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A parametric analysis of factors affecting acquisition and extinction of contextual fear in C57BL/6 and DBA/2 mice

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Abstract

Behavioral analyses of genetically modified and inbred strains of mice have revealed neural systems and molecules that are involved in memory formation. Many of these studies have examined memories that form in contextual fear conditioning, in which an organism learns that a particular context signals the occurrence of a footshock. During fear extinction, nonreinforced exposure to the context results in the loss of the conditioned fear response. The study of extinction has been instrumental for behavioral and molecular theories of memory. However, many of the transgenic, knockout, and inbred strains of mice that have been widely studied in memory have behavioral deficits in contextual fear conditioning, which makes the study of extinction in these mice particularly challenging. Here we explore several strategies for studying extinction in C57BL/6 and DBA/2 mice, two strains known to differ in contextual fear conditioning. First, we attempt to equate performance prior to extinction through several extensive conditioning protocols. Second, we examine extinction in subsets of mice matched for initial levels of context conditioning. Third, we examine within-strain effects of variables known to affect extinction. Differences between the strains persisted across extensive conditioning and extinction protocols, but both strains were sensitive to session duration and context manipulations during extinction. We describe the implications of our results for behavioral and neurobiological approaches to extinction, and we examine the general challenges in studying extinction in subjects that differ in learning or performance prior to extinction.

Keywords

Extinction; C57BL/6; DBA/2; fear; memory

Studies of genetically modified and inbred strains of mice have had an enormous impact on memory research because careful behavioral analyses have revealed consistent differences in memory and behavior as a function of genotype (e.g., Chen, Panksepp, & Lahvis, 2009; Stiedl, Radulovic, Birkenfeld, Palve, Kammermeier, Sananbenesi, & Spiess, 1999). Many of these studies have focused on the mechanisms that underlie memory in contextual fear conditioning, a hippocampus-dependent task in which a rodent learns to associate a physical context with a footshock (e.g., Rudy & O'Reilly, 1999). Studies of inbred and genetically modified mice have complemented pharmacological and lesion studies of hippocampal

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function by helping to isolate the cellular and molecular systems that are involved in hippocampus-dependent memory (e.g., Bucan & Abel, 2002; Chen, Kim, Thompson, & Tonegawa, 1996). For example, the study of the inbred DBA/2 (D2) and C57BL/6 (B6) mouse strains has been particularly informative about the genetic basis for hippocampus-dependent memory because of the consistent differences between these strains in hippocampal function at morphological, electrophysiological, and behavioral levels of analysis (e.g., Balogh, et al, 2002; Nguyen, et al, 2000; Paylor, et al, 1994).

Recent studies of memory have examined the mechanisms that occur during contextual extinction, as organisms learn that the previously established context-shock relation no longer occurs (e.g., Bouton, et al, 2006; Stafford, Raybuck, Ryabinin, & Lattal, 2012). Such learning does not erase the original memory, but instead suppresses behavior by laying down a new memory that inhibits the original context-shock memory (see Delamater, 2004; Lattal, Radulovic, & Lukowiak, 2006; Myers & Davis, 2007). Previous studies have demonstrated the involvement of the hippocampus in contextual extinction and others have shown that D2 mice have deficits in using contexts to retrieve an extinction memory (Waddell, Dunnett, & Falls, 2004). The challenge in investigating genetic contributions to extinction is that some of the most widely studied transgenics, knockouts, and inbred strains have impairments in initial memory formation. If genetic manipulations cause differences in initial memory formation, then the study of extinction becomes a challenge for the simple reason that there may be very little conditioned behavior to extinguish. Given that genetic strain cannot be regulated in the temporal fashion needed to isolate the effects of strain on extinction independent of initial memory formation, multiple approaches need to be explored for the behavioral genetic study of extinction.

The following experiments attempt to determine (1) if the B6-D2 strain difference in contextual fear can be eliminated by extensive conditioning, and (2) if these strains differ in extinction of contextual fear. If the B6-D2 difference in contextual fear conditioning can be overcome through extensive conditioning, we can investigate extinction in these strains from a common starting point in behavior, which allows effects of strain during extinction to be examined independent of differences in behavior that may have occurred prior to extinction. We combine these between-strain comparisons with a within-strain approach that allows us to ask whether B6 and D2 mice are sensitive to parametric manipulations during extinction that are independent of levels of conditioned behavior. Together, these between- and within-strain analyses provide a useful general approach for studying the genetic basis of extinction.

Materials and Methods

Subjects

Male and female C57BL/6J and DBA/2J mice bred in our animal facility from mice originally obtained from Jackson Laboratories were used in the experiments (1–3 generations of in-house breeding). They were 10–22 weeks old at the beginning of the experiments and were housed in groups of 2–4 per cage, where they had free access to food and water.

Apparatus

All experiments used four Coulbourn Instruments mouse conditioning chambers (H10-11M-TC), measuring 18 cm × 18 cm. The front and back walls were Plexiglas and the two side walls were metal. The floor consisted of 5.0 mm stainless steel grid rods spaced 6.4 mm apart. The chambers were housed in sound- and light-attenuating shells, where a fan provided background noise at 70 dB. A houselight (Coulbourn H11-01M) provided

continuous illumination during the sessions. A 2-s scrambled shock was delivered to the grid floor by a computer-controlled shock generator (Coulbourn H13-15). Mounted 18 cm above the floor of each chamber was an automated infrared activity monitor (Coulbourn H24-61). Prior to each session, the context was cleaned with distilled water. Experimental events were controlled by Graphic State 3.01 software. In the two context experiment (Figure 7), a second set of four conditioning chambers was used. These chambers were housed in an adjacent room. A clear Plexiglas cylinder (18 cm high, 15 cm in diameter) sat on top of the grid floor (Coulbourn H10-35M-08-SF with 5 mm rods variably spaced from 6.7 to 7.4 mm apart). The circular chambers were cleaned with 0.1% acetic acid and houselights and background fans were not activated.

Shock Intensity Experiment (Figures 1 & 2)

Sixteen mice from each strain were handled for 3 days prior to the experiment. On Day 1 of the experiment, mice received a 12-min conditioning session that consisted of a shock (0.35 or 1.75 mA) presented 2.5, 5, 9, and 11.5 min after placement into the context (a total of 4 shocks). On Day 2, mice were returned to the conditioning context and received a 24-min extinction test, in which no shocks were delivered. On each of the next 8 days, all mice received further conditioning, in which they received four 1.75 mA shocks in each 12-min session. Following this second phase of conditioning, mice received 12 daily extinction sessions, which consisted of a 12-min exposure to the context in the absence of shock. One B6 and one D2 mouse were not shocked on Day 1 due to an equipment malfunction and were excluded from the experiment. One D2 mouse died after the second extinction session and was excluded from the extinction analyses.

Shock Number Experiment (Figures 3–5)

Mice were handled for 3 days prior to the experiment. On Day 1 of the experiment, mice received 1, 4, or 8 shocks (2-s 0.35mA) in a 3, 12, or 24-min session, respectively. The inter-shock interval was a variable 3 min for the 4- and 8-shock groups. In the 1, 4, and 8 shock groups, there were 15, 14, and 14 B6 mice and 13, 17, and 14 D2 mice, respectively. On Days 2 and 3, all mice received a 24-min extinction test session. Beginning on Day 4, a subset of mice from each strain ($n=20$ per strain; mice were selected to match levels of test freezing as closely as possible between the two strains while also including an approximately equal number of mice from each conditioning group) received an additional 8 sessions of conditioning, in which 8 shocks were delivered in a 24-min session. These mice then received six sessions of extinction, which consisted of a nonreinforced 24-min exposure to the context each day. Mice received another 24-min extinction test session three weeks later.

Extinction Duration Experiment (Figure 6)

Mice were handled for 3 days prior to the experiment. On Days 1–4 of the experiment, B6 mice received a 12-min conditioning session that consisted of a shock (0.35mA) presented 2.5, 5, 9, and 11.5 min after placement into the context (a total of 4 shocks). D2 mice received the same shock schedule, except that the intensity was 1.75 mA. On Days 5–11, mice were returned to the conditioning context and received either a 3- (B6, $n=14$; D2, $n=16$) or 24-min (B6, $n=12$; D2, $n=15$) extinction session, in which no shocks were delivered.

Two Context Experiment (Figure 7)

Nine B6 and 10 D2 mice were handled for 3 days prior to the experiment. On Days 1–2 of the experiment, mice received nonreinforced pre-exposure to the two contexts (A and B; counterbalanced between the square and circular contexts). Each exposure was 12 min and was separated by a 2 hr interval. Order of exposure to the contexts was counterbalanced

across subjects. On Days 3 and 4 of the experiment, mice were conditioned in both contexts with a 2-hr interval separating the conditioning sessions (order again was counterbalanced within and between days). During conditioning, mice received a 12-min conditioning session that consisted of a 2-s shock presented 2.5, 5, 9, and 11.5 min after placement into the context (a total of 4 shocks). The shock intensity for B6 mice was 0.35 mA; for D2 mice, it was 1.75mA. On Days 5–12, mice received 24-min extinction sessions in Context A. On Day 13, mice received 12-min nonreinforced test sessions in Contexts A and B, separated by 2 hr (test order was counterbalanced).

Data Analysis

The dependent variable in all experiments was freezing, which was detected by the infrared activity monitors mounted on the ceiling of each chamber. Freezing was defined as a lack of movement detected by the activity monitors for > 3 s. This criterion has been validated in our previous work (Lattal, 2007; Lattal, Barrett, & Wood, 2007) and by other laboratories (e.g., Boatman & Kim, 2006). In the shock reactivity analysis (Figure 4), we recorded the number of movements detected by the activity monitors in 2-s time blocks. Comparisons were made between groups during the course of extinction to determine whether manipulations affected performance during extinction sessions. To assess the impact of the extinction manipulations on the retention of extinction, comparisons were made during test sessions, which also were extinction sessions. Data were analyzed using mixed design analysis of variance (ANOVA). Unless noted in the text, there were no reliable main effects or interactions involving sex. Student's t tests were used for planned comparisons during the beginning of extinction or test sessions. Post-hoc comparisons were made using the Bonferroni alpha-splitting correction.

Results

Extensive conditioning and extinction sessions reveal differences between B6 and D2 mice

Effects of shock intensity: Conditioning and Testing—Results from the initial conditioning and test sessions are shown in Figure 1. Both strains acquired context-evoked freezing and there appeared to be no effect of shock intensity on the rate of acquisition. Interestingly, relative to B6 mice, D2 mice appeared to acquire freezing quickly (Block 2 of Acquisition in Figure 1; strain $\times$ time block interaction: $F(3, 78)=9.3$, $p<0.001$), but asymptotic levels were similar (no reliable main effect of strain or intensity during Block 4, $F_s(1,26)<1.0$), suggesting that the strains differed in rate of acquisition, but not in terminal freezing levels. During testing, freezing was higher in B6 mice compared to D2 mice (main effect of strain: $F(1, 26)=10.9$, $p<0.005$). Although the overall interaction between strain and intensity was not reliable ($F(1, 26)=3.5$, $p=0.07$), there was a reliable strain $\times$ time block interaction during the test ($F(7, 182)=2.9$, $p<0.01$). Overall, more freezing occurred in B6 mice that received the strong shocks compared to the weaker shocks, $t(13)=2.4$, $p<0.05$. In contrast, D2 mice showed no sensitivity to shock intensity, suggesting that there may be limits to the amount of conditioning that is possible in this strain.

Effects of shock intensity: Extensive conditioning and extinction—The difference between strains during the test suggests that a strong conditioning protocol (four intense shocks) does not overcome the B6-D2 difference in context-evoked freezing. In the next phase of the experiment, we examined the effects of an extensive conditioning protocol to determine if freezing in D2 mice would increase to B6 levels, which would allow a more straightforward investigation of extinction. Results from the eight further conditioning sessions with four intense shocks and the 12 sessions of extinction are shown in Figure 2. There was no difference in reconditioning as a function of initial conditioning intensity, so

the different initial treatments were combined in each strain in Figure 2 (Extensive Conditioning). The B6-D2 strain differences in freezing persisted during extensive conditioning, though both strains showed increases during the first few sessions (main effect of strain: $F(1, 28)=29.1$, $p<0.001$; main effect of session: $F(7,196)=8.7$, $p<0.001$; no reliable interaction: $F(7, 196)<1.0$). Both strains showed a loss of freezing over the course of 12 extinction sessions (Extinction in Figure 2; main effect of session: $F(11,297)=35.0$, $p<0.001$). However, the strain differences that were evident after conditioning persisted over 12 extinction sessions (main effect of strain: $F(1, 27)=30.0$, $p<0.001$; no reliable interaction: $F(11,297)<1.0$). These results demonstrate that even very intense conditioning protocols cannot overcome the difference in context-evoked freezing between B6 and D2 strains, and that this difference persists over the course of 12 extinction sessions.

Effects of shock number: Initial conditioning—There was no evidence that a single (Figure 1) or multiple (Figure 2) conditioning sessions with multiple intense shocks could overcome the difference between B6 and D2 mice in context-evoked freezing. In the next experiment, we asked whether increasing both the number of shocks and the time in the context during a single and multiple conditioning sessions could increase the amount of conditioning in D2 mice. Initial acquisition (Acq in Figure 3) again appeared to occur more quickly in D2 mice (i.e., more freezing during the second block of three minutes in the 4 and 8 shock groups), but the asymptote of freezing was lower in D2 mice when 8 shocks were administered. Because the duration of the conditioning session differed in the 1, 4, and 8-shock groups, separate analyses were conducted on each shock number. There were no differences in freezing as a function of strain or sex during the 3-min single-shock conditioning session, $F_s(1,24)<3.4$. During the 12-min conditioning session with 4 shocks, there was a reliable main effect of 3-min time block, $F(3, 81)=48.4$, $p<0.001$, as well as a reliable block $\times$ sex interaction, $F(3, 81)=5.9$, $p<0.001$, which was caused by more freezing in female mice during the third time block, $t(29)=3.7$, Bonferroni-adjusted $p<0.01$. There were no reliable differences between the strains in rate of conditioning.

The use of a longer conditioning session in the eight shock groups allowed us to determine whether mice differ in freezing at the end of a session. As can be seen in the third panel of Figure 3, D2 mice that received 8 shocks during the acquisition session reached a lower level of performance, compared to B6 mice (reliable strain $\times$ block interaction, $F(7, 161)=10.0$, $p<0.001$; main effect of strain, $F(1,23)=7.4$, $p<0.05$). It is possible that these differences in freezing at the end of the session reflect differences in asymptotic performance, or at least the rate of approach to asymptote. In either case, this difference during the acquisition session suggests that the differences that were observed in the test sessions may not entirely reflect differences in memory, but instead may reflect differences either in the initial learning about the context or in the expression of that learning in performance.

Effects of shock number: Shock reactivity—To evaluate the response to the shock itself, we examined the burst of activity that occurred during the shock itself. We examined activity during five 2-s blocks immediately prior to the shock, during the 2-s shock itself, and during five 2-s blocks immediately after the shock in the group that received 8 shocks. As can be seen in Figure 4, which shows average shock reactivity collapsed across the session, D2 mice appeared to respond more during the shock compared to B6 mice. An ANOVA with strain and 2-s time block as factors revealed a trend toward a main effect of strain ($F(1,30)=4.0$, $p=.056$), a reliable main effect of time block ($F(10,300)=133.0$, $p<0.001$), and a reliable interaction ($F(10,300)=4.15$, $p<0.001$). Analysis of the interaction revealed that the strains differed in activity during the shock itself ($t(30)=5.3$, $p<0.001$). Further analyses of the effects on activity during the 10-s before and after the shock revealed a reliable main effect of time (before or after; $F(1, 30)=8.4$, $p<0.01$) and a time $\times$ strain

interaction ($F(1,30)=9.0$, $p<.001$), suggesting that although strains differed in activity prior to the shock, the strains showed equal response after the shock. Thus, although there were differences in response to the shock, the immediate after-effects of the shock were similar in the two strains (cf. Fanselow, 1982).

Effects of shock number: Testing—During the test (Test in Figure 3), D2 mice in all shock treatment groups froze less than did B6 mice, suggesting that increasing the number of shock presentations within a session did not overcome the strain difference in context-evoked freezing. An ANOVA with strain and sex as between subjects factors and 3-min time block as a repeated measure revealed reliable main effects of strain ($F(1, 80)=23.5$), shock ($F(2, 80)=11.3$), and time block ($F(7, 560)=44.4$), as well as reliable interactions between block and strain ($F(7, 560)=13.4$) and block and shock ($F(14, 560)=2.7$, all $ps<0.001$). There were no reliable main effects or interactions involving sex. These findings suggest that the B6-D2 strain difference in freezing persists across different numbers of shocks, and that, even though D2 mice froze less compared to B6 mice, they were sensitive to manipulations of shock number.

Effects of shock number: Extensive conditioning—Twenty mice from each strain received 8 further conditioning sessions, consisting of 8 shocks per session. Figure 5A shows the mean percent freezing averaged over the final two sessions of extensive conditioning (Cond in Figure 5A). As in the previous experiment with increased shock intensities, this experiment found that extensive conditioning with increased numbers of moderate shocks did not overcome the strain difference (main effect of strain, $F(1,33)=25.4$, main effect of session, $F(7,231)=31.9$, $ps<0.001$; no other reliable main effects or interactions).

Effects of shock number: Extinction after extensive conditioning—This difference between B6 and D2 mice was further evident during extinction trials, in which mice were exposed to the conditioning context in the absence of shock for six 24-min sessions (Ext 1–6 in Figure 5A). B6 mice showed rapid extinction within each session. Indeed, by the end of each extinction session, there were no differences in freezing between B6 and D2 mice. However, during the beginning of each subsequent session, the strain differences re-emerged with B6 mice showing spontaneous recovery of freezing. D2 mice showed rapid extinction during the first session but appeared to show little spontaneous recovery after that. A 3-way ANOVA with 6-min time block and session as repeated measures revealed a reliable 3-way interaction between strain, block, and session ($F(15,570)=2.5$, $p<0.001$). To explore the interaction, separate ANOVAs were performed on each extinction session. These analyses revealed reliable main effects of strain in Sessions 1–3 ($Fs(1, 38)> 11.2$, $ps<0.005$), and a reliable main effect of 6-min block and a reliable strain $\times$ block interaction in all extinction sessions ($Fs(3,114)>2.9$, $ps<0.05$). Further analyses revealed that the two strains differed during the first 6-min block of all but the fourth and sixth extinction session ($ts(38) >2.3$; Bonferroni-corrected $ps<0.05$), but did not differ during the last block of any session.

During the retention test (Retention in Figure 5A), which was conducted 21 days after the sixth extinction session, B6 mice appeared to freeze more compared to D2 mice during the session. An ANOVA with strain and block as factors revealed reliable main effects of strain ($F(1,38)=105$, $p<0.005$) and block, as well as a reliable interaction ($Fs(3,114)>4.6$, $ps<0.005$). This finding demonstrates that although the strain difference in freezing was eliminated in the sixth extinction session, it returned with time.

Effects of shock number: Extinction in mice matched for levels of conditioning—Analysis of the extinction results in Figure 5A demonstrates that any freezing that had occurred in D2 mice was rapidly and persistently reversed after a single extinction session. However, as in Figure 2, any apparent strain differences in the rate and persistence of extinction are complicated by differences in the levels of freezing at the beginning of extinction. Comparisons across different baselines or starting points of performance are extremely difficult, even when the data are normalized in some way (cf., Lattal, 1999). Consequently, Figure 5B shows extinction in a subgroup of animals from each strain matched in terminal levels of freezing during the final two conditioning sessions. Here, only the top 50% of D2 mice ($n=10$) and the bottom 50% of B6 mice ($n=10$) were included in the analysis.

As can be seen in Figure 5B, when strain performance during the end of conditioning was matched (Cond in Figure 5B), it appears that the persistence of extinction is greater in D2 mice compared to B6 mice, though these effects are small. A 3-way ANOVA with 6-min time block and session as repeated measures revealed reliable main effects of session ($F(5,90)=38.8$) and block ($F(3, 54)=63.7$, both $ps<0.001$), as well as reliable 2-way interactions between block and strain and between session and block ($F_s>13.4$, $ps<0.001$). Analyses of the differences in the first time block of each extinction session revealed a trend towards a difference in Session 3 ($t(18)=2.8$; Bonferroni-corrected $p=0.08$), but there were no other reliable differences during the first or last block of any extinction session. During the retention test (Retention in Figure 5), B6 mice appeared to freeze more than did D2 mice, but this difference again fell short of reliability ($t(18)=1.9$, $p=.07$). These findings suggest that when matched for performance, there is a tendency for D2 mice to show more persistent extinction, but this effect is small and not consistently reliable.

Together, these experiments demonstrated that the difference in context-evoked freezing between B6 and D2 strains persists across extensive conditioning sessions with either very intense shocks or a large number of moderate shocks and that this difference continues to persist during extinction. There was some evidence that D2 mice showed more persistent extinction, but such a conclusion must be made cautiously because of the strain differences in asymptotic freezing that resulted from conditioning.

B6 and D2 mice are sensitive to extinction session duration

The previous experiments demonstrated that extensive conditioning with the same shock parameters (intensity or number) did not bring B6 and D2 mice to common levels of freezing. Although we were able to examine extinction in a subset of mice matched for levels of performance, it is possible that the apparent enhancements in extinction in D2 mice reflected residual differences in initial learning that were masked by ceiling effects. Next, we attempted to equate levels of freezing by administering a strong conditioning protocol (four 1.75 mA shocks) to D2 mice and a weaker conditioning protocol (0.35 mA shocks) to B6 mice. These protocols brought freezing together during the final conditioning session in the two strains (B6 mean = 69.0; D2 mean = 63.7; $t(55)=1.4$), but there was a reliable strain difference during the first 3 min of the first extinction session (Ext 1 in Figures 6A & 6B; B6 mean=79.8; D2 mean=64.6; $t(55)=3.3$, $p<0.005$). These findings suggest that even when different conditioning protocols are used to match performance (freezing) during the final acquisition session, the retention of the contextual freezing was different in the two strains.

Although freezing began at different levels, it did appear that B6 and D2 strains were sensitive to the duration of the extinction session; more extinction occurred with 24-min sessions compared to 3-min sessions in both strains (Figures 6A & 6B). During the seven sessions of extinction, mice that received the 24-min exposure to the context showed decreases in freezing within each session. During the initial 3 min of the subsequent

extinction session, mice that received 24-min extinction generally showed less freezing compared to the 3-min groups after about the third extinction session. An ANOVA conducted on the extinction sessions (3 min session in the 3-min groups and first 3 min of the session for the 24-min groups) revealed main effects of strain ($F(1,53)=4.5$, $p<0.05$), extinction session duration ($F(1, 53)=24.3$, $p<0.001$), and extinction session ($F(6, 318)=18.0$, $p<0.001$), as well as reliable interactions between strain and duration ($F(1,53)=4.3$, $p<0.05$), extinction session and duration ($F(6, 318)=6.9$, $p<0.001$), and a reliable 3-way interaction ($F(6, 318)=2.9$, $p<0.01$). Separate analysis of the 3-min groups revealed reliable main effects of strain ($F(1, 28)=12.0$, $p<0.005$) and extinction session ($F(6, 168)=2.4$, $p<0.05$), but no reliable interaction, suggesting that the difference between the two strains at the beginning of extinction persisted throughout the course of the seven extinction sessions. Analysis of the 24-min extinction sessions revealed reliable main effects of extinction session ($F(6, 150)=51.6$) and 3-min extinction block ($F(7,175)=47.0$, $ps<0.001$), but did not reveal a reliable main effect of strain ($F(1, 25)<1.0$). The 3-way interaction between strain, extinction block, and extinction session was reliable ($F(42,1050)=2.1$, $p<0.001$).

To further explore these interactions, the effects of extinction session duration were examined within each strain separately. Again, to compare the impact of extinction under common testing conditions, only the first 3-min block was examined in the 24-min groups. C57BL/6 mice were sensitive to the duration of the extinction session (reliable main effect of session duration: $F(1,24)=22.5$, $p<0.001$); from Extinction Session 3–7, mice that received 3-min extinction sessions froze more than did mice that received the 24 min extinction sessions ($ts(24)>3.8$, Bonferroni-adjusted $ps<0.005$). DBA/2 mice, on the other hand, were much less sensitive to the duration of the extinction session; the difference between 3 and 24-min exposures was small. There was a reliable main effect of extinction session duration within the DBA/2 strain ($F(1,29)=4.5$, $p<0.05$), suggesting that overall, a 24-min session produced greater reduction in freezing compared to a 3-min session. Inspection of Figure 6B, however, suggests that the difference between 3- and 24-min context exposures was small and not present during each extinction session in D2 mice. This impression was confirmed as post-hoc analyses revealed that there was no reliable main effect of extinction duration in any individual extinction session. These analyses demonstrate that the amount of extinction produced by a 3- or 24-min exposure to the context is very different in B6 mice, but is similar in D2 mice.

These findings demonstrate that B6 and D2 mice are sensitive to the duration of the extinction session and that both strains showed spontaneous recovery at the beginning of each extinction session. It is possible that the spontaneous recovery observed in D2 mice in this experiment (compared to the little observed in Figure 5) was due to the use of more intense shocks during conditioning. Regardless of the spontaneous recovery effects, the impact of a short or long extinction session was greater in B6 mice, perhaps due to the lower levels of pre-extinction freezing in D2 mice being more vulnerable to the response-weakening effects of extinction.

B6 and D2 mice show specificity of extinction

In the next experiment, we attempted to examine whether the extinction that occurs in B6 and D2 mice is specific to the context that was extinguished. Mice in this experiment were conditioned in two contexts (A and B, counterbalanced), then received extinction in Context A, followed by a test in Contexts A and B. D2 mice received a strong conditioning protocol and B6 mice received a weaker conditioning protocol in an attempt to equate freezing prior to extinction. As can be seen in Figure 7, mice showed equivalently high levels of freezing during the final session of conditioning in both contexts (Acquisition in Figure 7). This demonstrates that the different conditioning protocols were effective in equating pre-extinction performance. During the initial extinction session in Context A, however, D2

mice froze less compared to B6 mice, again demonstrating poor retention of freezing established during conditioning. Both strains showed a reduction in freezing during the 8 extinction sessions (reliable main effects of strain ($F(1,17)=4.8$, $p<0.05$) and session ($F(7,119)=40.9$, $p<0.001$; no reliable interaction ($F(7,119)=2.0$, $p=0.06$). During the test, both strains froze less in the extinguished (Context A) compared to the nonextinguished context (Context B; $F(1,17)=30.4$, $p<0.001$; no reliable main effect of strain or interaction ($F(1,17)<1.0$). Thus, the decrease in freezing in both strains reflected a decremental process that was dependent on extinction in a given context. Further, the high level of freezing in the nonextinguished context confirms that the reduction in freezing in the extinguished context was not due to forgetting.

General Discussion

These experiments demonstrate some of the challenges in the study of extinction in mouse strains that differ in initial levels of conditioning. Despite extensive conditioning both within and between sessions, differences in context-evoked freezing persisted between C57BL/6 and DBA/2 strains of mice. This difference occurred when different numbers or intensities of shocks were administered within a conditioning session and they persisted over the course of conditioning sessions repeated for multiple days. Thus, identical conditioning protocols were unable to bring performance to common levels in B6 and D2 mice and these strain differences persisted during extensive extinction sessions.

Given that the differences in freezing between B6 and D2 mice could not be overcome through several extensive conditioning protocols, we examined other approaches to the study of extinction in these strains. One was to examine extinction in a subset of mice from each strain that were matched for performance during conditioning. This equated performance at the outset of extinction, which allowed the rate of extinction to be compared between strains without being affected by differences in freezing prior to extinction. This comparison revealed less spontaneous recovery at the beginning of some extinction sessions in D2 mice compared to B6 mice, even though performance at the end of the previous extinction session was similar. Thus, there was some evidence that extinction was enhanced in D2 relative to B6 mice. The difficulty is knowing how to interpret any enhanced behavioral extinction effect in D2 mice; enhanced extinction could reflect facilitated learning about the context-no shock contingencies, or it could simply reflect residual differences in initial learning or memory formation that become less obscured on lower parts of the behavioral scale during extinction. A further complication is that examining a subset of mice showing the strongest and weakest freezing responses in D2 and B6 strains, respectively, could produce sampling error that causes effects on extinction that are not reflective of an individual strain's typical extinction response. Thus, there are a number of challenges that complicate interpretation of between-strain differences during extinction.

Our second approach to extinction in these strains was to focus on within-strain effects of variables known to affect extinction. Both strains were generally sensitive to the duration of the extinction session; more extinction occurred with a 24-min exposure compared to a 3-min exposure to the conditioned context, although this difference was not as robust in D2 mice. Thus, just as D2 mice were somewhat sensitive to parametric manipulations during conditioning (shock number but not shock intensity), they also were somewhat sensitive to parametric manipulations of session duration during extinction. Together, these results suggest that D2 mice are capable of learning contextual conditioning and extinction, but at different overall levels compared to B6 mice. Further, our final experiment demonstrated that the loss of freezing that occurred to the conditioned context was specific to that context in both strains of mice. This context specificity in extinction is similar to results from studies of initial conditioning, which have found that even though D2 mice show lower levels of

freezing to a conditioned context, they are able to discriminate between contexts that have or have not been conditioned (Balogh, et al 2002).

Our findings that retention of initial conditioning differed between these strains are consistent with many other studies showing differences between B6 and D2 mice in levels of context-evoked freezing (e.g., Balogh et al, 2002; Logue, et al 1997; Nie & Abel, 2001; Paylor et al, 1994) and in the ability to use the context to retrieve memories of extinction of a discrete cue previously paired with shock (Waddell, et al. 2004). One of the reasons that these two strains have been popular choices for studying mechanisms of contextual fear conditioning is that there are well-documented differences in hippocampal function between B6 and D2 strains. Studies of the hippocampus in these strains have found morphological, biochemical, and electrophysiological differences that parallel the behavioral differences in some hippocampus-dependent tasks, such as contextual fear conditioning (e.g., Nguyen et al 2000). Thus, the behavioral data from these strains fit nicely with other genetic, pharmacological, and lesion studies of the hippocampus in contextual fear conditioning (Paylor, et al 1994). However, the role of the hippocampus in contextual fear conditioning is complicated because there is evidence that animals with hippocampal lesions can acquire contextual fear conditioning under some circumstances, particularly when those lesions occur prior to conditioning (e.g., Matus-Amat, et al, 2004; Wiltgen, et al, 2006).

Theories of hippocampal function and contextual fear conditioning suggest that there are hippocampus-dependent and independent ways that animals can form context-shock memories. Animals may rely on an elemental, hippocampus-independent strategy, in which individual elements of the context may become associated with shock (e.g., Estes, 1950), or a hippocampus-dependent configural strategy, in which the context as a configuration becomes associated with shock (e.g., Fanselow, 1999; Rudy, Huff, & Matus-Amat, 2004). In normal situations, the hippocampus may consolidate all of the individual features of a context into a configural representation that becomes associated with shock. Impairments in hippocampal function, such as those observed in D2 mice, may prevent configural representations from consolidating, causing animals to rely on a more basic, elemental strategy in which the feature of the context that receives attention just prior to the shock gets conditioned. This conditioning is limited only to those specific contextual features that are actively processed by the animal at the time of shock. Thus, under circumstances in which a limited number of shocks is used, animals with hippocampal impairments will only show a conditioned response when the elements that are directly associated with the shock are processed during a memory retrieval test; they will not show the conditioned response when other elements are processed because they do not have the configural representation or associative network that can activate those elements directly associated with the shock. However, with further conditioning, even animals that use an elemental strategy should show contextual conditioning because more and more of those individual elements would be associated with the shock. Thus, even if there is no hippocampus-dependent configural representation, the number of individual elements associated with shock should be quite large, resulting in an enhancement in contextual conditioning.

Wiltgen, et al. (2006) found that although hippocampal lesions impaired context conditioning, animals with lesions were sensitive to changes in conditioning parameters, such as the interval between placement into the context and shock. We found a similar sensitivity to conditioning parameters with D2 mice – although the strain difference was evident across shock numbers and intensity, increasing the number of shocks within a conditioning session increased context-evoked freezing during testing within each strain. However, the B6-D2 strain difference persisted even after extensive conditioning, which should have favored more associative learning between the context elements and the shock. This pattern is consistent with the effects of retrograde hippocampal lesions (e.g., Wiltgen,

et al 2006), even though any hippocampal impairments in D2 mice were present prior to conditioning.

The rapid speed at which extinction developed in D2 mice (see Figures 5 and 6) is consistent with the idea that the initial context-shock memory was not strongly formed, making it more vulnerable to the suppressive effects of extinction. However, a major challenge facing any inbred mouse strain model of memory is that there may be differences between strains that may influence the way learning is expressed in behavior. As with most studies of contextual fear conditioning, we used the behavioral response of freezing as a measure of fear, but there are other behavioral systems that may be engaged when a fearful stimulus is encountered (e.g., Fanselow & Lester, 1988, Siegfried et al, 1990). It is possible that in B6 mice, the defensive response of freezing is most likely to be engaged by stimuli associated with shock, whereas in D2 mice, escape response systems may become engaged, consistent with the increase in activity induced by the shock that we observed (Figure 4). Indeed, when the dependent variable is avoidance or escape from a previously shocked context, D2 mice perform just as well, if not better than B6 mice (e.g., Podhorna & Brown, 2002; Stavnes & Sprott, 1974; Wimer, et al 1968). Gerlai (1998) demonstrated that D2 mice showed less context-evoked freezing compared to B6 mice, but spent more time in the corners of the chambers, consistent with the idea that D2 mice searched for points of escape. If D2 mice are more likely to use an escape strategy, there may be a lower ceiling of freezing that they can reach, which would account for the different asymptotes of freezing that we observed in the extensive conditioning treatments.

The behavioral analysis of memory in inbred strains of mice is always faced with inherent challenges of determining when strain differences in performance reflect differences in memory storage or memory expression. This is particularly true in studies of extinction because, in the absence of temporal genetic regulation (e.g., Isiegas et al, 2006), genetic differences will necessarily be present during initial acquisition and during extinction. This makes it difficult to know if behavioral differences in extinction reflect genetic effects on extinction learning or reflect residual effects from initial memory formation (cf., Tarantino, et al 2000). Ultimately, an important direction for the behavioral genetics of extinction will be to reveal genotypes that differ only in some very specific aspects of extinction (e.g., Wellman, et al 2007) and to continue to develop techniques that allow for rapid modulation of gene expression. The ability to rapidly modulate gene expression allows for more direct comparisons to be made during extinction that are not complicated by potential differences in initial learning. Approaches that includes temporal regulation of gene expression and manipulations of variables within- and between-genetic manipulations will be helpful in ultimately elucidating genetic differences in extinction.

Highlights

We examine effects of different conditioning manipulations on the development and retention of conditioned freezing in C57BL/6 and DBA/2 mice.

We examine the effects of different extinction manipulations on the development and persistence of extinction in these mice.

Differences in extinction occurred despite various attempts to match levels of conditioning prior to extinction.

Despite the between-strain differences in extinction, both strains showed sensitivity to manipulations known to affect extinction (session duration and stimulus identity).

These findings highlight the difficulties inherent in the study of extinction in inbred mouse strains.

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Figure 1. Effects of shock intensity on acquisition and expression of contextual fear
Mean percentage of time spent freezing during a 12-min acquisition session consisting of four 0.35 mA or 1.75 mA and during a 24-min nonreinforced test session 24 hr later is shown. B6 = C57BL/6; D2 = DBA/2. Bars reflect standard error of the mean.

Figure 2. Effects of multiple sessions of conditioning and extinction

Mean percentage of time spent freezing during eight further conditioning sessions consisting of four 1.75 mA shocks and 12 extinction sessions consisting of 12-min nonreinforced exposures to the context. Bars reflect standard error of the mean.

Figure 3. Effects of shock number on acquisition and expression of contextual fear

Mean percentage of time spent freezing during acquisition sessions consisting of one, four, or eight 0.35 mA shocks and during a 24-min nonreinforced test session 24 hr later is shown. Bars reflect standard error of the mean.

Figure 4. Activity before, during, and after the shock

Mean activity counts recorded in the 8-shock groups from Figure 3 are shown. Data were recorded during the 10 seconds (Blocks 1–5) immediately before the shock, during the 2-s of shock (Block 6, indicated by the black bar), and during the 10-s (Blocks 7–11) immediately after the shock. Bars reflect standard error of the mean.

Figure 5. Extinction after extensive conditioning

Mean percentage of time spent freezing during extensive conditioning, extinction, and retention testing is shown. (A) Extinction in mice that were not matched for levels of freezing after conditioning ($n=20$ per strain). (B) Extinction in mice that were matched for levels of freezing after conditioning (top 50% of DBA/2 mice and bottom 50% of C57/BL6 mice ($n=10$ per strain). Cond = mean freezing during the final two conditioning sessions, Ext = extinction. Bars reflect standard error of the mean.

Figure 6. Effects of extinction session duration in (A) C57BL/6 and (B) DBA/2 mice
Mice received 3 or 24-min nonreinforced exposures to the context after conditioning. Bars reflect standard error of the mean.

Figure 7. Context specificity of extinction

Mice were conditioned in two contexts (A or B), followed by 24-min extinction sessions over eight days in one of those contexts (A, counterbalanced), followed by 12-min nonreinforced test sessions in Contexts A and B. Bars reflect standard error of the mean.