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Determining the Heritability of Ethanol-induced Locomotor Sensitization in Mice Using Short-term Behavioral Selection

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Abstract

Rationale—Sensitization to the locomotor stimulant effects of alcohol (ethanol) is thought to be a heritable risk factor for the development of alcoholism that reflects progressive increases in the positive motivational effects of this substance. However, very little is known about the degree to which genes influence this complex behavioral phenomenon.

Objectives—The primary goal of this work was to determine the heritability of ethanol-induced locomotor sensitization in mice using short-term behavioral selection.

Methods—Genetically heterogeneous C57BL/6J (B6) × DBA/2J (D2) F2 mice were generated from B6D2F1 progenitors, phenotyped for the expression of locomotor sensitization, and bred for high (HLS) and low (LLS) expression of this behavior. Selective breeding was conducted in two independently generated replicate sets to increase the confidence of our heritability estimates and for future correlated trait analyses.

Results—Large and significant differences in locomotor sensitization between HLS and LLS lines were evident by the fourth generation. Twenty-two percent of the observed line difference(s) were attributable to genes (h^2=.22). Interestingly, locomotor activity in the absence of ethanol was genetically correlated with ethanol sensitization; high activity was associated with high sensitization.

Conclusions—That *changes* in ethanol sensitivity following repeated exposures are genetically regulated highlights the relevance of studies aimed at determining how genes regulate susceptibility to ethanol-induced behavioral and neural adaptations. As alcohol use and abuse disorders develop following many repeated alcohol exposures, these data emphasize the need for future studies determining the genetic basis by which changes in response to alcohol occur.

Genetic factors contribute significantly to alcohol use and abuse disorders, with the risk of lifetime diagnosis of alcoholism split approximately 50% between genes and the environment (Johnson et al., 1998, Enoch and Goldman, 2001, Dick and Bierut, 2006). Thus, it is critical that we develop a better understanding of how genes regulate key features of alcoholism so that we may develop more effective and targeted treatment strategies.

One rather intuitive possibility is that genes drive increases in the subjective rewarding value of alcohol following repeated use. Known as behavioral sensitization, this progressive increase in the rewarding value of alcohol following repeated exposures has been hypothesized to contribute to the craving and compulsive use observed in alcohol addicted individuals (Newlin and Thomson, 1991, Hunt and Lands, 1992, Robinson and Berridge, 1993). Evidence in support of this view comes from research on alcohol's psychomotor (or locomotor) stimulating effects (Phillips et al., 1997). Because the locomotor stimulant effects of alcohol that occur shortly following its ingestion are thought to represent its positive subjective effects (Wise and Bozarth, 1987), a relative increase in this behavioral response following its repeated use has been hypothesized to be indicative of a positive shift

in its inherent rewarding value (Robinson and Berridge, 1993). Contradictory result on the relationship between the positive motivational effects of ethanol and the expression of locomotor sensitization do exist (Risinger et al., 1992, Cunningham et al., 2002). However, compelling reports in both humans and rodents continue to fuel interest in identifying the causes and consequences of this phenomenon.

Unfortunately, progress in identifying the role of genes on ethanol-induced sensitization in human populations has been hindered by interpretational confounds resulting from inconsistencies in drinking history as well as the inability to determine individual's true "baseline" responses due to ethical constraints. However, a landmark meta-analysis concluded that individuals with positive family history (and therefore genetic susceptibility) do indeed display locomotor sensitization to alcohol (Newlin and Thomson, 1990, 1991, 1999). Furthermore, several studies have shown that moderate and heavy drinkers display more robust stimulant-like responses to alcohol than do light drinkers, and that the magnitude of this stimulation is directly proportional to concurrent self-reports of 'wanting more' alcohol and 'liking' of the stimulating alcohol effects (King et al., 1997, Holdstock et al., 2000, King et al., 2011). Although these latter data do not speak to the relationship between acute stimulation and the development of sensitization, nor do they directly address the role of genes on the development of ethanol-induced sensitization, they do indirectly suggest that sensitization occurs in populations prone to increased alcohol consumption, and that the magnitude of this response is directly related to ethanol's positive motivational effects.

Conducting controlled genetic studies in humans would require imposing alcohol on those most susceptible to alcoholism – an ethically unjustifiable manipulation. For this reason, research into alterations in the locomotor response to alcohol in rodents (typically mice) has proven to be a very informative alternative. To date, three key studies utilizing over 20 recombinant inbred (RI) strains have confirmed that locomotor sensitization to alcohol is genetically mediated (Cunningham, 1995, Phillips et al., 1995, Phillips et al., 1996). All three studies found 1) differences in the development of sensitization dependent on genotype/genes and 2) specific regions of the genome, or quantitative trait loci (QTL), responsible for these differences. However, whereas there was much consistency between these three studies on basal locomotor activity and *acute* ethanol-induced stimulation in naïve mice, there was no significant convergence between the strain and QTL results related to the *sensitization* of this response (i.e. different strains and gene regions were implicated between studies). The authors suggested that these discrepancies may have been due to cross-experiment methodological differences such as the use of 3 different test apparatus, differences in the length and timing of ethanol and apparatus exposure, and differences in sex. Why and how these particular procedural differences would affect the relationship between alterations in ethanol-induced locomotor activity following repeated exposures but not basal locomotion or acute ethanol-induced locomotion is not immediately clear. Nevertheless, that each of these studies found strain differences associated with several regions of the genome suggests that this trait is indeed [poly]genetically regulated. Further support for polygenetic regulation of locomotor sensitization can be plainly observed by looking at the distributions of this behavior in the RI strains. If this trait was regulated by one gene then the distribution would be bimodal, with each strain resembling one of the parent strains (Crabbe et al., 1990). However, that sensitization was graded across strains, with some even below or above the parent strains, is a direct indication that more than one gene regulates this trait (Crabbe et al., 1990). Thus, although genetic factors regulate the development of locomotor sensitization to alcohol in both humans and rodents, there is still need to characterize the strength and significance of this relationship.

A better understanding of the role of genetics on the development of locomotor sensitization to ethanol and the influence of genes on the motivational properties of this substance are still needed. Given the available evidence suggesting that the development of locomotor sensitization in mice is regulated by several or many genes (Phillips, 1997), the use of 'short-term' behavioral selection provides a simple and straightforward means to accomplish this goal (Belknap et al., 1997).

The work detailed here was conducted to quantify the relative contribution of genes to the development of ethanol-induced locomotor sensitization in mice using short-term behavioral selection, with the hypothesis that 4 generations of selection pressure would produce mouse lines with wide divergence in the expression of locomotor sensitization.

Methods

Animals and Selective Breeding

The major difference in short-term selective breeding versus the more standard selective breeding strategies is that a less diverse but equally heterogeneous founding population is used. Detailed discussions of the molecular events surrounding the rate of line divergence are beyond the scope of this work. However, by using two homogeneous founding populations (versus 4 or 8), the probability that genes (including those regulating the selected behavior) will diverge between lines increases greatly (Belknap et al., 1997, Falconer, 1989), and in some instances allows researchers to evaluate the genetic regulation of traits that would otherwise be impractical to study in this fashion.

Short-term behavioral selection experiments have several additional strengths over other previously used tools/methods for assessing the heritability of locomotor sensitization (Cunningham, 1995, Phillips et al., 1995, Phillips et al., 1996). For example, although comparisons of many inbred mouse strains allows for the estimation of heritability (Crabbe, 1989, Crabbe et al., 1990), the fixation of genes within a given inbred strain is random. Therefore, the more genes that regulate a particular behavioral trait, the lower the probability any given inbred mouse strain will possess all or most of those genes. This is not the case with behavioral selection experiments in which only those genes that regulate a behavior are recruited and fixed in a homozygous state – excluding inbreeding. Thus, selective breeding strategies are often advantageous over inbred strain studies for accurately estimating heritability.

The C57BL/6J (B6) and DBA2/J (D2) mouse strains were chosen for the production of the founding population(s) to be consistent with other short-term selection studies for ethanol-related behavior phenotypes in mice (Belknap et al., 1997, Metten and Crabbe, 2005, Phillips et al., 2005) and to increase the likelihood of a rapid response to selection that might otherwise have been slower had we chosen a more heterogeneous population such as an outbred mouse stock (Belknap et al., 1997). Approximately 8 week (56±3 day) old offspring of a first filial generation cross between the B6 and D2 inbred mouse strains were ordered from Jackson Laboratory (Bar Harbor, ME) and shipped to the animal facility in the Purdue School of Science at Indiana University – Purdue University Indianapolis (IUPUI). These B6D2F1 (F1) mice were paired as breeders for the production of genetically heterogeneous B6D2F2 (F2) progeny. All F2 offspring (and mice derived from these F2 offspring) were weaned at 21 days of age and group housed by sex with littermates 2–5 to a cage where they remained throughout behavioral testing. Vivarium lighting was maintained on a 12/12 hour cycle with the lights turning on at 7:00 AM and the temperature and humidity were held at approximately 21°C and 50%, respectively. All mice had *ad lib* access to standard rodent chow and tap water except during behavioral testing which always occurred during the 'lights on' part of the light/dark cycle. Mice were naïve and between 60 and 74 days of age

on the first day of testing. We chose to test mice after postnatal day 60 as it is generally accepted that this time period corresponds to adulthood in rodents (Spear, 2000, Laviola et al., 2003) and we wished to minimize potential effects of development in our studies. In total, 261 adult F2 mice were tested for acute and repeated locomotor responses to 2.0 g/kg dose of ethanol as previously described (Boehm et al., 2008, Linsenbardt and Boehm, 2010), and used as the founding population for the selectively bred mouse lines (see "selective breeding" methods). From a subset of these F2 founding mice, 4 generations of selectively bred lines were generated with a total of 1,187 mice produced for locomotor sensitization phenotyping. Thus, a total of 1,448 mice were used. All procedures were approved by the Purdue School of Science Animal Care and Use Committee and conformed to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Academic Press, 2003).

Locomotor Activity Testing Chambers

Locomotor activity testing was conducted using the VersaMax Animal Activity Monitoring System (Accuscan Instruments Inc., Columbus, OH). Locomotor Activity was detected by the interruption of intersecting photocell beams evenly spaced along the walls of the 40 × 40 cm Plexiglas test chamber. This equipment was situated in sound-attenuating box chambers (inside dimensions, 53 cm across × 58 cm deep × 43 cm high) equipped with a house light and fan for ventilation and background noise. The locomotor activity testing equipment was interfaced with a Dell computer. Testing continued for 15 minutes during which time consecutive photocell beam interruptions were translated into distance traveled in cm by the VersaMax computer program. Data were collected in 1-min time intervals.

Alcohol Administration

190 proof Alcohol was purchased from Pharmco, Inc (Brookfield, CT) and diluted to 20% v/v in 0.9% physiological saline. Ethanol was administered by intraperitoneal (i.p.) injection manipulating volume injected according to body weight to administer a given dose (2.0 or 2.5 g/kg).

General Statistics

All analyses were first conducted with every possible factor included. These included sex, line, replicate, and selection generation. All significant higher order (3-way +) interactions were followed up with additional ANOVAs with one of the factors removed. We used an iterative process removing one factor at a time to determine which of the factors was most influential in driving the significant effects/interactions. In instances where we had specific hypotheses based on the results of these analyses and/or graphical observations, we conducted additional analyses on 1 or more factors. Our rationale for these additional analyses is stated directly in the statistics section. When appropriate, Tukey HSD post hoc analyses were conducted. Differences were considered significant at $p<.0.05$.

Procedures

Sensitization Procedure

We used a slightly modified version of an established mouse model of ethanol sensitization developed by Phillips et al. (1997) routinely used in our lab to evaluate ethanol-induced locomotor sensitization in mice (Boehm et al., 2008, Linsenbardt and Boehm, 2010). All mice were given identical treatment. The first two days of testing served to habituate the mice to the i.p. injections and testing in the locomotor activity chambers (day 1) and establish a baseline locomotor response (day 2). On these days mice were habituated to the testing room for 45–60 minutes, weighed, and then injected with sterile 0.9% saline, and

immediately placed in the center of the activity testing chambers. On day 3 mice were again allowed to habituate to the testing room as in the previous 2 days, only on this day mice received a 2.0 g/kg ethanol injection before being placed immediately into the activity testing chambers. On days 4–13 mice received a slightly higher 2.5 g/kg ethanol dose once daily. This slightly higher dose was chosen based on literature demonstrating its effectiveness at inducing robust locomotor sensitization in genetically heterogeneous (Phillips et al., 1997) and homogeneous (Boehm et al., 2008, Linsenbardt and Boehm, 2010, Linsenbardt and Boehm, 2010s2) mice. None of the mice were tested in the locomotor activity testing chambers following injection on days 4–13; mice were placed immediately into their home cage following injection. We did not test mice in the locomotor activity apparatus on days 4–13 in order to minimize any conditioned locomotor responses that might have developed following repeated ethanol/locomotor chamber pairings. On the final day (day 14) mice were treated identically to day 3; mice were given a 2.0 g/kg ethanol injection and locomotor activity was recorded. Thus, mice were habituated to the procedures (day 1), and then tested for baseline locomotion (day 2), acute ethanol-induced locomotion (day 3), and ethanol-induced locomotion following 11 daily ethanol exposures (day 14).

Selective Breeding

A cohort of 107 F2 mice were tested for the development of ethanol–induced locomotor sensitization using the above 14 day procedure. In order to determine the magnitude of change in ethanol-induced locomotion, we calculated a sensitization score for each individual F2 mouse based on the difference between its acute (Day 3) and repeated (Day 14) locomotor response to ethanol: Day 14 – Day 3 =‘sensitization score’ or ‘SENZ’. We then used an individual mass selection approach such that individual F2 mice were paired for breeding according to their individual sensitization score. The 14 male and 14 female F2 mice with the highest sensitization scores were paired to produce the first selection generation (S1) of High Locomotor Sensitization (HLS) offspring. The offspring of all future generations were tested for sensitization and then selected in an identical fashion to produce S2-4 HLS offspring with those mice with the highest sensitization scores chosen as breeders for each subsequent HLS generation. Identical procedures were used to produce the Low Locomotor Sensitization line (LLS), except that mice were chosen that had the lowest sensitization scores.

This entire breeding procedure was replicated in tandem in a second cohort of 154 F2 mice to produce a second replicate of HLS and LLS lines. The lines derived from the first cohort of F2 mice are referred to as HLS-1 and LLS-1, and the lines derived from the second cohort of F2 mice are referred to as HLS-2 and LLS-2. The production of 2 independently replicated lines allows for the strongest possible statistical evidence for heritability estimates and genetically correlated behavioral traits by ensuring the estimates and/or observed behavioral correlates are not driven by genetic drift (Crabbe et al., 1990). In some cases several mice were selected from the same litter. However, brother-sister pairing was avoided and pairing mice with similar grandparents was minimized to limit the fixation of trait-irrelevant alleles (inbreeding).

Blood Ethanol Concentrations (BECs)

Blood was sampled from all S4 mice phenotyped for the expression of locomotor sensitization to determine if differences in ethanol pharmacokinetics might explain any of the observed group differences. Peri-orbital sinus bloods (50 μl) were drawn immediately following behavioral testing and again 2 hours following ethanol injection on the 14th and final testing day. Samples were centrifuged and plasma was withdrawn and stored at −20°C. BECs were then determined using an Analox Alcohol Analyzer (Analox Instruments, Lunenburg, MA).

Heritability and Inbreeding

Calculations of heritability (h^2; the degree to which trait differences are due to genetic differences) and estimations of inbreeding were conducted using methods adapted from Falconer & Mackay (1996) and reviewed extensively by Dr. Crabbe and colleagues (Crabbe et al., 1990). The equation used for calculation of h^2 is as follows: $h^2=R/S$ [$R=x_1-x_0$; $S=x'_0-x_0$; x_1=mean of offspring of selected parents; x_0=phenotypic value of parent population; x'_0=mean of selected parents]. The inbreeding coefficient, or)F, was calculated as follows: $)F=1/(2N_e)$ [N_e=number of breeders +2]. Total realized heritability and cumulative inbreeding coefficients were calculated similarly upon completion of the final (4^{th}) selection generation.

Results

Sensitization in the F2 founder population

Characterizing the locomotor responses in the founding F2 populations for each replicate (S0-1 and S0-2) was of primary interest because these data have critical implications for the interpretation of behavioral responses observed following four generations of selection pressure. In order to characterize overall locomotion on all test days in these F2 populations, total mean locomotor activity in 15 minutes from days 1, 2, 3 and 14 were analyzed by 3-way mixed factor analysis of variance (ANOVA) with sex and replicate as the between-subjects factors, and day as the within-subjects factor. This analysis revealed significant main effects of sex [$F(1, 257)=33.00$ $p<.0001$], day [$F(3, 771)=837.47$ $p<.0001$], and replicate [$F(1, 257)=17.15$ $p<.0001$], as well as significant day*sex [$F(3, 771)=17.90$ $p<.0001$] and day*sex*replicate [$F(3, 771)=3.16$ $p<.05$] interactions. Post hoc tests of the significant day*sex and the non-significant sex*replicate interaction indicated that females had relatively higher locomotor responses than males on ethanol challenge days only (days 3 and 14; $p<.0001$), and that this higher female activity was more evident in replicate 1; females in replicate 1 had higher activity than females in replicate 2 on days 3 and 14 ($p's<.05$). These results were further supported by subsequent analyses with each sex of each replicate separately.

The results of replicate 1 (Figure 1A) indicated significant main effects of day for both males [$F(3, 153)=131.15$ $p<.0001$] and females [$F(3, 162)=192.59$ $p<.0001$]. Post-hoc tests revealed significant differences between days 3 and 14 ('sensitization') in both male and female groups ($p's<.0001$) and between days 2 and 3 ('stimulation') in the female group only ($p<.0001$).

The results of replicate 2 (Figure 1B) indicated significant main effects of day for both males [$F(3, 252)=292.49$ $p<.0001$] and females [$F(3, 204)=261.35$ $p<.0001$]. Contrary to replicate 1, where post-hoc tests revealed significant differences between days 2 and 3 ('stimulation') in females only, there were significant differences between days 2 and 3 in males and females in replicate 2 ($p<.05$). Consistent with replicate 1, there were also significant differences between days 3 and 14 in both sexes of replicate 2 ($p's <.0001$).

Although ethanol-stimulated locomotion was generally higher in female mice compared to males, the *change* in this response following repeated ethanol exposure was not different between sexes. Furthermore, across all of these studies we never detected any important behavioral sex interactions above and beyond what the F2 results indicated; that females generally display higher ethanol-stimulation locomotor behavior than males. We did observe 1 (non-behavioral) sex difference, but not in F2 mice (see description of Day 14 Blood Ethanol Concentrations below). Thus, although sex was included as a factor in subsequent statistical analyses, graphical representation/discussion of all data in the remainder of this document are collapsed on this factor.

STIM and SENZ Scores Over Generations

In addition to evaluating differences in sensitization (SENZ), we also evaluated alterations in locomotor activity following the first ethanol exposure compared to general baseline locomotor activity (i.e. acute ethanol-induced locomotor response). This score was calculated from the difference between the 2nd and 3rd test days (Day 3 – Day 2 = "STIM"). The STIM and SENZ scores were analyzed for each replicate separately using 3-way ANOVAs with line, sex, and selection generation as factors (see Figure 2).

The results of analysis of STIM scores can be seen in Figure 2A, B. Main effects of sex were detected in replicate 1 [$F(1, 566)=16.41$ $p<.0001$] and replicate 2 [$F(1, 589)=5.57$ $p<.05$]. Females displayed generally higher positive changes in locomotion compared to males. There was also a line*selection generation interaction in replicate 2 only [$F(3, 589)=4.29$ $p<.01$], which post hoc tests indicated was due to line differences at the final S4 selection generation only ($p<.001$). Thus, with the exception of this one effect at S4, there were no consistent differences between lines in STIM scores.

The results of analysis of SENZ scores can be seen in Figure 2C, D. There were significant main effects of sex [$F(1, 566)=5.26$ $p<.05$] [$F(1, 589)=12.13$ $p<.001$] and line [$F(1, 566)=173.04$ $p<.0001$] [$F(1, 589)=104.34$ $p<.0001$] for replicate 1 and 2 respectively. Females displayed generally higher SENZ scores than males, and as anticipated, HLS mice displayed higher SENZ scores than LLS mice. There was also a significant selection generation*line interaction [rep1; $F(3, 566)=11.70$ $p<.0001$] [rep2; $F(3, 589)=18.17$ $p<.0001$] in both replicates. Post hoc analysis of these interactions confirmed significant line differences all generations in replicate 1 (p's<.05) and in each of the final 3 selection generations in replicate 2 (p's<.0001). There were no line differences detected at the 1st selection generation ($p>.05$) in the 2nd replicate. Thus, with the exception of the first generation of selection in the replicate 2 mice, HLS lines consistently displayed larger magnitudes of sensitization than LLS mice in both replicates.

Response to Selection by Testing Day Over Generations

To characterize differences in each days locomotor activity as a function of selection generation, we analyzed each day separately with line, sex, and selection generation as factors. Day 1 (S1–S4): The results of day 1 analysis for each replicate can be seen in Figure 3A, E. Analysis of locomotor activity on day 1 in replicate 1 mice (Figure 3A) revealed significant main effects of sex [$F(1, 566)=13.31$ $p<.001$], generation [$F(3, 566)=17.21$ $p<.0001$], and line [$F(1, 566)=70.11$ $p<.0001$] with females displaying higher activity than males and HLS mice displaying higher activity than LLS mice. Analysis of locomotor activity on day 1 in replicate 2 mice (Figure 3E) revealed significant main effects generation [$F(3, 589)=3.13$ $p<.05$], and line [$F(1, 589)=19.72$ $p<.0001$], and a significant generation*line interaction [$F(3, 589)=4.99$ $p<.01$]. Post hoc tests revealed that there were line differences only in the 3rd ($p<.05$) and 4th ($p<.0001$) generations in this second replicate.

Day 2 (S1–S4): The results of day 2 analysis for each replicate can be seen in Figure 3B, F. Analysis of locomotor activity on day 2 in replicate 1 mice (Figure 3B) revealed significant main effects of sex [$F(1, 566)=5.38$ $p<.05$], generation [$F(3, 566)=10.22$ $p<.0001$], and line [$F(1, 566)=91.53$ $p<.0001$] and a significant sex*line interaction [$F(1, 566)=5.37$ $p<.05$] with females displaying higher activity than males and HLS mice displaying higher activity than LLS mice. Post hoc tests of the 2 way interaction indicated no differences between males and female HLS mice when collapsed on generation; HLS mice displayed higher activity than LLS mice regardless of sex. Analysis of locomotor activity on day 2 in replicate 2 mice (Figure 3F) revealed significant main effects of generation [$F(3, 589)=9.18$

p<.0001], and line [F(1, 589)=19.89 p<.0001], and a significant generation*line interaction [F(3, 589)=11.24 p<.0001]. Post hoc tests revealed that there were line differences only in the 3rd (p<.001) and 4th (p<.0001) generations.

Day 3 (S1–S4): The results of day 3 analysis for each replicate can be seen in Figure 3C, G. Analysis of replicate 1 mice (Figure 3C) revealed significant main effects of sex [F(1, 566)=5.38 p<.05], generation [F(3, 566)=10.22 p<.0001], and line [F(1, 566)=91.53 p<.0001] with females displaying higher activity than males and HLS mice displaying higher activity than LLS mice. However, the line effect was driven exclusively by the relatively higher locomotor response in the S3 generation of the HLS line compared to all other groups (p's<.05); there were only line differences in this S3 generation of this replicate. Analysis of locomotor activity on day 3 in replicate 2 mice (Figure 3G) revealed a significant main effect of sex [F(1, 589)=9.75 p<.01], with females showing generally higher ethanol-induced locomotor activity than males.

Day 14 (S1–S4): The results of day 4 analysis for each replicate can be seen in Figure 3D, H. Analysis of replicate 1 mice (Figure 3D) revealed significant main effects of sex [F(1, 566)=35.33 p<.0001], generation [F(3, 566)=12.61 p<.0001], and line [F(1, 566)=197.96 p<.0001] as well as a significant generation*line interaction [F(3, 566)=11.69 p<.0001]. Post hoc tests indicated that females displayed higher activity than males and HLS mice displayed higher activity than LLS mice. HLS and LLS lines were significantly different at generations 1 (p<.05), 2 (p<.0001), 3 (p<.0001), and 4 (p<.0001). Analysis of locomotor activity on day 14 in replicate 2 mice (Figure 3H) revealed significant main effects of sex [F(1, 589)=29.17 p<.0001] and line [F(1, 589)=93.62 p<.0001], and a significant generation*line interaction [F(3, 589)=17.28 p<.0001]. Post hoc tests indicated that HLS and LLS lines differed at generations 2, 3, and 4 (p's<.0001).

Day 14 Blood Ethanol Concentrations (S4)

The results of BEC analysis revealed significant main effects of sex [F(1, 164)=71.24 p<.0001]; [F(1, 138)=28.83 p<.0001], blood sample time point [F(1, 164)=16805.64 p<.0001]; [F(1, 138)=11061.87 p<.0001], and a significant sex*blood sample time point interaction [F(1, 164)=143.18 p<.0001]; [F(1, 138)=73.77 p<.0001] for replicates 1 and 2 respectively. Post hoc tests indicated that females had lower BECs at the 2 hour post-injection time point compared to the males; females metabolized ethanol more quickly than males. There were no significant line effects or interactions suggesting that ethanol pharmacokinetics were not responsible for the lines differences in locomotor behavior.

Estimates of Heritability and Inbreeding

Total realized heritability estimates calculated as the slope of the best fit line of R/S for each replicate can be seen in Figure 4. Remarkably, heritability estimates for replicates 1 and 2 were identical; h^2=.22. Thus, 22% of the difference in ethanol-induced locomotor sensitization between the HLS and LLS lines was attributable to genetic differences. The very high R^2 values for both replicates 1 (R^2=.94) and 2 (R^2=.97) indicate that additive genetic variability may not have been exhausted. In other words, had selection pressure been continued in these lines for additional generations, it is possible that the lines would have continued to diverge in the magnitude of sensitization. With one notable exception (HLS Replicate 1;R^2=.18), the R^2 values were similarly high when each line of each replicate was evaluated separately. This would suggest that selection pressure in the HLS replicate 1 line was much closer to exhaustion; additional generations of selection would not further increase the magnitude of the selection phenotype. Further discussion of this particular finding and how it relates to other similar findings in the literature are detailed in the "General Discussion" section.

Because each line represented a unique and fixed population of mice, inbreeding coefficients for each line within each replicate were calculated separately. Inbreeding coefficients increased by approximately .01 (or 1%) with each subsequent generation such that by the 4th generation cumulative inbreeding for each line was approximately.04 or 4%. The inbreeding coefficients for each line at the S4 generation were as follows: HLS-1 = 0.048, LLS-1 = 0.040, HLS-2 = 0.040, LLS-2 = 0.041. For short-term selection studies such as these this degree of inbreeding is extremely low. Thus, there were likely very few genes that were fixed that were unrelated to locomotor sensitization.

Discussion

These results support the hypothesis that locomotor sensitization to ethanol is genetically regulated. Short-term behavioral selection resulted in lines that displayed wide divergence in the expression of locomotor sensitization to ethanol in two independently generated replicates. HLS lines displayed high locomotor sensitization and the LLS lines displayed low locomotor sensitization. Calculations of the degree to which line differences were due to genetic factors were significant and virtually identical between replicates. Interestingly, there were also line differences in locomotor activity following saline injections prior to any ethanol exposure. HLS lines displayed higher locomotor activity than the LLS lines when the locomotor activity chambers were novel on the first day of testing and also on the second (identical) 'baseline' testing session 24 hours later. There were no consistent differences between lines in acute ethanol-induced locomotor activity following the first ethanol exposure. However, as would be expected based on the selection phenotype, there were large and significant differences in ethanol–induced locomotor activity following 11 daily ethanol exposures (day 14).

Locomotor Behavior in F2 Mice

The overall ethanol-induced increases in locomotor behavior (i.e. stimulation/sensitization) in the genetically heterogeneous F2 founding populations are consistent with previous reports using similar ethanol doses in genetically heterogeneous mice (Frye and Breese, 1981, Didone et al., 2008). However, despite the general observation that F2 mice displayed acute ethanol-induced locomotor stimulation and sensitization to this response, there was considerable inter- and intra-individual variability. These diverse locomotor responses to ethanol are important for the interpretation of the data, because on the basis of our previous definition, only those individuals who displayed an acute stimulant response could be considered as having developed sensitization to ethanol's stimulant effects. Individuals who displayed acute ethanol-induced locomotor sedation (decreases from day 2 baseline), but then increases in ethanol-induced locomotion following repeated exposures, might be considered as having developed tolerance to ethanol's sedative effects (if back to baseline) and/or sensitization to ethanol's stimulant effects (if above baseline). Obviously, there are many possible interpretational outcomes once each scenario has been considered. Nevertheless, because it is impossible to disambiguate possibly competing locomotor sedative/stimulating effects within an individual animal using only one behavioral endpoint (in this case locomotion), we chose to use group means to define the directionality of changes in locomotor behavior. Because in every generation all groups displayed overall acute ethanol-induced stimulation (see Figure 2A, B), all data are discussed as they relate to sensitization.

Heritability of Locomotor Sensitization

There is sufficient evidence from multiple reports that ethanol-induced locomotor sensitization in mice is genetically regulated (Phillips et al., 1997). However, to date only two published studies have directly evaluated the *magnitude* of genetic influence using

statistically estimated heritability calculations (Phillips et al., 1995, Phillips et al., 1996). Using recombinant inbred (RI) strains derived from C57BL/6J and DBA/2J progenitors, heritability of locomotor sensitization was calculated to be from 22% (Phillips et al., 1996) to 29% (Phillips et al., 1995). The heritability estimates in these studies (particularly the first) are consistent with the results of the work detailed here, which also suggests that 22% of the expression of locomotor sensitization is due to genetic factors. However, there are some important procedural and methodological issues surrounding these results that must be considered.

First, all of the published studies evaluating the heritability of locomotor sensitization, including the results presented here, used mice derived from the same 2 inbred mouse strain progenitors – C57BL/6J and DBA/2J. Because estimates of heritability might be expected to differ in populations with different genetic backgrounds, the generalizability of these data to the general mouse population may not be as strong as one would hope (Crabbe et al., 1990). Future studies designed to address this question specifically, for example using similar selective breeding strategies but with a more diverse founder population, would be useful in this regard. However, although published only in abstract form (Linsenbardt and Boehm, 2011), we have found remarkably similar (but slightly higher) heritability estimates to those found here using a panel of inbred strains. Thus, converging evidence suggests that at least 22% of ethanol-induced locomotor sensitization is due to genetic influences.

Out of the many ethanol-related behavioral phenotypes that have been evaluated using selective breeding, heritability estimates of some of the most successful have been similar or lower than the 22% calculated for locomotor sensitization. For example, in mice bi-directionally selected for hypnotic sensitivity to ethanol, and more recently, mice selected for drinking to high blood ethanol concentrations, heritability estimates were estimated at 0.18 (18%; (McClearn and Kakihana, 1981) and 0.10 (10%; (Crabbe et al., 2009) respectively. Despite the low genetic influence on these traits, the response to selection progressed greatly over generations. These studies emphasize a valid point- that although less than ¼ of an ethanol related response in mice may be driven by genes, the ability of those genes to influence a behavior can be profound and meaningful. This is particularly valid when one considers that individual animal models realistically only address one or a few diagnostic features of alcoholism at best (Crabbe, 2008).

A second important implication of these findings is that genes can influence *changes/alterations* in ethanol-evoked behavior. There are no known reports other than the current one of attempts to selectively breed for alterations in an ethanol-related behavior following repeated exposures. This is despite the fact that one of our ultimate goals is to model individuals who have, over the course of many alcohol drinking experiences, become excessive and often compulsive alcohol abusers. It is well established that although many individuals engage in frequent and excessive ethanol intake at some point in their lifetime (typically starting in young adulthood), only a certain percentage of those individuals continue this pattern of alcohol intake into adulthood (Gotham et al., 1997, Bennett et al., 1999). As one example, in a cohort of primarily young males diagnosed as alcohol abusers, approximately 50% continued to abuse alcohol and/or become alcohol dependent, whereas the other half discontinued alcohol abuse (Hasin et al., 1990). Although sensitization to alcohol's stimulant effects were not evaluated in this study, the selected lines in the current report may be analogous at some level to this type of phenomenon, and might give us some insight into potential genetic influences.

A third point worth discussion relates to the observed line differences in heritability. Because heritability estimates for the LLS lines were approximately twice what they were for the HLS lines, it would seem that the relative contribution of genes to the expression of

low locomotor sensitization was greater. Put another way, genetic factors were more influential than environmental factors in mediating *resistance* to changes in locomotion following repeated ethanol exposures. This resistance to changes in locomotion should not be confused with sensitization to the sedative effects of ethanol, as mean change scores in the LLS populations were always positive (i.e. LLS lines *did* develop locomotor sensitization). Under the assumption that locomotor sensitization does indeed reflect changes in the positive motivational effects of ethanol (Hunt and Lands, 1992, Robinson and Berridge, 1993), this finding might suggest that genes play a larger role in *protecting* individuals from developing maladaptive hedonics-driven ethanol-seeking. However, additional behavioral measures of the motivating effects of alcohol in these lines such as voluntary intake and/or conditioned place preference (CPP) assays are warranted.

The use of founding populations with 2 possible allelic combinations warrants one final discussion point. The probability of recruiting trait-relevant alleles is dependent on the frequency of those alleles in the population. Because the frequency of alleles in the 2 inbred strain founding F2 population is 50%, the odds of recruiting an allele(s) that influence the selected behavior early in selection is relatively higher than in one with a lower frequency. Therefore, where the likelihood of recruiting any given allele was the same for each individual in the F2 population, the calculated heritability estimates were influenced by the proportion of genetic variance accounted for by a particular allele. By way of comparison, the response to selection could have been due to 1) dozens of genes each with small effect or 2) only a few genes with cumulatively larger effect on the variance in the behavior. As genes and gene frequencies were not directly evaluated, we have no way of knowing the extent to which either of these occurred.

Response to Selection – Days 1–2 (Habituation/Baseline)

That selection for ethanol-induced locomotor sensitization was associated (genetically correlated) with general locomotor activity on the first and second days of testing was quite compelling. Whereas some studies have found basal locomotor activity to be positively genetically correlated with ethanol sensitization in mice (Phillips et al., 1996), others have found no association (Phillips et al., 1995). Our studies clearly support the former, as differences between HLS and LLS mice in locomotor activity on the first two tests days emerged at approximately the same rate as differences in the expression of locomotor sensitization. This finding is of potential interest if one considers locomotor activity in this relatively novel environment as an index of “novelty seeking” as some have suggested (Bardo et al., 1996). Genetic predisposition to novelty seeking has been associated with increased risk for drug abuse in humans (Heath et al., 1997, Prescott et al., 1997, Young et al., 2000, Knopik et al., 2004). Consistent with this view, rodent locomotor activity in a novel environment and/or preference for novel environments has been shown to predict self-administration of ethanol (Gingras and Cools, 1995, Nadal et al., 2002) as well as other commonly abused drugs (Piazza et al., 1989, Suto et al., 2001, Kalinichev et al., 2004, Cain et al., 2005, Mitchell et al., 2005, Belin et al., 2011). These data provide support for the hypothesis that locomotor sensitization reflects a genetically mediated index of susceptibility to increases in ethanol positive motivational effects. If selection for locomotor sensitization recruits genes that govern alterations in ethanol’s positive motivational effects, and similar genes regulate novelty-induced locomotor behavior, than the current results fit well with the findings cited above.

Response to Selection – Day 3 (First Ethanol Exposure)

There were no consistent differences in acute ethanol-induced locomotor activity between lines in these studies. The only instance when lines differed in overall locomotor activity following the first ethanol exposure was in the 3rd generation of replicate 1, where HLS

mice had generally higher locomotor activity following the first ethanol exposure compared to the LLS line (see Figure 3C). Interestingly, this line difference in overall locomotor activity did not translate to a line difference in the STIM response. That is, once baseline locomotor activity was taken into account, ethanol-induced stimulation did not differ between lines (see Figure 2A). This was due to the previously mentioned line differences in locomotor behavior on day 2. On the contrary, although there were no differences in overall locomotor activity following the first ethanol exposure in replicate 2 mice (see Figure 3G), there *was* a significant line difference in the degree of ethanol-induced stimulation (STIM) in the 4^{th} selection generation (see Figure 2B). Thus, the higher ethanol-induced stimulation score in the LLS lines was driven primarily by the relatively lower baseline locomotor response from the previous day. Together these results are in agreement with the majority of the literature in which no genetic association has been found between the acute locomotor response to ethanol and a change in this response following repeated exposures (Cunningham, 1995, Phillips et al., 1995, Phillips et al., 2005). The absence of this relationship for ethanol sets this substance apart from other drugs of abuse where this relationship has been reported more frequently.

Response to Selection – Day 14 (Final Ethanol Exposure)

Line differences in ethanol-induced locomotion following the final ethanol exposure were directly proportional to the magnitude and rate of the selection phenotype. This was not surprising, given that locomotor activity was higher on the final day compared to the first ethanol exposure. For this reason, locomotor activity on day 14 carried more relative weight in determining each individuals SENZ scores compared to data from day 3. An increase in ethanol-stimulated locomotion following repeated ethanol exposure in mice is a commonly reported phenomenon and directly illustrates the concept of locomotor sensitization.

Ethanol Pharmacokinetics

As there were no differences in BECs between lines, either immediately after behavioral testing or 2 hours following ethanol injections, line differences were not due to ethanol pharmacokinetics.

Summary and Conclusions

That *changes* in ethanol sensitivity following repeated exposures are in part genetically regulated highlights the relevance of studies aimed at determining how genes regulate susceptibility to ethanol-induced behavioral and neural *adaptations*. Together these studies provide clear evidence that genes are capable of regulating alterations in ethanol-induced locomotor behavior in mice.

Acknowledgments

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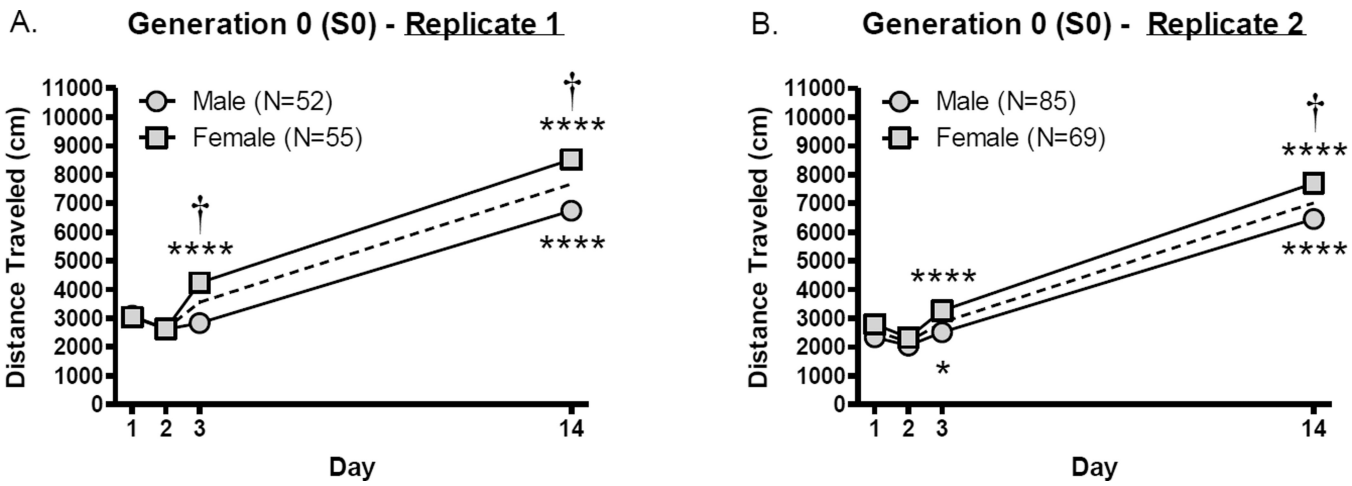

Fig. 1.

Total distance traveled in 15 minutes on all locomotor testing days in the two separate F2 founder populations used for the production of the 1st and 2nd replicates of selectively bred mouse lines. **A**. Replicate 1 female F2 mice displayed acute ethanol-induced locomotor stimulation (day 3) and an enhancement of this response following repeated ethanol exposures (day 14) whereas males displayed an enhanced locomotor response on day 14 compared to day 3 only. **B**. Replicate 2 male and female F2 mice both displayed acute ethanol-induced locomotor stimulation (day 3) and an enhancement of this response following repeated ethanol exposures (day 14). *'s indicate within-group differences from the previous test day when sex was analyzed separately (*<.05; ****<.0001). Day 3 differences indicate 'stimulation' whereas day 14 differences indicate 'sensitization'. †'s indicate between-sex difference on days 3 or 14 (p's<.01). Dotted line represents data collapsed on sex.

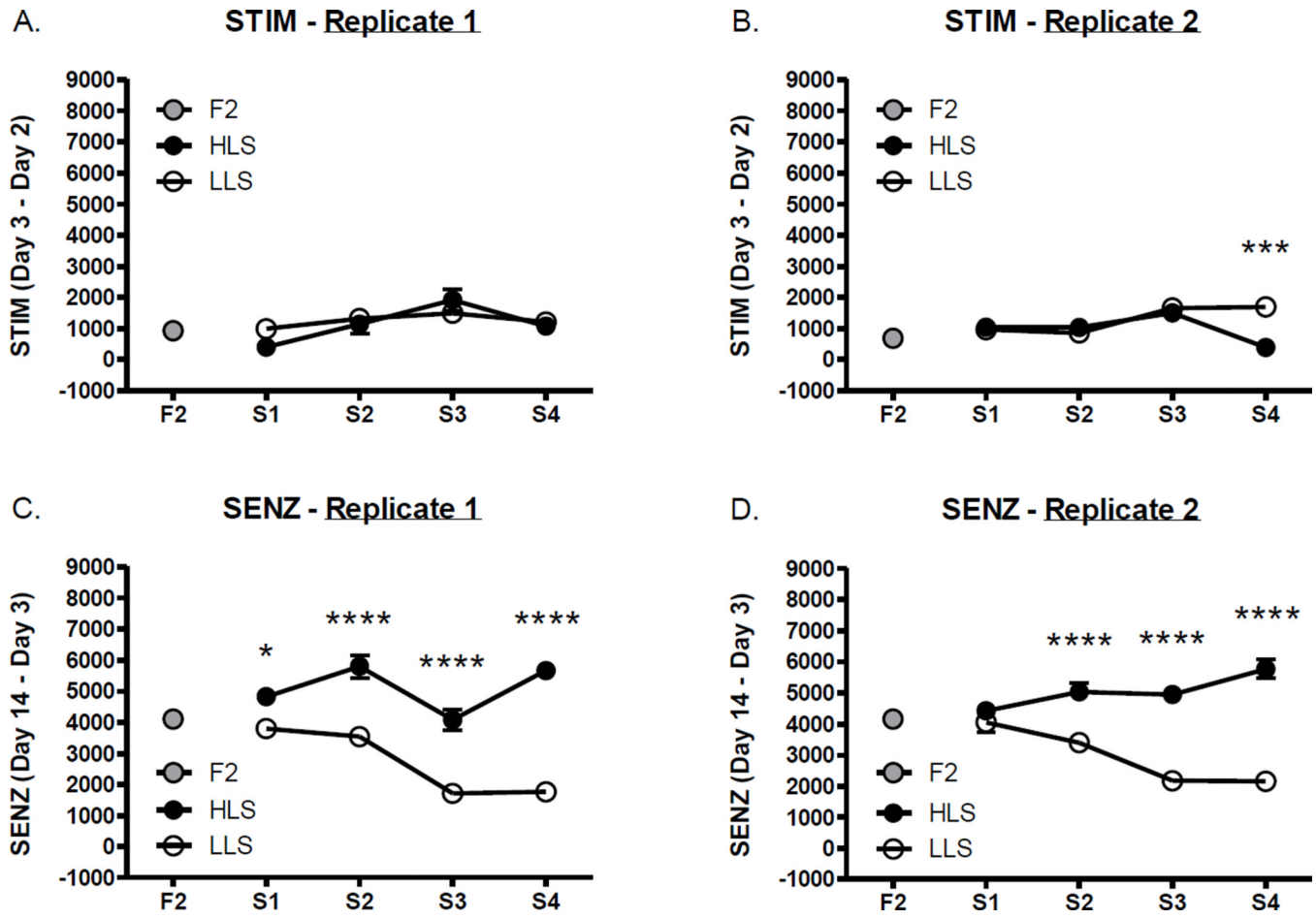

Fig. 2.

Stimulation (STIM) and sensitization (SENZ) scores over 4 generations of selection in replicates 1 (A+C) and 2 (B+D). **A–B**. There were no differences between lines in STIM scores in Replicate 1 mice whereas there were significant differences in STIM scores in replicate 2 mice but only in the final (4th) selection generation. **C–D**. Replicate 1 HLS mice displayed higher SENZ scores than LLS mice across all selection generations (S1–S4) whereas replicate 2 HLS mice displayed higher SENZ scores than LLS mice across only the final 3 selection generations (S2–S4). *'s indicate between-group (line) differences (*<.05; ***<.001; ****<.0001).

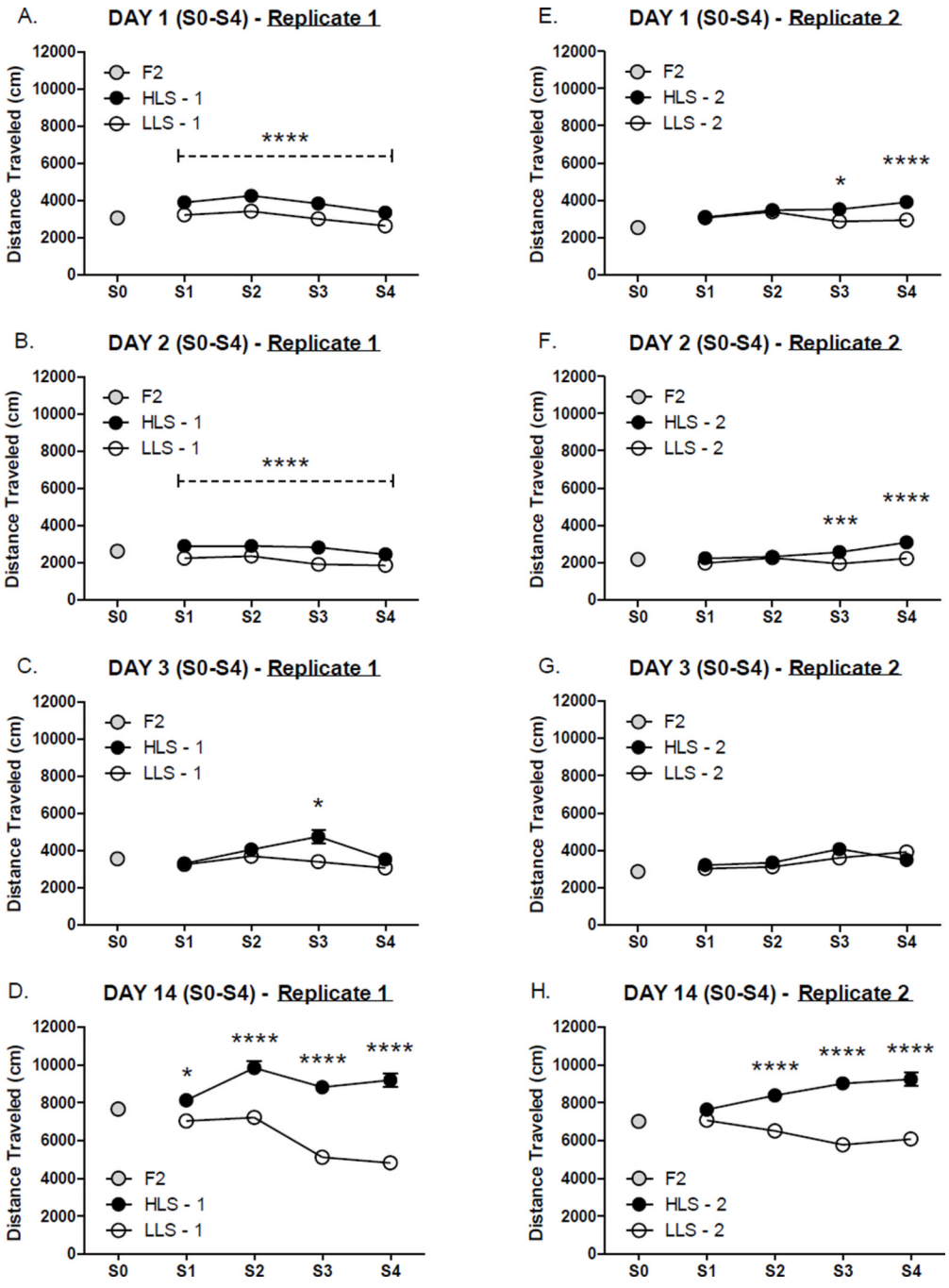

Fig. 3.

Total distance traveled in 15 minutes on testing days 1, 2, 3, and 14 over the course of four selection generations for replicates 1 and 2 separately. **A–D**. Replicate 1 HLS mice displayed higher overall locomotor activity than LLS mice on days 1 (A.), 2 (B.), and 14 (D) across all generations (S1–S4) but only on day 3 (C.) in the S3 selection generation. **E–H**. Replicate 2 HLS mice displayed higher overall locomotor activity than LLS mice on days 1 and 2 in the S3 and S4 selection generations, and on day 14 in the S2–S4 selection generations. There were no differences on day 3 in the 2nd replicate at any generation of selection. *'s indicate between-group (line) differences (*<.05;***<.001; ****<.0001).

Dotted line brackets indicate significant main effects of line and are present only in instances when main effects of line were detected in the absence of any significant line interactions.

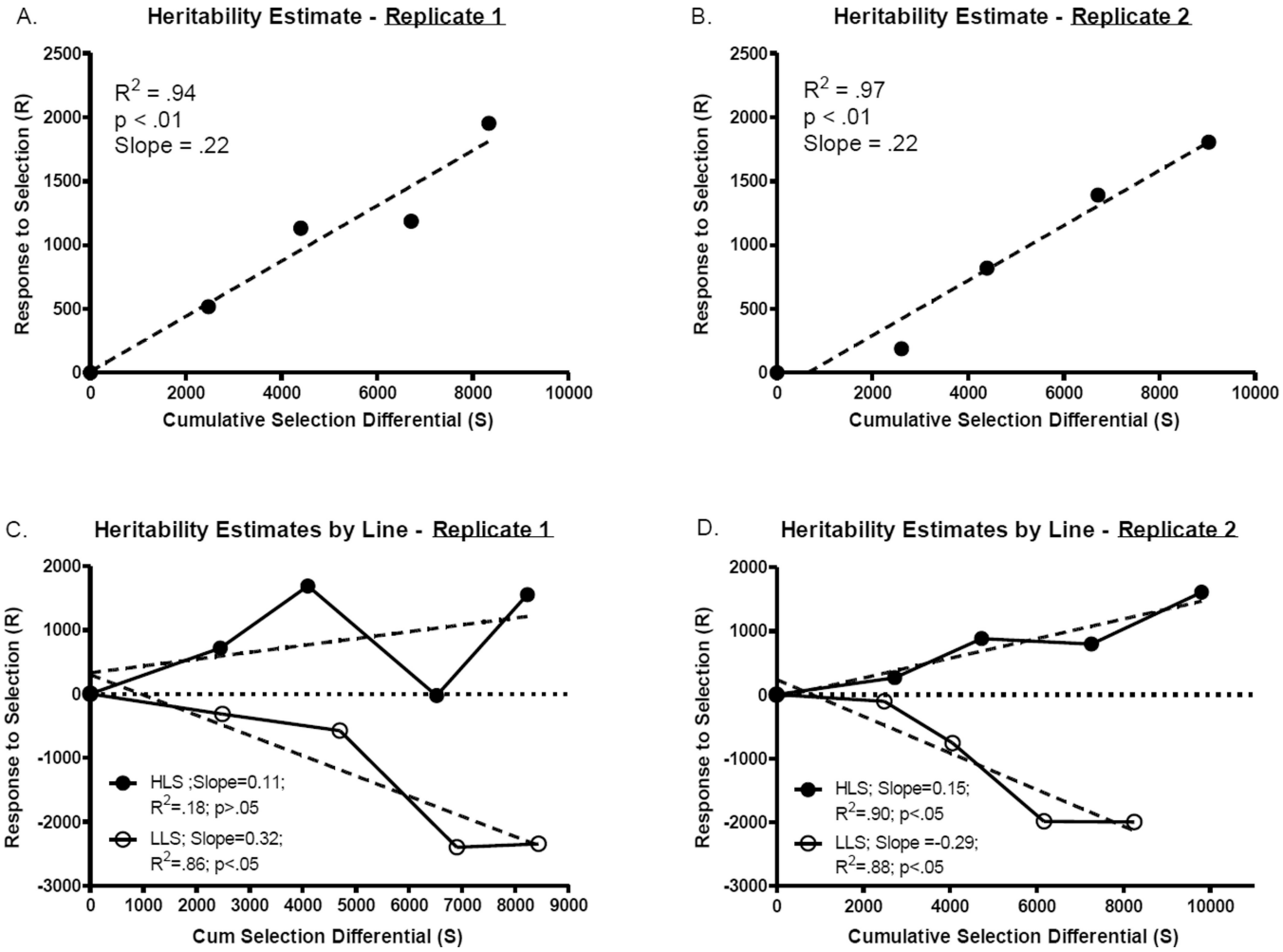

Fig. 4.

Total realized response to selection in lines selected for divergent ethanol-induced locomotor sensitization. Each selection differential value (S) represents the mean SENZ scores of the mice selected to breed the next generation (i.e. parents) minus the mean SENZ score of the population from which they were selected (ex. S2 parents - S1 offspring). Each realized response to selection value (R) represents the total change in mean SENZ scores from the S0 (F2) founder population; each value is added to the previous generations calculation to create a 'cumulative' S value. The slope of the best-fit line of R/S derived using linear regression analysis gives an estimate of heritability (h^2). Thus approximately 22% of the differences in ethanol-induced locomotor sensitization between the HLS and LLS lines is attributable to genetic differences in both replicates 1 (A.) and 2 (B.). Furthermore, the degree to which the R/S values deviate from the best-fit line gives us an indication of potential additive genetic variability. The very large R2 values indicate that additive genetic variability in each replicate had not been exhausted and that lines would continue to diverge had they continued to be selected in the same fashion. The relatively slow but significant increase in divergence over generations suggests that ethanol-induced locomotor sensitization is regulated by many genes, each with relatively small effect on the behavioral outcome.