

Impaired Pavlovian fear extinction is a common phenotype across genetic lineages of the 129 inbred mouse strain

M. Camp^{*,†}, M. Norcross[†], N. Whittle[‡],
M. Feyder[†], W. D'Hanis[§], D. Yilmazer-Hanke^{§,¶},
N. Singewald[‡] and A. Holmes[†]

[†]Section on Behavioral Science and Genetics, Laboratory for Integrative Neuroscience, National Institute on Alcoholism and Alcohol Abuse, NIH, Bethesda, MD, USA, [‡]Department of Pharmacology and Toxicology, Institute of Pharmacy and Center for Molecular Biosciences Innsbruck, University of Innsbruck, Austria, [§]Institute of Anatomy, Medical Faculty, Otto-von-Guericke University, D-39120, Magdeburg, Germany, and [¶]Department of Anatomy and Biosciences Institute, University College Cork, Cork, Ireland

*Corresponding author: M. Camp, PhD, Section on Behavioral Science and Genetics, Laboratory for Integrative Neuroscience, National Institute on Alcohol Abuse and Alcoholism, 5625 Fisher's Lane Room 2N09, Bethesda, MD, USA 20892-9304.
E-mail: campmc@mail.nih.gov

Fear extinction is impaired in psychiatric disorders such as post-traumatic stress disorder and schizophrenia, which have a major genetic component. However, the genetic factors underlying individual variability in fear extinction remain to be determined. By comparing a panel of inbred mouse strains, we recently identified a strain, 129S1/SvImJ (129S1), that exhibits a profound and selective deficit in Pavlovian fear extinction, and associated abnormalities in functional activation of a key prefrontal-amygdala circuit, as compared with C57BL/6J. The first aim of the present study was to assess fear extinction across multiple 129 substrains representing the strain's four different genetic lineages (parental, steel, teratoma and contaminated). Results showed that 129P1/ReJ, 129P3/J, 129T2/SvEmsJ and 129X1/SvJ exhibited poor fear extinction, relative to C57BL/6J, while 129S1 showed evidence of fear incubation. On the basis of these results, the second aim was to further characterize the nature and specificity of the extinction phenotype in 129S1, as an exemplar of the 129 substrains. Results showed that the extinction deficit in 129S1 was neither the result of a failure to habituate to a sensitized fear response nor an artifact of a fear response to (unconditioned) tone *per se*. A stronger conditioning protocol (i.e. five × higher intensity shocks) produced an increase in fear expression in 129S1, relative to C57BL/6J, due to rapid rise in freezing during tone presentation. Taken together, these data show that impaired fear extinction is a phenotypic feature common

across 129 substrains, and provide preliminary evidence that impaired fear extinction in 129S1 may reflect a pro-fear incubation-like process.

Keywords: Amygdala, anxiety, behavior, gene, genetics, incubation, prefrontal cortex, PTSD, rodent, sensitization

Received 13 March 2009, revised 11 May 2009 and 16 June 2009, accepted for publication 23 June 2009

Fear extinction, the learned inhibition of a conditioned fear response (Pavlov 1927), is impaired in anxiety disorders such as phobias and post-traumatic stress disorder (PTSD), as well as schizophrenia (Holt *et al.* 2008). This process is readily measurable in rodents and has emerged as a promising translational tool for studying the pathophysiology and treatment of anxiety disorders (Cryan & Holmes 2005; Kamprath & Wotjak 2004; Myers & Davis 2007; Quirk & Mueller 2008). However, while there is a considerable genetic contribution to individual variability in risk for anxiety disorders (Kendler 2001), the specific genetic factors underlying impaired fear extinction in these disorders are still poorly understood.

As an initial step toward the long-term goal of identifying genes contributing to variation in fear extinction, we recently surveyed a panel of inbred mouse strains and identified a common inbred mouse strain, 129S1/SvImJ (129S1), that exhibits impaired fear extinction learning coupled with functional abnormalities in a cortico-amygdala circuit mediating extinction (Hefner *et al.* 2008) (for commentary, see Sotres-Bayon *et al.* 2008). These neural and behavioral abnormalities were demonstrable by comparison with another inbred strain, C57BL/6J (B6), which shows good fear extinction. However, although the degree of genetic variation between the 129S1 and B6 strains is lesser than between two unrelated human individuals, variation is still likely to be extensive; hampering the identification of candidate genes underlying strain differences in fear extinction.

An extended family of 129 substrains has been produced by deliberate outcrossing of a single progenitor strain. On the basis of an analysis of breeding history and current genetic composition, Simpson and colleagues categorized three major 129 substrain lineages, each containing multiple substrains: parental (P), teratoma (T) and steel (S) (Festing *et al.* 1999; Simpson *et al.* 1997). A fourth line [contaminated (X)] consists of a single strain, and was produced by genetic contamination by an unknown donor strain (Adams *et al.* 2005; Threadgill *et al.* 1997; Wade & Daly 2005) (Fig. 1).

of age and tested between 10 and 20 weeks of age. All mice were housed (two per cage) side-by-side in a temperature ($72^{\circ}\text{F} \pm 5^{\circ}\text{F}$) and humidity ($45\% \pm 15\%$) controlled vivarium under a 12-h light/dark cycle (lights on 0600 h). The number of mice used in each experiment is given in the figure legends. All experimental procedures were approved by the National Institute on Alcohol Abuse and Alcoholism Animal Care and Use Committee and Austrian Ethical Committees on Animal Care and Use (Bundesministerium für Wissenschaft und Forschung) and followed the National Institute of Health guidelines outlined in 'Using Animals in Intramural Research' and the local Animal Care and Use Committees.

Strain survey of fear conditioning and extinction

The conditioning apparatus was a $27 \times 27 \times 11$ cm chamber with transparent walls and a metal rod floor, cleaned with a 79.5% water/19.5% ethanol/1% vanilla-extract solution to provide a distinctive olfactory cue. For conditioning, after a 180-second acclimation period, mice received three pairings (60–90 seconds variable interpairing interval) between a 30 seconds, 80 dB, white noise [conditioned stimulus (CS)] and a 2 seconds, 0.6 mA scrambled footshock [unconditioned stimulus (US)], in which the shock was presented during the last 2 seconds of the CS. There was a 120-second no-stimulus consolidation period after the final pairing before mice were returned to the home cage (Hefner & Holmes 2007). Twenty-four hours later, expression of the tone memory and within-session extinction of the memory was tested. Mice were placed in a novel context: Plexiglas cylinder with black/white-checked walls, solid-Plexiglas opaque floor, cleaned with a 99% water/1% acetic acid solution, housed in a different room from conditioning. After 180 seconds, mice received 50×30 seconds CS presentations (5 seconds no-stimulus interval). Twenty-four hours later, extinction retrieval was tested in the same context as extinction. After 180 seconds, mice received 3×30 seconds CS presentations (5 seconds no-stimulus interval). Freezing scores during extinction and extinction retrieval were binned into five- and three-trial averages, respectively, for analysis. Stimulus presentation was controlled by the Med Associates VideoFreeze system (Med Associates, Burlington, VT, USA). Freezing, manually scored every 5 seconds as no visible movement except that required for respiration, was measured as an index of fear (Blanchard & Blanchard 1969), and converted to a percentage [(number of freezing observations/total number of observations) $\times 100$].

The effects of strain and trial/trial-block on freezing during conditioning and extinction were analyzed using two-factor analysis of variance (ANOVA), with repeated measures for trial/trial-block, followed by Fisher's least significant difference (LSD) *post hoc* tests. As a measure of within-session extinction learning, paired *t*-tests compared freezing during the first and last trial-blocks of the extinction training session. Strain differences in freezing during extinction retrieval were analyzed using one-factor ANOVA followed by Newman-Keuls *post hoc* tests. As a measure of between-session extinction retrieval, paired *t*-tests compared freezing during extinction retrieval with freezing during the first trial-block of extinction training. The threshold for statistical significance for this and all other analysis was set at $P < 0.05$. All data were analyzed using Statview version 5.0.1 (SAS Institute, Cary, NC, USA).

Hot plate test for nociception

To test for possible differences in pain sensitivity that could contribute to strain differences in freezing, mice were assessed for nociception (more than 1 week after fear extinction) using the hot plate test as described previously (Karlsson *et al.* 2005). Mice were placed on a flat plate (Columbus Instruments, Columbus, OH, USA), heated to 55°C , and the latency to show a hindpaw shake or lick was manually timed (maximum response latency: 30 seconds). Strain differences were analyzed using one-factor ANOVA followed by Fisher's LSD *post hoc* tests.

Freezing to unconditioned tone

We conducted a control experiment to confirm that elevated freezing to tone during extinction training in 129S1 was not due to a freezing

response to the tone *per se* (i.e. no prior pairing with shock). B6 and 129S1 mice underwent a procedure identical to the fear conditioning and extinction procedure as above, with the exception that the CS but not the US was presented during conditioning (for schematic, see Fig. 3a). The effects of strain and trial/trial-block on freezing during 'conditioning' and 'extinction' were analyzed using two-factor ANOVA, with repeated measures for trial/trial-block. Strain differences in freezing during 'extinction retrieval' were analyzed using Student's *t*-test. Freezing during each phase was also compared to a hypothesized baseline of zero.

Fear sensitization and habituation

We next tested for possible fear sensitization under the same conditions that we examined fear extinction. We subjected B6 and 129S1 to a procedure identical to the fear conditioning and extinction procedure as above, with the exception that the US but not the CS was presented during conditioning (for schematic, see Fig. 3b). The effects of strain and trial/trial-block on freezing during 'extinction' were analyzed using two-factor ANOVA, with repeated measures for trial-block. Strain differences in freezing during 'extinction retrieval' were analyzed using Student's *t*-test. Freezing during each phase was also compared to a hypothesized baseline of zero.

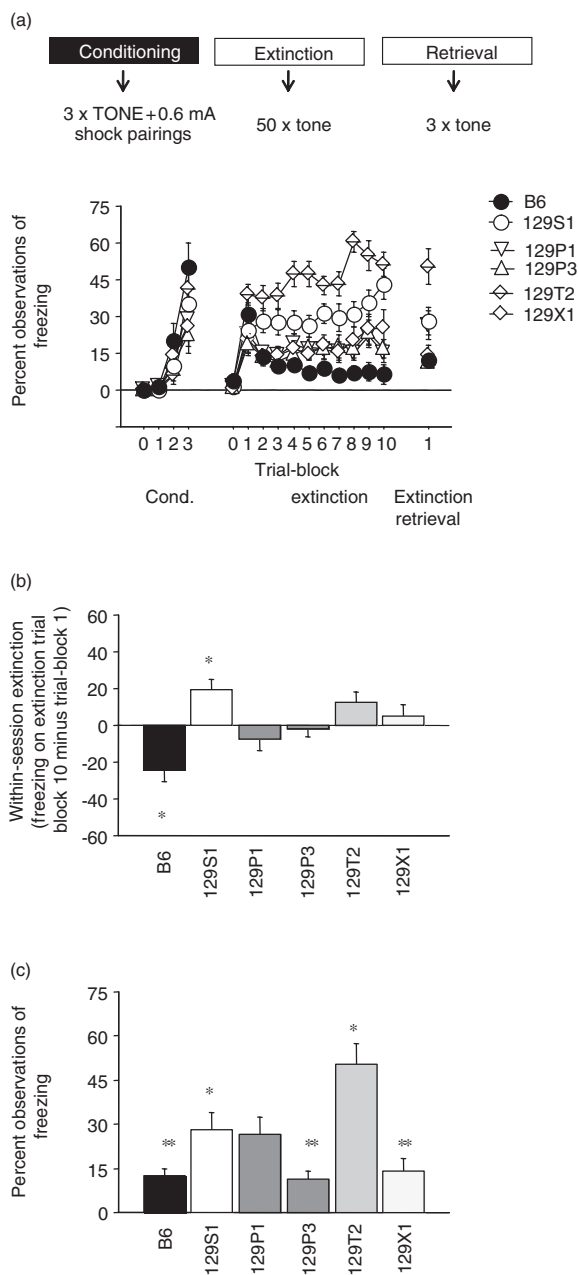
Stronger fear conditioning procedure

Here, we tested whether 129S1 would exhibit greater expression of the tone memory than B6 under a stronger conditioning protocol. To this end, mice underwent conditioning in the same manner as in the strain survey, but with the exception that there were five (as opposed to three) pairings of white noise and footshock, and footshock was increased to 0.7 (as opposed to 0.6) mA. The conditioning apparatus was a $26 \times 30 \times 32$ cm chamber with transparent walls and a metal rod floor cleaned with water. For conditioning, after a 180-second acclimation period, mice received five pairings between a 120 seconds, 80 dB, white noise CS and a 2 seconds, 0.7 mA scrambled footshock US, in which the shock was presented during the last 2 seconds of the CS. A fixed 120-second interpairing interval was used here given prior evidence that a fixed interval strengthens fear conditioning (Hymowitz 1973). There was a 120-second no-stimulus consolidation period after the final pairing before mice were returned to the home cage. Twenty-four hours later, the expression of memory for the tone and within-session fear extinction was tested. Mice were placed in a novel context: $26 \times 20 \times 13$ cm cage, illuminated to 10 lux cleaned with 100% ethanol, and housed in a different room from conditioning. After 180 seconds, mice received 15×120 seconds CS presentations (5 seconds no-stimulus interval). Twenty-four hours later, extinction retrieval was tested in the same context as extinction. After 180 second, mice received 1×120 second CS presentation. Stimulus presentation was controlled by the Habitest operant system (Coulbourn Instruments, Allentown, PA, USA). Freezing was scored and analyzed as above.

Results

Strain survey of fear conditioning and extinction

There was a significant effect of trial ($F_{2,172} = 65.74$, $P < 0.01$) but not strain and no trial \times strain interaction for freezing during conditioning, reflecting an increase in freezing across conditioning trials in all strains (Fig. 2a, left). There was a significant trial-block \times strain interaction for freezing during the extinction session ($F_{45,774} = 2.56$, $P < 0.01$). *Post hoc* tests found no significant differences in freezing between B6 and any 129 substrain during the first trial-block [i.e. during initial expression of the tone memory (Fig. 2a, middle, and Table S1)]. As compared to B6, freezing



was significantly higher in 129T2 on extinction trial-blocks 2–10, higher in 129S1 on trial-blocks 3–10 and higher in 129X1 on trial-block 10. In addition, in comparison to 129S1,

Table 1: Comparison of 129 substrains for nociception in the hot plate test

	B6	129S1	129P1	129P3	129T2	129X1
Latency	9.7 ± 0.8	12.5 ± 2.2	15.2 ± 1.9*	8.5 ± 1.1	14.6 ± 6*	13.0 ± 1.2

Hot plate response latencies were significantly higher in the 129P1 and 129T2 substrains than B6, but were no different from B6 in the other three 129 substrains. $n = 8–10$ /strain. Data are means ± SEM seconds to show a response.

* $P < 0.05$ vs. B6.

Figure 2: Comparison of 129 substrains for fear conditioning, extinction and extinction retrieval. (a) There was an increase in freezing across conditioning trials in all strains but not significant differences between strains. During extinction training, there were no significant differences between strains in freezing during the first trial-block. As compared to B6, freezing was significantly higher in 129T2 on extinction trial-blocks 2–10, higher in 129S1 on trial-blocks 3–10 and higher in 129X1 on extinction trial-block 10. (b) B6, but no other strain, showed within-session extinction, as measured by a significant decrease in freezing from the first to the final extinction trial-block. 129S1 showed a significant increase in freezing during extinction training (* $P < 0.05$ vs. extinction trial-block 1 in same strain). (c) During extinction retrieval [see last data point in panel (a) also], there was significantly higher freezing in the 129S1 and 129T2 substrains than B6 (* $P < 0.05$ vs. B6). B6, 129P3 and 129X1 showed a significant decrease in freezing on extinction retrieval relative to the first trial-block of extinction training (** $P < 0.05$ vs. extinction trial-block 1). For clarity, significant differences between strains are denoted in Table S1. $n = 14–16$ /strain. Data are means ± SEM.

there was significantly higher freezing in 129T2 on trial-blocks 4–9, and a significantly lower freezing in 129P1 on trial-blocks 2, 6 and 10, in 129P3 on trial-blocks 3, 6 and 10 and in 129X1 during trial-blocks 2, 3, 6 and 10. Finally, 129T2 froze significantly more than 129P1, 129P3 and 129X1 during extinction trial-blocks 2–10. Comparing freezing on the first and final extinction trial-blocks as a measure of within-session extinction learning, only B6 showed a significant decrease in freezing and 129S1 showed a significant increase in freezing over the session (Fig. 2b).

There was a significant effect of strain for freezing during extinction retrieval ($F_{5,86} = 8.60$, $P < 0.01$). *Post hoc* tests showed that freezing was significantly higher in 129S1 and 129T2 as compared to B6 (Fig. 2a,c for clarity). Comparison of freezing during extinction retrieval with freezing during the first trial-block of extinction training showed a significant decrease in freezing in B6 ($t = 3.62$, $df = 13$, $P < 0.01$), 129P3 ($t = 2.21$, $df = 15$, $P < 0.05$) and 129X1 ($t = 2.41$, $df = 13$, $P < 0.05$), while 129S1, 129P1 and 129T2 showed no change.

Hot plate test for nociception

There was a significant effect of strain for latency to respond ($F_{5,49} = 3.40$, $P < 0.01$). *Post hoc* tests showed that response latencies were significantly higher in 129P1 and 129T2 than B6 (Table 1), indicating that lower pain

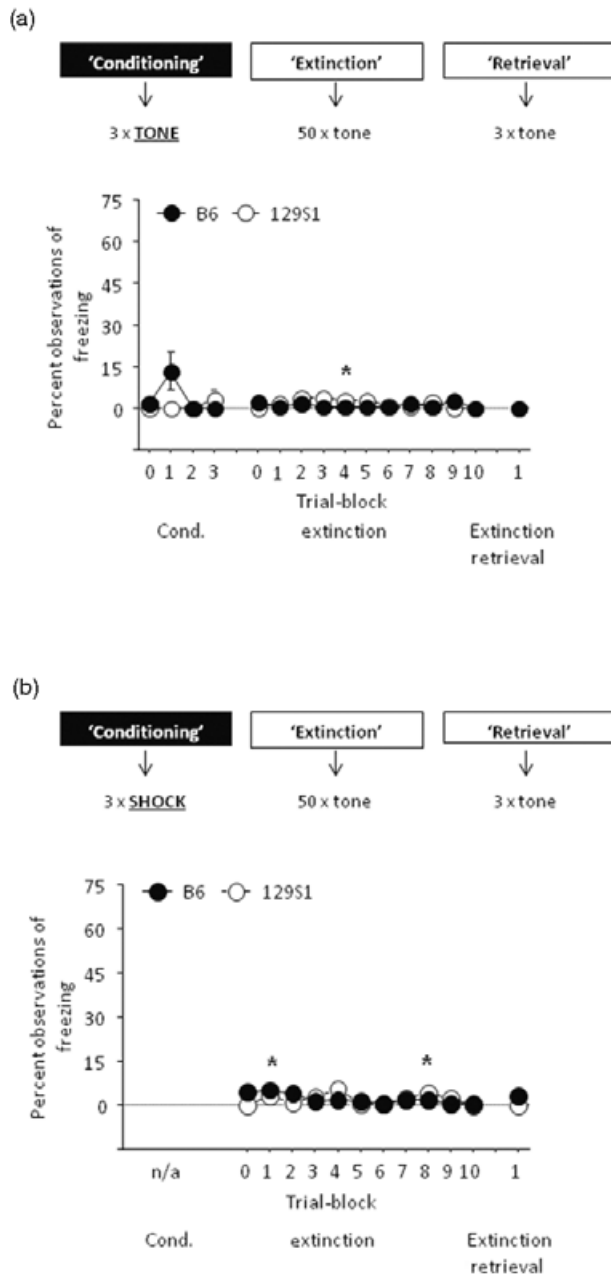


Figure 3: Comparison of 129S1 and B6 for freezing to unconditioned tone and for possible fear sensitization. (a) Freezing was negligible in 129S1 and B6 mice when tone but not shock was presented during ‘conditioning’ ($n = 6$ /strain) ($*P < 0.05$ vs. zero baseline in 129S1). (b) Freezing was negligible in 129S1 and B6 mice when shock but not tone was presented during ‘conditioning’ ($n = 6-8$ /strain) ($*P < 0.05$ vs. zero baseline in B6). Data are means \pm SEM.

sensitivity could have confounded fear conditioning in these two strains.

Freezing to unconditioned tone

Freezing to tone in mice previously exposed to the tone in the absence of the US was negligible and not different between strains during any phase of testing (Fig. 3a). One exception was significantly more freezing than a hypothesized baseline of zero on trial-block 4 in 129S1 ($t = 2.71$, $df = 5$, $P < 0.05$).

Fear sensitization and habituation

Freezing to the tone was negligible and not significantly different between 129S1 and B6 (Fig. 3b). Two exceptions were significantly more freezing than a hypothesized baseline of zero on trial-blocks 1 ($t = 3.97$, $df = 7$, $P < 0.05$) and 8 ($t = 2.38$, $df = 7$, $P < 0.05$) in B6.

Stronger fear conditioning procedure

There was a significant trial \times strain interaction for freezing during conditioning ($F_{4,44} = 4.85$, $P < 0.01$). *Post hoc* tests showed that freezing was significantly higher in 129S1 than B6 during the third, fourth and fifth trials (Fig. 4a). There was also a significant effect of trial/block \times strain interaction for freezing during extinction training ($F_{14,182} = 8.36$, $P < 0.01$). *Post hoc* tests found that freezing was higher in 129S1 than B6 on all trials except the second (all $P < 0.05$) (Fig. 4a). During extinction retrieval, freezing was higher in 129S1 than B6 ($t = 6.15$, $df = 11$, $P < 0.01$) (Fig. 4a).

These data show that 129S1 exhibits increased freezing following a relatively strong conditioning protocol. However, the CS duration was longer in this experiment (i.e. 120 seconds as opposed to 30 seconds) than in our experiments in which freezing was equivalent between B6 and 129S1. Therefore, we examined freezing during the first 30 seconds of the first 120-second extinction CS. Analyses of freezing in discrete 30-second timebins during this trial showed a significant strain \times bin interaction for freezing ($F_{3,33} = 5.68$, $P < 0.01$). *Post hoc* tests showed that freezing was significantly higher in 129S1 than B6 during the second, third and fourth, but importantly, not the first, timebins (all $P < 0.05$) (Fig. 4b). In addition, paired *t*-test comparisons of freezing during the first and last timebins indicated a significant decrease in B6 ($t = 3.20$, $df = 4$, $P < 0.05$), but a significant increase in 129S1 ($t = 4.63$, $df = 7$, $P < 0.01$). We also analyzed the extinction retrieval trial-block as three individual trials. This showed a significant effect of strain ($F_{1,11} = 41.90$, $P < 0.01$) but no trial and no strain \times trial interaction; showing higher freezing in 129S1 than B6 across all three retrieval trials, and no change across trials in either strain.

The finding that B6 showed a rapid decrease, and 129S1 a rapid increase, in freezing during a single but relatively lengthy CS, led us to reanalyze the data from our strain survey experiment by breaking down the first extinction trial-block and the extinction retrieval trial-block into individual trials. This analysis showed a significant effect of trial ($F_{4,344} = 3.40$, $P < 0.01$) but not strain and no trial \times strain interaction for freezing during extinction (Table S2).

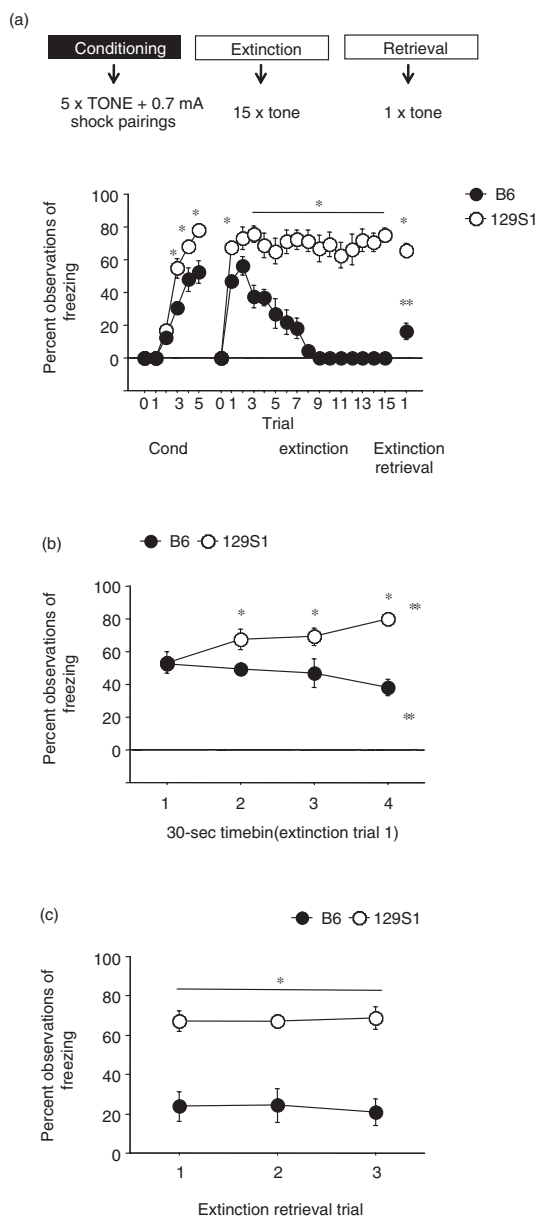


Figure 4: Comparison of 129S1 and B6 for fear conditioning, extinction and extinction retrieval using a stronger conditioning protocol. (a) Freezing was significantly higher in 129S1 than B6 on the third, fourth and fifth conditioning trials, on all trials except the second during extinction training and on all trials during extinction retrieval ($*P < 0.05$ vs. B6/same trial(s), $**P < 0.05$ vs. extinction trial-block 1 in B6). (b) Temporal analysis of the first extinction trial showed that freezing was not different between 129S1 and B6 during the first 30-second timebin, but significantly differed between strains on subsequent timebins ($*P < 0.05$ vs. B6/same timebin). Freezing significantly increased over timebins in 129S1 and significantly decreased in B6 ($**P < 0.05$ vs. timebin 1). (c) Temporal analysis of the extinction retrieval trial showed that freezing was significantly higher in 129S1 than B6 regardless of time bin. $n = 5-8$ /strain. Data are means \pm SEM.

This indicates that, statistically, freezing changed across the first five extinction trial-blocks but not in a manner significantly affected by strain. Analysis of the individual trials of extinction retrieval showed a significant effect of strain ($F_{5,86} = 8.60, P < 0.01$) but not trial and no strain \times trial interaction for freezing during extinction (Table S2). This indicates that the strain differences described earlier for the data averaged across the three trials did not statistically differ as a function of individual trial.

Discussion

We recently identified a common inbred mouse strain, 129S1, that exhibits impaired fear extinction and functional abnormalities in a cortico-amygdala circuit mediating extinction (Hefner *et al.* 2008; Sotres-Bayon *et al.* 2008). The major novel finding of the current study was that impaired fear extinction extends to four other 129 substrains. Similar to 129S1, these substrains showed normal fear (as measured by freezing) as compared to B6, but failed to show significant within-session extinction learning. Consistent with poor extinction learning, 129S1, 129P1 and 129T2 showed no evidence of extinction retrieval. Although 129P3 and 129X1 did display a reduction in freezing during the retrieval test relative to pre-extinction, this between-session reduction is most parsimoniously explained by decay of the original fear response unrelated to extinction because these two substrains showed no extinction learning.

Our data suggest that impaired fear extinction is a phenotypic abnormality common to the four separate genetic lineages of the 129 strains. This conclusion must be qualified by the fact that we only examined one representative substrain from the steel and teratoma lines and two substrains from the parental line. The other dozen or so substrains have yet to be tested for this behavior and we cannot exclude the possibility that some will exhibit intact extinction. Notwithstanding, the current data do not identify a 129 substrain that shows good fear extinction. Such a strain would be very useful to compare with the poor extinguishing 129S1 substrain as a means to expedite the discovery of the genes underlying the differences in extinction efficacy (e.g., see Ponder *et al.* 2007).

The finding that impaired fear extinction appears to be a common phenotypic feature of the 129 strain could have implications for the use of this strain as a source of embryonic stem (ES) cells in the generation of targeted gene mutant mice. The popularity of these strains for this purpose largely stems from the relative success with which 129 ES cells can be derived and then be incorporated into the germline of host blastocysts ('germline competency') (Evans & Kaufman 1981; Thomas & Capecchi 1987). Owing to various abnormal phenotypic features at the molecular (Koike *et al.* 2006), neuroanatomical (Wahlsten 1982) and behavioral level (Wolfer *et al.* 1997), 129 strains have not been favored as a genetic background for mutant mice (Crawley *et al.* 1997). Nonetheless, even with repeated backcrossing onto a more suitable strain, such as B6, a mutant line generated

with 129 ES cells will still harbor 129 genes in the region flanking the target mutation (Crusio *et al.* 2008; Wolfer *et al.* 2002). Thus, this raises the possibility that impairments in fear extinction attributed to a targeted gene mutation could in fact be a false positive caused by extant 129 flanking genes. It would seem prudent to at least bear this caveat in mind in future studies of fear extinction in mutant mice.

The current study replicates our recent observation (Hefner *et al.* 2008) of impaired fear extinction in the 129S1 substrain, relative to B6, and extends the finding in a number of ways. To ensure that elevated freezing in 129S1 during extinction training was not due to a fear response to the tone *per se*, we tested mice under conditions in which they received tone presentations, but not shocks, during conditioning, and found that freezing was negligible in both strains under these conditions. We also tested the possibility that impaired extinction in 129S1 was due to abnormalities in non-associative processes known as fear sensitization and habituation. Fear sensitization produces an increased fear response to a stimulus (e.g. tone) after exposure to an aversive event (e.g. footshocks), even though the tone has not been previously experienced (Kamprath & Wotjak 2004; Kamprath *et al.* 2006; McSweeney & Swindell 2002). A failure to habituate to this sensitized response with repeated tone presentations would manifest in the same way as impaired fear extinction. Indeed, impaired habituation has been found to contribute to apparent deficits in fear extinction in, e.g. cannabinoid CB1 receptor knockout mice (Kamprath *et al.* 2006). Current results indicate that the deficient extinction in these mice does not appear to reflect impaired habituation of a sensitized fear response, as exposure to the same number and intensity of footshocks (without pairing to tone) was not sufficient to produce sensitized fear in either 129S1 or B6. Taken together, these findings provide further support for the specificity of the fear extinction deficit in the 129S1 strain.

The extinction deficit in 129S1 was observed in the current study using a testing paradigm modified from that used in the Hefner *et al.* (2008) study which produces more modest levels of fear (e.g. see Norcross *et al.* 2008). This reduces the potential for 'ceiling' levels of fear to confound the assessment of extinction. It was interesting to note that against this modest level of fear, 129S1 showed not only a failure to reduce fear but also showed a modest increase in fear during extinction training. This profile is reminiscent of fear incubation; defined by Eysenck as 'an increment in conditioned responding over a period of time when the CS is applied once or a number of times, but without reinforcement' (Eysenck 1968). The mechanisms underlying fear incubation have not yet been as thoroughly described as for those mediating fear extinction (Quirk & Mueller 2008). Previous studies have shown that various experimental manipulations, such as spaced extinction training, administration of the β -adrenergic receptor agonist propranolol or extensive tone-shock conditioning, can produce fear incubation in rats and C57BL/6Tac mice (Cain *et al.* 2003; Pickens *et al.* 2009). It will be of great interest to apply these manipulations in future

studies to more directly test for a potential fear incubation phenotype in 129S1. Some initial insights into this issue, however, were made in the current study.

We have previously shown that 129S1 and B6 do not differ in fear expression using relatively mild conditioning protocols involving one or three pairings between tone and 0.6 mA footshock (Hefner *et al.* 2008). This is consistent with most previous studies that have also found either minimal differences or lesser fear in 129 substrains (including 129P1, 129P3, 129S1, 129S2/SvHsd, 129/SvevTacBr, 129S6/SvEvTac, 129T2, 129X1) as compared with B6 (Balogh & Wehner 2003; Bolivar *et al.* 2001; Bothe *et al.* 2004; Cook *et al.* 2002; Holmes *et al.* 2002; Nguyen *et al.* 2000; Owen *et al.* 1997; Schimanski & Nguyen 2005). These data do not, however, rule out the possibility that 129 substrains could exhibit increased fear under stronger fear conditioning protocols. Here, we found that 129S1 can exhibit increased freezing during fear conditioning and expression, relative to B6, under a stronger (i.e. more pairings, higher shock intensity) conditioning protocol. Importantly, however, close inspection of the increased freezing during the first extinction trial indicated that immediate fear during the first 30 seconds of the CS was normal (i.e. B6 like) in 129S1, but that fear in these mice rapidly increased above B6 levels over the next 90 seconds of the CS. Thus, elevated 129S1 freezing during the first CS presentation cannot simply be explained by an increase in fear, but instead reflects a more complex response involving a rapid rise in fear during exposure to a long continuous CS. This is again suggestive of an incubation of fear, similar to the effects seen across multiple short CS exposures discussed earlier. Alternative interpretations (e.g. timing of shock onset at tone termination) cannot be ruled out at this point, and further studies will be needed to more fully explore fear incubation and possible mechanisms underlying it (e.g. abnormal prefrontal-amygdala circuitry, see Vidal-Gonzalez *et al.* 2006).

In summary, we extend our recent finding that the 129S1 inbred mouse strain exhibits impaired Pavlovian fear extinction by showing that deficiency in this behavior is common across different genetic lineages of the 129 strain. Current results also show that the extinction deficit in 129S1 was not likely due to non-associative fear sensitization or a fear response to the auditory tone *per se*. Our data do, however, raise the intriguing possibility that impaired extinction in this strain may reflect (or unmask) a pro-fear incubation-like process. Further studies using this mouse model of impaired extinction could provide insight into the pathophysiology and improved treatment of extinction impairment in neuropsychiatric disorders such as PTSD (Holmes & Wellman 2009).

References

- Adams, D.J., Dermitzakis, E.T., Cox, T., Smith, J., Davies, R., Banerjee, R., Bonfield, J., Mullikin, J.C., Chung, Y.J., Rogers, J. & Bradley, A. (2005) Complex haplotypes, copy number polymorphisms and coding variation in two recently divergent mouse strains. *Nat Genet* **37**, 532–536.

- Balogh, S.A. & Wehner, J.M. (2003) Inbred mouse strain differences in the establishment of long-term fear memory. *Behav Brain Res* **140**, 97–106.
- Blanchard, R.J. & Blanchard, D.C. (1969) Crouching as an index of fear. *J Comp Physiol Psychol* **67**, 370–375.
- Bolivar, V.J., Pooler, O. & Flaherty, L. (2001) Inbred strain variation in contextual and cued fear conditioning behavior. *Mamm Genome* **12**, 651–656.
- Bothe, G.W., Bolivar, V.J., Vedder, M.J. & Geistfeld, J.G. (2004) Genetic and behavioral differences among five inbred mouse strains commonly used in the production of transgenic and knockout mice. *Genes Brain Behav* **3**, 149–157.
- Bothe, G.W., Bolivar, V.J., Vedder, M.J. & Geistfeld, J.G. (2005) Behavioral differences among fourteen inbred mouse strains commonly used as disease models. *Comp Med* **55**, 326–334.
- Cain, C.K., Blouin, A.M. & Barad, M. (2003) Temporally massed CS presentations generate more fear extinction than spaced presentations. *J Exp Psychol Anim Behav Process* **29**, 323–333.
- Cook, M.N., Bolivar, V.J., McFadyen, M.P. & Flaherty, L. (2002) Behavioral differences among 129 substrains: implications for knockout and transgenic mice. *Behav Neurosci* **116**, 600–611.
- Crawley, J.N., Belknap, J.K., Collins, A., Crabbe, J.C., Frankel, W., Henderson, N., Hitzemann, R.J., Maxson, S.C., Miner, L.L., Silva, A.J., Wehner, J.M., Wynshaw-Boris, A. & Paylor, R. (1997) Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies. *Psychopharmacology (Berl)* **132**, 107–124.
- Crusio, W.E., Goldowitz, D., Holmes, A. & Wolfer, D. (2008) Standards for the publication of mouse mutant studies. *Genes Brain Behav* **8**, 1–4.
- Cryan, J.F. & Holmes, A. (2005) The ascent of mouse: advances in modelling human depression and anxiety. *Nat Rev Drug Discov* **4**, 775–790.
- Evans, M.J. & Kaufman, M.H. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156.
- Eysenck, H.J. (1968) A theory of the incubation of anxiety-fear responses. *Behav Res Ther* **6**, 309–321.
- Festing, M.F., Simpson, E.M., Davisson, M.T. & Mobraaten, L.E. (1999) Revised nomenclature for strain 129 mice. *Mamm Genome* **10**, 836.
- Hefner, K. & Holmes, A. (2007) Ontogeny of fear-, anxiety- and depression-related behavior across adolescence in C57BL/6J mice. *Behav Brain Res* **176**, 210–215.
- Hefner, K., Whittle, N., Juhasz, J., Norcross, M., Karlsson, R.M., Saksida, L.M., Bussey, T.J., Singewald, N. & Holmes, A. (2008) Impaired fear extinction learning and cortico-amygdala circuit abnormalities in a common genetic mouse strain. *J Neurosci* **28**, 8074–8085.
- Herry, C. & Mons, N. (2004) Resistance to extinction is associated with impaired immediate early gene induction in medial prefrontal cortex and amygdala. *Eur J Neurosci* **20**, 781–790.
- Holmes, A. & Wellman, C.L. (2009) Stress-induced prefrontal reorganization and executive dysfunction in rodents. *Neurosci Biobehav Rev* **33**, 773–783.
- Holmes, A., Wrenn, C.C., Harris, A.P., Thayer, K.E. & Crawley, J.N. (2002) Behavioral profiles of inbred strains on novel olfactory, spatial and emotional tests for reference memory in mice. *Genes Brain Behav* **1**, 55–69.
- Holt, D.J., Lebron-Milad, K., Milad, M.R., Rauch, S.L., Pitman, R.K., Orr, S.P., Cassidy, B.S., Walsh, J.P. & Goff, D.C. (2008) Extinction memory is impaired in schizophrenia. *Biol Psychiatry* **65**, 455–463.
- Hymowitz, N. (1973) Comparisons between variable-interval and fixed-interval schedules of electric shock delivery. *J Exp Anal Behav* **19**, 101–111.
- Kamprath, K., Marsicano, G., Tang, J., Monory, K., Bisogno, T., Di Marzo, V., Lutz, B. & Wotjak, C.T. (2006) Cannabinoid CB1 receptor mediates fear extinction via habituation-like processes. *J Neurosci* **26**, 6677–6686.
- Kamprath, K. & Wotjak, C.T. (2004) Nonassociative learning processes determine expression and extinction of conditioned fear in mice. *Learn Mem* **11**, 770–786.
- Karlsson, R.M., Holmes, A., Heilig, M. & Crawley, J.N. (2005) Anxiolytic-like actions of centrally-administered neuropeptide Y, but not galanin, in C57BL/6J mice. *Pharmacol Biochem Behav* **80**, 427–436.
- Kendler, K.S. (2001) Twin studies of psychiatric illness: an update. *Arch Gen Psychiatry* **58**, 1005–1014.
- Koike, H., Arguello, P.A., Kvajo, M., Karayiorgou, M. & Gogos, J.A. (2006) Disc1 is mutated in the 129S6/SvEv strain and modulates working memory in mice. *Proc Natl Acad Sci U S A* **103**, 3693–3697.
- McSweeney, F.K. & Swindell, S. (2002) Common processes may contribute to extinction and habituation. *J Gen Psychol* **129**, 364–400.
- Myers, K.M. & Davis, M. (2007) Mechanisms of fear extinction. *Mol Psychiatry* **12**, 120–150.
- Nguyen, P.V., Abel, T., Kandel, E.R. & Bourtchouladze, R. (2000) Strain-dependent differences in LTP and hippocampus-dependent memory in inbred mice. *Learn Mem* **7**, 170–179.
- Norcross, M., Mathur, P., Enoch, A.J., Karlsson, R.M., Brigman, J.L., Cameron, H.A., Harvey-White, J. & Holmes, A. (2008) Effects of adolescent fluoxetine treatment on fear-, anxiety- or stress-related behaviors in C57BL/6J or BALB/cJ mice. *Psychopharmacology (Berl)* **200**, 413–424.
- Owen, E.H., Logue, S.F., Rasmussen, D.L. & Wehner, J.M. (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.
- Pavlov, I.P. (1927) *Conditioned Reflexes*. Oxford University Press, London.
- Pickens, C.L., Golden, S.A., Adams-Deutsch, T., Nair, S.G. & Shamm, Y. (2009) Long-lasting incubation of conditioned fear in rats. *Biol Psychiatry* **65**, 881–886.
- Ponder, C.A., Kliethermes, C.L., Drew, M.R., Muller, J., Das K., Risbrough V.B., Crabbe J.C., Gilliam T.C., Palmer A.A. (2007) Selection for contextual fear conditioning affects anxiety-like behaviors and gene expression. *Genes Brain Behav* **6**, 736–749.
- Ponnusamy, R., Nissim, H.A. & Barad, M. (2005) Systemic blockade of D2-like dopamine receptors facilitates extinction of conditioned fear in mice. *Learn Mem* **12**, 399–406.
- Quirk, G.J. & Mueller, D. (2008) Neural mechanisms of extinction learning and retrieval. *Neuropsychopharmacology* **33**, 56–72.
- Schimanski, L.A. & Nguyen, P.V. (2005) Mouse models of impaired fear memory exhibit deficits in amygdala LTP. *Hippocampus* **15**, 502–517.
- Siegmund, A., Langnaese, K. & Wotjak, C.T. (2005) Differences in extinction of conditioned fear in C57BL/6 substrains are unrelated to expression of alpha-synuclein. *Behav Brain Res* **157**, 291–298.
- Simpson, E.M., Linder, C.C., Sargent, E.E., Davisson, M.T., Mobraaten, L.E. & Sharp, J.J. (1997) Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. *Nat Genet* **16**, 19–27.
- Sotres-Bayon, F., Corcoran, K.A., Peters, J. & Sierra-Mercado, D. (2008) Neural correlates of individual variability in fear extinction. *J Neurosci* **28**, 12147–12149.
- Thomas, K.R. & Capecchi, M.R. (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51**, 503–512.
- Threadgill, D.W., Yee, D., Matin, A., Nadeau, J.H. & Magnuson, T. (1997) Genealogy of the 129 inbred strains: 129/SvJ is a contaminated inbred strain. *Mamm Genome* **8**, 390–393.
- Vidal-Gonzalez, I., Vidal-Gonzalez, B., Rauch, S.L. & Quirk, G.J. (2006) Microstimulation reveals opposing influences of prelimbic and infralimbic cortex on the expression of conditioned fear. *Learn Mem* **13**, 728–733.

- Wade, C.M. & Daly, M.J. (2005) Genetic variation in laboratory mice. *Nat Genet* **37**, 1175–1180.
- Wahlsten, D. (1982) Deficiency of corpus callosum varies with strain and supplier of the mice. *Brain Res* **239**, 329–347.
- Wolfer, D.P., Crusio, W.E. & Lipp, H.P. (2002) Knockout mice: simple solutions to the problems of genetic background and flanking genes. *Trends Neurosci* **25**, 336–340.
- Wolfer, D.P., Muller, U., Stagliar, M. & Lipp, H.P. (1997) Assessing the effects of the 129/Sv genetic background on swimming navigation learning in transgenic mutants: a study using mice with a modified beta-amyloid precursor protein gene. *Brain Res* **771**, 1–13.

Acknowledgments

Work supported by the National Institute on Alcohol Abuse and Alcoholism Intramural Research Program, the Fonds zur Förderung der Wissenschaftlichen Forschung, NFN-S102 and the German Academic Exchange Service (DAAD).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1: Numerical representation of comparison of 129 substrains for fear conditioning (Cond), extinction (Ext) and extinction retrieval (Ext Ret)

Table S2: Individual trial breakdown of comparison of 129 substrains for the first extinction trial-block (Ext) and the extinction retrieval trial-block (Ext Ret)

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.