effect $F(1,27) = 5.22$, $P = .03$]. An analysis of the HOT mice separately indicated that there was no significant effect of genotype [$F(2,40) = 0.36$, $P = .70$], and there were no differences among HOT-1 and -2 and IHOT-2 mice across time. There were no significant main effects when the ICOLD-2 were compared to the COLD-1 [$F(1,29) = 2.56$, $P = .12$], although there was a significant interaction [$F(1,29) = 29.635$, $P < .001$], indicating that they differed at 60 min.

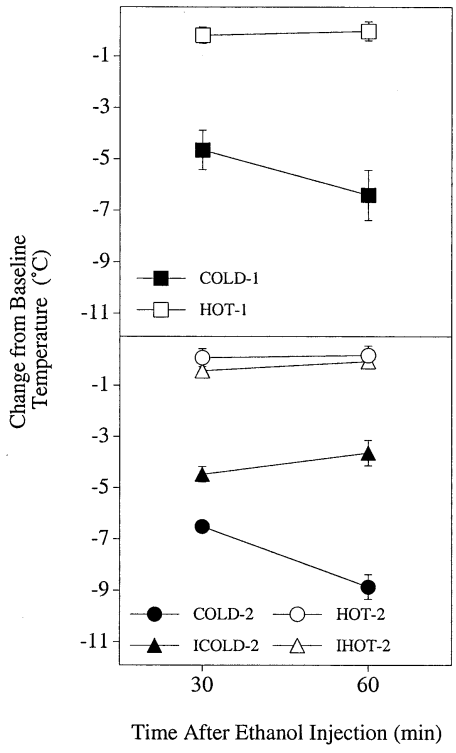

Fig. 4. Mean ± S.E.M. change from baseline temperature 30 and 60 min after an injection of 3.0 g/kg EtOH is shown for mice of all three replicates. Open symbols depict the response of HOT, and closed symbols illustrate the response of COLD mice. Replicate 1 (○) is depicted in the top panel, while Replicate 2 (□) and the inbred lines (●) are depicted in the bottom panel. S.E.M. larger than symbol size are shown. See text for statistical analyses.

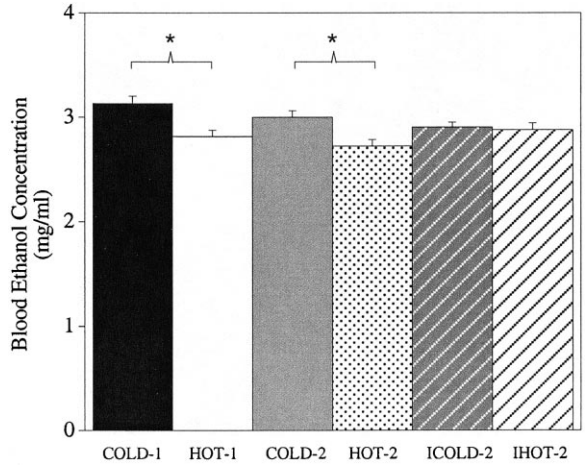

Fig. 5. Following the temperature measurement taken at 60 min (depicted in Fig. 4), a blood sample was taken from mice of each group (shown here). Plotted are mean ± S.E.M. blood ethanol concentrations (mg EtOH/ml blood). The dark bars depict the response of COLD mice, while the open bars depict the response of HOT mice. The * denotes statistically significant values between HOT and COLD Lines within the same Replicate. See text for statistical analyses.

Blood EtOH concentrations following the 60-min temperature measurements are depicted in Fig. 5. Data were analyzed with an ANOVA grouped on Line and Replicate. There was a significant Line × Replicate interaction [$F(2,81) = 3.41$, $P = .04$]; therefore, an ANOVA was performed on each HOT–COLD pair separately. There were no significant differences between the IHOT-2 and ICOLD-2, indicating these mice did not differ in blood EtOH concentrations at 60 min following an injection of 3.0 g/kg EtOH [$F(1,27) = 0.10$, $P = .76$]. The HOT and COLD mice of both Replicates 1 and 2 differed significantly from each other [$F(1,29) = 11.75$, $P < .01$ and $F(1,25) = 10.52$, $P < .01$, respectively], indicating that in each case the COLD line had greater blood EtOH concentrations.

3.5. Initial sensitivity and tolerance to equi-potent doses of EtOH

For the current experiment, doses of 1.25 g/kg (COLD-2), 1.5 g/kg (COLD-1 and ICOLD-2), 5.41 g/kg (HOT-1 and IHOT-2), and 5.55 g/kg (HOT-2) were used in an attempt to produce roughly equal initial hypothermia (see

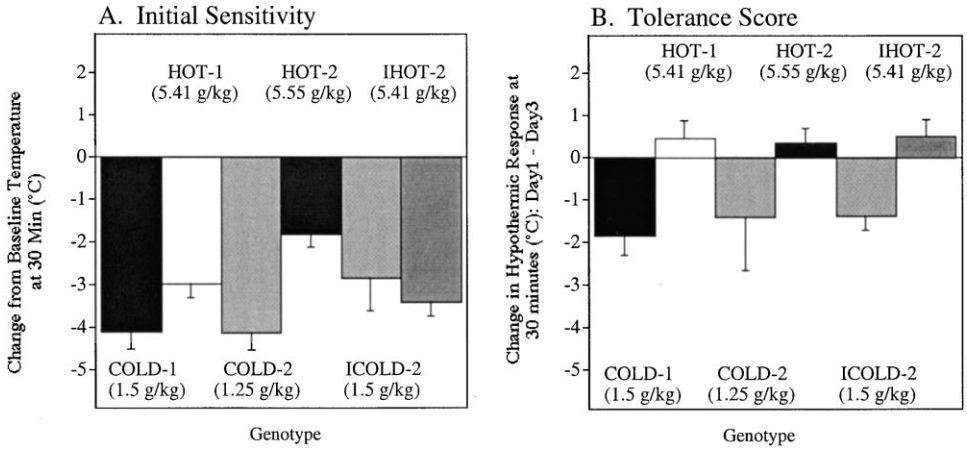

Fig. 6. Mean hypothermic response to different doses of EtOH depending on genotype. EtOH doses administered were 1.25 g/kg (COLD-2), 1.5 g/kg (COLD-1 and ICOLD-2), 5.41 g/kg (HOT-1 and IHOT-2), and 5.55 g/kg (HOT-2). Panel A: Hypothermic response (mean ± S.E.M. change from baseline at 30 min) on Day 1 following an injection of EtOH in HOT (open bars) and COLD (dark bars) mice. Panel B: Mean ± S.E.M. tolerance scores (difference between Days 1 and 3 hypothermic response at 30 min in the same animal) for HOT (open bars) and COLD (dark bars) mice. Negative difference scores indicate tolerance, and positive scores no tolerance. For statistical analyses, see text.

Fig. 6). Mean ± S.E.M. baseline temperatures (°C) were as follows: COLD1 (37.967 ± 0.112), COLD2 (37.511 ± 0.231), HOT1 (36.477 ± 0.206), HOT2 (36.464 ± 0.158), IHTSC2 (37.883 ± 0.185), and IHTSH2 (37.788 ± 0.161). There was a significant Line × Replicate interaction in basal body temperatures [$F(2,59) = 10.45$, $P < .001$]. Thus, data are expressed as change from baseline to avoid confounding the analysis of raw temperature data by these differential basal temperatures. However, it is important to note that when an ANOVA was performed on basal temperatures grouped on Line and Replicate with repeated measures for Day, there was no significant interaction of Line × Replicate × Day, indicating that the baseline temperatures of the genotypes did not change across days (data not shown).

When the initial sensitivity data (Day 1; Fig. 6A) were analyzed, a significant Line × Replicate interaction was present [$F(2,59) = 4.48$, $P < .02$]. To investigate this interaction, an ANOVA was performed on each replicate pair separately. There were no significant line differences in HOT-1 vs. COLD-1 or IHOT-2 vs. ICOLD-2 (P's = .06 and .48, respectively), indicating that these HOT and COLD genotypes showed similar initial hypothermia in response to the EtOH doses administered. However, there was a significant difference between the HOT-2 vs. COLD-2 mice ($P = .001$), indicating that despite the two very different doses administered (1.25 g/kg to COLD-2 and 5.55 g/kg to HOT-2), the COLD-2 line showed significantly greater hypothermia than its HOT-2 counterparts.

The development of tolerance can be observed by creating a difference score for each animal. These data are presented in Fig. 6B. A change in the negative direction indicates the development of tolerance, while a change in the positive direction indicates the no development of tolerance. The lines differed significantly [main effect $F(1,59) = 22.28$, $P < .001$; no significant interaction $P = .46$], with HOT mice showing an increase in temperature (reflecting no tolerance) while the direction of change in the COLD mice indicates tolerance.

4. Discussion

Selection for EtOH-induced hypothermia has been successful, and Fig. 1 suggests that the COLD lines have continued to respond to selection across generations. Selection for increased and reduced hypothermic response has been asymmetrical, with response occurring primarily in the COLD and not the HOT lines. Previous work from our laboratory, however, suggests that this is not due to any significant influence of natural selection ([17]; see Ref. [10] for possible reasons for asymmetry).

In response to the selection dose, both selected lines of COLD mice demonstrated greater hypothermia than the HOT mice. This was also true for the inbred ICOLD-2 and IHOT-2 strains. Furthermore, the COLD-1 and -2 mice showed a greater decrease in temperature at 60 compared to 30 min following EtOH administration. In contrast, the IHOT-2 and ICOLD-2 mice did not differ in temperature changes between 30 and 60 min following injection, which was the case after 14 selected generations [17]. The inbred HOT and COLD lines represent a unique opportunity to observe the degree of phenotypic difference that was present at Generations 20–30 and to compare this phenotypic difference with that currently characterizing the selected lines. It is important to keep in mind that during inbreeding, some genetic changes have undoubtedly occurred to make the inbred strains different from their HOT-2 and COLD-2 sources. That is, during inbreeding, some alleles for EtOH's thermal effects have been lost from the population that will

affect the degree of phenotypic difference fixed in the IHOT-2 and ICOLD-2 strains. Indeed, the ICOLD-2 strain is less sensitive to EtOH hypothermia (4.0°C reduction from baseline, see Fig. 4) than the COLD-2 selected line (6.0°C; see Fig. 1) when inbreeding was initiated.

Differences in metabolism of EtOH, which did not exist after Generations 7–11 of selection [4], have now developed. That is, because of continued selection pressure, genes contributing to differences in EtOH metabolism might have been fixed in either the HOT or the COLD lines. While the IHOT-2 and ICOLD-2 mice did not differ in blood EtOH concentrations at 60 min following injection, HOT and COLD mice of Replicates 1 and 2 now differ about 10% in blood EtOH concentrations. The inbred strains do not show this difference, suggesting that it occurred in later generations (i.e., after Generation 20). Previous research found that brain EtOH concentrations differed between the lines, although this difference was only apparent at 3–4-h post-injection [4]. Thus, the difference in brain EtOH concentration was apparent long after the maximal change in temperature had occurred. HOT mice, therefore, probably metabolize EtOH a little more rapidly than COLD mice, which in turn may be secondary to the greater hypothermia apparent in COLD mice. A test of this hypothesis in selected Generations 7–11 by administering 0.5 g/kg of EtOH and assaying brain EtOH concentrations 15–60 min later found no differences in rate of metabolism of this low EtOH dose [4]. Alternatively, HOT and COLD mice could differ in absorption and or distribution of EtOH. To address the issue of metabolic differences, further experiments need to be conducted. In any event, given the dose–effect relationships for EtOH-induced hypothermia, these small differences in blood EtOH concentrations can only account for a fraction of the very large difference in body temperature between HOT and COLD mice given the same fixed dose of EtOH.

There was a clear difference between the HOT and COLD lines in hypothermic tolerance development after repeated injections of EtOH. No evidence for tolerance was seen in the HOT mice, while the COLD lines developed substantial tolerance. Regardless of dosing schedule on Days 2–4 (see Fig. 2), the COLD mice developed tolerance and the HOT mice did not. It is interesting that such a low dose of EtOH can produce a robust hypothermic response in COLD mice. It is conceivable that the COLD mice are demonstrating a greater development of tolerance due to their increased response on Day 1, although when an attempt to find equi-potent doses of EtOH was made and the IHOT-2 and ICOLD-2 mice did not differ in their response on Day 1, ICOLD-2 mice developed tolerance while the IHOT-2 did not. Furthermore, that HOT mice failed to develop tolerance could not be explained by a failure to elicit central hypothermia, as HOT-1 and -2 and ICOLD-2 mice did not differ in hypothermic sensitivity in this experiment but only ICOLD-2 developed tolerance. Yet, on Day 3, HOT mice had more pronounced hypothermia than their response on Day 1 while COLD mice showed an attenuated hypothermia. This enhanced response in HOT mice has been reported previously [4]. Studies of inbred strains have also found that some strains show tolerance with chronic EtOH administration, while others show no change in hypothermia following chronic EtOH administration [6]. In previous studies, metabolism of EtOH could not account for the genetic differences between HOT and COLD mice and sensitive vs. insensitive strains in tolerance development [2,5].

These findings also support the existence of a genetic correlation between initial sensitivity to EtOH-induced hypothermia and the development of tolerance. This implies that some set of genes that enhances the sensitivity of mice to EtOH-induced hypothermia also enhances the attenuation of the hypothermic response elicited by repeated EtOH administration.

HOT and COLD mice provide a good tool for mechanistic examinations of EtOH's hypothermic effects. The HOT and COLD lines are also useful genetic models for identifying genes influencing EtOH-induced hypothermia. The inbred strains offer a novel approach for investigating how the HOT and COLD lines have diverged with selection. Studies have been successful in using these lines for initial investigations of neurotransmitter systems mediating observed differences between the HOT and COLD lines [11–13], and we plan to pursue this type of investigation.

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