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GENETIC DISSECTION OF INTERMALE AGGRESSIVE BEHAVIOR IN BALB/cJ AND A/J MICE

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Abstract

Aggressive behaviors are disabling, treatment refractory, and sometimes lethal symptoms of several neuropsychiatric disorders. However, currently available treatments for patients are inadequate, and the underlying genetics and neurobiology of aggression is only beginning to be elucidated. Inbred mouse strains are useful for identifying genomic regions, and ultimately the relevant gene variants (alleles) in these regions, that affect mammalian aggressive behaviors, which, in turn, may help to identify neurobiological pathways that mediate aggression. The BALB/cJ inbred mouse strain exhibits relatively high levels of intermale aggressive behaviors, and shows multiple brain and behavioral phenotypes relevant to neuropsychiatric syndromes associated with aggression. The A/J strain shows very low levels of aggression. We hypothesized that a cross between BALB/cJ and A/J inbred strains would reveal genomic loci that influence the tendency to initiate intermale aggressive behavior. To identify such loci, we conducted a genome-wide scan in an F2 population of 660 male mice bred from BALB/cJ and A/J inbred mouse strains. Three significant loci on chromosomes 5, 10, and 15 that influence aggression were identified. The chromosome 5 and 15 loci are completely novel, and the chromosome 10 locus overlaps an aggression locus mapped in our previous study that used NZB/B1NJ and A/J as progenitor strains. Haplotype analysis of BALB/cJ, NZB/B1NJ, and A/J strains revealed 3 positional candidate genes in the chromosome 10 locus. Future studies involving fine genetic mapping of these loci as well as additional candidate gene analysis may lead to an improved biological understanding of mammalian aggressive behaviors.

Keywords

aggression; mouse; behavior; genetics; genome scan; linkage; association

INTRODUCTION

Aggressive behaviors are disabling, and sometimes lethal, symptoms of several human neuropsychiatric and neurodevelopmental disorders (Appelbaum, 2006, Freedman *et al.*, 2007, Jensen *et al.*, 2007, Nemeroff & Schatzberg, 1999) and are frequent occurrences

among members of the general population (www.who.int/violence_injury_prevention/violence/en/). A clinically-important aspect of aggression is the threshold for the initiation of physical attack (Davidson *et al.*, 2000, Dumais *et al.*, 2005, Frankle *et al.*, 2005, Jensen *et al.*, 2007, Kessler *et al.*, 2006). The many biological and psychosocial factors that may inhibit or disinhibit the initiation of aggressive behaviors are only beginning to be elucidated (Caspi *et al.*, 2002, De Almeida *et al.*, 2005, Jensen *et al.*, 2007, Miczek *et al.*, 2002, Volavka, 2002).

There is strong evidence that genetic factors play a role, together with environmental factors, in predisposing humans and other mammals towards aggression (Beitchman *et al.*, 2006, Brodtkin *et al.*, 2002, Brunner *et al.*, 1993, Caspi *et al.*, 2002, Coccaro *et al.*, 1997, Eisenberger *et al.*, 2007, Kim-Cohen *et al.*, 2006, Meyer-Lindenberg *et al.*, 2006, Reif *et al.*, 2007). Mouse models are useful for the genetic dissection of complex behavioral traits, due to the experimental control and genetic resources they afford (Yalcin *et al.*, 2004). Although progress has been made in inducing single gene mutations that affect mouse aggression, less progress has been made in identifying alleles (gene variants) that contribute to individual or strain differences in aggression (Maxson, 2000, Miczek *et al.*, 2001, Nelson, 2005, Pfaff *et al.*, 2005, Roubertoux *et al.*, 1994). Only two whole genome scans to identify genetic loci that influence intermale aggression have been reported in mice (Brodtkin *et al.*, 2002, Roubertoux *et al.*, 2005). In one of these, we identified loci on chromosomes 10 and X that affect the initiation of intermale attack behavior in a population derived from the aggressive NZB/B1NJ and the relatively unaggressive A/J inbred strains (Brodtkin *et al.*, 2002). However, multiple crosses and genome scans, using other inbred strains, are needed to identify additional, as yet unknown, sources of genetic variation in aggressive behaviors (Flint & Mott, 2001).

BALB/c mice show moderate to high levels of intermale aggression, as well as other behavioral and brain phenotypes relevant to several neuropsychiatric syndromes associated with aggressive behaviors (Bouwknicht & Paylor, 2002, Brodtkin, 2007, Brodtkin *et al.*, 2004, Caldji *et al.*, 2004, Cook *et al.*, 2001, Crowley *et al.*, 2006, Dougherty *et al.*, 2004, Ducottet & Belzung, 2005, Dulawa *et al.*, 2004, Fairless *et al.*, 2008, Guillot & Chapouthier, 1996, Jensen *et al.*, 2007, Jones & Brain, 1987, Kessler *et al.*, 1977, McCracken *et al.*, 2002, Mineur *et al.*, 2003, Moy *et al.*, 2007, Panksepp *et al.*, 2007, Sankoorikal *et al.*, 2006, Southwick & Clark, 1968). We report here a genome-wide search for genetic loci that affect aggressive behavior in BALB/cJ and A/J mice. We hypothesized that we would find a partially overlapping, but partially novel set of loci relative to our previous cross between NZB/B1NJ and A/J mice (Brodtkin *et al.*, 2002).

MATERIALS AND METHODS

Animal housing and breeding protocol

Mice were housed and tested for aggressive behaviors at Princeton University, during the time that E.S.B. was a postdoctoral associate and then a visiting scientist and in the Department of Molecular Biology, Princeton University, laboratory of Lee M. Silver. All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Princeton University Animal Care and Use Committee. BALB/cJ (C), A/J (A), and 129T2/SvEmsJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME) at 7–8 weeks age. C and A mice obtained from the Jackson Laboratory were used for breeding additional C and A mice and for breeding reciprocal F1 hybrids. All mice used for behavioral testing (including C, A, F1, and F2 mice) were bred at Princeton. Males were removed from breeding cages prior to the birth of pups. Pups were weaned at ~4 weeks of age. Male pups were individually-housed immediately upon weaning and remained individually-housed through the end of behavioral

testing. Individually-housed males were moved in their cages from the breeding colony room to the home room several weeks prior to behavioral testing, and behavioral testing was carried out in the home room. With the exception of a weekly cage change, the mice were not handled until after the completion of all behavioral tests. Animals were housed in temperature-controlled rooms with a 12 hr light/12 hr dark cycle (lights on at 7 A.M.). They were given Purina 5015 LabChow (Ralston Purina Company, St. Louis, Missouri) and water ad libitum.

Measurement of aggressive behavior

Testing of mice for aggressive behavior was carried out by H.C.D. and E.S.B. and was videotaped, using previously described methods (Brodkin *et al.*, 2002). Male resident mice were tested for aggressive behavior starting at 63–71 days-of-age. Mice from a relatively non-aggressive inbred strain, the 129T2/SvEmsJ strain, were used as standard intruders in aggression tests (Maxson, 1992, Roubertoux & Carlier, 1987). 129T2/SvEmsJ mice were 56- to 63-days-of-age on the first day that they were used in aggression testing. Each individually-housed resident mouse was tested once a day on three consecutive days, and testing was conducted between 7 A.M. and 2 P.M. The order in which mice were tested was varied randomly from day to day over the three days. Testing was conducted in a small area of the home room that was enclosed by a curtain and that was dimly lit (7 lux). Aggression was measured, one mouse at a time, using a modification of the resident-intruder test in which the intruder was restricted to one part of the cage, and thus restricted in its ability to initiate an aggressive interaction (Brodkin *et al.*, 2002, Kessler *et al.*, 1977, Scott & Fredericson, 1951). The cage top was removed from the home cage, and replaced with a Plexiglas top. Two minutes later, an intruder mouse was lowered through a hole in the Plexiglas cage top. The intruder's tail was taped to the Plexiglas cage top, so that the intruder's front two paws were fully resting on the cage bottom, and its back two paws were lifted off of the cage bottom and were resting against the side of the cage. The positioning of the intruder allowed the resident full access to direct (in contact) sniffing of the entire body of the intruder, from the nose to the anogenital region and tail. The positioning of the intruder's body also enabled the resident to bite any part of the intruder's body, from the head to the tail. The resident mouse was observed to see whether it would display aggressive behavior towards the intruder within 5 minutes (300 seconds). The focus of this study was the initiation of the aggressive attack bite, because this is the core feature of mouse aggression that is likely to be relevant to the initiation of aggressive attack behavior across mammalian species, including humans. Only unequivocal attack bites of at least moderate intensity were considered to be "attacks." The judgment of whether an episode constituted an attack was determined by a decision by H.C.D. and E.S.B. Inter-rater reliability was determined to be > 0.95. The test was stopped in 300 seconds or within 3 seconds of the start of an attack, whichever came sooner. Thus, within a single testing session there could be a maximum of only one attack. No intruder was used in more than one test per day. Each resident mouse was tested with three different intruder males over the course of three consecutive days of testing. Intruders were used in only one three-day series of behavioral testing. Any intruder that was bitten with high intensity in a test was not used again for subsequent days of testing.

Phenotypic analysis

The phenotype of interest in this study was initiation of aggressive attack behavior, which either occurs or it does not occur within a defined time period (300 second test). Initiation of attack behavior in only one test could be due to a random, non-replicable event. If, however, initiation of attack by a single mouse is observed in repeated tests, one can have greater confidence that the attack phenotype is present and influenced by genetic predisposition (Miczek *et al.*, 2001) We conducted 3 tests of aggression on 3 separate days, and therefore a

mouse could show initiation of aggressive attack in either 0, 1, 2, or a maximum of 3 times over the course of 3 days of testing. As shown in Figure 1, the phenotype data (number of attacks over 3 tests) of the various populations (parental strains, F1, F2) were not normally distributed, and, in fact, could not be transformed to a normal distribution using standard transformations (logarithmic, square root, reciprocal), as conducted by one of the authors, H.L. Thus, analysis of the trait as a continuous variable with parametric methods would not have been appropriate. Therefore, we used nonparametric tests for statistical analysis. Fisher's exact test was used to compare the aggression scores of the two inbred strains (C vs. A), as well as to compare the aggression scores of reciprocal F1 hybrids (CAF1 vs. ACF1). The significance threshold for these phenotype comparisons was $P < 0.05$.

Genotypic analysis

Of the original set of 675 F2 mice, tails of 14 mice were lost (lost tails were from 12 mice with aggression score 0; 1 mouse with aggression score 1; and 1 mouse with aggression score 2). This left a sample of 661 F2 mice available for genotyping, with aggression scores of 0 ($n = 453$), 1 ($n = 86$), 2 ($n = 74$), and 3 ($n = 48$). One of the 74 mice with an aggression score of 2 had insufficient DNA to complete genotyping at all markers, which left a set of 121 animals that with aggression scores of either 2 or 3 (the most aggressive 18.3% of the F2 population) that underwent genotyping at microsatellite markers spaced at approximately 20–30 cM intervals throughout the genome in stage 1 of genotyping (Silver, 1995).

Genetic analysis was conducted in the laboratory of E.S.B. at the University of Pennsylvania School of Medicine as described previously (Brodkin *et al.*, 2002) at microsatellite markers spaced at approximately 20–30 centiMorgan intervals across the genome. Chromosomal centiMorgan (cM) map positions of microsatellite markers were obtained from The Jackson Laboratory Mouse Genome Informatics (MGI) website (<http://www.informatics.jax.org/genes.shtml>) and physical location was obtained from the Ensembl Genome Browser (www.ensembl.org), Mouse Genome Build 37 (see Table S3 for list of all markers used in the genome scan). There was one contradiction between the MGI and Ensembl databases in terms of the relative order of markers along a chromosome (markers D15Mit122, D15Mit133, D15Mit123, and D15Mit144)—in this case, the order provided by Ensembl was accepted for mapping.

Linkage and association analysis

Statistical analysis was carried out using the R package software (www.r-project.org). A two-stage genotyping strategy was used. In stage 1 (genome-wide linkage analysis), all mice that were classified as reproducibly aggressive (attacked in 2 or 3 out of three tests), i.e. the most aggressive mice in the F2 population, were genotyped at markers spaced at approximately 20–30 cM intervals throughout the genome, in order to identify the markers that were linked with reproducible initiation of aggressive attack behavior. Then, in stage 2 (association analysis), all F2 mice were genotyped at the chromosomal regions that showed any suggestion of linkage in stage 1. Two-stage genotyping procedures are widely-employed, standard genotyping protocols that successfully identify significant genomic loci with much greater efficiency than genotyping all mice at all markers (Boulton *et al.*, 2003, Downing *et al.*, 2003, Lander & Botstein, 1989, Reed *et al.*, 2003, Remmers *et al.*, 1996, Silver, 1995). The highly aggressive F2 generation animals (aggression scores of > 2) were the most genetically informative in the genome scan. The less or un-aggressive F2 mice (aggression scores of < 1) were much less genetically informative, because an un-aggressive phenotype can be seen with either a C or and A genotype, due to the incomplete penetrance of the aggression phenotype in the more aggressive C strain (see Figure 1) (Brodkin *et al.*, 2002). For the autosomes, the number of aggressive animals with C/C, C/A, and A/A genotypes at each marker were compared and tested for a significant departure from the

expected 1:2:1 ratio (based on the null hypothesis of no linkage) with the likelihood ratio test, using the R package (www.r-project.org). For the X chromosome, the number of aggressive animals with a C vs. A genotype at each marker were compared and tested for a significant departure from the expected 1:1 ratio with the likelihood ratio test.

In stage 2 (association analysis), all F2 mice were genotyped at all those markers that showed an *uncorrected* (not Bonferroni corrected) $P < 0.05$ in stage 1; i.e. we conducted this association analysis at all markers that showed even a slight (but not significant) suggestion of linkage in stage 1. Since the data were ordinal, rather than normally distributed, we tested whether these markers were associated with aggressiveness by using the Kruskal-Wallis test (Hollander & Wolfe, 1973) to obtain the chi-square statistic for comparing aggression scores (0–3) among the 3 genotype groups (C/C, C/A, and A/A), using the function “*kruskal.test*” in the R package www.r-project.org. The threshold value for significance for the association analysis (stage 2) was determined using a Bonferroni correction ($0.05/131 = 0.000381679$) based on the total number of markers analyzed in the whole genome scan (131 markers).

We performed interval mapping for the significant loci on chromosome 5, 10, and 15 using the R/qtl program (Broman *et al.*, 2003), using the nonparametric methods and treating the aggression scores (0–3) as the phenotypes. We obtained the 95% confidence intervals for each locus based on 1,000 bootstrap sampling. To determine the LOD score significance thresholds for the three loci identified, we performed 1,000 permutations of the aggression scores, and, for each permutation, we obtained the maximum LOD scores across all three chromosomes. We then obtained the value of the 95th percentile of these maximum LOD scores, 2.64, as our LOD score threshold at the adjusted significance threshold level of 5%. Note that since we only used marker data on three chromosomes for this followup interval mapping analysis, this LOD score cutoff value is smaller than the usual genome-wide threshold.

To quantify the effect of each genetic locus on the risk of aggressive behavior, we performed a logistic regression analysis to obtain the odds ratios of aggressive behavior for each locus. We treated the A/A genotype group as the baseline group, and compared the risk of aggression in mice with C/A genotype vs. A/A genotype and C/C genotype vs. A/A genotype at each locus. We also tested for two-way epistatic (non-additive) interactions between genetic loci linked with aggressive behavior using logistic regression.

Haplotype and candidate gene analyses

In order to better define the identified loci associated with aggressive behavior, we used a SNP comparison tool from The Jackson Laboratory (<http://cgd.jax.org/straincomparison/>) to compare haplotype blocks among BALB/cJ, A/J, and NZB/B1NJ (the latter strain was included because a cross between NZB/B1NJ and A/J inbred strains was used in our previous whole genome scan for aggression loci, which used exactly the same phenotyping methods (Brodtkin *et al.*, 2002)). Of the 3 loci (chromosome 5, 10, and 15) identified in the current cross between BALB/cJ and A/J, only the chromosome 10 locus was identified in our previously reported cross between NZB/B1NJ and A/J. Therefore, the haplotype analysis was aimed at identifying haplotype blocks within the chromosome 10 locus in which A/J differs from both BALB/cJ and NZB/B1NJ, as well as haplotype blocks within the chromosome 5 and 15 loci in which A/J differed from BALB/cJ but not from NZB/B1NJ (Burgess-Herbert *et al.*, 2008, Su *et al.*, 2009). With the SNP comparison tool (<http://cgd.jax.org/straincomparison/>), “Imputed Diversity Array—Build 37” was used, and we used two alternative conditions: haplotype blocks of 10 consecutive SNPs and haplotypes of 3 consecutive SNPs. Results of both conditions are presented in Tables S4, S5, and S6.

Because the haplotype analysis for chromosome 10 revealed a focused list of only 3 positional candidate genes (protein tyrosine phosphatase, receptor type, f polypeptide (*Ppfia2*), citrate synthase (*Cs*), and v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (*ErbB3*))--see "Results" section and Table S5), Ensembl (www.ensembl.org), NCBI (<http://www.ncbi.nlm.nih.gov/SNP/>), and The Jackson Laboratory (<http://cgd.jax.org/straincomparison/>) databases were searched for SNPs in the 3 candidate genes in which A/J differed from the other strains, including non-synonymous coding SNPs, synonymous coding SNPs, splice site SNPs, and 5' and 3' UTR SNPs. The Ensembl and NCBI databases have SNP information on A/J and BALB/cByJ strains, and the Jackson Laboratory database (<http://cgd.jax.org/straincomparison/>) has SNP information on the specific strains of interest (A/J, BALB/cJ, and NZB/B1NJ). All SNP information was confirmed with the Jackson Laboratory website for the specific strains used in our studies.

To confirm the Jackson Laboratory database (<http://cgd.jax.org/straincomparison/>) genotype of the SNP rs29358506 in the citrate synthase gene (the only relevant SNP that we identified in the Jackson Laboratory database), we first obtained sequence flanking the SNP using Ensembl.org, and then used the NEBcutter V2.0 software (<http://tools.neb.com/NEBcutter2/>) to identify a restriction site cut by the restriction enzyme *FatI* that is created by the C (cytosine) genotype and destroyed by the A (adenine) genotype at rs29358506. Next, we designed PCR primers flanking rs29358506 that amplified a 242 basepair (bp) fragment. The PCR primer sequences were 5'-TTCTCCACAGTAGGAAACCC-3' (forward primer) and 5'-GAACGCTTCTGAACCAAGAG-3' (reverse primer). The PCR cycle was 95°C for 3 min; then 35 cycles of 95°C for 45 sec, 56°C for 30 sec, 72°C for 45 sec; then 72°C for 5 minutes. The concentration of the PCR product was measured using a spectrophotometer, and 2.4 µg of DNA was digested by incubating 2 units of *FatI* restriction enzyme and buffer (New England Biolabs, Ipswich, MA) at 55°C for 1 hour. The digested and undigested PCR products were electrophoresed side-by-side on a 10% polyacrylamide gel and visualized using ethidium bromide staining.

The undigested, 242 bp PCR amplified fragment contained 2 potential *FatI* restriction sites (CATG), one that included the SNP at rs29358506, as well as a separate invariant *FatI* site. The C/C (cytosine/cytosine) genotype at rs29358506 expected for the BALB/cJ and NZB/B1NJ strains, based on the Jackson laboratory genotype database, would lead to cutting by *FatI* at that site, as well as at the invariant *FatI* site, leading to a *FatI* digest of the 242 bp fragment to 3 smaller fragments of 135 bp, 72 bp, and 35 bp. The A/A (adenine/adenine) genotype at rs29358506 expected for the A/J strain, based on the Jackson laboratory genotype database, would destroy the *FatI* site at that site, but would not affect the other invariant *FatI* site, leading to a *FatI* digest of the 242 bp fragment to 2 smaller fragments of 170 bp and 72 bp (see Figure S1).

RESULTS

Aggression in Inbred Strains, F1 hybrids, and F2 mice

C inbred mice ($n = 31$) showed a significantly higher frequency of aggressive attack behavior than A inbred mice ($n = 15$) (Fisher's exact test, $P < 0.0005$) (see Figure 1). The aggression trait, as measured here, was incompletely penetrant in C mice (not all C mice showed the trait). In contrast, all 15 A mice had aggression scores of 0. Moreover, in a previous study, we tested a separate set of 29 A mice, using exactly the same methods, in the same laboratory, and all of these 29 A mice had aggression scores of 0 (Brodtkin *et al.*, 2002). Thus, of 44 A mice tested to date in our laboratory, all have had aggression scores of 0. There was no significant difference in aggression scores between reciprocal F1 hybrid animals (ACF1 mice ($n = 17$) vs. CAF1 mice ($n = 52$)) ($P = 0.1909$) (see Figure 1). The

CAF1 group was larger than the ACF1 group because the relatively higher rate of successful breeding in the C $\times$ A cross than in the A $\times$ C cross. We tested 675 (CAF1 $\times$ CAF1) F2 animals for aggressive behavior, and, of these 465 (68.9%) attacked the intruder in 0 out of 3 tests; 87 (12.9%) attacked in 1 out of 3 tests; 75 (11.1%) attacked in 2 out of 3 tests; and 48 (7.1%) attacked in 3 out of 3 tests (Figure 1).

Stage 1 genotyping—genomewide linkage analysis

Table S1 shows all 35 markers included in the genome scan that showed *uncorrected* $P < 0.05$ (with no Bonferroni correction) in stage 1 of genotyping using the likelihood ratio test. All 35 of these markers were used in the Stage 2 genotyping and association analysis (see below).

Stage 2 genotyping—association analysis

In stage 2 of genotyping, all 661 F2 mice were genotyped at all markers with *uncorrected* $P < 0.05$ in Stage 1 of genotyping (all 35 markers from Table S1, plus some additional markers in the regions of the chromosome 5, 10, and 15 loci—a total of 131 markers used in stages 1 and 2—see Table S3 for a list of all markers genotyped in stages 1 and 2). We then tested for association between these markers and the aggression phenotype using the Kruskal-Wallis test (see Table S2). After Bonferroni correction for multiple comparisons (correction for tests at 131 markers throughout the genome), there were seventeen markers that showed genome-wide significance at the $P < 0.05$ level, which are shown in red and bold in Table S2: D5Mit132, D5Mit254, D5Mit290, D5Mit304, D5Mit201, D5Mit18, D10Mit134, D10Mit151, D10Mit145, D15Mit201, D15Mit220, D15Mit121, D15Mit46, D15Mit208, D15Mit122, D15Mit133, and D15Mit144. Figure 2 (lower panel, “Test of Association”) shows the all markers genotyped in stage 2, with the lower dashed line indicating the $P < 0.05$ threshold not corrected for multiple comparisons, and the upper dashed line indicating the $P < 0.05$ threshold with Bonferroni correction for 131 tests.

Figure 3 shows the interval mapping results for the chromosome 5, 10, and 15 loci obtained with the R/qtl program (Broman *et al.*, 2003). Based on 1,000 bootstrap analysis, the 95% confidence intervals for each locus are the following: chromosome 5, 0–144.4 Mb; chromosome 10, 117.3–122.3 Mb; chromosome 15, 41.1–112.5 Mb. To quantify the effect of the three chromosomal loci on the risk of aggressive behavior, we performed a logistic regression analysis on phenotype data and genotype data from markers D5Mit254, D10Mit145, and D15Mit46. These were chosen as representative markers from each of the loci because each of these markers had among the most highly significant linkage and association with the aggression phenotype (among the lowest P values in Tables S1 and S2). Logistic regression analysis revealed that the C/C genotype at each of these markers significantly increased the risk of aggressive behavior, relative to the A/A genotype in the F2 mice (see Table 1). At D5Mit254, the odds ratio for aggressive behavior with a C/C genotype was 2.48, which was significantly greater than 1 (95% confidence interval (C.I.) of 1.46, 4.33), but the odds ratio with a C/A genotype was 1.02, which was not significantly greater than 1 (95% C.I. 0.60, 1.76). At D10Mit145, the odds ratio for aggressive behavior with a C/C genotype was 7.37 (95% C.I. 3.39, 18.65), or with a C/A genotype was 4.30 (95% C.I. 2.05, 10.61), both of which were significantly greater than 1. At D15Mit46, the odds ratio for aggressive behavior with a C/C genotype was 2.46, which was significantly greater than 1 (95% C.I. 1.41, 4.43), but the odds ratio with a C/A genotype was 1.35, which was not significantly greater than 1 (95% C.I. 0.98, 2.34). There was no significant evidence for epistatic (non-additive) interactions between the loci in their affect on aggressive behaviors: D5Mit254 $\times$ D10Mit145 interaction, $P = 0.83$; D10Mit145 $\times$ D15Mit46 interaction, $P = 0.24$; and D5Mit254 $\times$ D15Mit46 interaction, $P = 0.57$.

Haplotype and candidate gene analyses

The chromosome 10 locus was identified both in a cross derived from BALB/cJ and A/J (current study), as well as a cross derived from NZB/B1NJ and A/J inbred strains (Brodtkin *et al.*, 2002), using identical methods to measure intermale aggression in both studies. Therefore, in an effort to narrow in on relevant regions and genes of interest within the chromosome 10 locus, we used haplotype analysis to identify haplotype blocks within the chromosome 10 locus in which A/J differs from both BALB/cJ and NZB/B1NJ (see Table S5 and Figure 4) (Burgess-Herbert *et al.*, 2008, Su *et al.*, 2009). This haplotype analysis using haplotype blocks of 10 consecutive SNPs reduced the size of the area of interest within the 95% confidence interval to 4.3 % of its original length (see Figure 4). In the entire chromosome 10 region significantly associated with aggression (see Tables S2 and S5), only three known genes were identified in haplotype blocks: protein tyrosine phosphatase, receptor type, f polypeptide (*Ppfia2*), citrate synthase (*Cs*), and, v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (*ErbB3*).

In the *Cs* gene, there is an interesting strain polymorphism in a non-synonymous coding SNP, rs29358506, at 127787558 bp on chromosome 10, which is in a haplotype block identified in our analysis (see Table S5). This SNP, rs29358506, in exon 3 of the *Cs* mRNA, encodes an amino acid in the α domain of the citrate synthase protein (Hayward & Berendsen, 1998). rs29358506 has the genotype A/A (adenine/adenine) encoding an asparagine (N) in the A/J strain but has the genotype C/C (cytosine/cytosine) encoding a histidine (H) in BALB/cJ and NZB/B1NJ strains (see www.ncbi.nlm.nih.gov/snp and www.ensembl.org and <http://cgd.jax.org/straincomparison/>). These strain genotypes at rs29358506 were empirically validated by the Jackson Laboratory (<http://cgd.jax.org/straincomparison/>), and we further confirmed the strain genotypes using RFLP analysis with the restriction enzyme *FatI* (see Figure S1). The C/C genotype at that position is highly conserved across mammalian species (see Figure S2). However, the A/J polymorphism is not a null mutation, as A/J mice have been reported to show citrate synthase enzyme activity in skeletal muscle (Leibowitz *et al.*, 2005). No other coding region, 5' UTR, 3' UTR, or splice site SNPs were identified in the pattern we sought (A/J different from both BALB/cJ and NZB/B1NJ) in *Cs*, *Ppfia2*, or *ErbB3*.

Because the chromosome 5 and 15 loci were identified in the cross derived from BALB/cJ and A/J, but not in the previous cross derived from NZB/B1NJ and A/J, we used haplotype analysis to identify regions within the chromosome 5 and chromosome 15 loci in which A/J differs from BALB/cJ but not from NZB/B1NJ. The results of these analyses are presented in Table S4, Table S6, and Figure 4. This haplotype analysis using haplotype blocks of 10 consecutive SNPs reduced the size of the area of interest within the 95% confidence interval for each chromosome to 12 % of its original length, in the case of chromosome 5, and to 15.7% of its original length, in the case of chromosome 15 (see Figure 4). A large number of genes were identified in these regions, some of which are considered in the Discussion section.

DISCUSSION

We have identified three significant genomic loci—on chromosomes 5, 10, and 15—that affect intermale aggression in BALB/cJ and A/J mice. We found a partially overlapping, but partially novel set of genetic loci relative to our previous cross between NZB/B1NJ and A/J mice (Brodtkin *et al.*, 2002). When two different mapping crosses use one strain in common (in this case A/J), it is typical to find some loci in common in both crosses and other loci that are unique to each cross, because of the differing pattern of polymorphisms between the two different sets of progenitor strains (Hitzemann *et al.*, 2003, Li *et al.*, 2005, Malmanger *et al.*, 2006).

The position of the distal chromosome 10 locus detected in the current study is essentially the same as that of the locus identified in our previous genetic study of aggression in NZB/B1NJ and A/J strains, with peak significance, in both studies, at the most distal (telomeric) markers on chromosome 10 (Brodkin *et al.*, 2002). This suggests the possibility that there is an A/J allele on distal chromosome 10 that contributes to lower levels of aggressive behavior. Only 3 known genes are contained within the relevant haplotype blocks on chromosome 10 in which A/J mice differ from BALB/cJ and NZB/B1NJ mice (see Table S5): *Ppfia2*, *Cs*, and *ErbB3*. Although the specific role of *Ppfia2* in brain is not well understood, receptor protein tyrosine phosphatases in general play a role in neuronal and glial development, axonal guidance, and synaptogenesis (Lamprianou & Harroch, 2006). *Cs* is expressed in cellular mitochondria in the brain and other tissues, and encodes the enzyme that catalyzes the first step of the citric acid (Krebs) cycle (Kurz *et al.*, 2005, Roccitano *et al.*, 2001). Thus, *Cs* plays an important role in brain energy metabolism. The non-synonymous coding SNP, rs29358506, that causes an amino acid difference in A/J vs. the other strains is not a null mutation, however, as A/J mice have been reported to show citrate synthase enzyme activity in skeletal muscle (Leibowitz *et al.*, 2005). Interestingly, neuronal nitric oxide synthase gene knockout mice, which are extremely aggressive (Nelson *et al.*, 1995, Trainor *et al.*, 2007), also show markedly elevated levels of mitochondrial citrate synthase expression and activity (Schild *et al.*, 2006). *ErbB3* is one of the epidermal growth factor (EGF)-like receptors whose ligands include neuregulin-1 (Nrg-1). ErbB signaling is involved in glial differentiation and neuronal migration, and *ErbB3* is expressed at high levels in white matter tracts of adult mice (Fox & Kornblum, 2005). The Nrg-1-ErbB signaling pathway has been implicated in the etiology of schizophrenia (Burden & Yarden, 1997, Corfas *et al.*, 2004, Walsh *et al.*, 2008). Although the genes within the chromosome 10 haplotype blocks can be considered candidate genes of highest priority, the arginine vasopressin receptor 1a gene (*Avpr1a*) at 121.9 Mb (within the chromosome 10 locus 95% confidence interval of 117.3–122.3 Mb) could also be considered as a candidate gene (Brodkin *et al.*, 2002), given the extensive literature implicating this gene in intermale aggressive behaviors (reviewed in (Ferris, 2005)) and other socioemotional behaviors in mammals (reviewed in (Donaldson & Young, 2008)).

The chromosome 5 and 15 loci identified in this study are novel. To our knowledge, no previous genome scans have linked these loci with aggressive behaviors in mice. The haplotype analysis to identify regions within these loci in which A/J differs from BALB/cJ but not from NZB/B1NJ revealed a large number of regions and candidate genes in both loci (see Tables S4 and S6). On chromosome 5, one candidate gene is the protocadherin 7 gene (*Pcdh7*), a member of the cadherin family of neural cell adhesion molecules, which are involved in synaptogenesis, synaptic function, and synaptic plasticity, and several members of which have been implicated in autism spectrum disorders (Junghans *et al.*, 2005, Morishita & Yagi, 2007, Morrow *et al.*, 2008, Wang *et al.*, 2009). *Pcdh7* is expressed in the amygdala and other brain regions (Vanhalst *et al.*, 2005). Other candidate genes on chromosome 5 include several GABA-A receptor subunit genes that are involved in inhibitory neurotransmission in the brain and are strongly implicated in various types of aggressive behaviors (De Almeida *et al.*, 2005, Dick *et al.*, 2009, Lee & Gammie, 2007, Liu *et al.*, 2007).

Possible candidate genes in the mouse chromosome 15 locus include several members of the cadherin gene family that was mentioned above. Another candidate gene is the catenin (cadherin associated protein) delta 2 gene (*Ctnnd2*) at 30.1 Mb, which has also been implicated in human schizophrenia and cri-du-chat syndrome (Medina *et al.*, 2000, Vrijenhoek *et al.*, 2008). Catenins bind cadherins and are involved in cell-cell adhesion and cell motility in the brain (Medina *et al.*, 2000). Another candidate is the adenylate cyclase 8 gene (*Adcy8*) at 64.5 Mb, which is one of the major calmodulin-stimulated adenylyl cyclases

in the brain, and which couples NMDA receptor activation to cAMP signaling pathways and affects various behaviors (Wei *et al.*, 2002).

The extremely low levels of aggression in young adult male A/J mice are not attributable to any known motor or sensory deficit in this strain (Moy *et al.*, 2007) nor to abnormalities of blood androgen levels (Hampl *et al.*, 1971). For example, relative to other inbred strains, young A/J mice show normal motor strength and coordination in the accelerating rotarod task (Moy *et al.*, 2007). A/J mice have a mutation in the dysferlin gene (on mouse chromosome 6) that first arose in the late 1970s or early 1980s, and that leads to a slowly progressive muscular dystrophy in older adult mice (Ho *et al.*, 2004). However, this muscular dystrophy phenotype does not account for reduced aggression of the young adult males in this study or our previous study (Brodkin *et al.*, 2002), because A/J mice in both studies were 9–10 weeks of age at the time of behavioral testing, which is younger than the very earliest onset of histological dystrophic muscle changes in A/J mice at 12–16 weeks (Ho *et al.*, 2004, Kobayashi *et al.*, 2010). Also, the extremely low aggression phenotype of A/J mice was observed in the 1960s (Southwick & Clark, 1968), prior to the occurrence of the dysferlin mutation in this strain (Ho *et al.*, 2004). Moreover, none of the aggression loci identified in our previous mapping study (Brodkin *et al.*, 2002) or this study mapped to the chromosomes containing dysferlin (mouse chromosome 6) or the dysferlin interacting protein 1 (mouse chromosome 11).

Both BALB/cJ and A/J mice have been found in previous studies to show relatively high levels of “anxiety-related behaviors” in behavioral assays (e.g. elevated plus maze, open field test) that employ high lighting levels (Bouwknicht & Paylor, 2002, Carola *et al.*, 2002). The current study and our previous study of aggression (Brodkin *et al.*, 2002) used very low lighting levels in all aggression tests to minimize light-induced anxiety. The relationship between anxiety and aggression is complex, because high aggression can be associated with either low or high anxiety levels (Neumann *et al.*, 2010). With regard to one measure of anxiety-related behavior, it does not appear that there was a substantial difference between the A/J and BALB/cJ strains in their latency to approach the intruder mouse in our studies. Latency to approach the intruder was measured in a subset of 20 BALB/cJ and 19 A/J mice from this study and our previous study (Brodkin *et al.*, 2002). The BALB/cJ mice showed a mean $\pm$ S.E.M. latency to first contact of the intruder of 3.8 ± 0.7 seconds, whereas A/J mice showed a mean $\pm$ S.E.M. latency to first contact of 5.4 ± 1.1 seconds; i.e. both strains rapidly approached and contacted the intruder.

The loci mapped in this study and our previous study do not overlap with aggression loci mapped in crosses derived from NZB/B1NJ and C57BL/J mice (Roubertoux *et al.*, 2005). The differences from the latter study are likely due to the fact that a different strain, C57BL/J, was used in the other cross (Roubertoux *et al.*, 2005). The differing results may also be due to differences in the nature of phenotypic measurements used in the different studies, as well as differences in rearing and testing conditions (Roubertoux *et al.*, 2005).

The mapping of genetic loci that affect aggression is a significant step toward the identification of alleles that influence aggression in the mouse. Fine genetic mapping of these loci and additional candidate gene analysis may lead to new insights into the neurobiological pathways that mediate aggressive behaviors (Clee *et al.*, 2006, Ferraro *et al.*, 2007, Flint *et al.*, 2005, Korstanje & Paigen, 2002, Yalcin *et al.*, 2004). An increased understanding of the genetics and neurobiology of aggression in a mammalian model organism may ultimately contribute to a better understanding of the factors that affect human aggression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Histograms of aggression scores of BALB/cJ, A/J, CAF1, ACF1, and (CAF1 $\times$ CAF1)F2 mice. The x-axis of each histogram shows the aggression score. The y-axis shows the number of mice displaying particular aggression scores. The aggression score corresponded to the number of aggressive attacks over 3 days of testing. A score of 0 signified no attacks over 3 days of testing; a score of 1 signified 1 attack over 3 days of testing; a score of 2 signified 2 attacks over 3 days of testing; and a score of 3 signified 3 attacks over 3 days of testing. A mouse could only have one attack per testing session, because the test was stopped after the initiation of an attack, therefore, 3 was the maximum possible aggression score over 3 days of testing. Aggression phenotype data were not normally distributed, and could not be transformed to a normal distribution using standard transformations. Data were analyzed using nonparametric methods that do not assume a normal distribution.

Figure 2.

Results stage 1 (genomewide linkage) and stage 2 (association) of genotypic analysis. In each plot, the x-axis represents a map of all chromosomes in the genome (chromosomes 1–19 and X), and the many vertical marks below the x-axis represent positions of markers genotyped for each chromosome. Vertical dotted lines within the plots represent boundaries between chromosomes. The y-axis represents Lod scores for the test of linkage or the Kruskal-Wallis Rank Sum chi square values for the test of association. In the “Test of Linkage” plot, the dashed horizontal line represents the *uncorrected* significance threshold (*uncorrected* $P < 0.05$ value). In the “Test of Association” plot, the lower dashed horizontal line represents the *uncorrected* significance threshold (*uncorrected* $P < 0.05$ value), and the upper dashed horizontal line represents the Bonferroni corrected significance threshold (Bonferroni corrected $P < 0.05$ value, corrected for testing at 131 markers). Significant loci were identified on chromosomes 5, 10 and 15 in the association analysis.

Figure 3.

Interval analysis. Interval mapping results are shown for the chromosome 5, 10, and 15 loci obtained with the R/qtl program (Broman *et al.*, 2003), using the nonparametric methods and treating the aggression scores (0–3) as the phenotypes. To determine the LOD score significance thresholds for the three loci identified, we performed 1,000 permutations of the aggression scores, and, for each permutation, we obtained the maximum LOD scores across all three chromosomes. We then obtained the value of the 95th percentile of these maximum LOD scores, 2.64, as our LOD score threshold at the adjusted significance threshold level of 5%. Based on 1,000 bootstrap analysis, the 95% confidence intervals for each locus are the following: chromosome 5, 0–144.4 Mb; chromosome 10, 117.3–122.3 Mb; chromosome 15, 41.1–112.5 Mb.

Figure 4.

Haplotype analysis. A graphical summary of haplotype blocks identified on chromosomes 5, 10, and 15 is shown. The intervals (horizontal lines) shown for each chromosome represent the 95% confidence intervals for each locus--that is, 0 to 144,379,186 bp on chromosome 5; 117,338,390 – 122,256,160 bp on chromosome 10; and 41,069,371–112,453,477 bp on chromosome 15. The small, vertical lines or blocks represent the haplotype blocks identified using the 10 consecutive SNP haplotype block analysis for each chromosome. The percent of each chromosomal interval that is encompassed by the summed length of the haplotype blocks for that chromosome is shown in parentheses after each chromosome name. Note that the intervals shown here for chromosomes 10 and 15 are smaller than the intervals for those chromosomes shown in Tables S5 and S6.

Table 1

The effect of each genetic locus on the risk of aggressive behavior. The results of logistic regression analysis are shown. For D5Mit254, D10Mit145, and D15Mit46, the odds ratio for risk of aggressive behavior is shown for the C/C genotype vs. the A/A genotype, and for the C/A genotype vs. the A/A genotype.

Locus	Genotype Comparison			
	C/C vs. A/A		C/A vs. A/A	
	Odds ratio	95% confidence interval	Odds ratio	95% confidence interval
Chr 5 (D5Mit254)	2.48	(1.46, 4.33)	1.02	(0.60, 1.76)
Chr 10 (D10Mit145)	7.37	(3.39, 18.65)	4.30	(2.05, 10.61)
Chr 15 (D15Mit46)	2.46	(1.41, 4.43)	1.35	(0.98, 2.34)