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ORIGINAL INVESTIGATION

A verification of previously identified QTLs for cocaine-induced activation using a panel of B6.A chromosome substitution strains (CSS) and A/J x C57Bl/6J F2 mice

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Abstract
Background The objective of this study was to confirm provisional quantitative trait loci (QTL) for cocaine-induced locomotor activation, on chromosomes 1, 5, 6, 9, 12, 15, 16, 17, and 18, previously identified in the AXB/BXA recombinant inbred (RI) and AcB/BcA recombinant congenic (RC) strains of mice derived from A/J (A) and C57BL/6J (B6) progenitors. This was accomplished through a genetic analysis of cocaine-induced activity in an AxB6 F2 cross and a phenotypic survey across a panel of B6.A chromosome substitution strains (CSS) mice. Mice were tested for cocaine-induced activity, following administration of saline and cocaine (20 mg/kg), utilizing an open-field procedure.
Results Among AxB6 F2 mice, differences in cocaine-induced activity were associated with loci on chromosome 1 (D1Mit305), 5 (D5Mit409), 16 (D16Mit131), and 18 (D18Mit189). A survey of the CSS panel confirmed cocaine QTLs on chromosomes 5 and 15, previously identified in RI or RC strains. Overall, the regions on chromosomes 5 and 18 represent verification of QTL previously identified in both the RC and RI strains. Additionally, the B6 allele for these QTL was consistently associated with greater relative cocaine activation.
Conclusions Collectively, chromosome 5 and 18 QTL have now been replicated in multiple independent crosses derived from the A/J and C57BL/6J progenitors. The use of an in silico analysis highlighted potential candidate genes on chromosomes 5 and 18. The present results complement the targeted gene approach currently prevalent in the study of cocaine and provide a broader empirically based focus for subsequent candidate gene studies.

Keywords Cocaine · F2 · CSS · QTL · Activity · Mice

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Introduction

Individual differences in the response to the psychomotor stimulant properties of cocaine have been shown to be mediated by genetic factors (George and Ritz 1990; Janowsky et al. 2001; Shuster et al. 1977). Quantitative trait loci (QTL) analysis continues to be one of the leading methods for the discovery of genes involved in complex neurobehavioral traits (Boone et al. 2008). A QTL approach, which can isolate multiple genes that individually account for small amounts of variance, has been used to identify chromosomal regions and potential candidate genes associated with the effects of multiple drugs of abuse including cocaine, ethanol, and morphine (Gill and Boyle 2008; Hitzemann et al. 2008; Radcliffe et al. 2007; Doyle

et al. 2008). To date, QTL analysis has been most extensively used in genetic analyses of alcohol-related phenotypes (Hitzemann et al. 2008; Bennett et al. 2008; Radcliffe et al. 2007; Ammons and Hunt 2008). A small number of studies have mapped QTLs related to cocaine-induced changes in locomotor activation and neurochemistry using the BXD recombinant inbred (RI) panel of strains derived from a cross between the C57BL/6J (B6) and the DBA/2J (D2) (Jones et al. 1999; Tolliver et al. 1994; Miner and Marley 1995; Phillips et al. 1998). Jones et al. (1999) used the BXD panel to examine the influence of cocaine (5–45 mg/kg) on several measures of activity including total distance travelled, nosepokes, repeated movements, and time spent in the center of an open-field apparatus. In addition to the behavioral phenotypes, Jones et al. (1999) examined dopamine receptor binding using [3H]-SCHH23390 (D1 receptors), [125I]-epidepride (D2 receptors), as well as [3H]-GBR12935 to measure dopamine transporter binding in the same panel of strains. QTLs associated with total cocaine activity were identified on chromosomes 5, 9, and 15. A large QTL region on Chr 15 (40–55 cM) was associated with variations in the Bmax for dopamine D1 and D2 receptors in the caudate-putamen, as well as cocaine-related behaviors. Utilizing the B6, D2, and 16 BXD RI strains, Tolliver et al. (1994) examined the effects of acute cocaine (1–56 mg/kg) on locomotor activity. The D2 progenitors were significantly more stimulated by cocaine than the B6, and putative QTL for acute cocaine-induced locomotion were identified on Chr 9, 11, 16, and 17. Miner and Marley (1995) measured the effects of cocaine (10 mg/kg) in 11 BXD RI strains. The largest QTLs for cocaine activation (cocaine−saline difference scores) were found on Chr 3 and 17. QTLs associated with cocaine-induced activation were identified on chromosomes 5, 8, 9, and 16. Finally, Phillips et al. (1998) used 25 strains from the BXD RI series to map genes associated with sensitivity to the acute stimulant and sensitizing effects of cocaine (5–40 mg/kg). QTLs for acute cocaine-induced activation were identified on all chromosomes except 6, 11, 17, and X.

The present authors have attempted to expand upon the database of putative QTL associated with cocaine-induced activation using crosses differentially derived from the B6 and the A/J strains. The confirmation of QTLs using additional crosses is necessitated by virtue of the fact that QTLs identified solely with RI lines should be considered provisional due to the large number of statistical tests conducted during QTL analysis and the potential for an inflated level of type 1 error (i.e., false positives). Generally, it is recognized that QTLs found in one genetic model need to be confirmed using a variety of models including the use of other RI sets, F2 intercrosses, and congenic mice (Dudek and Underwood 1993; Belknap et al. 1996). An analysis of the AXB/BXA series of RI strain in the present laboratory identified QTLs for cocaine-induced locomotor activation on Chr 5 (80 cM), 12 (23 cM), 15 (46.8 cM), 17 (4.1 cM), and 18 (30 cM; Boyle and Gill 2001). The B6 progenitor was shown to be significantly more activated by a 20-mg/kg dose of cocaine than the A/J. QTLs on chromosomes 12 and 15 for cocaine difference scores (cocaine−saline) were considered to be highly significant using conservative statistical criteria.

Subsequently, an independent analysis of cocaine-induced activation in the AcB/BcA recombinant congenic (RC) strains provided confirmation of a number of QTL previously identified in the AXB/BXA RI, as well as some novel loci (Gill and Boyle 2003). Significant regions were identified on chromosomes 1 (13–25.7 and 36.9–58.5 cM), 5 (1–28 and 84–86 cM), 6 (7–26.35 cM), 7 (9.4–27.8 cM), 9 (9–28 cM), 13 (21–37 cM), 16 (36–66 cM), 17 (22.5–24.5 cM), and 18 (45–48 cM), using 625 informative microsatellite marker. Multiple regression analysis demonstrated that a subset of four markers including D5Mit182 (24 cM), D5Mit409 (84 cM), D7Mit83 (26.5 cM), and D13Mit54 (35 cM) accounted for 90% of the genetic variance in cocaine activation difference scores.

This study represents one component of a multistage strategy for mapping QTLs, in which provisional associations are confirmed in independently derived crosses. It has been suggested that a multistage approach for linkage reduces the probability of false positives (McClearn et al. 1997).

More specifically, the objective of this study was to confirm the provisional QTL for cocaine-induced locomotor activity on chromosomes 1, 5, 6, 9, 12, 15, 16, 17, and 18 identified in the RI and RC strains of mice. This was accomplished by completing a genetic analysis of cocaine-induced locomotor activation in an AxB6 F2 cross and by conducting a phenotypic survey of the effects of cocaine across a panel of B6.A chromosome substitution strains (CSS) mice. The use of CSSs provides a statistically powerful approach for examining the genetic architecture of complex traits (Shao et al. 2008). The B6.A CSS strains have been used as a simple, rapid method of determining the chromosomal location of QTLs (Nadeau et al. 2000) for several complex traits, including prepulse inhibition (Leussis et al. 2009), sensitivity to methamphetamine (Bryant et al. 2009), anxiety (Singer et al. 2004), and cancer (Youngren et al. 2003). Methodologically, the CSS model has been demonstrated to be more efficient than traditional crosses in that it requires the testing of fewer progeny to detect a specific effect, or allows smaller effects to be detected with a given number of subjects (Belknap 2003). Furthermore, the ability to detect QTLs is enhanced as all, but the target chromosome, are fixed, eliminating variance owing to segregating QTLs on other chromosomes. The unique aspect of CSS (compared with RI or RC strains) is that the genetic background is homogeneous. The B6.A CSS, used in the present study, consists of a panel of 21 strains, each carrying

a different A/J chromosome transferred intact onto the B6 background. Application of the CSSs consists of phenotyping the entire panel of B6.A CCS strains; individual strains showing significant differences compared with the B6 progenitor are known to carry a QTL.

Materials and methods

Mice

Breeder pairs of the A/J, B6, and 20 strains of the B6.A CSS (19 autosomes and X chromosome) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Breeder pairs of A/JxB6 F2 and all CSS strains were set up in the Research Institute of the McGill University Health Centre, with strict adherence to protocols ensuring uniformity with regards to housing, lighting, handling, cage changing, noise, and exposure to stressors.

F1 and F2 mice were constructed using reciprocal crosses of the progenitor A/J and B6. For example, F1 crosses (A/J x B6 F1, B6 x A/J F1) were mated in all possible combinations to produce F2 (AB6F1 x AB6F1, AB6F1 x B6AF1, etc.), and sex and cross type were considered in all subsequent analyses. B6 and A/J progenitor mice were bred concurrently in order to assure a consistent exposure to environmental conditions. All mice were housed with same-sex litter mates until 8 weeks of age when testing commenced. All breeder pairs and pups were housed in an animal colony controlled for temperature and humidity on a 12-h day/night cycle (lights on between 0600 and 1800 hours), in standard (12.5 cm (H)×28.5 (W)×17.5 (D)) polycarbonate solid bottom cages with beta chip bedding and nestlet pads with Harlan Teklad (global 18% protein rodent diet) mouse chow and water ad libitum. Animals were tested under identical conditions in mixed squads (sex and strain) of approximately 24–32 mice over the course of a 2-year period. The Facility Animal Care Committee, in compliance with the Canadian Council on Animal Care, approved all procedures.

Locomotor activity testing

Locomotor activity was measured in open-field boxes constructed of Plexiglas, measuring 30×30×40 cm high. Six intersecting light-photocell assemblies placed inside the walls of the chambers, 3 cm above the floor, monitored the locomotor activity by automatic registration on a computer connected to the photocells. The hardware and software were custom designed and built in-house. Counts were automatically registered at 1-min intervals throughout the sessions. A custom-designed computer software program monitored activity on the photocells, providing measures of horizontal activity and stereotypy. Horizontal activity was operationally defined as the number of sequential beam breaks made over the course of the test period. Horizontal locomotor counts were automatically filtered in order to suppress activity resulting from the repetitive breaks of a single beam due to grooming. Testing was performed under dim red light (40 W) in a sound-insulated room between 1600 and 1800 hours immediately prior to lights out.

Response to cocaine in naive male and female mice was examined using a repeated measures design in injection procedures, as well as to the activity chambers by means of intraperitoneal (i.p.) injections of sterile 0.9% saline solutions (0.01 ml/g body weight) on two successive habituation days (days 1 and 2), followed by cocaine (20 mg/kg) on day 3. The dose of cocaine was chosen based on previous research with the A/J, B6, and AXB/BXA RI strains (Boyle and Gill 2001). The 20-mg/kg dose was found to be an activating dose in the B6 and to maximally separate the progenitors and RI strains. Locomotor activity was monitored for 15 min following each injection. Cocaine hydrochloride (2 mg/ml) was prepared in sterile saline and administered i.p. in a volume of 0.01 ml/g body weight. Cocaine-induced activity and procedures specific to each cross type are described below for the F2 and CSS mice.

Cocaine-induced activation testing in AxB6 F2 mice

Following habituation on days 1 and 2, F2 mice were tested for responses to cocaine on two test sessions spaced at 2-day intervals (test days 3 and 4). On each of these test sessions, mice received a single drug injection (cocaine, 20 mg/kg i.p.), and activity was monitored for 15 min. All F2 mice were tested twice for sensitivity to the effects of cocaine, in order to calculate a coefficient of variation and determine the reliability of the individual F2 scores.

Difference scores (total horizontal activity on cocaine day−total horizontal activity for saline day 2) were calculated for each cocaine test session (days 3 and 4), and the scores were averaged. Total horizontal activity was calculated as a function of the number of horizontal beam breaks observed over the 15-min period. A total of 405 F2 mice were initially phenotyped. One mouse was excluded for missing a datum. A correlational analysis indicated that cocaine-induced activation on days 3 and 4 (for the entire 404 mouse sample) was significantly correlated (cocaine total scores: $r=0.61$, $p<0.001$; cocaine difference scores $r=0.578$, $p<0.001$). Additionally, the mean cocaine difference scores (mean for days 3 and 4) were highly correlated with the individual day 3 and day 4 cocaine difference scores ($r=0.864$ and 0.910, respectively). Seventy-six mice were subsequently excluded by virtue of exceeding the test/retest reliability criteria as defined by a coefficient of variation >1.0. It should be noted that only 18 mice (out of the 76 excluded) exceeded a CV of 2.0. One hundred eighty mice representing the top and

bottom 28% from the extremes of the phenotypic distribution (328 mice) were genotyped (93 females and 87 males).

Genotyping

Genomic DNA samples were prepared from tail clips using standard extraction methods (tissue lysis, proteinase K, and ethanol precipitation) according to protocols described by Hofstetter et al. (1997). MIT mouse MapPair primers were purchased from Invitrogen (Carlsbad, CA, USA), and polymerase chain reaction (PCR) was conducted in 96-well microtiter plates using standard recommended conditions. PCR products were electrophoresed on agarose gels, stained, and scanned to a computer using a GeneGenius gel documentation system. Genotypes were scored independently by two individuals, entered into Microsoft Excel, and exported to Map Manager QTX (Manly et al. 2001). Previously identified QTL regions were targeted for genotyping in the AxB6 F2 mice. For each provisional QTL identified, at least three microsatellite markers were used for confirmation of the QTL. One marker was selected nearest the peak of the QTL, with two flanking markers within the 95% confidence interval (1-LOD support interval) of the QTL. A total of 32 markers on chromosomes 1, 5, 6, 9, 12, 15, 16, 17, and 18 were genotyped as shown in Table 1.

Table 1 A listing of the chromosomal positions of markers genotyped in the AxB6 F2 mice

Chromosome	Marker	cM	Genome coordinates (basepairs)
1	D1MIT82	36.9	73273629-73273775
1	D1Mit7	41	74999392-74999495
1	D1Mit415	52	88315827-88315981
1	*D1Mit305*	*55.1*	*90626498-90626642*
5	D5Mit148	18	32278263-32278411
5	D5Mit182	21	37920888-37921029
5	D5Mit31	78	138998202-138998421
5	D5Mit409	84	146673682-146673879
6	D6Mit159	7	29701222-29701334
6	D6Mit384	27.5	55149860-55149984
9	D9Mit297	15	33875129-33875232
9	D9Mit328	23	41695932-41696113
9	D9Mit4	29	51931283-51931404
12	D12Mit46	16	35725677-35725810
12	D12Mit236	22	46217345-46217465
12	D12Mit233	52	109494466-109494615
15	D15Mit156	39.1	71155976-71156119
15	D15Mit158	46.9	Not available
15	D15Mit245	58.9	94108982-94109100
15	D15Mit161	69.2	96841490-96841588
16	D16Mit131	4.3	7319135-7319274
16	D16Mit57	21.5	29406628-29406739
16	D16Mit84	34.2	45397811-45397949
16	D16Mit152	57	85804079-85804183
17	D17Mit66	24.5	47859524-47859654
17	D17Mit88	29.5	57368714-57368955
17	D17Mit142	47.4	79365240-79365386
18	D18Mit149	24	45157191-45157326
18	D18Mit51	37	61299030-61299225
18	D18Mit186	45	72180072-72180196
18	*D18Mit189*	*48*	*Not available*
18	D18Mit4	57	84295656-84295862

Data analysis

Each mouse is assigned a unique identifier that is used to track and coordinate all aspects of the data collection (study ID number, date, batch, cross type, phenotypic data, tail clip, DNA tube, and genotypes). Data were coded and entered into a central tracking database using Microsoft Excel and Prism (Graphpad Software Inc.). Subsequent statistical analysis was conducted using the micro-computer version of Statistical Package for the Social Sciences (SPSS; version 13.5 for Windows).

For the F2 mice, associations between genotype and phenotype were examined using a number of techniques including analysis of variance (ANOVA), single-locus association analysis, and composite interval mapping. Linkage analysis was performed to confirm previously identified QTLs using Windows QTL Cartographer 2.5 (Wang et al. 2006). Initially, an analysis at defined loci (simple marker associations) was performed. Confirmation in the F2 mice of previously identified QTLs was based upon a p value threshold of 0.01 (the nominal p value of 0.01 was corrected for multiple comparisons using the false discovery rate (FDR)), as proposed by Lander and Kruglyak (1995). Secondly, an analysis of positions inferred between loci with statistical control for other loci known to affect the trait (composite interval mapping (CIM)) was conducted. (Significant and suggestive QTL regions were selected as controls.) Permutation tests were performed on the data to empirically estimate the threshold for suggestive and significant loci (1,000 permutations at 1 cM intervals).

ANOVA was used to examine phenotypic differences among genotypes (homozygous A/J or B6, heterozygous) in F2 mice at each marker tested (including factors related to sex and cross type where appropriate). Each significant locus was re-tested for association using multiple regression.

Cocaine-induced activation testing in the CSS panel

Males and females for each of the 20 CSS and progenitors were assessed for sensitivity to the activating effects of

cocaine. Response to cocaine (20 mg/kg) in naive male and female CSS mice was assessed on day 3 following habituation to the handling and injection procedures as described above on days 1 and 2. Difference scores (total horizontal activity on cocaine day 3−total horizontal activity for saline day 2) and regression with the derived residuals were calculated. A total of 514 male and female CSS mice were evaluated (average of 25 mice/strain), along with 20 B6 and 17 A/J mice.

Data analysis

Each mouse was assigned a unique identifier that is used to track and coordinate the data collection as described above for the F2 mice. Subsequent statistical analysis was conducted using the micro-computer version of SPSS (version 13.5 for Windows). Data were tested in order to verify the absence of significant outliers using the SPSS explore function. Additionally, the SPSS explore function was used to assess the normality of the F2 distribution. Specially, normality plots with tests (Kolmogorov–Smirnov statistics) were used to test the null hypothesis that the distribution is normally distributed.

The statistical analysis of the phenotypic differences between the B6 and CSS strains was accomplished using a two factor (strain×sex) ANOVA. The identification of informative CSS strains (i.e., those strains carrying a donor A/J chromosome that produced a significantly different phenotype compared to the B6) was conducted using simple main effects contrasts (planned comparisons). A nominal p value of <0.01 was used as a threshold for establishing confirmation of locus a priori hypothesized to mediate the expression of cocaine-induced locomotor activity in the CSS strains. Novel QTL identified in the CSS were considered to be provisional, requiring further study for confirmation.

Results

AxB6 F2 mice: allelic influences on the expression of cocaine-induced activation

Overall, mean cocaine-induced activity (difference scores) for the F2 population was 635.174 (±22.52) and was normally distributed (Fig. 1a; Kolmogorov–Smirnov statistic (0.046, p=0.088)). Mean cocaine activation difference scores for the F2 mice were assessed as a function of genotype (A, B6, and heterozygote) at the specific MIT markers listed in Table 1. A series of two-way ANOVAs (sex×genotype) were used to determine whether there were significant differences in mean phenotype values of progeny with different genotypes. The results yielded a number of significant sex×marker

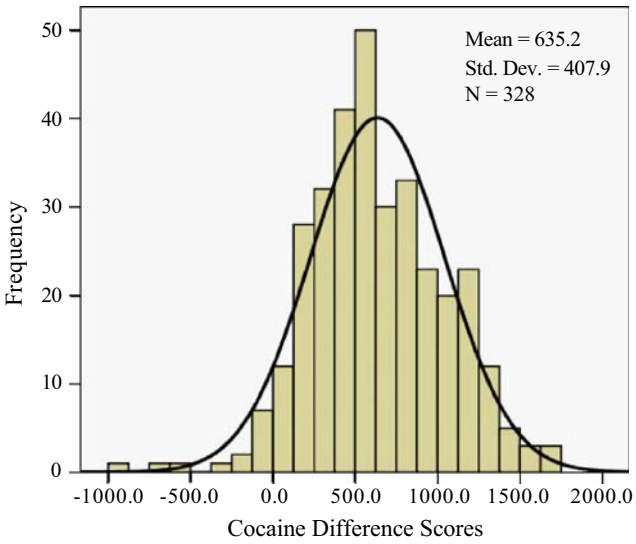

Fig. 1 Illustration of the frequency distribution of the mean cocaine difference scores for the entire sample of AxB6 F2 mice

genotype interactions, therefore, all subsequent analyses were conducted for males and females separately.

Significant differences in cocaine activation difference scores were observed as a function of allelic status for D18Mit189 (48.0 cM) [F(2,65)=4.15, p=0.020] on chromosome 18 in males. QTLs were identified in the females on chromosomes 5 (D5Mit409–84 cM; [F(2,80)=3.669, p=0.030]) and 16 (D16Mit131–4.3 cM; [F(2,81)=3.70, p=0.029]). These findings are summarized in (Table 2). In the QTLs identified in the AxB6 F2 on chromosomes 5, 16, 18, the B6 allele was associated with greater relative cocaine activity scores (increaser allele). The relationship between allelic status for representative loci on chromosomes 5 and cocaine-induced activity is presented in Fig. 2.

Quantitative trait loci analysis

A test of simple associations was conducted in order to identify associations between the genotyped markers and cocaine difference scores. Significant QTLs for cocaine difference scores detected by Windows QTL Cartographer are described in Table 2. The results of the analysis provided support for an association between the markers on chromosomes 1 and 18 in males and females and sex specific QTLs on chromosomes 5 and 16 (females). It should be noted that the marker identified on chromosome 1 (D1Mit305) was identified by Windows QTL Cartographer, but not by ANOVA. CIM analysis was used in order to control for the influence of unlinked QTLs. Target loci associated with cocaine difference scores were mapped while controlling for the influence of other QTLs. The 95% confidence intervals for the significant QTLs were estimated using 1-LOD score drop in the interval flanking the QTL

Table 2 Analysis of quantitative trait loci (QTL) in the AxB6 F2 mice assessed as a function of analyses using both analysis of variance (ANOVA) and Windows QTL Cartographer

Markers	Loci (cM)	Genome coordinates (basepairs)	1-LOD support	Chromosome	ANOVA[a]		QTL[b]	
					Sex	*p* value	Sex	*p* value
D1Mit305	55.1	90,626,498-642	13.7	1	–	NS	M, F	0.01
D5Mit409	84.0	146,673,682-879	3.9	5	F	0.03	F	0.016
D16Mit131	4.30	7,319,135-274	20.4	16	F	0.029	F	0.013
D18Mit189	48.0	Not available	25.7	18	M	0.020	M, F	*0.007*

[a] Independent one-way ANOVA were used to assess significant differences in mean phenotypic values of progeny with different genotypes (A, B6, and heterozygote) at specific markers

[b] Significant QTL for cocaine difference scores detected by Windows QTL Cartographer

Note that the QTL on chromosome 1 was only identified in analyses using Windows QTL Cartographer. ANOVA failed to indicate significant differences (NS) for males and females

and presented in Table 2. No additional QTL's were identified. The QTL on chromosome 18 confirms previously identified loci, based upon both the identification of informative strain (a nominal p value ≤ 0.01 adjusted for multiple comparisons through the use of the FDR) and the demonstration of concordance with the direction of allelic influences.

CSS strain survey

Males and females for each of the 20 strains of the CSS panel and parental strains were assessed for sensitivity to the activating effects of cocaine. Due to the significant sex effects displayed by the F2 mice, cocaine difference scores in males and females were analyzed separately, in addition to the combined analysis, using one-way ANOVA. Planned comparisons (simple main effect contrasts) were used to evaluate the significance of main effect differences between each CSS strain and the B6 parental strain.

The results of the one-way ANOVA among the male CSS indicated significant effect for strain [$F(21,281)= 3.442$, $p<0.001$]. An analysis of simple main contrast effects indicated that the CSS show decreased cocaine

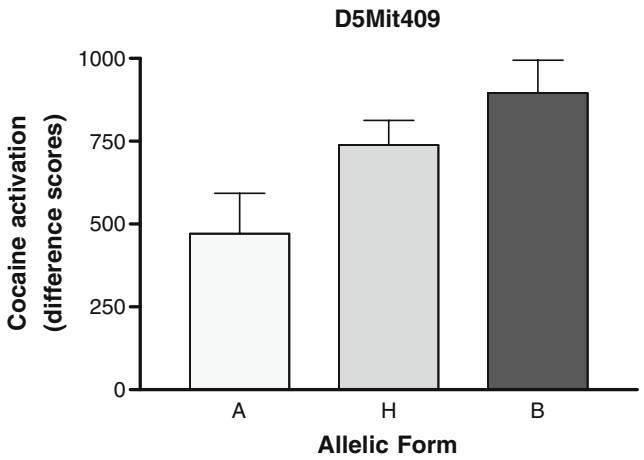

Fig. 2 Illustration of cocaine-induced activation in female AxB6F2 mice presented as a function of allelic status at D5Mit409. *B* designates alleles inherited from the B6 stain, *A* from the A/J, and *H* indicates heterozygous

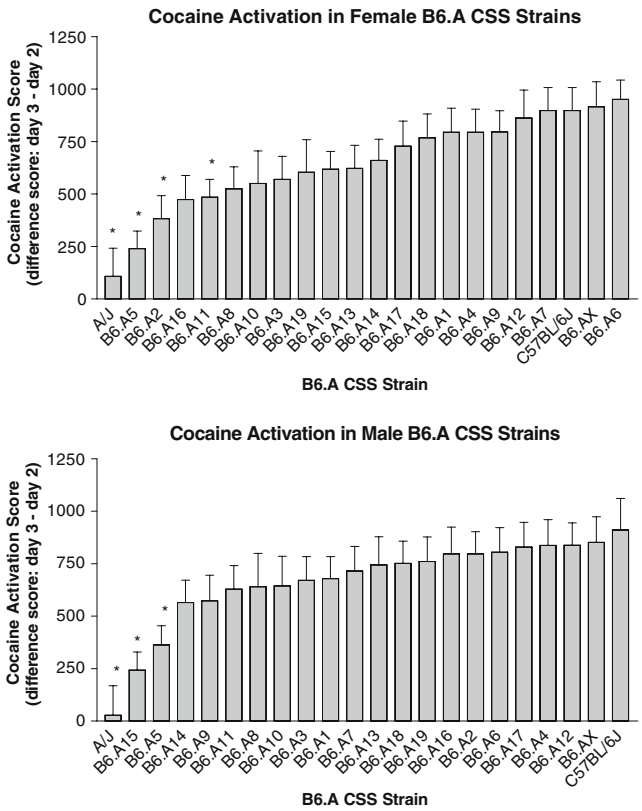

Fig. 3 Mean cocaine activation scores (+SEM) for female (*top panel*) and male (*lower panel*) chromosome substitution strains (CSS) mice. *Double asterisk* denotes CSS strains which are significantly different from the C57BL/6J background strain at a nominal corrected p value of <0.01. *Single asterisk* denotes informative strains with a p value of <0.05

activation on chromosomes 5, 15 ($p<0.01$), relative to the B6 progenitor strain. Similarly, a significant strain effect was observed in females [$F(21,252)=3.947$, $p<0.001$]. An analysis of simple contrast effects identified the CSS-2, -5, and -11 as informative strains ($p\leq0.01$). The CSS strain distribution patterns observed in the males and females independently are presented in Fig. 3. An analysis of the combined sample (males+females) indicated significant effect for strain [$F(21,533)=5.876$, $p<0.001$]. An analysis of simple main contrast effects indicated that the CSS show decreased cocaine activation on chromosomes 5, 8, 11, and 15 ($p<0.01$), relative to the B6 progenitor strain. The informative CSS-5 strain provided confirmation of QTLs previously identified in the AcB/BcA RC and/RI strains. Confirmation was established as function of both the identification of informative strains (a nominal p value of ≤0.01, corrected for multiple comparisons) and the demonstration of concordance with the direction of allelic influences on increasing or decreasing the phenotypic scores. The QTLs for cocaine-induced activity independently mapped in the AXB/BXA RI, AcB/BcA RCS, AxB6 F2, and the B6.A CSS panel are presented in Table 3. In the informative CSS strains, lower cocaine-induced activity scores were consistently observed in those strains carrying the A/J chromosome on a B6 background.

A potential confound in the use of drug change phenotypes, including difference and percent change scores, is the influence of strain differences in baseline activity measures. In order to address this issue, an analysis of derived regression residuals was performed to evaluate a measure of cocaine-induced changes in locomotor activity, which by definition is independent of baseline values. The resulting analysis of both males and females combined indicated a significant effect of strain [$F(21,533)=5.404$, $p<0.001$]. The CSS-5, -11, and -15 were identified ($p<0.01$) as informative strains. These strains are consistent with those observed following analysis of the cocaine difference data presented above. Thus, these findings increase the confidence in the reliability of the cocaine difference phenotype.

Discussion

The present analysis of AxB6 F2 and B6.A CSS mice confirmed multiple QTLs on chromosomes 1, 5, 15, 16, and 18 associated with cocaine-induced locomotor activity. Significantly, the regions associated with the QTL on chromosomes 5 and 18 confirm those reported previously in the AcB/BcA RC and/or AXB/BXA RI strains. B6 alleles were consistently associated with greater relative cocaine activity scores (representing increaser alleles). QTL on chromosomes 5 has now been replicated in multiple crosses (AXB/BXA RIS, AcB/BcA RCS, AxB6 F2 cross (female specific), and the B6.A CSS panel), derived from the A/J and C57BL/6J progenitors. Collectively, the confirmation of the QTL in multiple independent crosses suggests that they are unlikely to simply represent chance associations.

The locus confirmed in the present study on chromosome 5 (80 cM) is consistent with a report in the literature by Phillips et al. (1998). In this study, genes were mapped for sensitivity to the acute stimulant and sensitizing effects of cocaine. Sensitivity and sensitization to cocaine (5, 10, and 40 mg/kg) were measured in 25 BXD/Ty RI (BXD RI) strains and the progenitor B6 and DBA/2J (D2) strains. In contrast, the chromosome 18 QTL identified in both male and female AcB/BcA RCS and confirmed in the AxB6 F2 has not previously been associated with cocaine activation in the literature.

The identification of potential candidate genes underlying behavioral phenotypes is a significant challenge. Considerable difficulty is encountered in the effort to narrow QTLs to intervals that contain only a small number of candidate genes. In order to address this challenge, classical intercross mapping in combination with bioinformatics methods, including gene expression analysis, has been suggested to provide an effective means for narrowing QTL regions (Mackiewicz et al. 2008; DiPetrillo et al. 2005; Flint et al. 2005).

Chromosome 5 QTL

Initially, the Ensembl database (2009) was used in order to identify the base pair boundaries for the markers flanking the region defined by the 95% confidence interval for the chromosome 5 QTL identified in the RI strains. An interval from 125.931691 to 149.865583 Mbp was obtained. A search of the Mouse Genome Informatics (MGI 2009) located 376 genes within this region (map positions based upon the National Center for Biotechnology Information build 37). Subsequently, the Mouse Phenome Database (2009) was accessed in order to conduct a haplotype analysis using the RI strains in order to reduce the size of the region of interest. A bivariate analysis was conducted in order to identify significant ($p<0.01$) associations between cocaine activation and haplotype structure, and to identify regions without allelic variation across strains. The haplotype maps with the AXB/BXA RI strains arranged as a function of ascending cocaine activation values revealed that only the region between 132.505749 and 149.865583 Mbp showed significant correlations between the haplotype structure and cocaine activation. Within this region, a survey of polymorphic sites between the A and B6 produced a list of 24 unique genes producing non-synonymous substitutions in amino acids of proteins. A priori, the filtering of strong candidate genes is based upon the potential for changes in DNA sequence altering the amino acid make-up of the

Table 3 Significant quantitative trait loci (QTL) for cocaine-induced activity independently mapped in the AXB/BXA RI, AcB/BcA RCS, AxB6 F2 cross, and the B6.A chromosome substitution strains panel

AXB/BXA RI strains			AcB/BcA RC strains		AxB6 F2		B6.A CSS	Literature
Chr	Peak marker (cM)	Peak marker genome coordinates (bp)	Peak marker (cM)	Peak marker genome coordinates (bp)	Peak marker (cM)	Peak marker genome coordinates (bp)		Cocaine-related behaviors (includes crosses with B6 or A/J progenitors)
1			*D1Mit415* (*52*)	88,315,827-5981	*D1Mit305* (*55.1*)	90,626,498-642	NS	Jones et al. 1999; Phillips et al. 1998
5	*D5Mit32* (*78*)	136,395,980-6102	*D5Mit409* (*84*)	146,673,682-879	*D5Mit409* (*females*;*84*)	146,673,682-879	++	Phillips et al. 1998
6			*D6Mit159* (*7*)	29,701,222-1334	*NS*		NS	Jones et al. 1999
9			*D9Mit328* (*23*)	41,695,932-6113	*NS*		NS	Jones et al. 1999
15	*Pdgfb* (*48.6*)	79,826,330-45238			*NS*		++ (males)	Jones et al. 1999; Phillips et al. 1998
16			*D16Mit188* (*52.5*)	76,817,838-7961	*D16Mit131* (*females*; *4.3*)	7,319,135-274	NS	Phillips et al. 1998; Tolliver et al. 1994
17	D17J2 (4.1)	Not available	*D17Mit66* (*24.5*)	47,859,524-654	*NS*		NS	Tolliver et al. 1994; Miner and Marley 1995
18	Csf1r (30.0)	61,265,226-90788	*D18Mit186* (*45*)	72,180,072-196	*D18Mit189* (*48*)	Not available	NS	

Concordance in the directionality of allelic influences between all four test crosses was observed for the QTL on chromosome 5, suggesting that this chromosomal region harbors a gene that influences sensitivity to cocaine in mice

Results for AXB/BXA RI panel were published by Boyle and Gill (2001); results from AcB/BcA recombinant congenic panel were published by Gill and Boyle (2003)

Peak marker was identified by Map Manager QTX, and position was provided in recombinant distance in centimorgans from centromere

Significance of QTL was determined through permutation tests in Map Manager QTX, following the guidelines of Lander and Kruglyak (1995)

Analysis of AxB6 F2. Genotyping was conducted with 32 SSLP markers on chromosomes 1, 5, 6, 9, 12, 15, 16, 17, and 18 (n=180)

B6.A CSS ++ indicates that the B6.A CSS carrying the designated A/J chromosome displayed a significantly different phenotype compared to the B6 progenitor, indicating the presence of a QTL on the chromosome

translated protein. In part, this approach is based upon the literature which suggests that the value in identifying regulatory polymorphisms is hindered by limited knowledge of functional regulatory elements (DiPetrillo et al. 2005). A review of the Mouse Phenome Database (http://www.jax.org/phenome) was performed in order to identify those genes for which there was current evidence in support of gene expression in brain or brain subregions. Of the 24 genes with non-synonymous mutations, ten were found to be expressed in brain tissue (see Table 4).

Chromosome 18 QTL

The chromosome 18 QTL interval contained a limited number genotypic regions where the progenitor A and B6 differed, thus it was suitable for in silico analysis. Initially, the Ensemble database was used in order to identify the base pair boundaries for the markers flanking the region defined by the 95% confidence interval for the chromosome 18 QTL (see Table 3) confirmed in the RC and F2 mice. It should be noted that the directionality of the chromosome 18 QTL influence on behavior in the RI was not concordant with those detected in the RC and F2 mice. As such, the interval for the RI chromosome 18 QTL was excluded from the present in silico analysis. An interval from 69.19 to

Table 4 Genes within the significant quantitative trait loci (QTL) regions on chromosomes 5 and 18 for which there is current evidence of central nervous system expression

SNPs data downloaded from Mouse Phenome Database (http://www.jax.org/phenome)		Description
Mbp location (build 37)	NCBI gene annotation	
Chr5: 134713707	Gtf2i	General transcription factor II I
Chr5: 134965256	Clip2	CAP-GLY domain containing linker protein 2
Chr5: 135095746	Eif4h	Eukaryotic translation initiation factor 4H
Chr5: 138580930	Zfp113	Zinc finger protein 113
Chr5: 139045844	Zfp68	Zinc finger protein 68
Chr5: 145908383	Ptcd1	Pentatricopeptide repeat domain 1
Chr5: 145965431	Zkscan5	Zinc finger with KRAB and SCAN domains 5
Chr5: 146890843	Cyp3a59	Cytochrome P450, subfamily 3A, polypeptide 59
Chr5: 148142317	Flt3	FMS-like tyrosine kinase 3
Chr5: 148373180	Flt1	FMS-like tyrosine kinase 1
Chr18: 71418392	DCC	Deleted in colorectal carcinoma
Chr18: 74427945	Mbd1	Methyl-CpG binding domain protein 1

76.00 Mbp was identified. A search of the MGI database located 38 genes within this region. A survey of polymorphic sites produced a list of five unique genes producing non-synonymous substitutions in amino acids of relevant proteins. Of the five genes with non-synonymous mutations, two were found to be expressed in brain tissue, specifically deleted in colorectal carcinoma (DCC) and Mbd1 (Table 4).

DCC candidate gene

The DCC gene is a potentially interesting candidate gene in relation to cocaine-related phenotypes. Cocaine is a powerful dopamine reuptake inhibitor, and its primary site of action is on presynaptic neurons in the mesolimbic dopamine system (Koob and Le Moal 2008; Leshner and Koob 1999). The DCC gene is highly expressed in dopamine neurons and is also likely to play a role in the development and organization of dopaminergic circuitry (Flores et al. 2005). Netrins form a family of proteins that regulate the migration of neurons and axonal growth during brain development. The attraction and repulsion responses produced by Netrin-1 are mediated by specific receptors in the DCC and UNC-5 family of proteins (Barallobre et al. 2005). Through the use of mice heterozygous for a null mutation of DCC, it has been demonstrated that alterations in netrin-1 receptor levels during development lead to major changes in the functional organization of mesocorticolimbic DA circuitry. DCC heterozygous mice exhibit sizeable increases in basal as well as amphetamine-induced extracellular concentrations of DA in medial prefrontal cortex, but decreased amphetamine induced DA activity in nucleus accumbens (Flores et al. 2005). Additionally, the DCC mutant mice exhibit blunted amphetamine-induced locomotion and reward, resistance to amphetamine-induced deficits in sensorimotor gating, and absence of amphetamine sensitization (Flores et al. 2005; Grant et al. 2007). Future research will examine the role of the DCC gene in cocaine-related behaviors, such as locomotor activation and intravenous cocaine self-administration. Ultimately, the relevance of the DCC gene, or any other candidate genes in general, in the expression of cocaine-induced behavioral effects, will require future proteomics research. Specifically, an analysis of protein expression across brain regions and developmental stages will be required.

The confirmation of QTL and the identification of novel candidate genes mediating the expression of cocaine-induced behavioral effects in the present study are significant. The study of genetic influences on the expression of cocaine-induced behavioral effects appears to have transitioned to an essentially targeted gene approach. In the past 5 years, only one report has been published describing a QTL analysis of cocaine-related phenotype in animals (Gill and Boyle 2008). In contrast, during the last 2 years alone, at least 20 papers have been published which used a targeted candidate gene approach (Imbesi et al. 2009; Pulipparacharuvil et al. 2008; Brunk et al. 2008). The choice of candidate genes for analysis appears to be based largely upon the known neurobiological mechanisms of cocaine, including dopamine receptors (Imbesi et al. 2009) and the circuitry of reward (Brunk et al. 2008). While candidate gene studies are crucial in testing specific hypotheses regarding the involvement of target genes in the expression of cocaine' behavioral effects, the approach is limited in its capacity to identify novel genes and pathways previously unknown to play a role in the behavioral effects of cocaine. As noted by McClearn et al. (1997), the value of QTL studies resides in using heritable differences in inbred strains to identify the genes underlying specific vulnerabilities. The identification of novel candidate genes through QTL and in silico analyses compliment the prevalent targeted gene approach and provide a broader empirically based focus for subsequent targeted gene studies.

The results of this study confirm previously reported QTL for cocaine-induced activity in AXB/BXA RI and AcB/BcA RC strains. Collectively, the loci on chromosomes 5 and 18 have been replicated across multiple independent crosses derived from the A/J and C57BL/6J progenitors. The use of in silico analyses reduced the number of candidate genes within these QTL regions. The DCC gene was proposed as a potential candidate for targeted genetic analysis. Significantly, the present QTL and in silico analyses have identified potential candidate genes previously unknown to play a role in the behavioral effects of cocaine.

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