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Rapid Selection Response for Contextual Fear Conditioning in a Cross Between C57BL/6J and A/J: Behavioral, QTL and Gene Expression Analysis

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Abstract We used short-term selection to produce out-bred mouse lines with differences in contextual fear conditioning. Within two generations of selection all low selected mice were homozygous for the recessive tyr^c allele and showed the corresponding albino coat color. Freezing differed in the high and low selected lines across a range of parameters. We identified several QTLs for the selection response, including a highly significant QTL at the tyr locus ($p < 9.6^{-10}$). To determine whether the tyr^c allele was directly responsible for the response to selection, we examined B6 mice that have a mutant tyr allele (tyr^{c-2j-}) and an AJ congenic strain that has the wild-type B6 allele for tyr. These studies showed that the tyr allele had a small influence on fear learning. We used Affymetrix micro-arrays to identify many differentially expressed genes in the amygdala and hippocampus of the selected lines. We conclude that tyr is one of many alleles that influence fear conditioning.

Keywords Fear conditioning · Albino · Tyrosinase (tyr) · Selection · QTL · Gene expression

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Introduction

The processing of emotionally salient information is a fundamental feature of the mammalian nervous system and is likely to be involved in a variety of psychiatric disorders (Gershenfeld and Paul 1998). Fear learning has been extensively studied for several decades in both mice and humans (LeDoux 2003) and provides a framework for the investigation of emotional regulation. In particular, there is a positive relationship between fear learning as measured in a laboratory setting and anxiety in humans (Lissek et al. 2005), suggesting that fear learning provides a means of probing the activity of brain circuits related to anxiety. In an effort to identify the genetic basis of emotionality in mice, a number of previous studies have examined fear learning (Caldarone et al. 1997; Gershenfeld and Paul 1997; Owen et al. 1997; Ponder et al. 2007a, 2007b; Radcliffe et al. 2000; Talbot et al. 2003).

Short-term, bi-directional selection can be used to create lines of mice with differences in the selection phenotype while limiting inbreeding and fixation of trait-irrelevant alleles (Belknap et al. 1997). Short-term selected lines (STSL) are thus particularly well suited to assess correlated phenotypes and can be used to directly address questions about the nature of the selected phenotypic difference (Phillips et al. 2002). We and others have previously used short-term selection to produce reliable differences in fear conditioning in a cross between C57BL/6J and DBA/2J (Ponder et al. 2007a; Radcliffe et al. 2000). In the present study we created an STSL for contextual fear conditioning based on an F_2 cross between C57BL/6J (B6) and A/J (AJ) mice.

After creating STSL lines we used a number of tests to determine whether additional phenotypes were correlated with selection. The effect of the number of CS–US pairings

and the effect of different shock intensities on fear conditioning was examined to determine whether the difference in fear conditioning was the result of specific experimental parameters. Next, extinction of freezing to context was examined in both lines. In addition, fear potentiated startle was used to gauge fear learning in a freezing-independent manner. We also measured acute sensitivity to foot shock to determine whether selection had altered shock sensitivity. We then evaluated fear conditioning in isogenic strains that had opposite tyr alleles to directly evaluate the role of tyr in fear conditioning. Finally, we used a combination of QTL analysis and gene-expression microarrays to identify other genes that are differentially expressed between the selected lines.

Materials and methods

Environment and housing

All experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by Columbia University's (most studies) or University of Chicago's (studies of B6-albino and A/J-pigmented mice) Institutional Animal Care and Use Committees. Mouse colony rooms were maintained on a 12/12 light/dark cycle with lights on at 07:00 h. Two to five same-sex littermates were housed in clear plastic cages with standard corn-cob type bedding. All mice were maintained with food and water ad libitum, except during testing. Certain procedures were followed in all behavioral experiments: testing was conducted during the light phase between 09:00 and 17:00 h, mice were brought into the testing room in their home cages and allowed to adapt for a minimum of 30 min before testing.

Fear conditioning

Fear conditioning procedures were identical to those described previously in Ponder et al. (2007a). Fear conditioning chambers obtained from Med Associates (St. Albans, VT) had inside dimensions of 29 cm × 19 cm × 25 cm with metal walls on each side, clear plastic front and back walls and ceilings, and stainless steel bars on the floor. A fluorescent light provided dim illumination (~3 lux) and a fan provided a low level of masking background noise. Chambers were cleaned with 80% ethanol (New York) or 10% isopropanol (Chicago) between animals. Behavior was recorded with digital video and analyzed with FreezeFrame software from Actimetrics (Evanston, IL). Mice were between 7 and 14 weeks old on the first day of testing.

Testing for fear conditioning was conducted over 3 days. On test day 1 baseline activity was measured, beginning 30-s after the mice were placed into the test chambers, and ending 150-s later. The mice were then exposed twice to the conditioned stimulus (CS), which consisted of an 85 dB, 3 kHz tone that persisted for 30 s and co-terminated with the unconditioned stimulus (US), which was a 2-s, 0.5 mA foot shock, with a 30 s inter-trail interval (ITI) between pairings.

On day 2, the testing environment was identical to day 1; however neither tones nor shocks were presented. Freezing in response to the test chamber (context) was measured beginning 30-s after the mice were placed into the test chambers and ended 150-s later. Selective breeding was based on a freezing score which was the percentage of time spent freezing on day 2 minus the percentage of time spent freezing on day 1, both during the 150-s periods mentioned above.

On day 3, the context was altered in several ways: a different experimenter wore a different style of gloves, the transfer cages had no bedding, the metal shock grid was covered with a white plastic floor, a bent white plastic wall was inserted into the test chamber, a yellow light filter was placed over the chamber lights, chambers were cleaned with 0.1% acetic acid solution, and the vent fan was partially obstructed to change the background noise. The CS was presented at the same times as on day 1, but no foot shock was administered. The "freezing to tone" score is an average of the percentage of time spent freezing during the two 30-s CS presentations. Data from day 3 were *not* used to determine which animals would be selected as breeders.

Selective breeding

Procedures for mass selection to create STSL are described in Ponder et al. (2007a). The foundation population was an F_1 cross between C57BL/6J (B6) females and A/J (AJ) male mice obtained from The Jackson Laboratory (Bar Harbor, ME). The B6AJF$_1$ mice were intercrossed at Columbia University to obtain an F_2 population. One hundred and forty-four F_2 mice (72 of each sex) were tested for FC. From these mice, the 12 males and 12 females with the highest freezing score were bred to create the high selected lines regardless of family origin (brother-sister breeder pairs were avoided). In addition, three alternate breeder pairs were created from the mice with next highest freezing scores, and their offspring used for the subsequent line only when primary breeder pairs failed to produce offspring. The same procedure was repeated with the lowest freezing animals from the F_2 population in order to create the low selected line. From the high-freezing breeder pairs, 96 offspring from 12 families (as close to four male and four female animals per family as

possible) constituted the first selected generation (denoted S_1). Mice were selected in the same manner, until the S_4 litter had been produced. The procedure to produce the low selected line was identical, except that mice with the lowest freezing scores were bred in each generation. The line names reflect the number of generations removed from the F_2 population, and their freezing behavior (L for low, H for high), so that the offspring of the 4th selected breeders for the high freezing line were called S_4H. Forty-eight mice per line from the S_4 generation were phenotyped for FC. All phenotypes were measured in naïve mice between 7 and 14 weeks old. Equal numbers of male and female mice were tested. The lines were terminated after the S_4 generation.

Effect of number of CS/US pairings on freezing behavior

The effect of number of CS–US pairings on the freezing exhibited on day 2 was tested by using different numbers of tone-shock pairings (0, 1, 2, 4 and 8) on the training day in an approximately equal number of male and female mice from the S_3 generation ($n = 40$ per line; age = 8–14 weeks). All sessions were identical to those described above, except that the session length was shortened or extended to accommodate the different number of CS/US pairings. On day 1 the 0 CS–US group was presented with two 30-s tones but received no shocks. Day 2 lasted 5-min regardless of day 1 treatment group.

Effect of shock magnitude on learning

To test the effect of the shock magnitude (in mA) on the freezing behavior exhibited on day 2, we varied the intensity of the shocks presented on day 1 in male and female mice from the S_4 generation ($n = 42$–45 per line; age = 8–10 weeks). All groups contained approximately equal numbers of male and female mice. The training session was identical to the session used for selection except that the different shock intensities were used (0, 0.1, 0.3, 0.5, 0.7 or 0.9 mA; $n = 8$ per line per intensity).

Extinction of freezing to context

Extinction of freezing to context was tested in male and female mice from the S_4 generation ($n = 12$ per sex/per line; age = 7–12 weeks). The training session was identical to the session used for selection except that a "no-shock" control group was also included. The "no-shock" control group received a training session that was identical to day 1 except that it did not include any shocks. The testing sessions for days 3–9 were identical to day 2. Corrected freezing scores for each day were obtained by

subtracting the baseline freezing on training day from time spent freezing on each subsequent testing day.

Fear potentiated startle

Fear potentiated startle (FPS) was tested in male and female mice from the S_4 generation ($n = 36$ low; $n = 35$ high; age = 7–14 weeks). Two-thirds of the mice in this study were male and only one-third were female, this was a result of availability, this ratio was uniform across treatment groups and lines; none of our analyses detected main effects or interactions involving sex. The protocol and apparatus are similar to those that have been described previously (Ponder et al. 2007a). On day 1 mice from each line (low or high) were randomly presented with one of three training sessions. The paired training session (P) consisted of 20 light + shock pairings (30-s chamber light co-terminating with 0.5-s 0.14 mA shock). The unpaired training session (UP) consisted of 10 light + shock pairings, 10 shock alone trials (0.5-s 0.14-mA shock) and 10 light alone trials (30-s chamber light, no shock). The no shock training session (NS) consisted of 20 light alone trials—no shocks were presented to mice in this treatment group. On day 2 all mice were presented with the same set of stimuli, which consisted of 12 light plus startle trials and 12 trials that consisted of that startle stimuli alone, in the absence of the light cue. The P and NS groups consisted of 12 mice, and the UP group 11 mice. The maximum response for each trial in the 65 ms following the onset of the startle stimulus was used for all calculations.

Shock sensitivity

Sensitivity to the foot shock was assessed using the startle apparatus described above in male and female mice from the S_4 generation ($n = 14$ per line; age = 11–13 weeks). After a 5-min adaptation period, five startle trials were presented, separated by an average of 15 s, with pseudo-random ITI. Each startle trial consisted of a 40-ms burst of 100-dB white noise. Next, a total of 25 shocks were presented in five blocks that consisted of one shock of each of the five intensities (0.1, 0.3, 0.5, 0.7, and 0.9 mA; all shocks were 500 ms in duration) in a pseudorandom order. Each shock trial was separated by a pseudo-random ITI that averaged 90-s. After the last shock, a final block of five startle trials was presented, after which the session ended. The startle response to each shock trial was measured in the same manner as in FPS except that the response window was 500 ms and both the maximum and average responses over that time window were used for analysis.

Fear conditioning in B6, B6-albino,
AJ and AJ-pigmented strains

Mice for these studies were obtained from Jackson Laboratories and bred in house at the University of Chicago. These were the only studies conducted at the University of Chicago. All equipment, analysis and personnel were identical to those used for the selection studies conducted at Columbia University. Fear conditioning was tested in C57BL/6J (B6), C57BL/6J$^{-Tyrc-2J-}$ (B6-Albino), A/J (AJ) and A.B6$^{-Tyr+/J}$ (AJ-pigmented). B6-albino mice are B6 inbred strain with a point mutation inactivating the tyrosinase gene and AJ-pigmented are a congenic A/J strain with the B6 allele for tyrosinase. Testing procedures were identical to those used for selection except that we employed a 0.7 mA shock for some of the studies, in an effort to elicit greater freezing behavior from the low-freezing B6 mice. A total of 20 B6, 20 B6-albino, 22 AJ and 21 AJ-pigmented mice were used at the 0.5 mA shock intensity. For the 0.7 mA shock intensity, 20 B6, 20 B6-albino, 13 AJ and 6 AJ-pigmented mice were used. Males and females were approximately equally represented in all groups.

Gene expression

We compared gene expression between the high and low selected lines using Affymetrix MOE430v2 microarrays. Gene expression was measured in the amygdala or hippocampus of experimentally naïve male mice from the S$_4$ generation. We collected a total of 72 samples, 36 per selected line, with 18 representing the hippocampus and the other 18 representing the amygdala. Collection of tissue has been described previously (Ponder et al. 2007a).

A second microarray experiment measured gene expression differences in the hippocampus of 7-week-old B6 and B6-Albino inbred strains again using Affymetrix MOE430v2microarrays. Total RNA was isolated from the 18 naïve male mice, 9 per inbred strain. We did not examine gene expression in the amygdala in these mice because we had previously observed much greater experimental noise in amygdala samples which we believe is partially due to difficulties in reproducibly micro-dissecting the same piece of amygdala relative to the consistency in hippocampal dissections.

In both experiments, total RNA from three male siblings was pooled and hybridized to a single Affymetrix MOE430v2 array. The concentration of the purified total RNA was quantified using a spectrophotometer and combined such that each individual sample was represented equally in each pool. Total RNA samples were sent to the NINDS/NIMH Microarray Consortium (http://arrayconsortium.tgen.org) where they were processed, labeled and hybridized to the arrays using standard Affymetrix protocols.

Array data were normalized within each experiment using Bioconductor 1.8 and the GCRMA algorithm (Gentleman et al. 2004; Irizarry et al. 2003). The normalized expression levels from the S$_4$ selected mice were then subject to a two-way ANOVA with brain region and line as factors. The normalized expression levels from the B6 and B6-Albino mice were subject to a two-tailed t-test. We then used the program QVALUE (http://faculty.washington.edu/~jstorey/qvalue) to calculate q-values from the p-values from the ANOVAs and t-tests (Storey and Tibshirani 2003) and used a $q < 0.05$ threshold for significance. Present, marginal or absent (P/M/A) calls were calculated in R using the "mas5calls" command to verify all probe sets were expressed. In the selected line comparison, the fold change for each significant transcript was determined for each brain region to find the probe sets with largest differences in expression.

QTL mapping

Breeders from the S$_3$ generation were genotyped by KBiosciences (Hoddesdon, Hertfordshire, UK) with 165 informative markers selected from Petkov et al. (2004). We searched for quantitative trait loci (QTL) by comparing the frequency of each marker allele in the high and low selected populations from each generation to the probability distribution defined by genetic drift. Allele frequencies that are unlikely to be the results of genetic drift are presumed to reflect the effects of selective breeding (Belknap et al. 1997).

Results

Creation of high and low fear conditioning selected lines

Bi-directional selection for freezing score (defined as the difference between freezing to context on day 2 and pre-training freezing observed on day 1) resulted in a highly significant divergence between the high and low lines (Fig. 1a). A three-way ANOVA with line, generation and sex as factors, showed significant effects of line ($F_{[1,651]} = 769.8$; $p < 0.001$), generation ($F_{[3,651]} = 7.09$; $p < 0.001$) and sex ($F_{[1,651]} = 7.82$; $p < 0.01$). Females froze more then males. The only significant interaction was between generation and line ($F_{[3,651]} = 8.94$; $p < 0.001$). The difference between the lines was significant from the S$_1$ generation ($F_{[1,190]} = 120.6$; $p < 0.001$) and behavior progressively diverged in each subsequent generation. To determine the significance of the response to selection, one-way ANOVAs were performed within each line for generation (F$_2$–S$_4$). There was a significant effect of generation
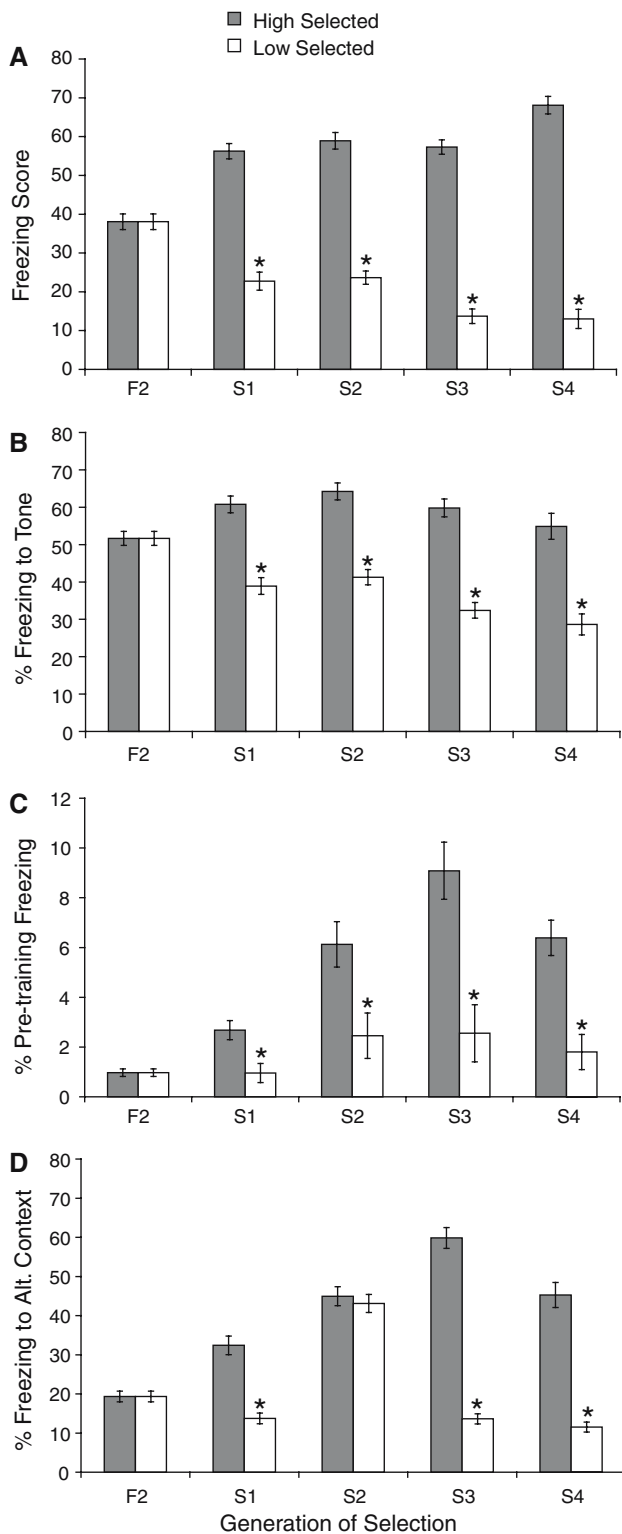
Fig. 1 Response to selection. (**a**) Freezing score (% freezing to context − % pre-training freezing), (**b**) % freezing to tone, (**c**) % pre-training freezing, and (**d**) % freezing to the altered context F$_2$ is a single population common to both selected lines represented by two bars. Each other bar is a single high or low selected generation. Bars are mean ± SE. * $p < 0.05$

in the low line ($F_{[4,471]} = 37.0$; $p < 0.001$) and in the high line ($F_{[4,474]} = 23.9$; $p < 0.001$).

Fear conditioning was tested in the F$_2$ population in three 2-h sessions with 48 animals each, with a 30 min break between each session. A one-way ANOVA of freezing score by session number (1st, 2nd, 3rd) showed no effect of time of day on freezing behavior ($F_{[2,141]} = 1.76$; $p = 0.18$).

Selection for freezing to context also altered freezing in response to tone (Fig. 1b). The freezing to tone was analyzed with a three-way ANOVA for sex, generation (S$_1$–S$_4$) and line. There was a significant effect of line ($F_{[1,648]} = 193$; $p < 0.001$), generation ($F_{[3,648]} = 6.69$; $p < 0.001$) and sex ($F_{[1,648]} = 16.2$; $p < 0.001$). Females froze more then males. Although there were no significant interactions, we chose to perform one-way ANOVAs for generation within each line; these tests showed significant effects of generation in both the low line ($F_{[4,471]} = 17.4$; $p < 0.001$) and the high line ($F_{[4,471]} = 5.4$; $p < 0.001$). Had we applied a correction for freezing in the altered context (prior to the presentation of the tone; Fig. 1d), the two selected lines would not have been nearly as divergent.

Pre-training freezing changed in response to selection (Fig. 1c), which suggests a difference unrelated to learning. A three-way ANOVA for sex, line and generation found no significant effect of sex, so data were then collapsed on sex for further analysis of pre-training freezing. A two-way ANOVA of generation (S$_1$–S$_4$) and line showed a main effect of line ($F_{[1,664]} = 73.4$; $p < 0.001$) and generation ($F_{[3,664]} = 14.7$; $p < 0.001$) and their interaction ($F_{[3,664]} = 5.31$; $p < 0.01$). Planned comparisons showed a difference in pre-training freezing in the S$_1$ ($F_{[1,664]} = 4.0$; $p < 0.05$), the S$_2$ ($F_{[1,664]} = 18.1$; $p < 0.001$), the S$_3$ ($F_{[1,664]} = 57.4$; $p < 0.001$) and the S$_4$ ($F_{[1,664]} = 14.2$; $p < 0.001$). These differences confound the interpretation of the selection phenotype as a measure of learning.

Selection also affected freezing in the altered context (Fig. 1d). A three-way ANOVA with line, generation and sex as factors, showed significant effects of line ($F_{[1,648]} = 230.1$; $p < 0.001$), generation ($F_{[3,648]} = 7.09$; $p < 0.001$) and sex ($F_{[1,648]} = 5.59$; $p < 0.05$; females froze more), with a significant interaction between line and generation ($F_{[3,648]} = 41.4$; $p < 0.001$). A one-way ANOVA with generation showed a significant effect in the low line ($F_{[4,471]} = 59.3$; $p < 0.001$) and in the high line ($F_{[4,471]} = 54.8$; $p < 0.001$).

Effect of number of CS/US pairings on freezing behavior

The difference in freezing behavior between the selected lines was consistent over a range of tone-shock pairings
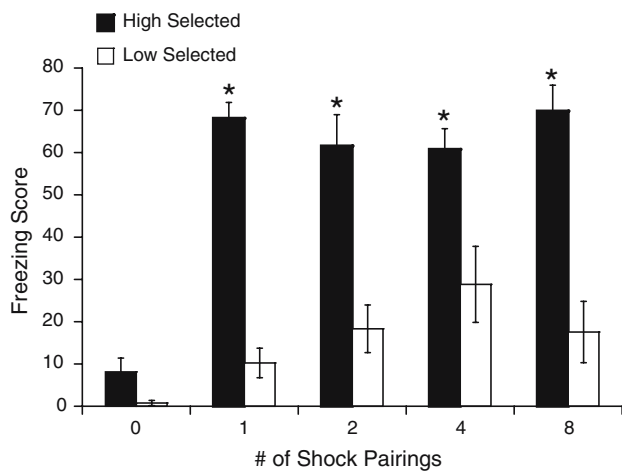
Fig. 2 Freezing score as a function of the number of CS/US pairings. Mice from the high and low lines were exposed to 0, 1, 2, 4, or 8 shocks of 0.5 mA. Bars are mean ± SE. * $p < 0.05$
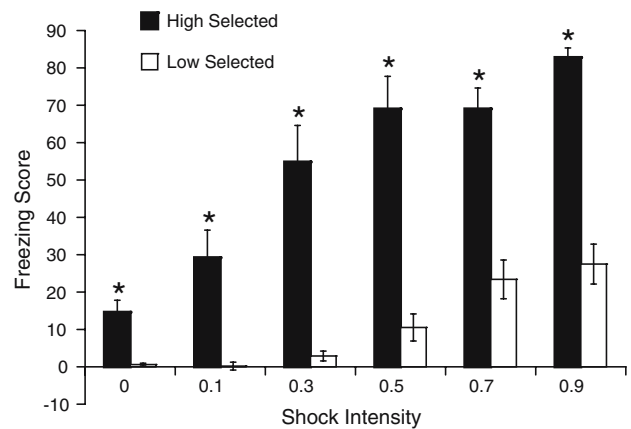

Fig. 3 Freezing score as a function of shock intensity. The control group (marked 0) were not exposed to any shocks. All other groups were exposed to two shocks of the indicated intensity. Bars are mean ± SE. * $p < 0.05$

(Fig. 2). In a three-way ANOVA there was a significant effect of line ($F_{[1,60]} = 125.0$; $p < 0.001$) and number of shocks ($F_{[4,60]} = 19.8$; $p < 0.001$), but not sex. There was a significant interaction between line and shock ($F_{[4,60]} = 6.61$; $p < 0.001$) and sex and shock ($F_{[4,60]} = 2.79$; $p < 0.05$). Separate one-way ANOVAs were then used to test the effect of number of shocks in each line individually. There was a significant effect of number of shocks in both the low line ($F_{[4,35]} = 3.02$; $p < 0.05$) and the high line ($F_{[4,35]} = 23.6$; $p < 0.001$). In the low line one shock ($F_{[1,35]} = 1.28$; $p = 0.27$) was not different from no shocks, but two shocks ($F_{[1,35]} = 4.4$; $p < 0.05$), four shocks ($F_{[1,35]} = 10.9$; $p = 0.01$) and eight shocks ($F_{[1,35]} = 4.0$; $p = 0.05$) were significantly different. In the high line one shock ($F_{[1,35]} = 63.9$; $p < 0.001$), two shocks ($F_{[1,35]} = 50.7$; $p < 0.001$), four shocks ($F_{[1,35]} = 49.2$; $p < 0.001$), and eight shocks ($F_{[1,35]} = 67.6$; $p < 0.001$) were all different from no shocks. Planned comparisons between the lines were not different for no shocks ($F_{[1,60]} = 0.94$; $p = 0.33$), but were different for one shock ($F_{[1,60]} = 53.1$; $p < 0.001$), two shocks ($F_{[1,60]} = 32.4$; $p < 0.001$), four shocks ($F_{[1,60]} = 17.2$; $p < 0.001$) and eight shocks ($F_{[1,60]} = 47.4$; $p < 0.001$).

Effect of shock magnitude on learning

The difference in freezing behavior between the selected lines was observed at all shock intensities tested in the S_4 generation (Fig. 3). In a three-way ANOVA for line, sex and shock, sex had no significant main effect or interactions, so sex was collapsed in further analyses. A two-way ANOVA revealed a significant effect of line ($F_{[1,75]} = 157.9$; $p < 0.001$), shock intensity ($F_{[5,75]} = 19.3$; $p < 0.001$) and their interaction ($F_{[5,75]} = 4.1$; $p < 0.001$).

One-way ANOVAs were then used to test the effect of increasing shock intensity in each line individually. There was a significant effect of shock intensity in the low line ($F_{[5,39]} = 11.1$; $p < 0.001$) and the high line ($F_{[5,36]} = 10.6$; $p < 0.001$). Increasing shock intensity resulted in increased freezing in both lines. In the low line 0.1 mA ($F_{[1,75]} = 0$; $p = 0.96$), 0.3 mA ($F_{[1,75]} = 0.1$; $p = 0.78$), 0.5 mA ($F_{[1,75]} = 1.5$; $p = 0.22$) were not different than no shock, but 0.7 mA ($F_{[1,75]} = 8.5$; $p < 0.01$) and 0.9 mA ($F_{[1,75]} = 11.9$; $p < 0.001$) were. In the high line the 0.1 mA shock was not different from no shock ($F_{[1,75]} = 2.6$; $p = 0.11$), but the 0.3 mA ($F_{[1,75]} = 20.4$; $p < 0.001$), 0.5 mA ($F_{[1,75]} = 37.4$; $p < 0.001$), 0.7 mA ($F_{[1,75]} = 35.5$; $p < 0.001$) and 0.9 mA shock intensities ($F_{[1,75]} = 55.7$; $p < 0.001$) were different from no shock. Line was significantly different at all shock intensities, including the 0.0 mA (no shock) intensity.

Extinction of freezing to context

Despite the difference between the selected lines in freezing behavior, the number of no stimulus presentations required for extinction of freezing was nearly identical (Fig. 4). The high selected lines showed much higher freezing on day 2, but shocked and un-shocked mice were similar after 4 days of exposure to the context. The low selected lines showed much less freezing behavior after training on day 2, but they did show elevated freezing for the 3 days subsequent to training. An initial four-way ANOVA for sex, treatment, line and day showed no significant effects or interactions with sex. A three-way ANOVA of treatment, line and day revealed significant three-way interaction between treatment, line and day ($F_{[7,308]} = 4.4$; $p = 0.001$).
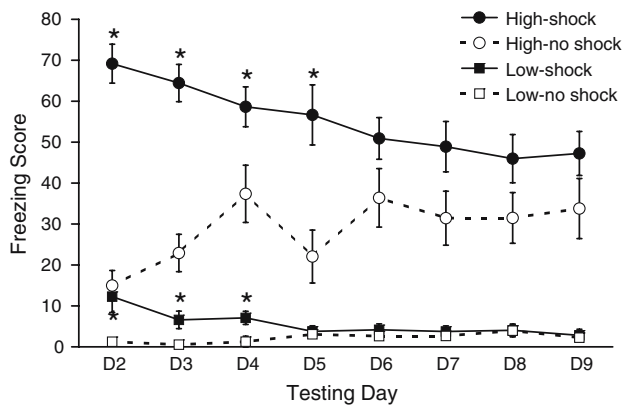
Fig. 4 Extinction of contextual fear conditioning. Solid lines are groups exposed to foot shock. Dashed lines are control groups not exposed to foot shock. High freezing line is in black and the low freezing line in grey. Each point represents the freezing to context on that day minus pre-training freezing on day 1. Points are mean ± SE. * $p < 0.05$

Fig. 5 Fear potentiated startle. Grey bars are high selected mice and white bars are low selected mice. Two control groups, No shock (NS) and unpaired (UP) are included along with fear conditioned potentiated groups (P). Bars are mean ± SE

Planned comparisons to investigate the source of the line*treatment*day interaction first compared the two treatment groups in a single line on day 2. Mice exposed to shock froze more on day 2 in the low line ($F_{[1,22]} = 8.0$; $p < 0.01$) and high line ($F_{[1,22]} = 81.3$; $p < 0.001$), but again the high line froze more than the low line in the no shock treatment group ($F_{[1,22]} = 13.7$; $p < 0.01$). The two treatment groups were first equivalent on day 5 in the low freezing line, and day 6 in the high freezing line indicating only a minor difference in extinction. The rate of extinction in the high freezing line is driven as much by the increasing inactivity of the no-shock control as it is by the daily decrease in freezing of the shocked group. The no-shock control for the high freezing line "freezes" much more than either treatment groups in the low line on day 3 and beyond. This is another indication of differential activity levels between the selected lines.

Fear potentiated startle

Fear potentiated startle (FPS) measures fear learning by assessing the increase in the startle response in the presence of a CS after it has been paired with the US, relative to the startle response in the absence of that CS. Fear potentiated startle was exhibited by the low freezing line in the paired treatment group (P), but was not observed in the P groups from the high line or in the no shock (NS) or the uncorrelated (UC) negative control groups from either line. A two-way ANOVA for line and treatment showed no effect of line, but a significant effect of treatment ($F_{[2,65]} = 4.93$; $p = 0.01$). The low line showed a trend towards greater FPS (Fig. 5), though the effect was non-significant ($F_{[1,22]} = 2.66$; $p = 0.12$). This non-significant trend was opposite to the expected direction; we would have
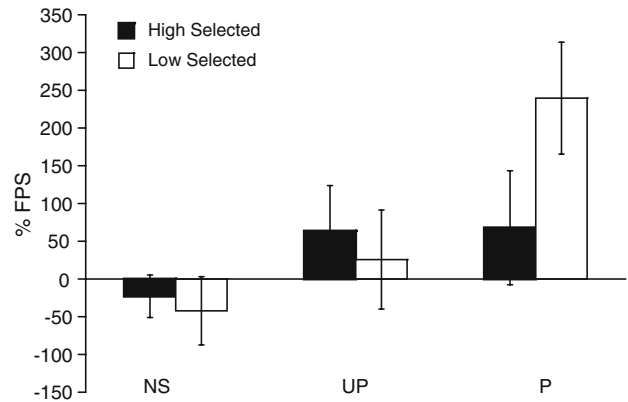
predicted that fear conditioning as measured by freezing to context would have been correlated with fear potentiated startle, as had been observed in our previous study (Ponder et al. 2007a).

Shock sensitivity

Selection for high or low freezing could have changed the frequency of alleles that influence sensitivity to shock, resulting in differential fear conditioning due to differential sensitivity to the US, rather then a difference in learning. We measured the acute startle response at each of five shock intensities in both high and low selected mice. There was a main effect of sex for both the max ($F_{[1,24]} = 9.45$; $p < 0.01$) and average measure ($F_{[1,24]} = 12.4$; $p < 0.01$) but no significant interaction involving sex and line, so sex was removed for all further analyses (males showed somewhat greater shock sensitivity compared to females). There was no main effect of line, or interaction between line and shock intensity for max measures (Fig. 6a), however, for the average measure there was a significant interaction between line and shock intensity ($F_{[4,104]} = 4.59$; $p < 0.01$), which reflected greater shock sensitivity in the low line at all shock intensities (Fig. 6b). While selection was associated with altered sensitivity to foot shock as measured by the average (but not the max) measure, the low line appeared to be *more* sensitive than the high freezing mice, which is opposite to the observed fear learning differences. There was a clear main effect of shock intensity for both the max ($F_{[4,104]} = 117$; $p < 0.001$) and average measures ($F_{[4,104]} = 197$; $p < 0.001$), indicating greater response among both lines to increasing shock intensities. Because the low line appeared more sensitive to the shock, it appears that shock sensitivity is unrelated to the robust response to selection observed in these mice.
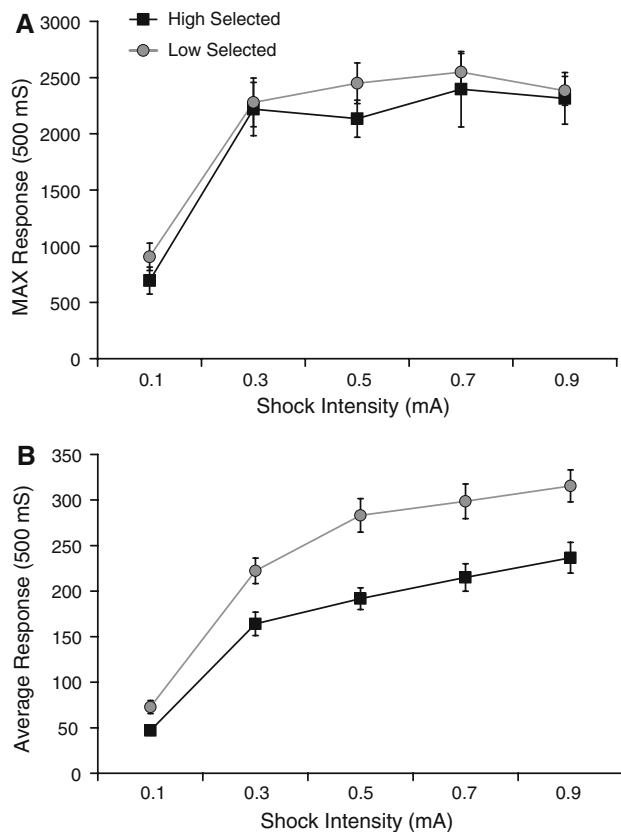
Fig. 6 Shock sensitivity. Sensitivity to shock was determined by measuring the acute motor response to shocks. (a) Max response to shock. (b) Average response to shock. Points are mean ± SE
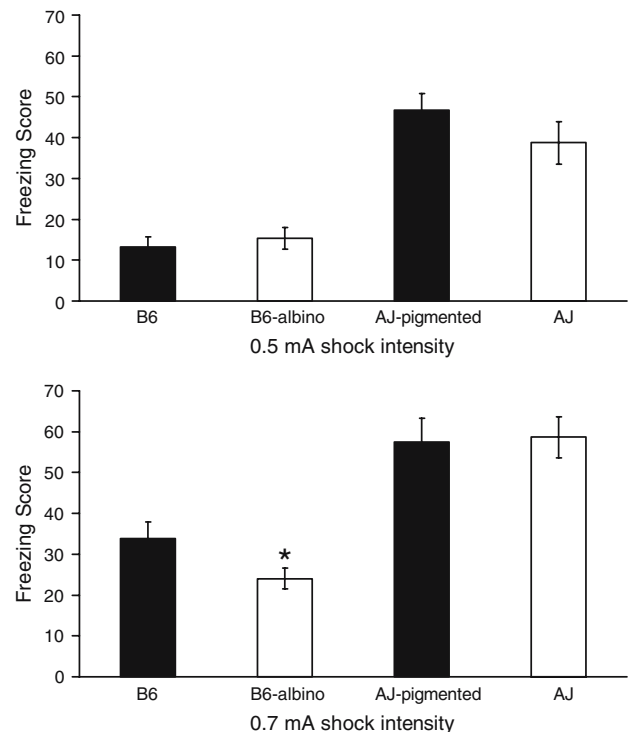

Fig. 7 Freezing score in mice with different and the tyr alleles. Behavior of B6, B6-albino, AJ, and AJ-pigmented mice trained with 0.5 or 0.7 mA shocks. Bars are mean ± SE. * p < 0.05

Fear conditioning in albino and congenic strains

We tested the inbred strains used to create the selected lines, along with an albino B6 strain and an AJ with a functional tyrosinase allele (Fig. 7) with a shock intensity of 0.5 and 0.7 mA. An ANOVA for line with B6 versus B6-albino showed no significant difference in freezing to context at the 0.5 mA shock intensity ($F_{[1,38]} = 0.41$, $p = 0.53$) but a significant decrease in freezing to context in the B6-albino mice relative to the B6 mice at the 0.7 mA shock intensity ($F_{[1,38]} = 4.15$, $p < 0.05$). An ANOVA for line with the AJ versus AJ pigmented strain showed no significant difference for either the 0.5 mA shock intensity ($F_{[1,41]} = 1.52$; $p = 0.22$) or the 0.7 mA shock intensity ($F_{[1,17]} = 0.00$; $p = 0.97$). We used the 0.7 shock intensities because the B6 mice have very low freezing at 0.5, and thus it was difficult to detect the expected decrease in freezing caused by the albino allele when it was expressed on the B6 background.

Gene expression

The results of a two-way ANOVA of microarray data from the S_4 selected lines for the factors brain region and line

identified 364 probe sets that showed a main effect of line and that had q-values <0.05. Of the 364 probe sets identified using the AVOVA, 76 had a fold change of 1.5 or greater in at least one brain region (Table 1). The list contained seven genes represented by two probe sets; coiled-coil domain containing 91 (Ccdc91), galactokinase 2 (Galk2), glial fibrillary acidic protein (Gfap), heat shock protein 1 (Hspb1), myocyte enhancer factor 2C (Mef2c), Musashi homolog 2 (Msi2) and suppressor of Ty 16 homolog (Supt16h) such that the 76 probesets corresponded to 69 unique genes.

The results of the t-test of microarray data from the B6 and B6 albino inbred strains did not identify any genes with a main effect of line and a q-value <0.05. This experiment was fundamentally different from the selected line comparison because all the mice within each inbred strain were genetically identical. RNA from three identical mice was combined for each chip, and each group was measured with three chips. We used q-values to address the multiple testing issues associated with the 45,101 probe sets contained on the MOE430 2.0 array, however, had we found a gene that was non-significantly differentially expressed between these two inbred strains but was significantly differentially expressed between the selected lines, we would have accepted an uncorrected p-value of <0.05. Even with this relaxed threshold, none of the genes

Table 1 Differentially expressed genes in the amygdala and hippocampus of high and low selected lines

Gene title	Gene symbol	Probe set ID	Ch	Mb (UCSC Feb 2006)	q-Value			Fold change			P/A calls			
					Line	Reg	Interact	A	H	R	AH	AL	HH	HL
Transcribed locus	–	1455699_at	6	144950985–144951995	0.035	0.008	0.818	1.4	1.8	1.3	P	P	P	P
RIKEN cDNA 1700020114 gene	1700020I14Rik	1437774_at	2	119293397–119295340	0.038	0.025	0.818	1.5	1.9	1.3	P	P	P	P
RIKEN cDNA 2610207I05 gene	2610207I05Rik	1457262_at	7	117924635–117925002	0.004	0.216	0.818	1.7	2.0	1.0	P	P	P	P
RIKEN cDNA 6330531I01 gene	6330531I01Rik	1453306_at	14	76554825–76556577	0.030	0.011	0.819	1.5	1.8	1.3	P	P	P	P
RIKEN cDNA A630005I04 gene	A630005I04Rik	1438862_at	7	113033601–113034933	0.044	0.062	0.819	1.4	1.7	1.2	P	P	P	P
RIKEN cDNA A930005H10 gene	A930005H10Rik	1441264_x_at	3	115879779–115880101	0.049	0.142	0.825	1.4	1.6	1.1	A	A	A	P
Activin A receptor, type 1	Acvr1	1457551_at	2	58327879–58328513	0.045	0.000	0.818	1.4	2.2	2.1	A	A	A	P
AK033525 (probable pol polyprotein-related protein 4)	AK033525	1438754_at	14	49800521–49800947	0.021	0.000	0.818	2.5	1.8	2.0	P	P	P	P
Arylsulfatase J	Arsj	1457827_at	3	126430644–126432397	0.045	0.000	0.818	1.0	1.9	1.9	A	A	P	P
Expressed sequence BB182297	BB182297	1436585_at	2	51948197–51948776	0.004	0.000	0.818	1.4	1.6	1.3	P	P	P	P
16 days neonate cerebellum cDNA, RIKEN library, clone: 9630018I10	BE955100	1447723_at	7	78058528–78058624	0.033	0.000	0.818	2.3	1.6	2.1	P	P	P	P
Expressed sequence BF642829	BF642829	1440227_at	16	91973182–91974179	0.047	0.000	0.867	1.5	1.5	2.1	P	P	P	P
Coiled-coil domain containing 91	Ccdc91	1418459_at	6	147433040–147589781	0.015	0.085	0.818	1.2	2.0	1.1	P	P	P	P
Coiled-coil domain containing 91	Ccdc91	1449083_at	6	147433040–147589781	0.024	0.028	0.818	1.4	1.6	1.2	P	P	P	P
Cd99 antigen-like 2	Cd99l2	1456746_a_at	X	67681609–67681869	0.045	0.002	0.825	1.6	1.9	1.6	P	P	P	P
Component of oligomeric golgi complex 1	Cog1	1433774_x_at	11	113477227–113478491	0.030	0.021	0.818	1.7	2.1	1.3	P	P	P	P
COMM domain containing 9	Commd9	1438644_x_at	2	101702222–101702484	0.030	0.186	0.841	2.5	2.2	1.1	P	P	P	P
Cathepsin C	Ctsc	1416382_at	7	88153306–88186067	0.005	0.000	0.818	3.3	1.9	3.0	P	P	P	P
CUE domain containing 1	Cuedc1	1451447_at	11	87985758–88009234	0.026	0.004	0.818	1.8	1.4	1.4	P	P	P	P
RIKEN cDNA D230012E17 gene	D230012E17Rik	1431169_at	1	54422654–54424318	0.024	0.034	0.818	1.5	1.1	1.1	P	A	A	P
Dephospho-CoA kinase domain containing	Dcakd	1447105_at	11	102840719–102841110	0.024	0.157	0.818	1.5	2.1	1.1	P	P	P	P
Diacylglycerol kinase, gamma	Dgkg	1419756_at	16	22383733–22572475	0.048	0.000	0.770	2.4	1.1	6.2	P	P	P	P
RIKEN cDNA E430018J23 gene	E430018J23Rik	1425024_at	7	127163676–127184767	0.015	0.039	0.818	1.2	1.8	1.1	A	A	A	P
Ectonucleoside triphosphate diphosphohydrolase 4	Entpd4	1447900_x_at	14	68319819–68320014	0.045	0.009	0.827	1.5	1.4	1.3	P	P	P	P
Fumarylacetoacetate hydrolase	Fah	1417220_at	7	84461357–84482054	0.004	0.000	0.818	1.6	1.4	2.1	P	P	P	P
FGFR1 oncogene partner 2	Fgfr1op2	1427526_at	6	146535124–146544319	0.015	0.026	0.818	1.4	1.7	1.2	P	P	P	P
Furry homolog (Drosophila)	Fry	1458985_at	5	150615381–150615756	0.047	0.126	0.818	1.3	2.1	1.1	P	P	A	P
Galactokinase 2	Galk2	1438642_at	2	125574922–125675156	0.045	0.004	0.818	2.2	1.5	1.6	P	P	P	P
Galactokinase 2	Galk2	1439195_at	13	91973906–91974688	0.024	0.004	0.818	2.2	3.5	1.9	P	P	A	P

Table 1 continued

Gene title	Gene symbol	Probe set ID	Ch	Mb (UCSC Feb 2006)	q-Value			Fold change			P/A calls			
					Line	Reg	Interact	A	H	R	AH	AL	HH	HL
Glycerophosphodiester phosphodiesterase domain containing 1	Gdpd1	1424077_at	11	86849987-86890315	0.019	0.203	0.845	1.6	1.6	1.0	P	P	P	P
glial fibrillary acidic protein	Gfap	1426508_at	11	102703425-102713221	0.015	0.000	0.834	1.5	1.6	1.9	P	P	P	P
Glial fibrillary acidic protein	Gfap	1440142_s_at	11	102706248-102706701	0.010	0.069	0.818	4.3	7.6	1.5	P	P	A	P
Glia maturation factor, beta	Gmfb	1417069_a_at	14	45730027-45744039	0.016	0.001	0.818	1.6	1.2	1.3	P	P	P	P
Glutamate receptor interacting protein 1	Grip1	1445561_at	10	119022083-119022607	0.024	0.193	0.821	4.5	3.0	1.1	P	P	P	P
HD domain containing 3	Hddc3	1428692_at	7	80216644-80220227	0.017	0.067	0.863	2.3	2.4	1.2	P	P	P	P
Hepatoma-derived growth factor, related protein 3	Hdgfrp3	1450924_at	7	81757473-81807986	0.012	0.063	0.818	1.5	1.7	1.1	P	P	P	P
Histidine acid phosphatase domain containing 2A	Hisppd2a	1442466_a_at	2	121038464-121039612	0.045	0.229	0.829	2.3	1.9	1.0	P	P	P	P
Histone 2, H3c2	Hist2h3c2	1421155_at	3	96323526-96323788	0.045	0.026	0.818	1.5	1.1	1.1	P	P	P	P
Heat shock protein 1	Hspb1	1422943_a_at	13	45089642-45089804	0.045	0.000	0.834	1.6	1.4	2.0	P	P	P	P
Heat shock protein 1	Hspb1	1425964_x_at	5	136172734-136173998	0.026	0.000	0.863	1.6	1.5	2.0	P	P	A	P
Kit oncogene	Kit	1415900_a_at	5	75856658-75938391	0.049	0.000	0.818	1.8	1.0	4.5	P	A	P	P
Kelch domain containing 5	Klhdc5	1426988_at	6	147065543-147069946	0.036	0.030	0.818	1.3	1.8	1.2	P	P	P	P
Lectin, galactose binding, soluble 7	Lgals7	1436356_at	14	46024007-46025027	0.027	0.000	0.855	1.6	1.6	3.1	P	P	P	P
mbt domain containing 1	Mbtd1	1441100_at	11	93701917-93729104	0.024	0.006	0.873	1.7	1.7	1.4	P	P	P	P
Malic enzyme 3, NADP(+)-dependent, mitochondrial	Me3	1429071_at	7	89708886-89727155	0.029	0.001	0.843	1.7	1.6	1.6	P	P	P	P
Myocyte enhancer factor 2C	Mef2c	1421028_a_at	13	83981483-84141260	0.049	0.046	0.818	1.2	1.8	1.2	P	P	P	P
Myocyte enhancer factor 2C	Mef2c	1451506_at	13	84051102-84145021	0.047	0.049	0.818	1.3	2.0	1.2	P	P	P	P
MAX gene associated	Mga	1431232_a_at	2	119649754-119656802	0.030	0.199	0.872	1.9	1.9	1.0	P	P	P	P
Musashi homolog 2 (Drosophila)	Msi2	1435521_at	11	88157881-88159583	0.007	0.000	0.846	2.1	2.2	2.5	P	P	P	P
Musashi homolog 2 (Drosophila)	Msi2	1441373_at	11	88336290-88337105	0.004	0.001	0.818	3.6	2.3	1.8	A	P	A	P
Myosin VIIa	Myo7a	1421385_a_at	7	97926518-97984440	0.007	0.000	0.818	1.9	2.6	2.4	P	P	P	A
NAD(P) dependent steroid dehydrogenase-like	Nsdhl	1451799_at	X	69193598-69194109	0.044	0.017	0.827	1.8	2.2	1.5	P	P	A	P
Nuclear protein 1	Nupr1	1419665_a_at	7	126414395-126416623	0.016	0.066	0.818	1.4	1.6	1.1	P	P	P	P
Poly (A) polymerase alpha	Papola	1424217_at	12	106185760-106214931	0.042	0.018	0.818	1.3	1.6	1.2	P	P	P	P
Protocadherin 20	Pcdh20	1451454_at	14	87312753-87315324	0.047	0.000	0.818	1.8	1.3	8.1	P	P	P	P
Purkinje cell protein 4-like 1	Pcp4l1	1452913_at	1	173009937-173032915	0.029	0.000	0.818	1.5	1.9	23	P	P	P	P

Table 1 continued

Gene title	Gene symbol	Probe set ID	Ch	Mb (UCSC Feb 2006)	q-Value Line	q-Value Reg	Interact	Fold change A	Fold change H	Fold change R	P/A calls AH	P/A calls AL	P/A calls HH	P/A calls HL
Protein disulfide isomerase associated 3	Pdia3	1445330_at	2	121129242–121130125	0.045	0.031	0.818	1.3	1.6	1.2	A	P	P	P
Phosphatidylinositol binding clathrin assembly protein	Picalm	1455773_at	7	90011929–90012380	0.015	0.001	0.836	2.7	2.3	1.8	P	P	P	P
Pallidin	Pldn	1457088_at	2	122440178–122440916	0.032	0.124	0.825	1.6	1.9	1.1	P	P	P	A
Prolylcarboxypeptidase (angiotensinase C)	Prcp	1452190_at	7	92750463–92809218	0.024	0.017	0.818	1.7	1.4	1.2	P	A	A	A
RAP2B, member of RAS oncogene family	Rap2b	1448885_at	3	61452501–61455671	0.043	0.009	0.825	1.6	1.9	1.4	P	P	P	P
Ring finger protein 41	Rnf41	1432003_a_at	10	127818126–127842375	0.024	0.001	0.825	1.4	1.5	1.4	P	P	P	P
Sterile alpha motif domain containing 4	Samd4	1429449_at	14	45990853–46024100	0.015	0.000	0.818	2.0	1.2	1.7	P	P	P	P
Spermatogenesis associated 13	Spata13	1438543_at	14	59647410–59647998	0.007	0.000	0.818	2.4	1.3	1.6	P	P	A	P
Spermatogenesis associated glutamate (E)-rich protein 4b	Speer4b	1429993_s_at	5	27826996–27827189	0.044	0.000	0.818	1.8	1.0	1.5	A	P	A	A
Spermatogenesis associated glutamate (E)-rich protein 7, pseudogene 1	Speer7-ps1	1453391_at	5	15192533–15226422	0.045	0.000	0.818	1.4	1.8	4.3	P	P	P	P
Spondin 1, (f-spondin)	Spon1	1424415_s_at	7	1135557320–113833605	0.026	0.000	0.837	1.7	1.5	2.1	P	P	P	P
single-stranded DNA binding protein 2	Ssbp2	1429951_at	13	91935401–92039452	0.026	0.024	0.818	2.4	5.1	1.7	P	P	A	P
Suppressor of Ty 16 homolog (S. cerevisiae)	Supt16h	1419741_at	14	51082397–51119173	0.022	0.092	0.848	1.6	1.5	1.1	P	P	P	P
Suppressor of Ty 16 homolog (S. cerevisiae)	Supt16h	1456449_at	14	51116892–51117567	0.047	0.093	0.818	1.7	1.2	1.1	P	P	P	P
TAF15 RNA polymerase II	Taf15	1438130_at	11	83315201–83315525	0.005	0.035	0.818	1.8	1.6	1.2	P	P	P	P
Transmembrane protein 159	Tmem159	1426436_at	7	119893501–119912110	0.010	0.087	0.818	1.3	1.9	1.1	A	A	A	A
Transmembrane protein 87A	Tmem87a	1424454_at	2	1200046778–1200095557	0.045	0.235	0.840	4.2	5.5	1.0	P	P	P	A
Transformation related protein 53 binding protein 1	Trp53bp1	1457812_at	2	120918850–120919204	0.045	0.204	0.841	2.1	2.4	1.0	A	P	A	P
Translin-associated factor X	Tsnax	1430045_at	8	127899138–127916359	0.047	0.000	0.818	1.6	1.3	1.5	P	P	P	P
Thioredoxin-like 4	Txnl4	1419179_at	18	80368857–80385588	0.024	0.169	0.818	2.3	1.3	1.1	A	P	A	P

All transcripts that had a statistically significant main effect of selected line (high or low) with a q-value <0.05 are listed, along with the chromosome and location (in MB). The q-values were derived from the p-value distribution of a two-way ANOVA for the factors line and region. The fold change for just the amygdala or just the hippocampal samples are shown; white cells are low > high and italicized cells are high > low. The fold change between the brain regions (with both amygdala and hippocampal samples included) is also shown; white cells are amygdala > hippocampus and shaded cells are hippocampus > amygdala. Finally, the presence or absence of the gene in each group (e.g. "A L" = amygdala, low selected) as determined by the "MAS5Calls" command in Bioconductor is shown; "P" denotes a present call in at least four of the six microarrays, "A" denotes an absent or marginal call in four or more of the six microarrays

Table 2 QTLs based on allele frequency changes following selection

Chr	Mb	cM	SNP	p-Value
2	113	64	rs3681694	0.0007
3	151	84	rs3725806	0.0005
5	145	84	rs3713156	0.0022
7	81	46	rs3686613	9.6^{-10}
10	122	69	rs3653850	0.0025
13	82	45	Rs3716022	0.0023
14	69	42	Rs3693589	0.0023

The chromosome, and the location of the marker on the physical and genetic maps are listed, along with the RS number for each SNP and the p-value obtained according to the method of Belknap et al. (1997)

detected in the selected lines showed expression differences in between the B6 and B6-albino mice.

Quantitative trait loci (QTL)

We used the method of Belknap (1997) to calculate p-values for each marker that was typed in the selected lines (Table 2). Marker rs3686613, which is on Chromosome 7 and within 4 MB of the tyr allele was significant at the genome-wide level with a $p < 9.6^{-10}$. Chromosomes 2, 3, 5, 10, 13 and 14 can be considered suggestive at the genome-wide level with $p < 0.0034$.

Discussion

Short-term selection for fear conditioning in a cross between B6 and AJ strains produced a divergence in behavior after the first generation of selection, and behavior continued to diverge over four generations of selection (Fig. 1). While the difference in freezing to context was robust, a number of correlated phenotypes suggest that the observed difference might not be completely accounted for by a difference in learning. A difference in the pre-training activity was observed between the selected lines in the later selected generations. Freezing is used to measure fear learning, so differences in pre-training activity might be expected to confound this measure of learning. This was not the case in our prior selection study in which B6 and D2 mice were used as the foundation for selection (Ponder et al. 2007a). Additionally, fear potentiated startle did not differ between the selected lines; this suggests that the response to selection was specific to freezing as a measure of learning and raises concerns as to whether freezing can be interpreted unambiguously in these lines. Again, this result differed from our prior selection experiment (Ponder et al. 2007a). Microarray and QTL analysis identified many significantly differentially expressed genes and chromosomal regions. While there was strong initial reason

to think the QTL on chromosome 7 was due to the albino allele itself, we identified only modest evidence in support of this idea. Microarray studies showed that none of the gene expression differences were related to the tyrosinase allele. Thus, while there are reasons to be cautious about the nature of the behavioral differences between the high and low selected lines, we have identified QTL regions and genes that may be involved in fear learning.

Evidence of non-learning related changes in response to selection

Several tests indicated a difference in the activity levels between the two selected lines. A difference was observed in pre-training freezing in the first selected generation, and the difference became more pronounced with each subsequent generation. The experiments for number of tone-shock pairings (Fig. 2), shock magnitude (Fig. 3), and extinction (Fig. 4) all included no-shock controls for each line and each indicate that the high freezing line was less active than the low freezing line in the absence of fear conditioning. Thus, part or all of the response to selection may be due to differences in activity rather than differences in learning.

Whereas our prior study of lines selected for differences in freezing behavior (Ponder et al. 2007a) showed a corresponding difference in FPS, no such differences were observed in the present study (Fig. 5). This raises questions as to whether there were true differences in fear learning between the two lines. Unlike rats, mice do not readily show FPS; consistent with this, neither the high nor the low lines showed a difference between the paired and either of the two control groups. It is therefore difficult to interpret these data because, unlike our prior study (Ponder et al. 2007a), the parameters we selected failed to induce significant FPS in either line.

A test of the shock sensitivity in the startle apparatus suggests a difference in shock sensitivity between the selected lines (Fig. 6), but the low freezing line appears more sensitive to the shock than the high freezing line. Since it was the high freezing mice that were less sensitive to the shock, this difference is unlikely to play a role in the response to selection.

Microarray and QTL studies in the selected lines

A number of the genes that are differentially expressed are located on chromosomes where we also identified QTLs. A subset of these genes could be differentially expressed because there are cis-acting expression QTLs (eQTLs) located on these chromosomes causing the behavioral QTLs. We have previously used such evidence to implicate Csnk1e in methamphetamine response (Palmer et al.

2005), and have gone on to show that this same gene is involved in human sensitivity to amphetamine (Veenstra-VanderWeele et al. 2006). Other genes may also have eQTLs and be differentially expressed simply because they are in linkage disequilibrium with the true QTL allele as a result of the few crossovers present after only four generations of recombination, and thus were altered by selection pressure in the same manner as the SNP alleles that we used as markers. Such undesirable linkage is especially prevalent in short term selected lines derived from F_2 crosses between inbred strains. Thus, many of the genes in our list are likely to be unrelated to fear conditioning. As an example of this phenomenon, 15 of the 69 genes in Table 1 are located on chromosome 7.

Three genes were identified as differentially expressed between the high and low selected lines that had also been identified in our previous study of selected lines (Ponder et al. 2007a): suppressor of Ty 16 homologue (Supt16h), endonucleoside triphosphate diphosphohydrolase4 (Entpd4; aka Lysal1) and diacylglycerol kinase, gamma (Dgkg). These genes may provide particularly interesting candidates for future study.

The results of the QTL analysis show that the region of chromosome 7 that contains the tyr^c allele was a factor in mediating the response to selection. Additional QTLs were identified at suggestive levels. We have previously examined fear conditioning in chromosome substitution strains (CSS) lines, in which individual AJ chromosomes have been backcrossed onto a B6 background (Ponder et al. 2007b). The present study shows only limited concordance with those results. Chromosomes 3, 7, 10 and 14 are identified in both studies. The present results also identified chromosomes 2, 5 and 13 at suggestive levels of significance. The CSS study identified genome-wide significant QTLs on chromosomes 6, 11 and 18 that were not observed in the present study. We have previously reported the results of another STSL study in which we used short-term selection for fear conditioning but started with an F_2 cross between B6 and DBA/2J (D2) mice (Ponder et al. 2007b). In that study we identified several of the same chromosomes as reported in the present study. Specifically, chromosomes 5, 10, 13 and 14 are implicated in both studies. The mapping precision of this technique is likely to be rather poor, however it is worth noting that the chromosome 5 and 10 QTLs both appear more proximal in our previous study compared to this one, while the chromosome 13 and 14 QTLs identify similar regions of those chromosomes. There are several possible explanations for the inconsistent results observed in these studies. For the comparison between the present results and our previous study of CSS lines, differences are likely due to a combination of limited power and epistatic interaction that are present in the selected lines, but do not exist in the much simpler CSS lines. Differences between results from CSS lines and F_2 crosses have been reported previously for physiological phenotypes (Stylianou et al. 2006). For the comparison between the present data and our previous selection study, different F_2 populations (B6xAJ versus B6xD2) were used as a foundation for selection, and hence it is likely that alleles unique to either AJ or D2 are the cause of at least some of the observed differences. In all cases, the limited power, and suggestive nature of the QTLs provide additional explanations for the inconsistent results.

The albino allele and the response to selection

We identified some evidence that suggests the albino allele is partially responsible for the response to selection. An observation made during the creation of the selected lines was that all low selected mice were albino after the second selection generation. This indicated that the tyr^c allele, which causes albinism, had become fixed in the low selected line. QTL analysis confirmed that the frequency of a marker 4 MB away from the tyr allele changed over the course of selection in a manner that is extremely unlikely to be accounted for by genetic drift (Table 2). This is interpreted as evidence of a nearby QTL for freezing behavior. In our previous selected line study, no alleles achieved complete fixation after four generations of selection (Ponder et al. 2007a). The result was even more surprising because the tyr^c allele is recessive, and hence would generally be slower to respond to selective pressure as compared to additive or dominant alleles. These data suggested the presence of a strong QTL at or near the tyr^c allele.

In order to examine the effect of the tyr^c allele in isolation, we compared B6 and B6-albino mice. B6-albino mice are the result of a spontaneous loss-of-function mutation in tyr. B6-albino mice showed significantly decreased freezing when using the 0.7 mA shock compared to B6 mice, however there was no difference between the groups when the 0.5 mA shock was used (Fig. 7). Contextual fear conditioning was also tested in a congenic AJ strain containing a functional tyr allele (AJ-pigmented). The AJ-pigmented strain showed a non-significant trend towards more freezing to context than the albino strain at 0.5 mA shock intensity, but no difference when the 0.7 mA shock was used. In a previous study of inbred CSS we identified the CSS-7 strain (which carries both the albino allele as well as the rest of the AJ version of chromosome 7) as having significantly lower freezing behavior as compared to B6 when training used the 0.5 mA shock intensity (Ponder et al. 2007b). Thus, when all of chromosome 7 is derived from AJ, the effect on freezing behavior is observable at the 0.5 mA intensity and is also greater in magnitude than when just the tyr^c allele is

different. This observation suggests the possibility that there are additional, closely linked alleles on chromosome 7 that also underlie the response to selection. Thus, this study has provided some evidence that tyr[c] reduces freezing behavior and may have been partially responsible for the change in marker allele frequency observed over the course of selection (Table 2); however there is also evidence that suggests the existence of additional alleles.

We were initially concerned that the automated video scoring system might incorrectly score freezing in white (albino) mice. To investigate this possibility, we asked several human observers unfamiliar with the issue to score a selection of video files from albino and pigmented mice. We found the observations of human scorers were highly correlated with automated video scoring. The r^2 correlation between automated and human scoring were (0.92; 0.79; 0.87; 0.85 and 0.52; average = 0.79). This demonstrates that the difference in freezing was not due to our use of automated scoring.

AJ mice are albino, due to the tyr[c] allele of the tyrosinase gene located on chromosome 7 (87.3 MB) which inhibits the production of melanin as a result of a single nucleotide mutation. Therefore, mice with the tyr[c] allele lack melanin in the hair, skin and eyes, resulting in white fur and red eyes. Albino mice have fewer rod photoreceptors, defects in visual projections at the optic chasm, an increased occurrence of glaucoma, and spatiotemporal defects in neuronal development of the retina (Beermann et al. 2004; Rachel et al. 2002). A visual task measured in the Morris water maze showed that albino strains in general, and the AJ strain in particular, show long latencies to locate a visual platform (Owen et al. 1997), indicating a general visual deficiency in these strains. The effect of visual deficit on contextual fear conditioning is unknown; however, AJ mice show a very high level of contextual fear conditioning compared with B6 mice (Ponder et al. 2007b), suggesting a robust ability to learn the association between shock and context despite potential visual impairments. On the surface, the high freezing in the AJ mice may seem to contradict our evidence that the tyr[c] allele (present in AJ but not B6) decreases freezing. Multiple alleles are expected to influence fear learning; while the tyr[c] allele may decrease fear learning in the AJ line, it apparently harbors other alleles that result in higher freezing behavior compared to B6. Thus, the two observations are not contradictory.

It has been known for decades that the tyr[c] allele is associated with behavioral differences (Churchill et al. 2004; Crabbe et al. 1999; DeFries 1969; Henry and Schlesinger 1967; Katz and Doyle 1981; Meier and Foshee 1965; Rhoades and Henry 1977; Talbot et al. 2003; van Abeelen and Kroes 1968; Winston and Lindzey 1964). A QTL for behavior on the plus maze on chromosome 7 at the tyr locus has been previously reported (Cohen et al. 2001).

Interestingly, a recent study reported that pigmentation is related to social inhibition in human subjects (Moehler et al. 2006), raising the interesting possibility (though clearly quite speculative) that these findings may model a source of human behavioral variability.

Microarrays identified numerous gene expression differences in the amygdala and hippocampus of these selected lines. A second microarray experiment was used to assess the gene expression differences between B6 and B6-Albino strains to see how many of the differentially expressed genes in the selected lines were a result the tyr allele specifically. The data indicated that no genes were differentially expressed in the brain as a result of the tyr[c] allele on a B6 background. Additionally the data indicated that tyrosinase gene expression is undetectable in the brain regions examined.

Because of the apparent effect of tyr on fear learning, as well as the potential (unrealized in the present study) for video-based automated scoring systems to be confounded by coat color, these results could be taken as a warning against using populations where different tyr alleles are segregating for the study of complex behavior where examination of tyr is not the objective. Had we based our cross on an F_2 between B6 and AJ-pigmented, or between B6-albino and AJ, we would have been better able to assess the effect of other alleles on fear conditioning.

Conclusions

The results of this study provide some support for the hypothesis that the tyr allele alters fear learning. The extent to which the overall response to selection reflects differences in learning is not perfectly clear. Additional findings of this study include the identification of QTLs and the identification of specific genes that are differentially expressed in the brains of selected mice. Taken together these results provide a foundation for the systematic, unbiased identification of genes that mediate differences in fear learning.

References

Beermann F, Orlow SJ, Lamoreux ML (2004) The Tyr (albino) locus of the laboratory mouse. Mamm Genome 15:749–758

Belknap JK, Richards SP, O'Toole LA, Helms ML, Phillips TJ (1997) Short-term selective breeding as a tool for QTL mapping: ethanol preference drinking in mice. Behav Genet 27:55–66

Caldarone B, Saavedra C, Tartaglia K, Wehner JM, Dudek BC, Flaherty L (1997) Quantitative trait loci analysis affecting contextual conditioning in mice [see comments]. Nat Genet 17:335–337

Churchill GA, Airey DC, Allayee H, Angel JM, Attie AD, Beatty J, Beavis WD, Belknap JK, Bennett B, Berrettini W, Bleich A, Bogue M, Broman KW, Buck KJ, Buckler E, Burmeister M, Chesler EJ, Cheverud JM, Clapcote S, Cook MN, Cox RD, Crabbe JC, Crusio WE, Darvasi A, Deschepper CF, Doerge RW, Farber CR, Forejt J, Gaile D, Garlow SJ, Geiger H, Gershenfeld H, Gordon T, Gu J, Gu W, de Haan G, Hayes NL, Heller C, Himmelbauer H, Hitzemann R, Hunter K, Hsu HC, Iraqi FA, Ivandic B, Jacob HJ, Jansen RC, Jepsen KJ, Johnson DK, Johnson TE, Kempermann G, Kendziorski C, Kotb M, Kooy RF, Llamas B, Lammert F, Lassalle JM, Lowenstein PR, Lu L, Lusis A, Manly KF, Marcucio R, Matthews D, Medrano JF, Miller DR, Mittleman G, Mock BA, Mogil JS, Montagutelli X, Morahan G, Morris DG, Mott R, Nadeau JH, Nagase H, Nowakowski RS, O'Hara BF, Osadchuk AV, Page GP, Paigen B, Paigen K, Palmer AA, Pan HJ, Peltonen-Palotie L, Peirce J, Pomp D, Pravenec M, Prows DR, Qi Z, Reeves RH, Roder J, Rosen GD, Schadt EE, Schalkwyk LC, Seltzer Z, Shimomura K, Shou S, Sillanpaa MJ, Siracusa LD, Snoeck HW, Spearow JL, Svenson K, Tarantino LM, Threadgill D, Toth LA, Valdar W, de Villena FP, Warden C, Whatley S, Williams RW, Wiltshire T, Yi N, Zhang D, Zhang M, Zou F, Complex Trait C (2004) The Collaborative Cross, a community resource for the genetic analysis of complex traits. Nat Genet 36:1133–1137

Cohen RM, Kang A, Gulick C (2001) Quantitative trait loci affecting the behavior of A/J and CBA/J intercross mice in the elevated plus maze. Mamm Genome 12:501–507

Crabbe JC, Wahlsten D, Dudek BC (1999) Genetics of mouse behavior: interactions with laboratory environment [see comment]. Science 284:1670–1672

DeFries JC (1969) Pleiotropic effects of albinism on open field behaviour in mice. Nature 221:65–66

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5:R80

Gershenfeld HK, Paul SM (1997) Mapping quantitative trait loci for fear-like behaviors in mice. Genomics 46:1–8

Gershenfeld HK, Paul SM (1998) Towards a genetics of anxious temperament: from mice to men. Acta Psychiatr Scand Suppl 393:56–65

Henry KR, Schlesinger K (1967) Effects of the albino and dilute loci on mouse behavior. J Comp Physiol Psychol 63:320–323

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4:249–264

Katz RJ, Doyle RL (1981) The albino locus and locomotor behavior in the mouse: studies using extended test intervals. Behav Genet 11:167–172

LeDoux J (2003) The emotional brain, fear, and the amygdala. Cell Mol Neurobiol 23:727–738

Lissek S, Powers AS, McClure EB, Phelps EA, Woldehawariat G, Grillon C, Pine DS (2005) Classical fear conditioning in the anxiety disorders: a meta-analysis. Behav Res Ther 43:1391–1424

Meier GW, Foshee DP (1965) Albinism and water escape performance in mice. Science 147:307–308

Moehler E, Kagan J, Brunner R, Wiebel A, Kaufmann C, Resch F (2006) Association of behavioral inhibition with hair pigmentation in a European sample. Biol Psychol 72:344–346

Owen EH, Logue SF, Rasmussen DL, Wehner JM (1997) Assessment of learning by the Morris water task and fear conditioning in inbred mouse strains and F1 hybrids: implications of genetic background for single gene mutations and quantitative trait loci analyses. Neuroscience 80:1087–1099

Palmer AA, Verbitsky M, Suresh R, Kamens HM, Reed CL, Li N, Burkhart-Kasch S, McKinnon CS, Belknap JK, Gilliam TC, Phillips TJ (2005) Gene expression differences in mice divergently selected for methamphetamine sensitivity. Mamm Genome 16:291–305

Petkov PM, Ding Y, Cassell MA, Zhang W, Wagner G, Sargent EE, Asquith S, Crew V, Johnson KA, Robinson P, Scott VE, Wiles MV (2004) An efficient SNP system for mouse genome scanning and elucidating strain relationships. Genome Res 14:1806–1811

Phillips TJ, Shen EH, McKinnon CS, Burkhart-Kasch S, Lessov CN, Palmer AA (2002) Forward, relaxed, and reverse selection for reduced and enhanced sensitivity to ethanol's locomotor stimulant effects in mice. Alcohol Clin Exp Res 26:593–602

Ponder CA, Kliethermes CL, Drew MR, Muller J, Das K, Risbrough VB, Crabbe JC, Gilliam TC, Palmer AA (2007a) Selection for contextual fear conditioning affects anxiety-like behaviors and gene expression. Genes Brains Behav 6:736–749

Ponder CA, Munoz M, Gilliam TC, Palmer AA (2007b) Genetic architecture of fear conditioning in chromosome substitution strains: relationship to measures of innate (unlearned) anxiety-like behavior. Mamm Genome 18:221–228

Rachel RA, Mason CA, Beermann F (2002) Influence of tyrosinase levels on pigment accumulation in the retinal pigment epithelium and on the uncrossed retinal projection. Pigment Cell Res 15:273–281

Radcliffe RA, Lowe MV, Wehner JM (2000) Confirmation of contextual fear conditioning QTLs by short-term selection. Behav Genet 30:183–191

Rhoades RW, Henry KR (1977) Effects of single albino gene substitutions on the performance of mice in a compound avoidance-discrimination task. Physiol Behav 19:87–92

Storey JD, Tibshirani R (2003) Statistical methods for identifying differentially expressed genes in DNA microarrays. Meth Mol Biol 224:149–157

Stylianou IM, Tsaih SW, Dipetrillo K, Ishimori N, Li R, Paigen B, Churchill G (2006) Complex genetic architecture revealed by analysis of high-density lipoprotein cholesterol in chromosome substitution strains and f2 crosses. Genetics. 174:999–1007

Talbot CJ, Radcliffe RA, Fullerton J, Hitzemann R, Wehner JM, Flint J (2003) Fine scale mapping of a genetic locus for conditioned fear. Mamm Genome 14:223–230

van Abeelen JH, Kroes HW (1968) Albinism and mouse behaviour. Genetica 38:419–429

Veenstra-VanderWeele J, Qaadir A, Palmer AA, Cook EH Jr, de Wit H (2006) Association between the casein kinase 1 epsilon gene region and subjective response to D-amphetamine. Neuropsychopharmacology 31:1056–1063

Winston HC, Lindzey G (1964) Albinism and water escape performance in the mouse. Science 144:189–191