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Fear-Potentiated Startle Response in Mice: Genetic Analysis of the C57BL/6J and DBA/2J Intercross

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MCCAUGHRAN, J. A., JR., J. BELL III AND R. J. HITZEMANN. *Fear-potentiated startle response in mice: Genetic analysis of the C57BL/6J and DBA/2J intercross.* PHARAMCOL BIOCHEM BEHAV **65**(2) 301–312, 2000.—The role of genetic factors in the fear-potentiated startle (FPS) response was examined in the inbred C57BL/6J (B6) and DBA/2J (D2) mouse strains. Mice in the D2 strain displayed a significant potentiation in the acoustic startle response (ASR) when presented with a visual condition stimulus (CS) previously paired with an aversive unconditioned stimulus (US). The maximal FPS response was observed following 20 conditioning trials but a near maximal response was noted following as few as five trials. Forty conditioning trials produced a significant reduction in the FPS response that may be related to overtraining. The FPS response in the B6 strain was significantly lower than the D2 strain, regardless of the number of conditioning trials. The contrasting FPS responses were not related to differences in auditory sensitivity known to exist between these strains. Analysis of a full Mendelian cross formed from the B6 and D2 strains found that the FPS response was a highly heritable trait, best described by a simple additive model of inheritance and with a broad-sense heritability of 0.46. The distribution of the FPS response in F2 hybrids formed from the intercross of the D2 and B6 strains was continuous which suggests a multigenic substrate. The light + noise and noise-alone trial types were highly correlated, but no association was detected between the baseline ASR amplitude and the FPS response. Mice from the phenotypic extremes of the F2 distribution displayed FPS responses that were more extreme than either of the progenitor strains. However, both baseline startle amplitude and the salience of auditory stimuli did not differ in these groups. The results of this study confirm an early report by Falls et al. (1997), and provide additional quantitative genetics information necessary for the eventual mapping of the chromosomal regions or genes associated with the FPS response in mice. © 2000 Elsevier Science Inc.

Fear-potentiated startle Conditioned fear Inbred mice Genetics C57BL/6J DBA/2J

FEAR conditioning can be used to study the mechanisms underlying emotional learning, anxiety, and anxiety-related behaviors (14,31–33). The FPS response, first described by Brown et al. (4) and later by Davis and Astrachan (11), can assess fear conditioning in experimental animals and humans. In the typical FPS paradigm, the degree of conditioned fear is reflected by the amplitude of the ASR elicited in the presence of a CS previously paired with an aversive US [for a review, see (14)]. In humans, the ASR is enhanced in anticipation of an aversive shock (24) or in association with certain disorders such as posttraumatic stress disorder (25,26,40). FPS is inhibited by anxiolytic drugs, some atypical neuroleptics, and a diverse number of other drugs such as clonidine and morphine (1,12–14,28,30). Conversely, anxiogenic compounds enhance the FPS response and can interfere with the normal acquisition or extinction of conditioned fear (2,3,13,21,56). The re-

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sults of these studies suggest that the FPS response may provide a valuable tool for the investigation of the neuromechanisms underlying anxiety- and/or fear-related behaviors (14).

Fear conditioning and the FPS response have been studied extensively in the rat (3,15,22,28,30,51–53,56). However, relatively few studies have examined these behaviors in mice. Mice have certain advantages over the rat, particularly with regard to the study of the genetic basis of complex traits. The availability of a large number of highly polymorphic inbred strains provides a relatively simple screen for the detection of genetic factors. The availability of dense molecular genetic maps can subsequently facilitate the detection of genetic loci associated with behaviors. Finally, the ability to manipulate genomic structure and/or function using knock-out strategies provides additional means to study the functional correlates of these loci or the role of candidate genes. The B6 and D2 strains are ideally suited to examine the role that genetic factors play in the FPS response. They are highly polymorphic (17), display significant variability in learning ability, emotionality, behavioral responses to stress, startle response amplitude, and prepulse inhibition (5,7–10,16,23,29,34,37,38,43–45,48,50,55). A number of studies reported that the B6 and D2 strains also differ in their locomotor responses to fear conditioning (41,44,54). Falls et al. (20) reported that the FPS response in D2 mice was significantly greater than B6 mice. Although these results provide additional support for the hypothesis that genetic factors play a significant role in fear conditioning and the FPS response, additional quantitative genetic information is required to characterize the inheritance of the response and to precisely define the behavioral phenotype in a large segregating population of mice that may eventually be used in a mapping study [e.g., quantitative trait loci analysis (QTL analysis)].

Falls et al. (20) used the repeated pairing of an auditory stimulus with an aversive footshock to produce conditioned fear in the B6 and D2 strains. In view of the genetic variability in auditory sensitivity known to exist across inbred mouse strains [see (27)], the use of acoustic stimuli in fear conditioning could confound the interpretation of the results. The B6 and D2 strains are especially prone to age-related hearing loss (AHL) and cochlear pathology (6,42,57–60). The D2 strain, in particular, is characterized by a loss of sensitivity to high frequencies beginning at 4 weeks of age and becoming severe by 3–5 months. The B6 strain also display progressive hearing loss, albeit at a much slower rate (42,47,58,59,60). Falls et al. (20) convincingly demonstrated that the strain-related differences in the FPS response were not related to AHL. They noted that the confounding effects of differences in auditory sensitivity could be largely overcome by using young mice and acoustic stimuli with equal salience for each strain.

In the following study, the FPS response was examined in young B6 and D2 mice. Fear conditioning in these mice was accomplished following repeated pairing of a visual CS (house lamp) with an aversive US (footshock). The acoustic stimulus chosen for the FPS paradigm was based on frequency response curves obtained from 5-week-old B6, D2, C3H, and 6–7-week-old F2 hybrid mice. In addition, the FPS response was defined as the difference between the ASR observed in the presence of the CS (i.e., light + noise trial type) and the baseline startle response (noise-alone trial type). Because the acoustic stimulus is fixed for each trial type, the difference between the two trials types is more likely to reflect the effects of conditioning than the effects of strain-related differences in auditory sensitivity. A full Mendelian cross was formed using the B6 and D2 strains, and the inheritance of the FPS response was examined. A large segregating population of F2 was also produced in order to characterize the extreme FPS response phenotypes.

METHOD

Animals

Male B6, D2, and C3H mice, as well as male and female B6D2F1 hybrids (F1), between 5–8 weeks of age, were obtained from Jackson Laboratory, Bar Harbor, MA. F1 hybrids were intercrossed to form B6D2F2 progeny (F2) . Backcross progeny were obtained by crossing F1 females with either B6 (BcB6) or D2 (BcD2) males. Reciprocal crosses were not formed in order to examine maternal effects, and only male offspring were used in the following studies. Litters were not culled, and mice were weaned between 19 and 21 days of age. Mice were housed two to four to a cage in a constant-temperature colony room with a 12 L:12 D cycle. Single housing of mice was avoided. Food and water were provided ad lib. Mice obtained from Jackson Laboratory were allowed a minimum of 2 weeks to acclimatize to the colony environment before behavioral testing or breeding. Testing was conducted between 1000 and 1600 h. All animal care and testing protocols were approved by the Laboratory Animal Users Committee at the State University of New York at Stony Brook, and conformed to the NIH Guidelines for Using Animals in Intramural Research.

Fear Conditioning Apparatus

Fear conditioning was conducted in one chamber of a Coulbourn Instruments mouse shuttlebox (18 × 16 × 21 cm high). The floor of the chamber consisted of 3.0-mm steel rods spaced at 1.0-cm intervals and connected to a constant-current shock generator (Coulbourn Instrument Model E13-12). A 1.0 s 500-μA scrambled foot shock was used as the US. The CS comprised a 7-W house lamp mounted on the wall, 20 cm above the floor. The shuttlebox was housed within a sound-attenuating chamber. A fan mounted on one wall of the chamber was used for ventilation and background noise. Six shuttleboxes were connected to a Zeos 386 computer via the Coulbourn Instruments environmental interfaces (E91-12) and data ports (L91-12). The L2T2 Lablinc Test Table software (Coulbourn Instruments) was used to construct the fear-conditioning training schedules and for operant control of the shuttleboxes.

Startle Response Apparatus

A Coulbourn Instruments startle response acoustic test system was used to evaluate the FPS response. Startle platforms were coupled to strain gauge transducers for detection of the response. The signal from each platform was digitized and a 200-ms portion of the signal, initiated by the startle stimulus, was analyzed. The strain gauges were calibrated over a 10–100-g range, with the animal restraining cages (8 × 15 × 5 cm high) in place. The startle stimuli were generated by a voltage controlled oscillator, amplified by a Coulbourn Instruments acoustic pulse power amplifier, and delivered to the test chamber by speakers mounted in the floor and ceiling. Stimulus amplitude was determined by a Klark-Tecknik DN 60 Real-Time Sound Analyzer. The stimulus waveform was shaped with a rise/fall gate to conform to a linear envelope

with a 2.0 ms rise/fall time. The four startle platforms and speakers were housed within a single test chamber (50 × 50 × 30 cm high) lined with 4 cm of acoustic foam. The Coulbourn apparatus was modified slightly to accommodate the FPS test. A 7-W lamp identical to that used in the fear conditioning trials was mounted in a specially constructed holder 20 cm above each platform. Two output ports on the Coulbourn system were used to control the CS presentation. A signal through one port started a Coulbourn Universal timer (S53-21) connected to a 28-V power driver (S61-05) that controlled the CS. The startle stimulus was delivered during the last 80 ms of the 10-s CS presentation. A signal through the second port was used to switch off and reset the timer following the startle stimulus. A fan mounted in the floor of the chamber provided ventilation during the intertrial intervals. The background noise level within the chamber was less than a 30-dB sound pressure level (SPL).

PROCEDURE

Fear Conditioning

The effect of 1, 5, 20, or 40 fear conditioning trials on the FPS response was examined in naive male B6 and D2 mice (n = 5–12 mice/group) and male F2 mice (n = 796) between 6 and 7 weeks of age. Each mouse was placed in one chamber of the dark shuttlebox and allowed to habituate for 5 min. The 7-W house lamp (CS) was then presented for 10 s. During the last 1.0 s of the CS interval, the US (1.0 s scrambled 500-μA foot shock) was delivered through the steel rods of the floor. Each CS-US trial was followed by an intertrial interval (ITI) that ranged from 30–110 s (mean duration = 70 s). Additional groups of naive male 6–7-week old B6 and D2 mice (six to eight mice/group) were used as controls for the fear conditioning. These mice were subjected to a modified fear conditioning paradigm consisting of 20 trials on which the CS and US presentations were randomized to minimize the formation of CS-US associations [noncontingent CS-US group; (46)]. All conditioning or control trials were delivered within a single session. Mice from the C3H strain were not subjected to fear conditioning.

Salience of Acoustic Stimuli

Frequency response curves were generated using the B6 and D2 strains to select a stimulus with equal saliency for use in the FPS paradigm. In addition, a group of male C3H mice of similar age were included for comparison because this strain does not suffer from the age-related hearing deficits found in B6 and D2 mice (36,27). The effect of white noise, 5-, 10-, 15-, 20-, and 25-kHz startle stimuli (100 dB, SPL; 80-ms duration) on ASR amplitude in groups of naive male 5-week-old B6, D2, C3H, and 6–7-week-old F2 mice was examined. Mice were placed in the holders, put in the startle chamber, and allowed to habituate for 2.0 min. The test session consisted of an orienting 120-dB (SPL) noise burst (60 ms duration) followed by eight blocks of seven trial types delivered in pseudorandom order, with a mean intertrial interval of 20 s (range = 10–30 s). Trial types 1 through 6 comprised white noise, 5-, 10-, 15-, 20-, or 25-kHz stimuli delivered at 100 dB (SPL) for 80 ms. Trial type 7 was a null trial on which no stimuli were presented. Transducer output on this trial was considered baseline, and was used in the calculation of ASR amplitude. ASR amplitude was expressed as a percent of the null trial: [ASR (g)/null trial (g)] × 100 for each trial type. For comparisons, the ASR associated with each frequency was normalized to the response evoked by the 10-kHz tone.

FPS Paradigm

The FPS response was assessed in all mice 24 h following the conditioning trials. The acoustic stimulus used in this paradigm was based on the frequency response curves described in the preceding section. Each FPS session was initiated by a 5-min habituation period followed by a 120- dB (SPL) 60-ms noise burst. Data from this trial were not used in the analysis. A session consisted of 30 trials divided into 10 blocks of three trial types/block. Trial type 1 (noise-alone) consisted of an 80-ms 120-dB (SPL) noise burst, and was considered the baseline startle response trial. Trial type 2 was a light + noise trial in which the CS was presented for 10 s and the 80-ms 120-dB (SPL) noise burst was delivered during the last 80 ms. Trial type 3 was a null trial on which neither the startle stimulus nor the CS were presented. Trial types were presented in pseudorandom order separated by an ITI of 15–55 s (mean = 35 s). ASR amplitude for each trial type was recorded in grams, but was converted to a percent relative to the null trial: [ASR(g)/null trial (g)] × 100. The FPS response was defined as: light + noise ASR − noise-alone ASR. In some mice, the FPS response was negative, indicating that the light + noise ASR was lower than the noise-alone ASR.

Biometrical Genetic and Statistical Analysis

The inheritance of the FPS response was assessed using the genotypic means and variances observed in the parental strains and hybrid groups. Quantitative genetic analysis, consisting of joint scaling tests with theoretically defined contrast vectors as predictors of additive, dominance, and epistatic genetic effects were used to assess the inheritance of the FPS response, and are presented in Table 1 [for a detailed description of these, see (36)]. The goodness-of-fit of the observed FPS response to each of these models was assessed using the χ^2 statistic. Broad-sense heritability (h^2B), the proportion of phenotypic variance accounted for by all sources of genetic variance, was estimated by:

$$\mathrm{Var}_{F2} - (\mathrm{Var}_{B6} + \mathrm{Var}_{D2} + \mathrm{Var}_{F1})/\mathrm{Var}_{F2}.$$

Multivariate analysis of variance procedures were most frequently used for the analysis of the data (GB-STAT: Dynamic Microsystems, Inc.). The light + noise and noise-alone trial types were routinely treated as repeated measures. The analysis of the FPS response (i.e., the difference between the light + noise and noise-alone ASRs) was analyzed using a one-way ANOVA. Post hoc comparisons were done using the Tukey–Kramer procedure.

RESULTS

Salience of Acoustic Stimuli

The effect of stimulus frequency on the ASR is shown in Fig. 1. The amplitude of the ASR varied according to the group, $F(3, 138) = 14.9$, $p < 0.0001$, stimulus frequency, $F(5, 690) = 69.6$, $p < 0.0001$, and the interaction of group × frequency, $F(15, 690) = 8.6$, $p < 0.0001$. Stimuli of 5 or 25 kHz did not elicit a measurable ASR in any of the groups ($p > 0.05$). The highest ASRs were observed in the B6 strain following the noise burst, 10-, and 15-kHz stimuli ($p < 0.01$ compared to the C3H, D2, and F2 groups; Fig. 1). The F2 group generally displayed the lowest startle responses and a relatively flat fre-

TABLE 1

GENETIC MODELS USED FOR THE BIOMETRICAL ANALYSIS OF THE INHERITANCE OF FPS

Genotype	Additive-Dominance Parameters	Additive-Dominance and Epistasis
B6 (P_1)	$m + [d]$	$m + [d] + [i]$
BcB6 ($F_1 \times P_1$)	$m + 1/2[d] + 1/2[h]$	$m + 1/2[d] + 1/2[h] + 1/4[i] + 1/4[j] + 1/4[j]$
F_1	$m + [h]$	$m + [h] + [l]$
F_2	$m + 1/2[h]$	$m + 1/2[h] + 1/4[l]$
BcD_2 ($F_1 \times P_2$)	$m - 1/2[d] + 1/2[h]$	$m - 1/2[d] + 1/2[h] + 1/4[i] + 1/4[j] + 1/4[l]$
$D_2(P_2)$	$m - [d]$	$m - [d] + [l]$

Models used to analyze the inheritance of the FPS response: m = constant; d = additive genetic effects; h = dominant genetic effects; i = epistatic interaction of homozygous pairs of alleles at different loci; j = epistatic interaction of heterozygous and homozygous pairs of alleles; l = epistatic interactions between heterozygous pairs of alleles. A least-squares technique was used to fit the observed genotypic means and their weighting factors ($1/(SEM)^2$) to each of these models. The goodness-of-fit to each model was assessed using a χ^2 statistic. A detailed description of these models can be found in Mather and Jinks (36).

quency response curve (Fig. 1), but no statistically significant differences were found between these mice and the D2 and C3H strains ($p > 0.05$).

Normalization of the frequency response curves to the ASR evoked by the 10-kHz tone was used to assess the saliency of the acoustic stimuli (Fig. 1; bottom panel). The normalized ASR varied according to the group, $F(3, 138) = 6.38$, $p < 0.0005$, frequency of the stimulus, $F(4, 552) = 69.62$, $p < 0.0001$, and the interaction of group $\times$ frequency, $F(12, 552) = 5.86$, $p < 0.0001$. The ASR elicited by 15- and 25-kHz stimuli decreased in all groups but the F2 group. The F2 group displayed a greater ASR at this frequency than the other groups ($p < 0.01$; Fig. 1). The ASRs in B6, D2, and C3H strains were similar following the noise burst, 5-, and 15-kHz stimuli ($p > 0.05$; Fig. 1). F2 mice displayed similar responses following the noise burst or 15-kHz stimuli but greater responses following the 5-kHz stimulus ($p < 0.01$). The curves obtained from the D2 and C3H strains were similar, although the C3H curve was somewhat flatter (Fig. 1). The response was lower in the B6 strain than the C3H and D2 strains following the 20- and 25-kHz tones ($p < 0.01$). Although the normalized ASRs suggested that the groups displayed similar responses up to 15 kHz, the noise burst was eventually selected as the acoustic stimulus for use in the FPS paradigm. This was largely based on the observation that this stimulus is most frequently used in FPS studies that involve rats.

Acquisition of the FPS Response

The effect of varying the number of fear conditioning trials on the amplitude of the light + noise and noise-alone ASR, and the difference between these trial types is shown in Fig. 2. Multivariate analysis using strain and the number of trials as single factors and the noise-alone and light + noise trial types as repeated measures found significant main effects for strain, $F(1, 82) = 20.6$, $p < 0.0001$, the number of conditioning trials, $F(3, 82) = 8.1$, $p < 0.0001$, and the interaction of strain $\times$ number of trials, $F(3, 82) = 5.5$, $p < 0.001$. No main effects for trial type were detected, $F(1, 82) = 0.3$, $p > 0.05$, but interactions were found between trial type $\times$ number of conditioning trials, $F(3, 82) = 12.1$, $p < 0.0001$, and strain $\times$ number of conditioning trials $\times$ trial type, $F(3, 82) = 28.9$, $p < 0.0001$. Subsequent analysis determined that the noise-alone ASR was lower in the D2 strain than the B6 strain, regardless of the

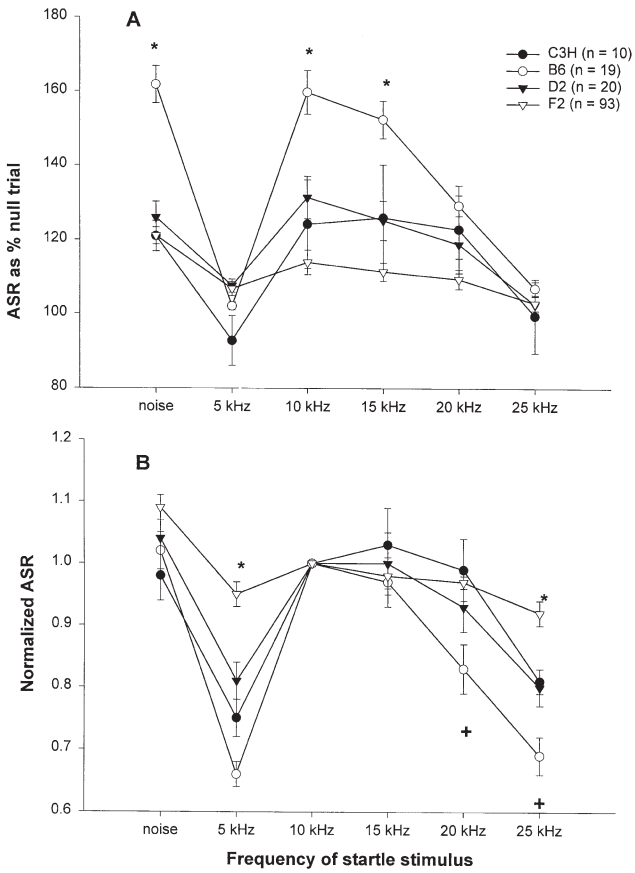

FIG. 1. (A) The effect of 100 dB (SPL) startle stimuli of varying frequencies on the amplitude of the ASR in the C3H, B6, D2, and F2 mice. (B) The startle response at each frequency was normalized to the ASR observed following the 10-kHz stimulus (normalized ASR at 10 kHz = 1.0). *$p < 0.01$ compared to each of the other groups; +$p < 0.01$ compared to the D2 and C3H strains.

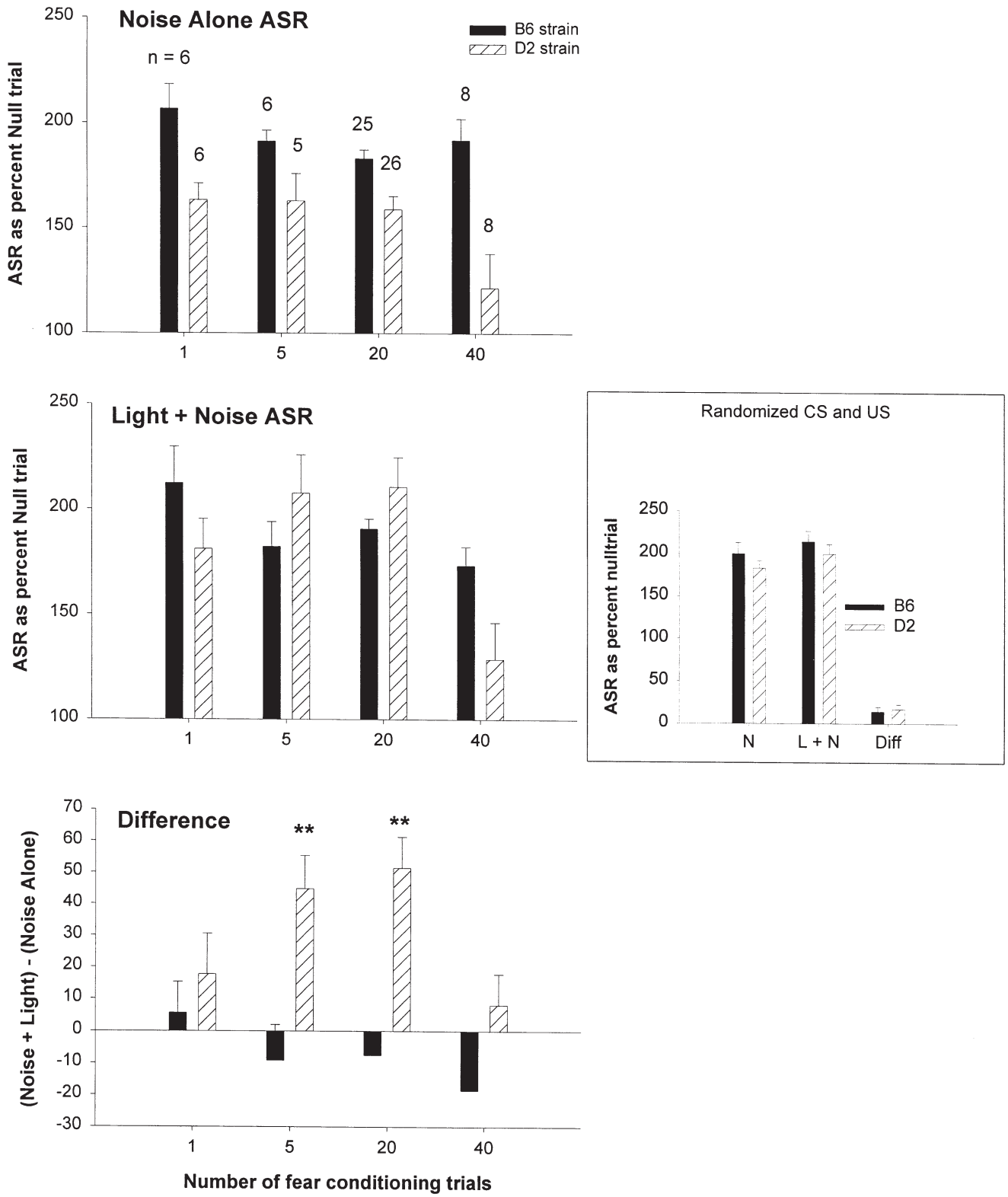

FIG. 2. The effect of 1, 5, 20, and 40 fear conditioning trials on light + noise and noise-alone ASRs in the B6 (n = 6, 6, 12, and 8, respectively) and D2-alone strains (n = 6, 5, 12, and 8, respectively). The effect of randomized CS and US presentations on the noise-alone (N) and light + noise (L+N) trial types and their difference (Diff) is presented in the small panel. All data are expressed as the mean ± SEM. *$p < 0.05$; **$p < 0.01$.

number of conditioning trials. Within each strain, the noise-alone ASR did not vary with the number of conditioning trials ($p > 0.01$). Furthermore, the two trial types were similar in B6 mice, regardless of the number of conditioning trials ($p > 0.05$; Fig. 2), while differences were noted in D2 mice following 5–20 conditioning trials ($p < 0.01$). In D2 mice subjected to 40 conditioning trials, the amplitude of the light + noise ASR was lower than mice subjected to 1, 5, or 20 trials ($p < 0.01$). The amplitude of the light + noise ASR also varied in B6 mice following 40 trials, but only compared to the ASR following a single trial ($p < 0.05$; Fig. 2).

The FPS response, defined as the difference between the light + noise and noise-alone trial types was analyzed using a two-way ANOVA. Significant effects were detected for strain, $F(1, 82) = 29.5$, $p < 0.0001$, the number of conditioning trials, $F(3, 82) = 3.2$, $p < 0.03$, and the interaction of strain × conditioning trials, $F(3, 82) = 4.58$, $p < 0.005$. In the D2 strain, a single fear conditioning trial did not result in a significant FPS response ($p > 0.05$). The greatest FPS response in this strain was observed following 5 or 20 conditioning trials ($p < 0.01$). D2 mice subjected to 40 conditioning trials displayed a lower FPS response than mice subjected to 5 or 20 trials ($p > 0.05$; Fig. 2). The B6 strain did not display an FPS response, regardless of the number of conditioning trials.

The effect of randomizing the order of the CS and US on the FPS response is shown in the inset in Fig. 2. The amplitude of the light + noise and noise-alone ASR did not vary within either strain ($p > 0.05$). Similarly, the difference between these trial types did not vary ($p > 0.05$). The difference in each group was subsequently compared to mice subjected to the conventional conditioning paradigm consisting of 20 fear conditioning trials. The response in B6 mice was similar between the experimental and control groups ($p > 0.05$). In the D2 strain, the difference between the two trials was greater in mice subjected to 20 fear conditioning trials than mice subjected to the control paradigm ($p < 0.01$).

Inheritance of the FPS Response

The inheritance of the FPS response was examined in the parental strains, F1 hybrids, backcross (BcD2 and BcB6), and F2 progeny following 20 fear conditioning trials. The distribution of the genomic means for the noise-alone and light + noise ASRs and the difference between these is shown in Fig. 3. The amplitude of the ASRs, regardless of the trial type, was markedly lower in each of the hybrid groups than the parental strains. Analysis of these trial types using them as repeated factors in a multivariate design found group and trial type effects, $F(5, 792) = 25.3$, $p < 0.0001$, and $F(1, 792) = 29.6$, $p < 0.0001$, respectively, and a group × trial type interaction, $F(5, 792) = 15.8$, $p < 0.0001$. The noise-alone ASR was greater in the B6 strain than any of the other groups ($p < 0.01$). Analysis of the difference in ASR amplitude between these trial types (i.e., the FPS response) found significant variability, $F(5, 792) = 16.4$, $p < 0.0001$. Post hoc analysis found that the difference was greater in the D2 group than all other groups but the BCD2 group ($p < 0.05$).

The inheritance of the difference in ASR amplitude between the two trial types was examined using the biometrical models described in Table 1. A least-squares technique was used to fit the mean FPS response from each group to each of these models. The distribution of means was best described by a simple additive genetic model of inheritance (Table 2). The broad-sense heritability (H^2B), a measure of the proportion of phenotypic variance accounted for by all sources of genetic variance, was estimated at 0.46.

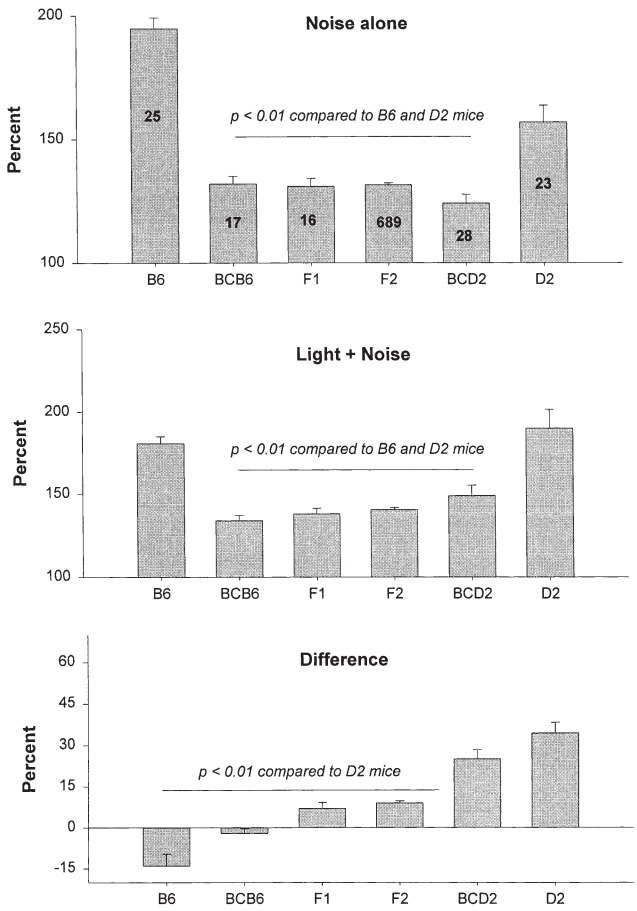

FIG. 3. Noise-alone and light + noise ASRs and the difference following 20 fear-conditioning trials in the B6, D2, and hybrid groups of mice. The number of mice/group is presented within each bar. All measures represent the mean ± SEM.

FPS Response in the F2 Mice

The FPS response was examined in a total of 796 F2 males. Eleven mice were dropped from the study because the noise-alone ASR was less than 100%. Furthermore, it was unclear if

TABLE 2

BIOMETRICAL GENETIC ANALYSIS OF FPS RESPONSE

Genotype	Observed Mean*	Observed SEM	*n*	Predicted Mean*
B6 (P_1)	−14.4	3.3	28	−15.5
BcB6 ($F_1 \times P_1$)	−2.0	2.0	31	−3.3
F_i	7.0	2.2	20	8.9
F_2	8.7	0.8	689	8.9
BcD2 ($F_1 \times P_2$)	25.0	3.3	22	29.1
D_2 (P_2)	34.4	3.8	26	33.2

*At least-squares technique (36) was used to fit the observed genotypic means and their weighting factors ($1/(SEM)^2$) to each of the models described in Table 1. The observed genotypic means were most closely described by a simple additive model of inheritance with a constant value $m = 8.9 \pm 0.7$ and an additive genetic parameter $d = 24.4 \pm 4.0$ ($\chi^2 = 2.97$, $df = 3$, $p > 0.05$). Broad-sense heritability (H^2_B) was estimated as 0.46.

a low noise-alone ASR was the result of low startle reactivity or a reflection of impaired hearing. Because it is generally better to adopt a conservative approach to the censoring of data than to risk assigning an incorrect phenotype to a mouse, mice with noise-alone ASRs less than one standard deviation below the F2 mean were dropped from the study. Because the magnitude of the FPS response was defined by the difference between the two trial types, there was a tendency for mice with low noise-alone ASRs to display high FPS responses. This bias resulted from the floor effects associated with ASR amplitude (i.e., ASR amplitudes could not be less than 100%). The elimination of mice with low baseline startle amplitudes served to minimize this bias. The F2 mean and standard deviation were 132 and 22%, respectively. Therefore, mice with noise-alone ASRs $< 112\%$ ($n = 96$) were excluded from the study. Of these, 20 of 689 were considered high FPS response phenotypes (see the following section for a description of these phenotypes), while only 12 of 689 were considered low FPS response phenotypes. The remaining 62 mice were intermediate FPS response phenotypes.

The distribution of the noise-alone ASR, light + noise ASR, and the difference between these trial types is shown in Fig. 4. The noise-alone and light + noise ASRs were not normally distributed (noise alone $p < 0.003$, and light + noise $p < 0.007$, Kolmogorov–Smirnov two tailed). However, the difference between these was normally distributed (Kolmogovov–Smirnov $p > 0.05$, two tailed). The association between the two trial types and their difference was examined across the F2 sample. The light + noise and noise-alone ASRs were highly correlated ($r = 0.73$, $p < 0.0001$; Fig. 5).The light + noise ASR was also highly correlated with the difference measure ($r = 0.70$, $p < 0.0001$; Fig. 5). However, no correlation was detected between the noise-alone ASR and the difference measure ($r = 0.02$, $p > 0.05$; Fig. 5).

Extreme FPS Response Phenotypes in the F2 Distribution

The entire F2 sample was divided into groups of mice characterized by the magnitude of their FPS response: each tail of the F2 distribution comprised mice with extremely high or extremely low FPS responses. These phenotypic extremes were defined as mice with FPS responses greater than one standard deviation from the F2 mean. Thus, 33% of the FPS distribu-

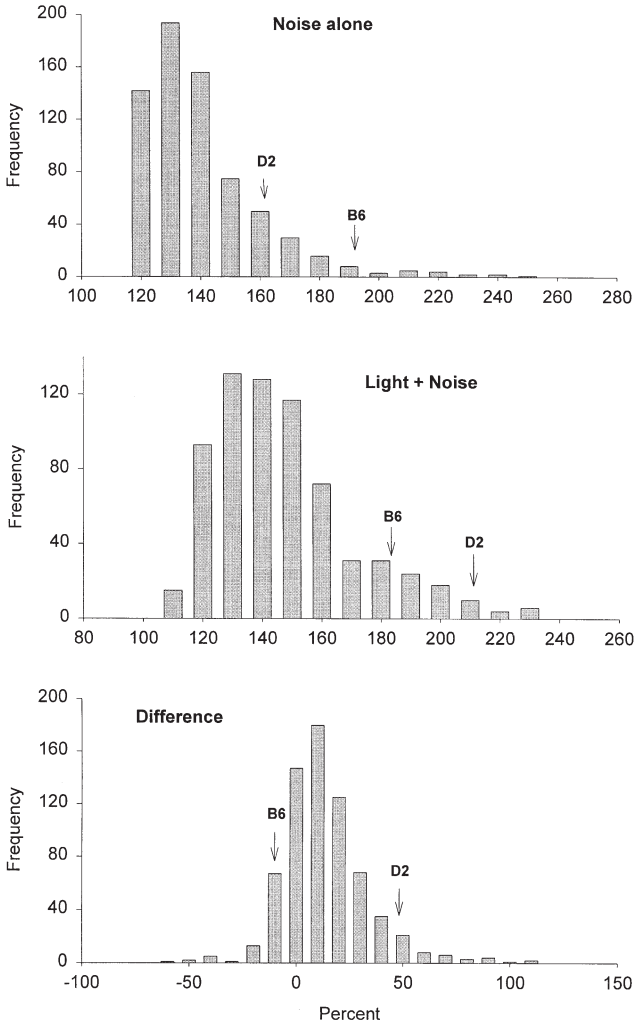

FIG. 4. The distribution of the noise-alone ASR, light + noise ASR, and the difference in F2 mice. The approximate positions of the D2 and B6 means are indicated by the arrows.

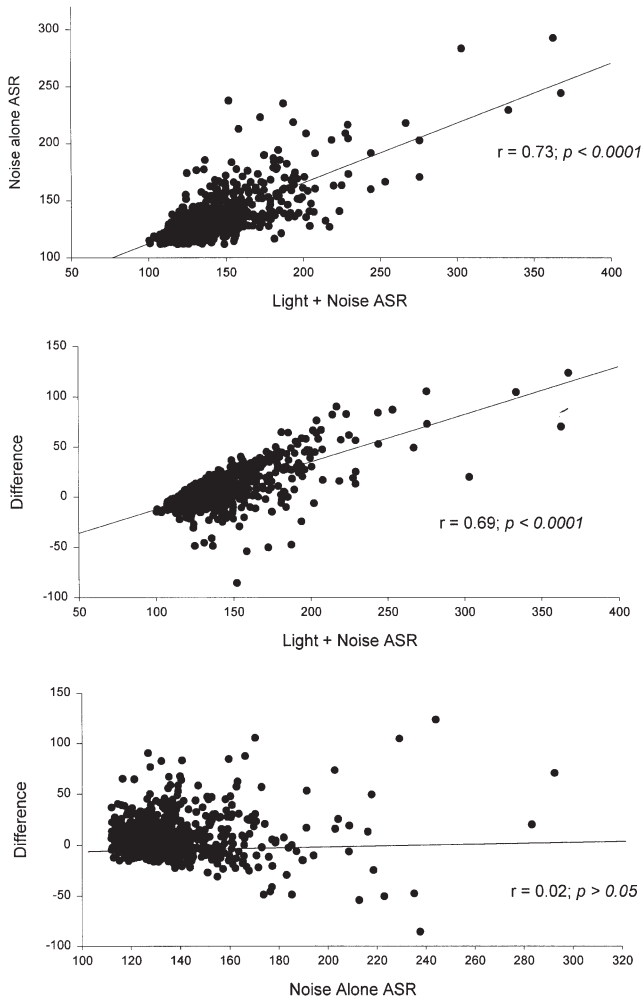

FIG. 5. Correlations between the noise-alone ASR, light + noise ASR, and the difference in F2 mice ($n = 689$).

TABLE 3
FPS RESPONSE PHENOTYPES IN F_2 MICE.

Phenotype		Trial Type: Noise alone	Trial Type: Light + Noise	Difference
HFPS	mean	143.9	186.4†‡	42.5†
$n = 114$	SD	26.8	39.1	19.3
	range	(112.0–244.2)	(128.1–368.0)	(20.0–123.8)
Intermediate	mean	131.1	137.6	6.5
$n = 461$	SD	17.7	19.9	8.4
	range	(112.1–283.3)	(100.0–303.4)	(−12.5–22.5)
LFPS	mean	147.44	131.0‡	−16.3
$n = 114$	SD	24.7	18.4	11.7
	range	(112.6–237.7)	(100.5–187.7)	(−86.6–7.1)
Total F_2 group	mean	135.9	144.7	8.7
$n = 689$	SD	21.8	30.4	20.7
	range	(112.0–283.3)	(100.0–368.0)	(−86.6–123.8)
F_2 mice censored	mean	107.7	115.8	8.1
from the	SD	2.8	10.3	10.0
data set	range	(100.0–111.9)	(100.0–158.5)	(−7.7–46.9)
$n = 96$*				

*F_2 mice with noise-alone ASRs < 112% that were eliminated from the study (see Results for details)
†$p < 0.01$ compared to the LFPS phenotype; ‡$p < 0.01$ compared to the noise-alone startle response.

tion consisted of mice with high (HFPS phenotype; $n = 114$ mice) or low FPS responses (LFPS phenotype; $n = 114$ mice). A total of 16.5% of the entire distribution comprised each of the tails. Mice that displayed FPS responses between these were classified as intermediated phenotypes ($n = 461$ mice). The ASR for each trial type and the FPS response in each of these groups is shown in Table 3. Analysis of the noise-alone and light + noise ASRs found group and trial type effects, $F(2, 686) = 90.9$, $p < 0.0001$, and $F(1, 686) = 416.1$, $p < 0.0001$, respectively, and a group × trial interaction, $F(2, 686) = 1019.1$, $p < 0.0001$. The noise-alone ASR did not differ between HFPS and LFPS groups ($p > 0.05$; Table 3). However, the ASR was greater in each of these groups than the Intermediate FPS group ($p < 0.05$; Table 3). As expected, the light + noise ASR was greater than the noise-alone ASR in the HFPS group ($p < 0.05$) and lower than the noise-alone ASR in the LFPS group ($p < 0.01$; Table 3).

Mice censored from the study because of low noise-alone ASRs are also presented in Table 3. As expected, the noise-alone ASR was significantly lower in this group than the extreme and intermediate FPS phenotype groups ($p < 0.0001$). In addition, the amplitude of the light + noise ASR from the censored mice was also lower than that observed in each of these groups ($p < 0.0001$). However, the difference measure in this group was similar to that observed in the total F2 group and in mice with an intermediate FPS phenotype ($p > 0.0001$).

To determine if the phenotypic differences in the FPS response were possibly related to variability in auditory sensitivity, frequency response curves were generated using a small number of F2 mice from each of the extreme and the intermediate phenotype groups. The amplitude of the normalized ASR in mice representing the HFPS ($n = 12$), LFPS ($n = 12$), and Intermediate FPS phenotypes ($n = 54$) is presented in Fig. 6. No significant differences were detected between these groups ($p > 0.05$).

DISCUSSION

Falls et al. (20) used the repeated pairing of an auditory CS with an aversive foot shock to produce fear conditioning in the B6 and D2 strains. The present study, in which conditioned fear was elicited by the repeated pairing of a visual CS with an aversive US, confirms these results and provides additional information concerning the inheritance of the FPS response in these strains. As noted by Falls et al. (20), the D2 strain display a significantly greater FPS response than the B6 strain. Indeed, a number of B6 mice displayed light + noise ASRs less than the noise-alone response. A number of control conditions were examined to determine the role, if any, that reactivity to the testing procedures or other unconditioned effects may play in the strain-related differences in FPS response. Because neither strain displayed a significant FPS response following a conditioning paradigm in which the CS and US were explicitly unpaired (46), the results strongly sug-

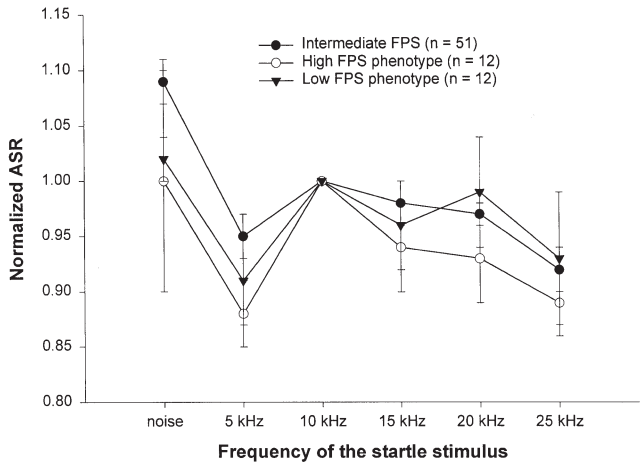

FIG. 6. The effect of 100 dB (SPL) startle stimuli of varying frequencies on the normalized ASR (see text or Fig. 1) in F2 mice from the LFPS, HFPS, and Intermediate FPS phenotypes. The normalized ASR observed at each of the frequencies did not differ between these groups and the Intermediate FPS group.

gest that the formation of the CS-US association is crucial for the response.

Falls et al. (27) used the same auditory stimulus in the fear-conditioning paradigm as the FPS paradigm, which could be problematic without the appropriate controls. Because these strains differ markedly in their susceptibility to age-related auditory deficits (6,27,42,57–60), it is crucial that possible strain-related differences in the saliency be eliminated from the study. In the present study, a visual CS was used in the conditioning paradigm, and the potentiation of the ASR in its presence was used to assess the FPS response. This paradigm largely avoids the problems caused by differences in auditory sensitivity. Furthermore, by using the difference between the noise-alone and light + noise trial types to define the magnitude of the FPS response, it is unlikely that strain-related differences in FPS are related to auditory sensitivity. Nevertheless, frequency response curves from each strain were examined, and the acoustic stimulus used in this study was selected based on these. Analysis of these curves indicated that the salience of the noise burst was similar between B6 and D2 mice. More importantly, a similar analysis of the phenotypic extremes (i.e., the LFPS and HFPS groups) also failed to detect a relationship between the magnitude of the FPS response and salience of acoustic stimulus. Finally, a recent article by McCaughran et al. (39) demonstrated that only a weak relationship exists between the amplitude of the ASR and the age of onset of high-frequency hearing loss in mice derived from the intercross of the B6 and D2 strains. Although a number of methodological differences may exist between the present study and that of Falls et al. (20), the results of these studies are generally compatible.

Elucidation of the mechanisms underlying the strain-related differences in the FPS response will require additional studies. Because the FPS response is considered to reflect the degree of fear and/or anxiety associated with fear conditioning (14,15,52), it is reasonable to suggest that differences in emotionality may contribute to the contrasting FPS responses. For example, open-field activity and the number of light–dark transitions are greater in B6 mice (8–10,37). These studies suggest that D2 mice have a higher baseline level of emotionality than B6 mice, and that this would be consistent with a greater FPS response. Alternatively, given that the strains also differ markedly in their performance on simple and complex learning tasks (35,43,45,49,50,55), differences in the acquisition of conditioned fear should also be considered. In D2 mice, the magnitude of the FPS response was clearly related to the number of fear conditioning trials. In contrast, the B6 strain displayed little evidence of FPS, regardless of the number of conditioning trials. Although this is consistent with a deficit in the acquisition of fear conditioning, this hypothesis is not supported by existing research. For example, a number of studies demonstrate that the B6 strain is extremely susceptible to fear conditioning, as measured by the effect of explicit or contextual cues on locomotor activity (41,44,54). In fact, the cue fear response is similar in the B6 and D2 strains (41,44). Because the FPS response, under the present experimental conditions, is akin to the cued fear response in a locomotion paradigm, these observations are at odds with the present study. The most reasonable hypothesis to explain these differences is that the locomotor correlates and the FPS response assess different emotional components of fear conditioning. Locomotor activity is clearly more sensitive to the effects of fear conditioning than the FPS response, at least with regard to the D2 and B6 strains. In general, decreased locomotor activity is evident following a single fear conditioning trial in mice (41), whereas 5–20 trials were required to evoke a maximal FPS response in the D2 strain. The lack of contextual cues in the FPS paradigm may, in part, contribute to the differences between these measures of fear, although the mechanism by which this might occur is currently unknown.

The inheritance of the FPS response was best described by a simple additive model. The response was highly heritable with a broad sense heritability of 0.46. By comparison, the heritability of the cued and contextual fear responses is approximately 0.29 (41). The continuous distribution of the two trial types and the difference measure in the F2 population further suggests that each is under the control of multiple genes. The nonnormality of the light + noise and noise-alone distributions may, in part, be due to floor effects encountered because the startle amplitude could not be less than 100%. Over dominance may also be an important factor [i.e., the heterozygote phenotype (the ASR response in hybrid mice) lies outside the phenotypic range of the homozygous parental strains]. The reduction in startle amplitude observed in the hybrid groups is consistent with a previous observation from this laboratory (56).

Although the strain-related deficits in auditory sensitivity can be minimized by following the procedures described in this report, analysis of the FPS response and the characterization of the phenotypic extremes in a large group of F2 mice could be problematic. For example, Willott and others (6,27,42,57–60) provide convincing evidence that the D2 and B6 strains are not only predisposed to age-related hearing deficits but that a number of "deafness" genes exist that contribute to this trait (18,19). Although only a weak relationship has been found between the ASR and hearing-related deficits like cochlear pathology in strains derived from the B6 and D2 strains [e.g., the BXD recombinant inbred strains; (39,61)], the transgressive segregation of "deafness" genes in F2 mice could result in some mice displaying auditory deficits that are more extreme than either parent. Because this group of F2 mice will eventually be used for the mapping of chromosomal loci associated with the FPS response (i.e., QTL analysis), it is crucial that the behavioral phenotype be accurate and not confounded by extraneous variables like auditory sensitivity. The censoring of the data set removed approximately 12% of the mice from the study. Although mice that displayed both noise-alone ASRs $<112\%$ and light + noise ASRs $<112\%$ (the difference score $\sim 0\%$) may reflect a valid FPS response phenotype, they could also reflect mice with poor hearing. Other than to switch to strains that do not harbor auditory deficits or to test all mice using evoked potential techniques, this may be the only effective way to eliminate mice with poor hearing. Alternatively, auditory thresholds could be examined in those mice dropped from the study to gauge the effectiveness of the censoring process. This approach is currently being examined in this laboratory. The censoring of the data set also served to minimize a bias that was detected in the study. Because the ASR amplitude could not be less than 100%, a disproportionate number of mice with low startle responses displayed high FPS responses. For example, 20 of the mice censored from the study were HFPS phenotypes, while only 12 were LFPS phenotypes. Although the censoring of these mice from the study may reduce the statistical power of the QTL analysis, their inclusion will present a similar problem if they do, in fact, represent a phenotype that differs from the HFPS and LFPS phenotypes defined in this study. As shown in Table 3, mice censored from the study not only displayed a mean noise-alone ASR that was lower than the other phenotypic groups, but the mean light + noise ASR in these animals

was also lower. Together, these results support the hypothesis that the mice censored from the study may reflect a unique phenotype characterized by low startle responding possibly as a result of auditory deficits.

Falls et al. (20) speculated that low baseline ASRs were more readily potentiated in a fear-conditioning/FPS paradigm than high baseline ASRs. Therefore, mice with low ASRs like the D2 strain would tend to show greater FPS responses than a strain with a high-baseline ASR like the B6 strain. Analysis of the light + noise and noise-alone ASRs and the FPS response in F2 mice failed to support this hypothesis. Although significant correlations were detected between the light + noise and noise-alone ASR and between the light + noise and difference measure in F2 mice, no correlation was detected between the noise-alone and difference measure. The noise-alone ASR in the LFPS and HFPS groups were also similar, which supports the hypothesis that the baseline startle response has little predictive power with regard to the FPS response. The extreme phenotypes displayed by the HFPS and LFPS groups could not be attributed to differences in auditory sensitivity. The frequency response curves obtained from the LFPS group, HFPS group, and mice with intermediate FPS phenotypes were similar, which suggests that the phenotypic differences in the FPS response are not the result of differences in the saliency of the startle stimulus.

Although the LFPS and HFPS groups represent a relatively small portion of the F2 population (33%), they contain much of the genetic information necessary for the eventual detection of loci or candidate genes associated with this behavior. The contrasting FPS responses found in the B6 and D2 strains, or the extreme FPS response phenotypes identified in F2 mice, could be used to examine the biobehavioral substrates of the fear conditioning process. Considerable evidence is currently available that implicates a number of forebrain and brainstem structures (3,14,15,22,32). In view of the crucial role played by the amygdala in fear conditioning (31,32), it is reasonable to speculate that the strain-related differences in the FPS found in the present study may be related to differences in amygdala function. Studies recently conducted in this laboratory found (1) a greater number of c-*fos*–reactive neurons in the central amygdaloid nucleus of the D2 strain than the B6 strain following moderate locomotor-activating doses of ethanol (29), and (2) the phenotypic extremes for this response were also characterized by marked differences in c-*fos* activity in the central amygdaloid nucleus (16).

Of the extreme phenotypic groups, the LFPS group was particularly interesting because the presentation of the CS to these mice was associated with a reduction in startle amplitude. It should be emphasized that this was not a "freezing" response. Reactivity to the CS was reduced but the light + noise ASR in some mice was relatively high. The emotional correlate of this response is speculative. The response displayed by the HFPS group is not unlike that reported in rat studies [e.g., (14)]. However, the reduction in startle reactivity in the presence of the CS has only been reported in rats following pharmacological manipulation [e.g., (12,30)]. The reduction in startle reactivity displayed by the LFPS group could, in fact, represent a more adaptive response to the CS. Predators are explicit cues within the environment and mice generally do not startle in their presence. As one final note, Walker et al. (51,53) reported that overtraining reduced the FPS response in rats. The reduction in the FPS response observed in D2 mice following 40 conditioning trials could reflect a similar phenomenon. If we assume that the ability to acquire conditioned fear varies continuously in F2 mice, it is reasonable to expect that some mice will rapidly acquire the response while others will not. If mice that rapidly acquire the response are subjected to additional conditioning trials, the FPS response may decline. It is possible that the LFPS group contains a large number of these animals.

In conclusion, the present study demonstrates that genetic factors play an important role in the FPS response in mice. More importantly, it provides evidence that the inheritance of the response is largely additive, highly heritable, and independent of the underlying genetic differences in auditory sensitivity. The variation in the response in F2 mice is continuous and indicative of a multigenic complex trait. The phenotypic extremes of the FPS response distribution consist of two groups of mice: the HFPS group and LFPS group. Selective genotyping of these extreme phenotypes is currently underway to determine the association between the FPS response and chromosomal loci containing genes that may underlie the behavior.

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