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ORIGINAL RESEARCH

Differences in Aggressive Behavior and DNA Copy Number Variants Between BALB/cJ and BALB/cByJ Substrains

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Abstract Some BALB/c substrains exhibit different levels of aggression. We compared aggression levels between male BALB/cJ and BALB/cByJ substrains using the resident intruder paradigm. These substrains were also assessed in other tests of emotionality and information processing including the open field, forced swim, fear conditioning, and prepulse inhibition tests. We also evaluated single nucleotide polymorphisms (SNPs) previously reported between these BALB/c substrains. Finally, we compared BALB/cJ and BALB/cByJ mice for genomic deletions or duplications, collectively termed copy number variants (CNVs), to identify candidate genes that might underlie the observed behavioral differences. BALB/cJ mice showed substantially higher aggression levels than BALB/cByJ mice; however, only minor differences in other behaviors were observed. None of the previously reported SNPs were verified. Eleven CNV regions were identified between the two BALB/c substrains. Our findings identify a robust difference in aggressive behavior between BALB/cJ and BALB/cByJ substrains, which could be the result of the identified CNVs.

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Introduction

Some substrains of BALB/c mice have previously been suggested to exhibit robust differences in aggressive behavior, although the genetic basis for this behavioral difference has not been identified (Ciaranello et al. 1974). The BALB/c substrains were derived from an initial BALB/c stock established by 1935 (Les 1990); this BALB/c stock was then acquired by several other laboratories and were maintained and bred as independent stocks including BALB/cJ, BALB/cN, and BALB/cByJ. The resulting BALB/c substrains may exhibit genetic and phenotypic differences due to breeding errors that introduced new alleles, and/or spontaneous genetic mutations. With regard to new mutations, attention has traditionally been focused on single nucleotide polymorphisms (SNPs), and public databases indicate the existence of multiple SNPs that distinguish the BALB/cByJ and BALB/cJ substrains (www.jax.org/phenome). More recently, attention has focused on genomic deletions or duplications among closely related inbred strains, which are collectively termed copy number variations (CNVs) (Egan et al. 2007; Watkins-Chow and Pavan 2008). In particular, evidence of spontaneous changes in copy number and correlations between phenotype and CNVs have recently been reported in inbred mouse strains.

Ciaranello et al. (1974) reported that male mice of the BALB/cJ substrain exhibited robust increases in aggression compared to those of the BALB/cN substrain. Specifically, BALB/cJ mice showed short latencies to attack a BALB/cN intruder following 2 weeks of isolation and acute tail pinch, while BALB/cN mice did not attack intruders under the

same conditions. Similarly, we have observed that group-housed male BALB/cJ mice often fight extensively in the home cage resulting in severe injury, while group-housed male BALB/cByJ mice do not (unpublished observations). We therefore sought to determine whether BALB/cJ mice show robust increases in offensive aggression compared to BALB/cByJ mice under controlled experimental conditions. We assessed these two BALB/c substrains in the resident intruder paradigm, a commonly used test of offensive aggression for male rodents that quantifies behavior using ethological methods (Blanchard et al. 2003; Miczek et al. 2001; Miczek and O'Donnell 1978; Olivier and Mos 1992). To thoroughly examine offensive aggression in these substrains, we assessed aggression of an isolated resident toward an intruder, since isolated mice share characteristics with territorial mice (Brain 1975). We assessed aggression following both short-term (48 h) and long-term (4 weeks) isolation and following long-term housing with a female (Crawley et al. 1975).

To examine the specificity of the behavioral difference in aggression between the BALB/cJ and BALB/cByJ substrains, mice were also assessed for other measures of emotionality and information processing including the open field, the forced swim test, fear conditioning, startle reactivity, and prepulse inhibition. We then sought to identify potential candidate genes that could be responsible for the robust and specific differences in aggressive behavior we observed between these two substrains. We first evaluated whether the .18% SNP variation reported between the BALB/cJ and BALB/cByJ substrains (Petkov et al. 2004) was correct, or the result of genotyping error. We then assessed these substrains for CNVs in an effort to identify possible genetic substrates for their observed phenotypic difference in aggressive behavior.

Methods

Animals

Male BALB/cJ and BALB/cByJ resident mice (Jackson Laboratories, Bar Harbor, ME) were 10–12 weeks of age and weighed 22–30 g when testing began. C57BL/6J intruder males were 9–11 weeks of age and weighed 20–25 g at the start of testing. Separate groups of resident and intruder mice were used for each experiment assessing aggression. Mice were housed in groups of five except for resident mice, which were singly housed for 4 weeks upon arrival for long-term isolation studies, or for 48 h following 1 week of group housing for short-term isolation studies. One female mouse of the same age and substrain was housed with each resident male for breeder aggression studies. Mice were maintained on a 12L:12D schedule with food and water provided ad libitum. Resident intruder and open field testing occurred during the dark phase, and all other behavioral testing occurred during the light phase. No testing occurred within one hour of the transition between light and dark cycles. Animal testing was conducted in accord with the NIH laboratory animal care guidelines and with IACUC approval.

Behavioral apparatus

Resident intruder test

All aggression testing occurred in the home cage of the resident in a dark sound attenuated room with red overhead lighting. Behavior was videotaped using a tripod-mounted camera on an infrared setting. No other mice except for the resident and intruder were present in the room during testing.

Open field

Locomotor activity was quantified using 42 × 42 × 30 cm Plexiglas activity chambers (Accuscan, Columbus, OH). Chambers were equipped with sixteen infrared beams (2.5 cm apart) along each wall to record the paths taken by mice. Paths were stored as x–y coordinate sequences. The computer defined a square center region within each open field using grid lines 8.3 cm from each wall.

Forced swim

Plastic buckets 24 cm high and 19 cm in diameter filled 19 cm high with 23–25°C tap water were positioned directly below a tripod-mounted camera.

Prepulse inhibition

Startle chambers were nonrestrictive Plexiglas cylinders 5 cm in diameter resting on a Plexiglas platform in a ventilated chamber (San Diego Instruments, San Diego, CA) as described elsewhere (Shanahan et al. 2008). Sixty-five consecutive 1-ms readings were recorded beginning at startling stimulus onset to obtain the amplitude of the animals' startle response to each stimulus (Geyer and Dulawa 2004). Sound levels were measured as described elsewhere using the A weighting scale (Mansbach et al. 1988).

Fear conditioning

Fear conditioning (FC) chambers (Med Associates, St. Albans, VT, USA) had inside dimensions of 29 × 19 × 25 cm with metal walls on each side, clear plastic front and back walls and ceilings, and stainless steel bars on the floor. A fluorescent light provided dim illumination (~10 lux)

and a fan provided a low level of background noise. Behavior was recorded with digital video and analyzed with FreezeFrame software from Actimetrics (Evanston, IL, USA).

SNP sequencing

In the course of our investigation of genetic differences between BALB/cJ and BALB/cByJ mouse strains, we examined SNP data available from available from the JAX Phenome Database (www.jax.org/phenome). Specifically, we used the "Low data density strain set" to identify SNPs that are polymorphic between these two strains. This database indicated 37 polymorphisms existed between these two strains. These SNPs were unevenly distributed into several small clusters. We amplified and sequenced 20 of these regions in an effort to verify that the SNPs were truly polymorphic; SNPs were selected from each of the available data sources (dbSNP, TJL2, TJL3).

Detection of copy number variants

DNA was collected from 5 BALB/cJ and 5 BALB/cByJ mice obtained directly from Jackson Labs (Bar Harbor, ME). DNA was extracted, processed, hybridized to Affymetrix Mouse Exon arrays, and analyzed using a Hidden Markov approach as described previously (Williams et al. 2009). Rather than using the C57BL/6J as a reference strain, comparisons were between the two BALB/c substrains; differences between these substrains may indicate either a duplication in one or a deletion in the other. The exact boundaries of each CNV can only be estimated using this approach. For each identified feature, we recorded the inner and outer boundaries for the beginning and end of each region. We have previously verified the duplication on chromosome 17 (Table 1) using a PCR based approach (Williams et al. 2009) as well as real time PCR (data not shown).

Experimental procedures

Resident intruder (4 week isolation)

Following 4 weeks of isolation, each BALB/cJ and BALB/cByJ resident mouse was confronted in its home cage with a C57BL/6J intruder. Intruder tests were conducted every 3 days until a stable level of aggression was achieved for both substrains; stability was defined as no more than 15% variation in the frequency of attacks from the previous test. Testing lasted for 5 min following the first attack. Videotapes of fighting behavior were scored later by an experienced observer for attack burst incidence, attack latency, and frequency of attacks, threats, and tail rattles (Miczek and O'Donnell 1978). An attack was defined as a bite directed at the back or flanks of the intruder; a threat was defined as approaching the intruder with short steps, lateral rotation of the body, and piloerection. The observer was blind to experimental conditions, as BALB/cJ and BALB/cByJ mice appear identical.

Table 1 Table shows copy number differences observed between BALB/cJ and BALB/cByJ

Chr#	Gene	Start outer	Start inner	Stop inner	Stop outer	Min probe#	Max probe#	Direction
2	Gm711, Rexo4, Surf2, Surf4	26741619	26741793	26784707	26786017	291	311	BALB/cJ < BALB/cByJ
2	1700029J11Rik, 2410001C21Rik, F730031O20Rik, Intergenic	172118179	172119149	172173858	172193566	226	252	BALB/cJ > BALB/cByJ
3	Intergenic	35297234	35302596	35751109	35756904	595	619	BALB/cJ > BALB/cByJ
4	5930403L14Rik, Intergenic	60167313	61187586	61318659	61319330	17	47	BALB/cJ < BALB/cByJ
4	Foxd2, Foxe3, Intergenic	138119931	138131135	138179360	138185512	134	164	BALB/cJ > BALB/cByJ
7	Intergenic, LOC435953, LOC620537, LOC620758, LOC621620, LOC628664, LOC667067, LOC667215, LOC667240, LOC667273, Nalp4e	19208032	20136499	23030541	23051816	21	31	BALB/cJ < BALB/cByJ
11	Intergenic, Pscd1	117962130	117980500	118013743	118021230	112	147	BALB/cJ > BALB/cByJ
12	Nrxn3	90121131	90128821	90217995	90222368	163	177	BALB/cJ < BALB/cByJ
17	Btbd9, Dnahc8, Glo1, Glp1r, Intergenic	30173661	30174462	30651572	30652599	1058	1064	BALB/cJ < BALB/cByJ
17	Intergenic	39416429	39451281	39456763	39473608	123	131	BALB/cJ < BALB/cByJ
X	2610020O08Rik, Akap14, Intergenic, Rpl39, Upf3b	6892401	6897107	6946855	6951219	184	251	BALB/cJ > BALB/cByJ

Columns indicate chromosome number, gene(s) involved, the outer and inner boundaries of the predicted beginning and end of the feature in question and the minimum and maximum number of probes involved. All coordinates are in terms of Build 36 of the mouse genome

Resident intruder (48 h isolation)

All aspects of testing were identical to those described above, except that residents were isolated for only 48 h before testing began.

Resident intruder (4 weeks breeding)

Testing was identical, except that each resident was housed with a female for 4 weeks before testing began.

Open field

BALB/cJ and BALB/cByJ mice were placed into the corner of an open field and activity was recorded for 30 min. Testing occurred during the dark cycle to determine whether any differences in locomotor activity between substrains confounded aggression measures.

Forced swim

BALB/cJ and BALB/cByJ mice were placed into swim buckets for 6 min on two consecutive days, which increases sensitivity for detecting antidepressant behavioral effects. Swim sessions were videotaped from a tripod-mounted camera positioned directly above the swim buckets. Behavior was analyzed by a blind scorer using a time sampling technique in which the predominant behavior (swimming, immobility, or climbing) was scored every five-seconds in the last 4 min of the test (Cryan et al. 2002).

Prepulse inhibition

BALB/cJ and BALB/cByJ mice were placed into startle chambers and assessed for PPI and startle reactivity in a 22 min session. For each session, mice were exposed to five different types of discrete stimuli or "trials": a 40-ms broadband 120 dB burst (Pulse Alone trial); three different Prepulse + Pulse trials in which either 20-ms long 3, 6, or 12 dB above background stimuli preceded the 120 dB pulse by 100 msec (onset to onset); and a No Stimulus trial, in which only background noise (65 dB) was presented. Trials were presented in a non-systematic order. An average of 15 s (range: 9–20 s) separated trials. The test session began with a 5-min acclimation period, followed by four consecutive blocks of test trials. Blocks one and four consisted of six consecutive Pulse Alone trials, while blocks two and three each contained six Pulse Alone trials, five of each kind of Prepulse + Pulse trial, and four no stimulus trials.

Fear conditioning

Testing was conducted over 3 days as we have described previously (Ponder et al. 2007). On day 1, baseline activity was measured beginning 30 s after mice were placed into the chambers, and terminated after 180 s (pretraining). Mice were then exposed twice to the CS, which consisted of a 30 s long 85 dB, 3 KHz tone which co-terminated with the US, which was a 2 s, 0.5 mA foot shock. A 30-second intertrial interval (ITI) separated paired stimuli. On day 2, the testing environment was identical to the first day, but neither tones nor shocks were presented. Freezing in response to the test chamber (context) was measured beginning 30 s after the start of the test and ending at 180 s. On day 3, the context was altered in several ways and aspects of the testing procedures were also changed, as described previously (Ponder et al. 2007). Freezing to the altered context was defined as freezing that occurred between 30 and 180 s. The CS was presented twice beginning at 180 and 240 s, but no foot shock was delivered. The 'freezing to tone' score was an average of the percent time spent freezing during the two 30-s CS presentations.

Statistical analysis

Behavioral studies

For all studies, ANOVAs were applied to each measure after confirming homogeneity and normal distribution of the data. Significant interactions were resolved using post-hoc ANOVAs for within subjects variables and Newman Keuls post-hoc tests for between subjects variables. Substrain was a between-subjects factor for all analyses. For aggression studies, dependent measures were attack incidence, attack latency, attack frequency, threat frequency, and tail rattle frequency. Attack incidence reflects the percentage of mice exhibiting at least one attack. Attack frequency reflects the mean number of attacks exhibited per mouse. For open field studies, total distance traveled, center distance, center time, and rearing were dependent measures, and block (5 min intervals) was a within-subjects factor. For PPI studies, PPI was calculated as [100—(prepulse-Pulse trial/averaged Pulse Alone) × 100]. Pulse Alone values were calculated as the mean of startle values from blocks two and three. Startle reactivity was calculated as the average response to all 24 startle trials. PPI and startle reactivity were dependent measures, and block and prepulse intensity were within-subjects factors. For FST studies, immobility was the dependent measure, and block (1 min. intervals) was a within-subjects factor. For fear conditioning experiments we applied a series of within-subjects ANOVAs to compare the two substrains at

different stages of testing (freezing to context, freezing to tone, etc.). Effect sizes of significant substrain differences were calculated using Cohen's d, which is the difference between two means divided by the pooled standard deviation.

Results

Resident intruder tests

Following 4 weeks of isolation, BALB/cJ mice showed high levels of aggression towards intruders compared to BALB/cByJ mice (Fig. 1). Both substrains reached a plateau for frequency of attacks on test day 4. ANOVAs revealing substrain x test day interactions and post-hoc tests showed that BALB/cJ mice exhibited a higher attack frequency ($F(3,48) = 3.27$; $P < .05$), shorter attack latency ($F(3,48) = 9.19$; $P < .0001$), and higher attack incidence ($F(3,48) = 5.88$; $P < .01$) than BALB/cByJ mice on test days 2, 3, and 4. Additionally, BALB/cJ mice showed a higher frequency of threats ($F(3,48) = 4.18$; $P < .01$) and tail rattles ($F(3,48) = 4.39$; $P < .01$) than BALB/cByJ mice on test days 3 and 4.

BALB/cJ mice also showed increased aggression levels toward the intruder compared to BALB/cByJ mice following only 48 h of isolation (Fig. 2a). While BALB/cByJ mice never fought and therefore reached a plateau for attack frequency by day 2, BALB/cJ mice reached a plateau for attack frequency on day 6. For day 6 values, BALB/cJ mice exhibited a lower attack latency ($F(1,27) = 4.36$; $P < .05$), higher attack incidence ($F(1,27) = 4.74$; $P < .05$), and higher threat ($F(1,27) = 4.51$; $P < .05$), and tail rattle frequency ($F(1,26) = 8.74$; $P < .01$) compared to BALB/cByJ mice. A trend was also found for BALB/cJ to show more attacks than BALB/cByJ mice ($F(1,26) = 3.22$; $P = .08$).

BALB/cJ mice housed with a female for 4 weeks showed robust increases in aggression towards an intruder compared to BALB/cByJ mice housed under the same conditions (Fig. 2b). BALB/cJ mice exhibited substantially higher levels of aggression beginning on day 1 (BALB/cJ attack freq: 10.9 ± 3.8; BALB/cByJ attack freq: .5 ± .3), and both substrains reached a plateau for attack frequency on day 2. On day 2, BALB/cJ mice exhibited a higher attack frequency ($F(1,16) = 8.58$; $P < .01$), lower attack latency ($F(1,18) = 10.06$; $P < .01$), higher attack incidence ($F(1,18) = 6.82$; $P < .05$), and higher threat ($F(1,16) = 6.87$; $P < .05$) and tail rattle frequency ($F(1,16) = 5.18$; $P < .05$) compared to BALB/cByJ mice. Four more days of testing revealed that these differences in aggressive behavior persisted over time.

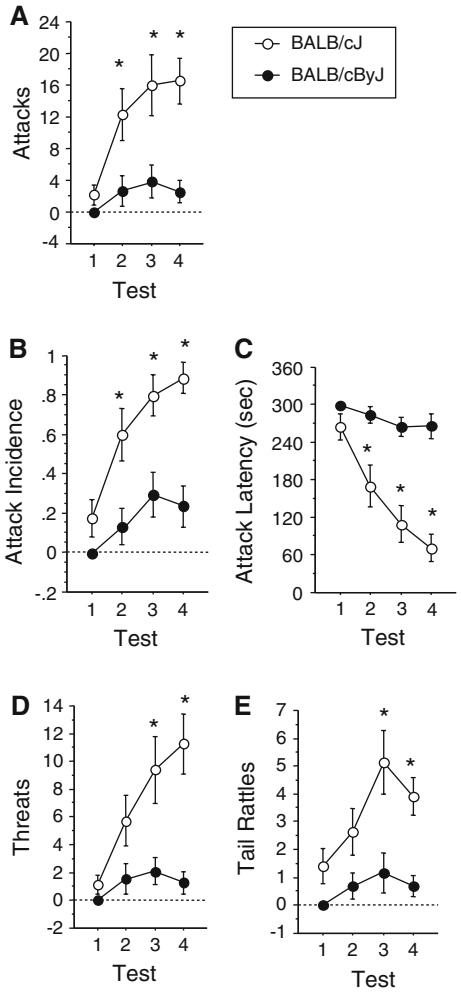

Fig. 1 Aggressive behavior of BALB/cJ ($n = 18$) and BALB/cByJ ($n = 19$) in resident-intruder test following 4 weeks of isolation. Attack frequency (**a**), attack incidence (**b**), attack latency in seconds (**c**), threat frequency (**d**), and frequency of tail rattles (**e**) are shown for residents. Four tests were required to establish stable levels of attacks in both strains. Values are means ± SEM. An asterisk (*) indicates a significant difference between substrains

Open field

The two BALB/c substrains showed no differences in the total distance traveled, distance traveled in the center, or time spent in the center of the open field (Fig. 3). A main effect of block indicated that mice spent more time in the center ($F(5,170) = 8.07$; $P < .0001$) and traveled more distance in the center ($F(5,170) = 3.21$; $P < .01$) over time during open field testing. A main effect of strain indicated that BALB/cByJ mice exhibited a greater frequency of rearing than BALB/cJ mice ($F(1,34) = 9.39$; $P < .01$).

Prepulse inhibition

No differences in PPI were found between the two BALB/c substrains. ANOVA indicated a main effect of prepulse

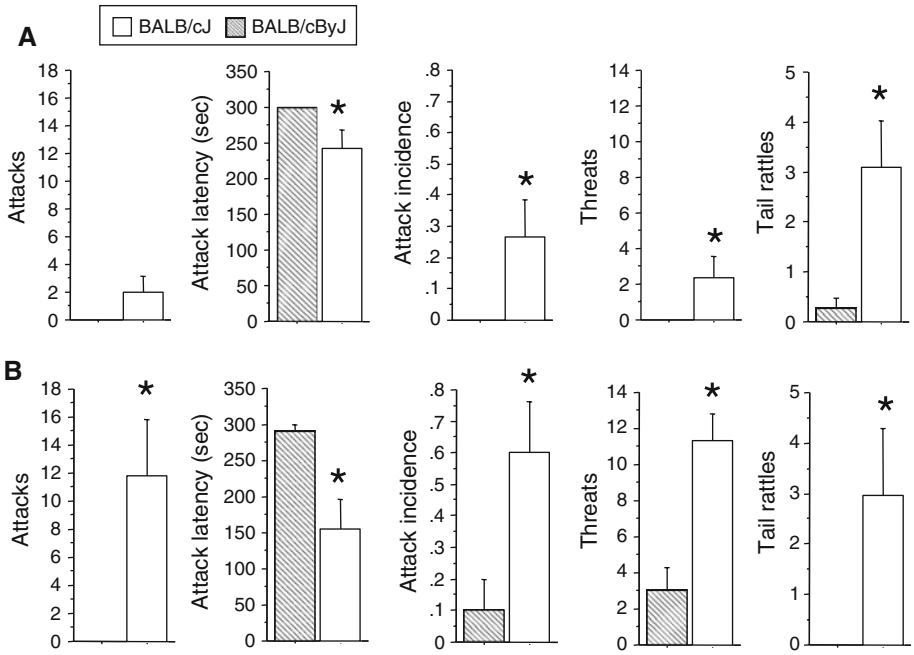

Fig. 2 Aggressive behavior of mice in resident-intruder test following **a** 48 h of isolation ($n = 15$, BALB/cJ; $n = 14$, BALB/cByJ) or **b** 4 weeks of housing with a female ($n = 10$, BALB/cJ; $n = 10$, BALB/cByJ). Attack frequency, attack latency in seconds, attack incidence, threat frequency, and frequency of tail rattles are shown for residents. Values are means ± SEM. An asterisk (*) indicates a significant difference between substrains

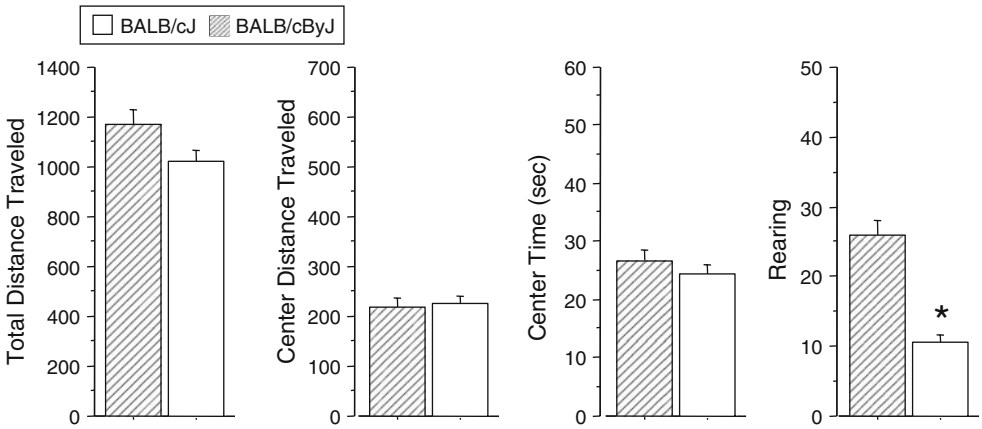

Fig. 3 Open field measures for BALB/cJ ($n = 17$) and BALB/cByJ ($n = 19$) mice. Total distance traveled, center distance traveled, time in the center, and rearing frequency are shown. Values are means ± SEM. An asterisk (*) indicates a significant difference between substrains

intensity ($F(2,38) = 33.95$; $P < .0001$), indicating that PPI increased with increasing prepulse intensity. Overall startle reactivity was higher in BALB/cByJ mice compared to BALB/cJ mice ($F(1,19) = 5.00$; $P < .05$) (Fig. 4a).

Forced swim test

No differences in immobility were found between the two BALB/c substrains, although a main effect of block ($F(3,48) = 2.97$; $P < .05$) indicated that mice exhibited more immobility over time (Fig. 4b). However, the two BALB/c substrains exhibited differences in active behavior in the FST. BALB/cByJ mice showed increased swimming ($F(1,16) = 21.19$; $P < .001$) relative to BALB/cJ mice, while BALB/cJ mice showed increased climbing ($F(1,16) = 12.99$; $P < .01$) relative to BALB/cByJ mice. Both swimming ($F(3,48) = 6.94$; $P < .001$) and climbing ($F(3,48) = 2.32$; $P = .09$) behavior decreased over time across strains.

Fear conditioning

We observed a significant difference between BALB/cJ and BALB/cByJ on day 1 (pretraining) prior to the administration of any shocks; BALB/cJ mice spent approximately 1% more time freezing during the 150 s pretraining period than did BALB/cByJ mice (Fig. 5). This finding can not be interpreted as a difference in learning, because it occurred prior to the administration of any foot shocks. Additionally, this difference in pretraining freezing

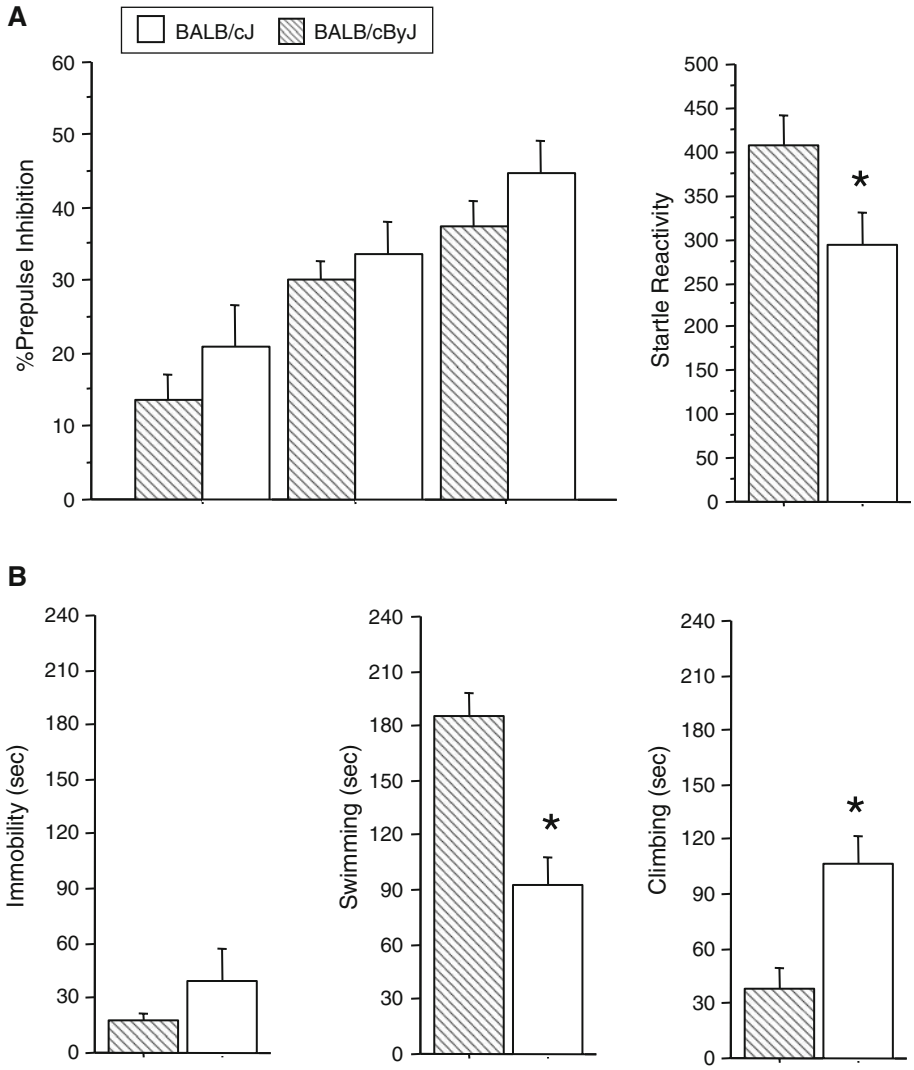

Fig. 4 **a** Startle reactivity and prepulse inhibition and **b** forced swim test measures for BALB/cJ ($n = 9$) and BALB/cByJ ($n = 12$) mice are shown. Values are means ± SEM. Values are means ± SEM. An asterisk (*) indicates a significant difference between substrains

is highly unlikely to contribute to the large differences in aggressive behavior observed between the two substrains. Furthermore, the activity of these two strains was similar in the open field test. Thus, the large differences in aggressive behavior observed between these two strains do not appear to be an artifact of either locomotor or freezing differences.

Substrain differences in freezing to tone, context, and altered context were not significant.

SNPs

We resequenced 20 regions that were predicted to be polymorphic between these two strains based on data from the JAX Phenome Database. To our surprise we found that none of these polymorphisms could be verified. These false positives are likely due to genotyping errors recorded in the database. The clustering of multiple SNPs in small genomic regions was apparently the result of error prone sequence data deposited in these databases, and the remaining polymorphisms were apparently due to genotyping errors as well. Based on these data we conclude that few or none of the 37 SNPs available in public databases for BALB/cJ and BALB/cByJ are real. This indicates that historical accounts of the relationship of these two strains are accurate and suggests that phenotypic differences may instead be due to novel mutations.

CNVs

In an effort to identify new mutations, we examined CNVs between the two BALB/c substrains. We identified a total of 11 CNV regions (Table 1). Several of the regions were quite large and contained multiple genes. A total of 30 genes or predicted genes were partially or completely contained within these identified CNVs. We have previously identified the region on chromosome 17 (Williams et al. 2009) and have verified this finding using both PCR and real time PCR. The other regions have not been

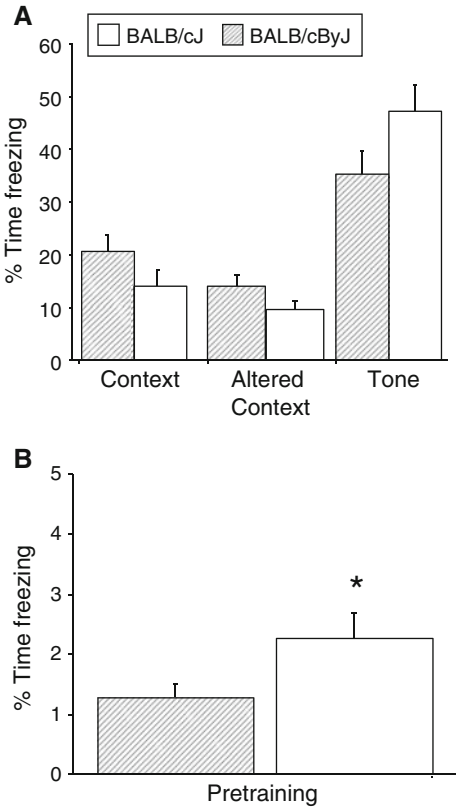

Fig. 5 Percent time BALB/cJ ($n = 20$) and BALB/cByJ ($n = 20$) mice spent freezing to the context, the altered context, or the tone (**a**), or during the pretraining period (**b**). Values are means ± SEM. An asterisk (*) indicates a significant difference between substrains

validated and may be false positives. It is also likely that additional CNVs exist between these two strains but that our discovery strategy was not sufficiently sensitive to detect them.

Discussion

Here we show that male BALB/cJ mice exhibit robust increases in offensive aggression compared to BALB/cByJ mice in the resident intruder paradigm. Furthermore, we found that these two substrains show only more modest differences in other behavioral paradigms examined, including tests of exploration and startle reactivity. Finally, we identified 11 CNV regions between the two substrains containing 30 genes. These genes represent novel candidate genes for the modulation of murine offensive aggression; however, further experiments will be required to determine whether any of these identified genes are responsible for the differences in aggressive behavior between the substrains.

The BALB/cJ substrain has previously been suggested to exhibit robust increases in aggression compared to the BALB/cN substrain. BALB/cJ mice show shorter latencies to attack an intruder following 2 weeks of isolation and acute tail pinch than BALB/cN mice (Ciaranello et al. 1974, but see Maengwyn-Davies et al. 1973). However, the use of painful stimuli such as tail-pinch have been reported to generate aggression in mouse strains that do not normally exhibit aggressive behavior, suggesting problems with the validity of this method (Brain 1975; Miczek et al. 2001). Furthermore, BALB/cJ mice have not previously been compared to the BALB/cByJ substrain for aggressive behavior. We found that male BALB/cJ mice show increases in aggressive behavior relative to BALB/cByJ mice that are robust and reliable (Figs. 1 and 2). Both substrains showed low initial levels of aggression as measured by attack number, attack incidence, attack latency, threat number, and tail rattles following short or long term isolation in the resident intruder test. However, BALB/cJ mice showed larger progressive increases in aggression than BALB/cByJ mice during additional tests. Following long term housing with a female, BALB/cJ mice exhibited substantially higher levels of aggression than BALB/cByJ starting on the first test day (Fig. 2b), and aggression levels plateaued on day 2 for both strains. Thus, we found that BALB/cJ mice consistently show elevated levels of offensive aggression compared to BALB/cByJ mice in several variants of the resident intruder paradigm.

We found few differences in other behavioral measures of emotionality or information processing between the two BALB/c substrains. The effect sizes of substrain differences in the resident intruder test were substantially greater than those obtained for startle reactivity or rearing in the open field test. For example, rearing, which provides a measure of exploratory behavior, was the only measure which differed between the two substrains in the open field test. BALB/cByJ mice exhibited more rearing than BALB/cJ mice (Fig. 3) (effect size = .82). BALB/cByJ mice also exhibited greater startle magnitude compared to BALB/cJ mice (Fig. 4a) (effect size = .89), although PPI was comparable between the substrains. Differences were observed between BALB/c substrains in swimming and climbing behavior in the FST; however, these findings do not indicate differences in depression-related behavior. Furthermore, no differences in active versus passive (immobility) behavior was found, indicating that depression-like or coping behavior was similar between the substrains. Similarly, no differences in fear conditioning were observed between the two substrains. Thus, BALB/cJ and BALB/cByJ mice showed similar levels of anxiety–like behavior in both the open field and fear conditioning tests. The effect sizes observed for these differences in exploratory behavior and startle reactivity were smaller than those found in resident aggression toward an intruder following either 4 weeks of isolation or after housing with a female. For

example, the average effect size across all measures for aggression following 48 h of isolation, 4 weeks of isolation, and 4 weeks of breeder housing were .88, 1.65, and 1.62, respectively. Thus, BALB/cJ mice exhibit higher levels of offensive aggression than BALB/cByJ mice in the resident intruder test that are robust and relatively specific.

We identified 11 relatively large CNVs that might account for the robust difference in aggressive behavior between BALB/cJ and BALB/cByJ substrains (Table 1). At the same time, we discovered that none of the SNPs available in public databases could be confirmed by sequencing. None of the genes identified have been directly implicated in aggressive behavior; however, several of these genes, including *Foxd2*, *Nrxn3*, and *Upf3b* have reported functions which might influence aggressive behavior. For example, *Foxd2* is a transcription factor which plays a critical role in adrenal development; disruption of *Foxd2* results in hypoplastic adrenal glands (Else and Hammer 2005; Kume et al. 2000). Our results suggest that BALB/cJ mice possess more copies of this gene relative to BALB/cByJ mice (Table 1). *Nrxn3*, which is present in additional copies in BALB/cByJ compared to BALB/cJ mice, is an adhesion molecule thought to regulate reward-related learning by modulating the synaptic plasticity of neurons in the indirect pathway of basal ganglia (Kelai et al. 2008), and has recently been associated with cocaine and alcohol dependence (Hishimoto et al. 2007; Rodd et al. 2008). Mutations in *Upf3b*, a member of the nonsense-mediated mRNA decay complex, cause syndromic and nonsyndromic mental retardation in humans (Tarpey et al. 2007). This gene is present in additional copies in BALB/cJ vs. BALB/cByJ mice. Several genes associated with mental retardation in humans alter aggressive behavior when mutated in mice (D'Adamo et al. 2002; Frints et al. 2003). Another gene that we identified within CNV regions, *Glo1,* has been implicated in the modulation of anxiety-like behavior in mice, but is unlikely to be responsible for the large differences in aggression observed between these two strains because many inbred mouse strains have the same number of copies as either BALB/cJ or BALB/cByJ but do not show the high levels of aggression seen in BALB/cJ (Williams et al. 2009).

Our present findings regarding CNVs identified between BALB/cJ and BALB/cByJ mice should be interpreted with caution. Due to the microarray used, our approach can only detect CNVs that span exons, as discussed in Williams et al. (2009). Thus, additional CNVs may exist between the two BALB/c substrains. Of the CNVs identified here, we have confirmed only the CNV on chromosome 17 which contains *Glo1* using a PCR-based strategy (Williams et al. 2009). Although it is possible that one or more genes within the identified CNVs are responsible for the large differences in aggressive behavior, an unidentified SNP or CNV could also responsible for the phenotypic difference observed. Furthermore, it could be that two or more mutations are required to produce the aggressive phenotype. Additional experiments in which these two substrains are crossed to produce F_2 or backcross progeny would be required to determine whether any of the identified CNVs co-segregate with the aggressive phenotype.

In conclusion, we have shown that male BALB/cJ mice exhibit high levels of offensive aggression relative to BALB/cByJ mice in several variants of the resident intruder test. These two substrains show relatively small differences in some measures of exploratory behavior and startle reactivity, suggesting that the difference in aggression represents a relatively specific behavioral phenotype. CNVs identified between these two substrains contain gene(s) which might be responsible for the differences in aggressive behavior; alternatively, an unidentified locus may be responsible. Future experiments are needed to determine whether any of the novel candidate genes identified here play a role in modulating aggressive behavior.

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