

A major QTL on chromosome 11 influences psychostimulant and opioid sensitivity in mice

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The identification of genes influencing sensitivity to stimulants and opioids is important for determining their mechanism of action and may provide fundamental insights into the genetics of drug abuse. We used a panel of C57BL/6J (B6; recipient) × A/J (donor) chromosome substitution strains (CSSs) to identify quantitative trait loci (QTL) for both open field activity and sensitivity to the locomotor stimulant response to methamphetamine (MA). Mice were injected with saline (days 1 and 2) and MA (day 3; 2 mg/kg i.p.). We analyzed the total distance traveled in the open field for 30 min following each injection. CSS-8, -11 and -16 showed reduced MA-induced locomotor activity relative to B6, whereas CSS-10 and -12 showed increased MA-induced locomotor activity. Further analysis focused on CSS-11 because it was robustly different from B6 following MA injection, but did not differ in activity following saline injection and because it also showed reduced locomotor activity in response to the mu-opioid receptor agonist fentanyl (0.2 mg/kg i.p.). Thus, CSS-11 captures QTLs for the response to both psychostimulants and opioids. Using a B6 × CSS-11 F₂ intercross, we identified a dominant QTL for the MA response on chromosome 11. We used haplotype association mapping of cis expression QTLs and bioinformatic resources to parse among genes within the 95% confidence interval of the chromosome 11 QTL. Identification of the genes underlying QTLs for response to psychostimulants and opioids may provide insights about genetic factors that modulate sensitivity to drugs of abuse.

Keywords: A/J, C57BL/6J, chromosome 11, chromosome substitution stains, CSS, F₂, fentanyl, locomotor

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Drugs of abuse, including both psychostimulants and opioids, increase locomotor activity in rodents (Wise & Bozarth 1987). This behavior is partially mediated by dopamine release in the nucleus accumbens (NAc) (Di Chiara & Imperato 1988; Koshikawa *et al.* 1989), a brain region critical for drug reward. Differences in the sensitivity to the locomotor activating effects of methamphetamine (MA) are heritable (Phillips *et al.* 2008), and we and others hypothesize that some of the genes that mediate differences in MA-induced locomotor activity may also modulate the rewarding effects of drugs. Consistent with this hypothesis, we previously identified an expression polymorphism in casein kinase 1 epsilon (*Csnk1e*) that modulates MA-induced locomotor activity in mice (Bryant *et al.* 2009a; Palmer *et al.* 2005) and translated this finding to humans by showing that a polymorphism in *CSNK1E* predicted the subjectively euphoric effects of amphetamine in healthy human volunteers (Veenstra-VanderWeele *et al.* 2006). More recently, a polymorphism in *CSNK1E* that is associated with heroin dependence has been reported (Levrin *et al.* 2008). These data support the hypothesis that genes that modulate the locomotor stimulant response to MA in mice may also influence the rewarding effects of drugs and the risk for developing drug abuse in humans.

Several studies have identified chromosomal regions, termed quantitative trait loci (QTL), that are associated with differential sensitivity to the locomotor stimulant effects of psychostimulant drugs in mice (Phillips *et al.* 2008). Chromosome substitution strains (CSSs) provide a rapid means for identifying QTLs for complex traits such as the locomotor stimulant response to MA (Nadeau *et al.* 2000; Singer *et al.* 2004). Each CSS has been bred such that a single chromosome from the donor strain (A/J) has been introgressed onto an otherwise homogenous, recipient background (B6). Phenotypic differences between a CSS and B6 indicate the presence of a QTL on the substituted chromosome. The advantages and disadvantages of using CSS for QTL mapping have been previously discussed (Nadeau *et al.* 2000); two notable advantages include the elimination of genotyping and the ability to fine map by creating an intercross between B6 and the relevant CSS. Disadvantages include lower initial QTL localization (e.g.

1 compared to an F_2) and the inability to detect epistatic
2 interactions.

3 We surveyed a panel of C57BL/6J (B6) \times A/J mouse CSS
4 for open field activity on days 1 and 2 and the locomotor
5 response to MA on day 3. We also used a B6 \times CSS-11
6 F_2 population to further map a QTL on chromosome 11.
7 Following localization of this QTL to the distal region of
8 chromosome 11, we used haplotype association mapping
9 to identify cis expression quantitative trait loci (eQTLs)
10 for several brain regions. Finally, we used bioinformatic
11 resources to identify non-synonymous coding SNPs between
12 B6 and A/J within the chromosome 11 QTL.

13 Methods

14 Subjects

15 All experiments were performed in accordance with the National
16 Institutes of Health Guidelines for the Care and Use of Laboratory
17 Animals and were approved by the University of Chicago's
18 Institutional Animal Care and Use Committee. Breeder pairs for B6,
19 A/J and each CSS were obtained from the Jackson Laboratories (Bar
20 Harbor, ME, USA) and offspring were generated for testing at the
21 University of Chicago. The number following CSS (CSS-#) indicates
22 which B6 chromosome has been substituted with the corresponding
23 A/J chromosome. CSS-13 was not included in the study because
24 of poor breeding, which prevented us from obtaining the necessary
25 sample size. F_2 mice were generated by crossing B6 females with
26 CSS-11 males to create an F_1 generation, and then intercrossing
27 female and male F_1 mice to create F_2 mice.

28 Mouse colony rooms were maintained on a 12/12-light/dark cycle
29 with lights on at 0600 h. All mice were provided unlimited access
30 to food and water, except during testing. Two to five same-sex
31 littermates were housed in clear plastic cages with standard corn
32 cob-type bedding; cage mates were always tested on the same
33 days. Testing was conducted between 0900 h and 1600 h. Mice
34 were transported from the vivarium next door into the test room and
35 were allowed to habituate for at least 30 min before testing.

36 Behavioral testing

37 Mice were tested over the course of 15 three-day sessions; not all
38 strains were tested in all three-day sessions due to the breeding
39 limitations. Approximately 4.5 times more B6 mice were phenotyped
40 compared to any individual CSS, as recommended by (Belknap 2003).
41 The exact number of mice tested is listed in Table 1. B6 mice were
42 included in almost all 3-day sessions, whereas individual CSS and
43 A/J mice were tested in only a fraction of 3-day sessions. Care was
44 taken to randomize and balance the order and sex of strains in each
45 3-day session; an average of six strains were included in each 3-day
46 session. CSS mice ranged from 7 to 14 weeks old on the first day of
47 testing; F_2 mice ranged from 7 to 11 weeks old on the first day of
48 testing.

49 The procedures for behavioral testing have been described
50 previously (Bryant *et al.* 2009a; Palmer *et al.* 2005). Just before
51 testing, mice were removed from their home cages and placed in
52 clean holding cages for approximately 5 min after which they received
53 an intraperitoneal injection of saline (10 ml/kg; days 1 and 2) or MA
54 (2 mg/kg i.p.; day 3) and were immediately placed in the center of
55 the open field; total distance traveled over the subsequent 30 min
56 was recorded. The 12 open fields were cleaned before and after
57 each 30 min recording on each of the 3 days with 10% isopropanol.
58 Locomotor activity was measured using automated Versamax open
59 field (AccuScan, Columbus, OH, USA). Each open field arena was
made of a clear acrylic arena (40 \times 40 \times 30 cm) placed inside a frame
containing evenly spaced photocells and receptors making a grid
of infrared photobeams from the front to the back and from the

left to the right of the arena. The floor of the open field is white.
Beam breaks were recorded on a computer and converted into total
distance traveled (cm). Each open field was surrounded by a sound
attenuating PVC/lexan environmental chamber (AccuScan). In each
open field, overhead lighting provided dim illumination (~80 lux)
and a fan provided ventilation and masking of background noise.
In a separate series of experiments (described in following text),
we examined the effects of fentanyl (0.2 mg/kg i.p., day 3; Sigma,
St. Louis, USA), a selective mu-opioid receptor agonist, on B6 and
CSS-11 strains; the protocol was identical except that on day 3,
fentanyl was injected instead of MA. This fentanyl dose produces a
robust increase in locomotor activity in the open field over 30 min in
C57BL/6J mice (Bryant *et al.* 2009b).

19 Analysis

20 The dependent measure for all analyses was total distance traveled
21 over 30 min. In some analyses we treated days 1, 2 and 3 as repeated
22 measures, whereas other analyses examined each day separately.
23 In addition, we considered the difference in activity between days 3
24 and 2 (day 3–day 2), which we and others have previously used to
25 identify the response to MA treatment (e.g. Palmer *et al.* 2005).

26 For the comparisons involving B6 vs. A/J and B6 vs. CSS-11
27 mice, we used a three-way repeated measures analysis of variance
28 (ANOVA) to examine the factors such as day (within group repeated
29 measures), strain and sex. For analysis of all CSS, we used two-way
30 ANOVAs to examine the effect of strain and sex for each day (1, 2
31 or 3) in separate analyses using age as a covariate. Because there
32 was never a main effect or interaction of sex or age, these variables
33 were collapsed out of the analysis and we followed up with one-way
34 ANOVAs for the factor strain. Main effects and interactions between
35 strain and day were further examined with *post hoc* tests (unpaired
36 or paired *t*-tests) as appropriate.

37 We converted behavioral data into Z-scores and used these values
38 to compare each strain to B6, using Equation (3) from Belknap
39 (2003). The Z-score reflects the number of standard deviations that
40 separate the means of B6 and each CSS. Based on Dunnett's test,
41 a significance threshold of $Z > 2.9$ ($P < 0.004$) was employed to
42 correct for multiple comparisons against a single B6 background
43 strain; whereas $Z > 1.96$ ($P < 0.05$) was considered suggestive
44 (Belknap 2003). We also used the Z-scores to estimate the effect
45 size (proportion of the variance explained, v_{CSSB}^2), using Equation (4)
46 of Belknap (2003). This value indicates the proportion of total variance
47 explained by fitting the genetic effect. Thus, by generating Z-scores
48 we were able to determine which strains captured significant QTLs
49 and the effect of those QTLs on the phenotype.

50 For the analysis of the F_2 cross between B6 \times CSS-11, 87 F_2
51 mice (39 females, 48 males) were tested in the same manner as
52 the CSS. DNA was extracted from the tail and used for genotyping.
53 We selected single nucleotide polymorphism (SNP) markers with an
54 average spacing of 7.6 cM as estimated by a previously published
55 genetic map (Shifman *et al.* 2006). Mb positions are based on
56 build 37 of the mouse genome: rs13480888 (16.9 Mb), rs13480921
57 (25.7 Mb), rs6280308 (32.6 Mb), rs13481015 (48.1 Mb), rs13481044
58 (57.6 Mb), rs13481117 (79.1 Mb), rs13481170 (95.5 Mb), rs3023315
59 (99.4 Mb), rs13481220 (108.4 Mb) and rs13481256 (118.0 Mb). All
assays were conducted using Applied Biosystems TaqMan[®] SNP
Genotyping Assays, according to the manufacturer's instructions.

Quantitative trait loci analysis of the F_2 mice was conducted using
interval mapping (step = 1 cM) and the estimation maximization
procedure as implemented in *R/qtl* (Broman *et al.* 2003). We used a
genetic map that was generated from our marker data for mapping;
this map showed good agreement with the previously published
maps. The significance threshold was set at $P < 0.05$ as determined
by 1000 permutations. The 95% confidence interval was estimated
by calculating the Bayes credible interval using *R/qtl*. Effect plots
were generated for the marker with the highest LOD score.

60 Haplotype association mapping of eQTL

61 An eQTL is a locus that modulates the expression of a particular gene.
62 Quantitative trait loci for complex traits can be mediated by gene

Table 1: The *n*, mean age (shown in days), SEM of the mean age and the age range for B6, A/J and each CSS is listed for each sex

Strain	<i>n</i> (females)	Age (females) (mean ± SEM and range)	<i>n</i> (males)	Age (males) (mean ± SEM and range)
B6	55	67.0 ± 1.2 (50–82)	53	65.9 ± 1.3 (52–79)
A/J	9	63.4 ± 1.9 (57–73)	10	71.3 ± 1.2 (66–75)
CSS-1	4	72.5 ± 0.5 (71–73)	6	55.0 ± 1.3 (53–59)
CSS-2	10	66.7 ± 3.6 (52–81)	10	72.6 ± 4.7 (51–83)
CSS-3	8	62.0 ± 1.9 (55–69)	14	60.0 ± 1.5 (54–67)
CSS-4	10	68.1 ± 1.4 (60–72)	9	63.4 ± 2.0 (58–72)
CSS-5	14	64.1 ± 2.2 (54–73)	10	58.9 ± 1.9 (54–73)
CSS-6	10	66.8 ± 1.7 (62–74)	9	71.6 ± 3.6 (60–89)
CSS-7	10	63.2 ± 1.7 (59–71)	11	59.0 ± 0.5 (57–61)
CSS-8	12	64.8 ± 1.4 (60–71)	8	69.1 ± 2.2 (63–75)
CSS-9	10	67.8 ± 2.1 (58–72)	9	66.6 ± 2.1 (60–72)
CSS-10	13	62.8 ± 1.5 (58–70)	16	62.7 ± 1.4 (57–71)
CSS-11	14	60.0 ± 2.7 (48–76)	23	67.7 ± 2.2 (50–78)
CSS-12	15	80.5 ± 2.0 (72–92)	34	78.7 ± 2.0 (62–92)
CSS-14	9	71.4 ± 0.2 (71–72)	5	74.6 ± 5.0 (63–85)
CSS-15	10	62.8 ± 1.5 (54–65)	13	65.7 ± 1.5 (54–70)
CSS-16	13	71.2 ± 1.8 (65–80)	9	71.2 ± 1.6 (65–77)
CSS-17	3	74.3 ± 3.7 (67–78)	9	66.3 ± 0.9 (62–69)
CSS-18	5	62.4 ± 0.6 (60–63)	9	61.4 ± 1.0 (55–64)
CSS-19	10	61.6 ± 2.1 (55–69)	10	66.0 ± 2.2 (56–72)
CSS-X	12	75.3 ± 1.2 (72–82)	6	54.0 ± 0.0 (54–54)

expression differences due to polymorphisms in regulatory regions near the gene (cis acting) or elsewhere in the genome (trans acting). We were interested in identifying cis-eQTL within our chromosome 11 QTL, because such eQTL could be causally related to the QTL for differential locomotor response to MA. Expression QTL can be detected in standard mapping populations, including F₂ crosses and recombinant inbred strains (Chesler *et al.* 2004). However, no brain eQTL data are publicly available for crosses between B6 and A/J. Therefore, we conducted haplotype association mapping using a panel of inbred strains (23–29 per brain region) chosen from the Mouse Phenome Project priority strains (Bogue 2003) for which we had also measured gene expression. This method takes advantage of the fact that laboratory inbred strains were largely derived from the same founders and share regions of the genome that are largely identical by descent (IBD) (Wiltshire *et al.* 2003). Therefore, at some

locations, B6 and A/J will be IBD with respect to each other, and thus *unlikely* to segregate opposite alleles of an eQTL. At other loci, B6 and A/J will not be IBD with respect to one another, but may be IBD with respect to many other inbred strains. By comparing the gene expression of A/J-like strains with B6-like strains at regions where A/J and B6 are not IBD, it can be determined whether the tested location is correlated with a difference in gene expression.

Gene expression in the inbred strains was measured in five brain regions (striatum, NAc, prefrontal cortex, amygdala and hippocampus). Adult (10- to 12-week-old) mice were euthanized and brain regions were collected from groups of three experimentally naive male mice. The brain was positioned in a 1-mm brain block with the anterior surface abutting a single-edged razor blade placed in the first slot and ventral surface visible. The slot nearest the boundary of medulla was used as the first landmark. Razor blades

were placed in slots 1 and 2 mm anterior to that boundary. Additional razor blades were placed in remaining anterior slots. Prefrontal cortex was dissected from the section +1.7 to +1.2 mm to Bregma at the anterior surface by taking a triangular section formed by two incisions made 45° from the midsagittal plane using the corpus callosum as a ventral boundary. From the section +1.3 to +0.7 mm to Bregma at anterior surface, NAc was dissected by using a 1 mm punch to remove tissue ventrolateral to anterior commissures and striatum was extracted using a 1 mm punch between the corpus callosum and anterior commissure. Two 2-mm thick sections -2.0 mm to Bregma at posterior surface were dissected. In the first section, a horizontal cut was made at the ventral boundary of the external capsule. Another cut in line with the external capsule was made to separate the piriform cortex from the amygdala. In the remaining 2-mm thick section, the cortex was peeled apart from the hippocampus. Tissues were quickly frozen on dry ice. Tissues were pulverized while frozen, and total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA), then further processed by using the RNeasy Miniprep Kit according to manufacturer's protocols (Qiagen, Chatsworth, CA, USA). The quality of all samples was determined with an Agilent Bioanalyzer (Palo Alto, CA, USA).

Five micrograms of total RNA was used to synthesize cDNA that was then used as a template to generate biotinylated cRNA. cRNA was fragmented and hybridized to Affymetrix MOE430 gene expression arrays, according to the manufacturer's instructions. The arrays were then washed and scanned with a laser scanner, and images were analyzed by using the MAS5 algorithm. Arrays were normalized by using global median scaling.

A total of 10 990 SNPs spaced at ~300-kb intervals were chosen for genotyping the strains and inferring haplotypes. A 3-SNP window was used to assign strains to a haplotype, with a minimum requirement of five strains per haplotype to be considered at a locus (McClurg *et al.* 2006; Pletcher *et al.* 2004). A marker association algorithm combined with family-wise error rate (gFWER) analysis was used to identify associations and to account for relatedness among strains and thus, decrease the rate of false positive associations (McClurg *et al.* 2006). We report only eQTLs that were located in regions where B6 and A/J were assigned to different haplotypes groups and these eQTLs had $-\log(P) > 3.5$, with a QTL peak within 50 kb of the gene whose expression is being measured.

Results

A/J vs. B6

The B6 and A/J strains were significantly different from each other on all three test days (Fig. 1) as reflected by main effects of strain ($F_{1,34} = 112.09$; $P < 0.0001$), day ($F_{2,68} = 93.37$; $P < 0.0001$) and an interaction between strain and day ($F_{2,68} = 37.33$; $P < 0.0001$). A/J showed less activity than B6 on all 3 days ($t_{34} = 10.86, 8.68$ and 8.38 , for days 1, 2 and 3, all $P < 0.0001$). Paired *t*-tests comparing activity on day 1 vs. day 2 indicated that B6 mice showed a significant decrease in activity ($t_{16} = 3.09$; $P = 0.007$), whereas A/J mice showed a trend for an increase in activity ($t_{18} = 1.93$; $P = 0.069$). Both B6 and A/J strains showed an increase in activity from day 2 to day 3 ($t_{16} = 10.21$, $P < 0.0001$; $t_{18} = 3.26$, $P = 0.0044$, respectively), reflecting the effect of MA treatment on day 3.

CSS

Table 1 lists the *N*, mean age \pm SEM and the age range for B6, A/J and each CSS for each sex; all data are available at the Mouse Phenome Database (www.jax.org/phenome). We found a significant effect of strain on day 1 activity

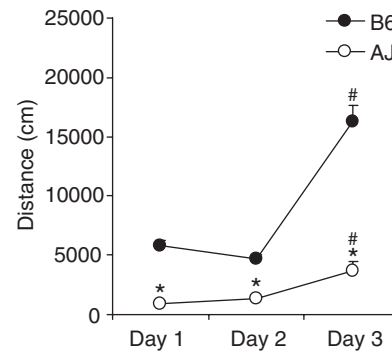


Figure 1: Locomotor response in B6 and A/J. B6 ($n = 10$ females, 7 males) and A/J mice ($n = 9$ females and 10 males) received saline injections (i.p.) on days 1 and 2 and MA (2 mg/kg i.p.) on day 3 before placement in the open field. Total distance traveled was recorded over 30 min. Data are presented as the mean \pm SEM. *, A/J significantly different from B6 for that day and #, significant difference between days 2 and 3.

($F_{19,500} = 14.20$; $P < 0.0001$; Fig. 2a). CSS-1, -10, -12, -15 and -16 showed significantly less locomotor activity on day 1 relative to B6 mice, whereas CSS-7 and -9 showed significantly more locomotor activity compared to B6. We also found a significant effect of strain on activity on day 2 ($F_{19,500} = 13.85$; $P < 0.0001$; Fig. 2b). In general, the mean activity of the strains on day 1 and 2 was highly correlated ($r = 0.93$; $P < 0.0001$) as shown in the inset of Fig. 2b. Strains CSS-3, -10, -12 and -16 showed significantly less locomotor activity relative to B6 mice, whereas strains CSS-7, -9 and -14 showed significantly more locomotor activity compared to B6.

The main focus of our study was to assess activity following treatment with MA, which was measured on day 3. There was a significant main effect of strain on day 3 ($F_{19,500} = 6.68$; $P < 0.0001$; Fig. 2c). The strains CSS-8, -11, and -16 showed significantly less locomotor activity following MA administration as compared to B6, none of the CSS showed significantly increased locomotor activity compared to B6.

We also examined the difference between activity on day 3 and day 2 (Fig. 2d), which we and others have used in the past as a way to distinguish between differences that are specific to drug treatment vs. those that are secondary to differences in basal locomotor activity and occur even in the absence of drug treatment. There was a significant effect of strain ($F_{19,500} = 8.22$; $P < 0.0001$). The results were generally similar to those shown in Fig. 2c; however, CSS-10 and -12 showed significantly more locomotor activity compared to B6. Thus, CSS-10 and -12 are greater than B6 only when the difference between day 3 and day 2 is used as the dependent measure. This difference stems from the fact that these strains showed significantly lower activity on days 1 and 2 and a non-significant trend towards higher activity on day 3. Interestingly, there was little correlation between

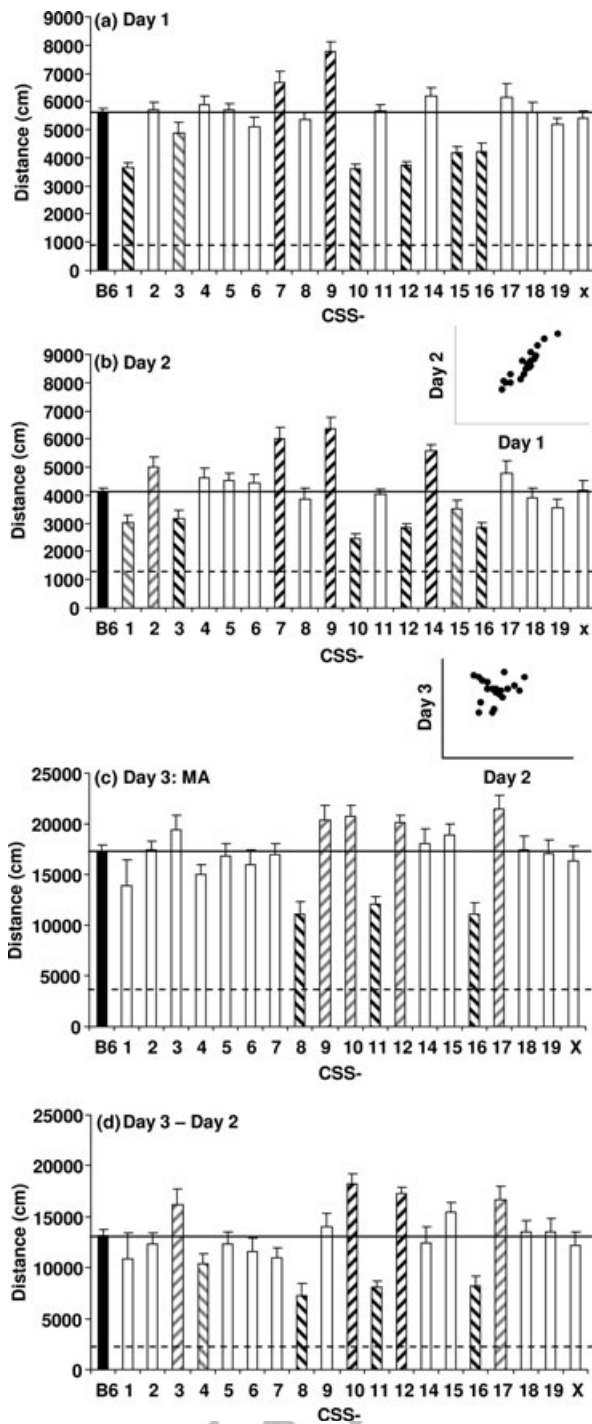


Figure 2: Locomotor response in CSS on days 1, 2, 3 and day 3 minus day 2. Total distance traveled over 30 min is shown for (a): day 1 (saline), (b): day 2 (saline), (c): day 3 (MA; 2 mg/kg i.p.) and (d): day 3 minus day 2. The *n* for each strain and sex and their mean age \pm SEM are listed in Table 1. The solid, horizontal line indicates the mean value for B6. The dashed, horizontal line indicates the mean value for A/J. The inset of panel (b) illustrates the high degree of correlation between day 1 and day 2 activity ($r = 0.93$; $P < 0.0001$). The inset of panel (d) illustrates the lack of correlation between day 3 and day 2 activity. Significant differences as compared to B6 are indicated by a black and white hatched pattern. Suggestive differences are indicated by a gray and white hatched pattern. Data are presented as the mean value for each CSS \pm SEM.

on a given chromosome (Belknap 2003). We also examined the correlations between the strain means of the variables shown in Fig. 2 and Table 3. In general, there were strong correlations between days 1 and 2, and between day 3 and day 3–day 2; however, activity on days 1 and 2 was not significantly correlated with the response to MA on day 3.

B6 vs. CSS-11

CSS-11 stood out because it had a significantly lower response to MA on day 3 as compared with B6, but was virtually identical to B6 on days 1 and 2 (Fig. 2a,b). In addition, CSS-11 had the highest Z-score for MA-induced activity on day 3 (-4.80), accounting for 14% of the trait variance. Thus, we chose to examine this strain in greater detail. We compared the response of a subset of B6 mice that were tested on the same days as CSS-11 mice across all 3 days of treatment, treating day as a repeated measure (Fig. 3a). We identified a significant interaction between strain and day ($F_{2,176} = 20.36$; $P < 0.0001$). To investigate the source of the interaction between strain and day, we compared the two strains for each day. As stated earlier, B6 and CSS-11 were not different on days 1 and 2, but CSS-11 showed a significantly lower response to MA on day 3 ($t_{88} = 5.29$; $P < 0.0001$). Both strains showed a significant increase in activity from day 2 to day 3 in response to MA (B6: $t_{52} = 17.57$; $P < 0.0001$; CSS-11: $t_{36} = 12.38$; $P < 0.0001$).

We were interested to know if these differences would generalize to opioids that are mechanistically distinct for MA but are also commonly abused by humans. We examined the response to the selective mu-opioid receptor agonist fentanyl in a separate cohort of B6 and CSS-11 mice, using a procedure that was identical to that used to examine the response to MA, except that fentanyl (0.2 mg/kg) was given on day 3 in place of MA (Fig. 3b). We observed a significant interaction between strain and day ($F_{2,38} = 14.85$, $P < 0.0001$). To investigate the source of this interaction, we examined the difference between strains at each day separately. B6 and CSS-11 were similar on days 1 and 2, but CSS-11 showed a significantly lower response to fentanyl on day 3, as compared with B6 ($t_{19} = 3.78$; $P = 0.0013$). Both strains showed a significant

activity on days 2 and 3, as shown by the inset in Fig. 2c ($r_s = 0.16$; $P > 0.05$).

To better understand the data shown in Fig. 2, we calculated Z-scores for each of these phenotypes that are shown in Table 2. Table 2 also lists v_{CVSB}^2 , which is the proportion of phenotypic variance accounted for by the QTL

Table 2: Significant (bolded) and suggestive (unbolded) Z-scores and v_{CvsB}^2 for the phenotypes of CSS relative to the B6 strain

CSS-	Day 1 activity (saline)		Day 2 activity (saline)		Day 3 activity (MA)		Day 3–day 2 (MA–saline)	
	Z	v_{CvsB}^2	Z	v_{CvsB}^2	Z	v_{CvsB}^2	Z	v_{CvsB}^2
1	-4.36	0.14	-2.47	0.050				
2			2.74	0.056				
3	-2.32	0.041	-3.12	0.071			2.37	0.042
4							-2.06	0.033
5								
6								
7	3.22	0.075	5.87	0.21				
8					-4.44	0.14	-4.45	0.14
9	6.35	0.24	6.68	0.26	2.21	0.038		
10	-7.15	0.27	-6.03	0.21	2.90	0.058	4.50	0.13
11					-4.80	0.14	-4.93	0.15
12	-8.05	0.30	-5.55	0.17	2.85	0.050	4.34	0.11
14			3.74	0.10				
15	-4.69	0.15	-2.03	0.031				
16	-4.41	0.13	-4.13	0.12	-4.65	0.14	-3.84	0.10
17					2.42	0.047	2.14	0.037
18								
19								
X								

Table 3: Correlation matrix of the average phenotypes for each CSS and B6

Variable	Day 1	Day 2	Day 3	Day 3–day 2
Day 1	—	—	—	—
Day 2	0.93	—	—	—
Day 3	0.12	0.16	—	—
Day 3–day 2	-0.21	-40.19	0.94	—

increase in activity from day 2 to day 3 in response to fentanyl (B6: $t_{10} = 5.94$; $P = 0.0001$; CSS-11: $t_9 = 2.57$; $P = 0.03$). Thus, CSS-11 has a blunted response to both MA and fentanyl as compared to B6, despite similar behavior in the absence of drug administration as measured on days 1 and 2.

B6 × CSS-11 F₂ intercross

We phenotyped and genotyped 87 B6 × CSS-11 F₂ mice in an attempt to better define the QTL for day 3 activity on chromosome 11. Figure 4 shows the QTL and effect plots for day 1 (panels a, b), day 2 (panels c, d) and day 3 activity (panels e, f). There was no effect of sex or age, nor an interaction of sex with age for activity on day 1, 2 or 3 ($P > 0.05$). For day 1 activity, a QTL at 6.5 cM (95% confidence interval = 0–37 cM) with an LOD score of 2.34 (significance threshold = 2.33) was observed; this QTL showed an additive mode of inheritance. The LOD score for day 2 activity was not significant. For day 3 activity, a QTL was observed at 52.4 cM (95% confidence interval = 45–65 cM) with an LOD score of 2.79 (significance

threshold = 2.20) and a dominant mode of inheritance. We used the Equation $1 - 10^{-2LOD/n}$ to estimate the proportion of trait variance accounted for by this QTL (n = sample size; Broman *et al.* 2003). As with CSS-11, the proportion of variance accounted for by the QTL was 14%.

Haplotype association mapping of eQTL

A total of 32 cis-eQTLs between A/J and B6 were identified (Table 4) in the 95% confidence interval (79–109 Mb; build 37) derived from our F₂ study. We did not explore the possibility that SNPs within a probe set might be responsible for the eQTL that we detected.

Non-synonymous coding SNPs

There are 30 810 reported synonymous (non-coding) and non-synonymous (coding) SNPs between B6 and A/J on chromosome 11 between 79 and 109 Mb (www.jax.org/phenome), and there are likely to be others that have yet to be identified. Of these, 204 are non-synonymous SNPs (Table S1). A total of 117 genes containing coding SNPs

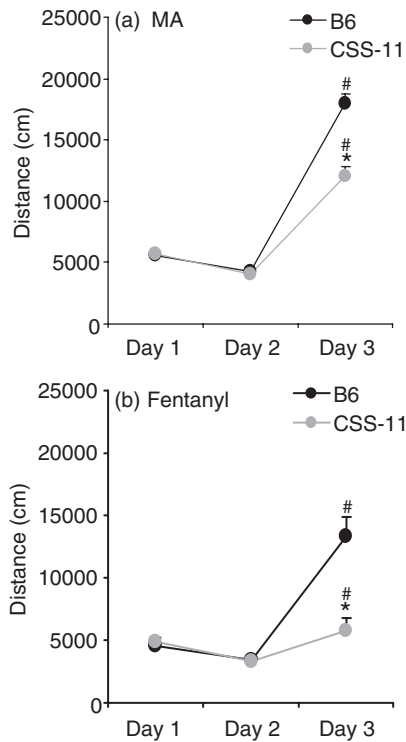


Figure 3: Locomotor response in B6 and CSS-11 to MA and fentanyl. Two different cohorts of mice received saline injections on days 1 and 2, and received either MA (2 mg/kg i.p.; panel a) or fentanyl (0.2 mg/kg i.p.; panel b) on day 3. For panel (a), $n = 23$ females, 30 males for B6 (average age = 67.8 ± 0.9) and $n = 14$ females, 23 males for CSS-11 (average age = 64.8 ± 1.8). For panel (b), $n = 5$ females, 6 males for B6 (average age = 66.2 ± 1.8) and $n = 6$ females, 4 males for CSS-11 (average age = 69.6 ± 0.7). Total distance traveled was recorded over 30 min. Data are presented as the mean \pm SEM. *, CSS-11 significantly different from B6 for that day and #, significant difference between days 2 and 3.

were identified, many of which contain multiple coding SNPs. Seven of these genes, *Stxbp4* (90.4 Mb), *Nme2* (93.8 Mb), *Rsad1* (94.4 Mb), *Atp5g1* (95.9 Mb), *Snx11* (96.6 Mb), *Mpp3* (101.9 Mb) and *Gfap* (102.8) also contain eQTLs (Table 4). The QTL for MA sensitivity could be due to coding SNPs, non-coding SNPs or to copy number differences; the latter two categories would presumably act via changes in gene expression (Williams *et al.* 2009).

Discussion

B6 and A/J mice show striking differences in locomotor activity both in the presence and absence of drug treatment (Fig. 1). Screening B6 \times A/J CSS showed multiple QTLs influencing saline-induced and MA-induced locomotor activity (Fig. 2 and Table 2). For CSS-11, we showed a blunted response to both MA and fentanyl (Fig. 3), suggesting

that a single QTL on chromosome 11 may influence the response to both drugs. We used a B6 \times CSS-11 F₂ intercross to more accurately map and determine the mode of inheritance of the QTL for MA-induced locomotor activity (Fig. 4). Bioinformatic procedures were then used to identify candidate genes within the 95% confidence interval of this QTL. Taken together, these data identify many QTLs for multiple phenotypes that may be relevant for understanding genetic variability in the response to drugs of abuse.

The genetic relationship between locomotor activity following saline vs. MA injections can be elucidated using our data. In general, we did not observe any strong relationship between activity on days 1 and 2 compared with activity following MA on day 3. One exception is CSS-16 which showed lower activity on all 3 days, suggesting that the lower activity following drug treatment may reflect a general tendency toward hypolocomotion. However, both CSS-10 and -12 showed lower activity after saline on days 1 and 2 but significant higher activity following MA on day 3 (using the day 3–day 2 measure) showing that in some strains, activity following saline is inversely correlated with MA-induced activity. These different relationships reflect the lack of overall correlation between saline- and MA-induced activity that is shown in Table 3. In some instances, a single QTL may pleiotropically influence activity in both conditions. In other cases, two or more distinct QTLs may be present. We have previously reported MA-induced locomotor stimulation as the difference between day 3 and day 2 (e.g. Palmer *et al.* 2005). However, Table 3 shows that day 3 and day 3–day 2 are highly correlated with each other. In examining Fig. 2c,d, the only minor differences between these two approaches are observed. Overall, we conclude that both measures are similar and that subtraction of day 2 neither significantly adds nor detracts from the utility of the measure.

We conducted follow-up studies on an F₂ cross between B6 and CSS-11 because of the observed strain difference was specific to the locomotor response to MA and because CSS-11 was the most divergent strain from B6 when considering the MA response (Fig. 2c,d) with Z scores of -4.80 and -4.93 (Table 2). CSS-11 was also much less sensitive to fentanyl-induced hyperlocomotor activity (Fig. 3b), suggesting a single QTL on CSS-11 may influence sensitivity to both psychostimulants and opioids (alternatively, there could be two separate QTLs, one that influences MA and the another that influences fentanyl). We have previously reported a QTL on chromosome 11 for decreased MA response using a cross between B6 and DBA/2J (Palmer *et al.* 2005). This raises the possibility that the same polymorphism causes both QTLs. Interestingly, a previous finding using B6 and A/J progenitor strains localized a QTL for sensitivity to the locomotor stimulant effect of nicotine to the same region of chromosome 11 (95% CI: 48–54 cM; Gill & Boyle 2005), perhaps indicating a QTL with pleiotropic effects on both MA and nicotine-induced locomotor activity. Alternatively, there could be separate genes on chromosome 11 regulating sensitivity to MA, fentanyl and nicotine.

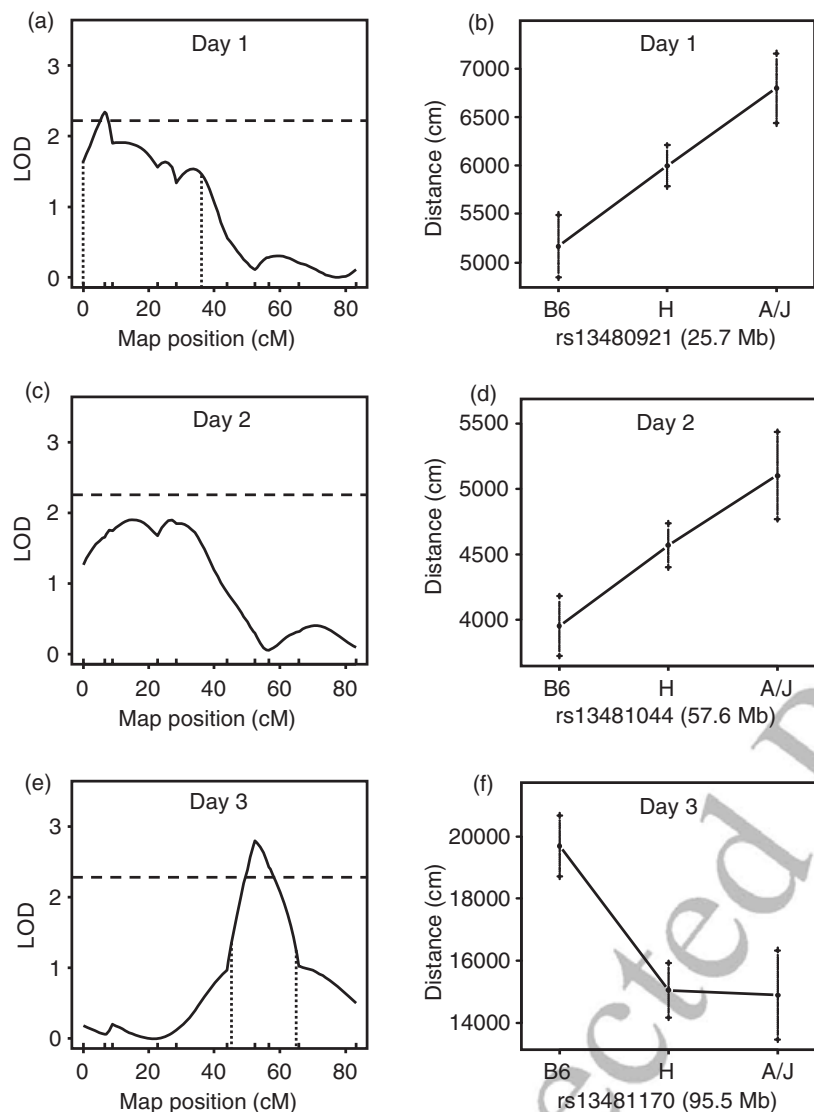


Figure 4: QTL mapping of the locomotor response using a B6 × CSS-11 F₂ population. Thirty nine females and 48 males were used for this study. Panels (a), (c) and (e): QTL plots for locomotor activity on day 1, day 2 and day 3, respectively. The dashed, horizontal lines indicate significance threshold (1000 permutations). The vertical, dotted lines indicate the 95% confidence interval (Bayes credible interval). Panels (b), (d) and (f): effect plots of the marker with the highest LOD score showing the mean and standard error for each genotype class. B6, homozygous for B6; H, heterozygous for B6 and A/J alleles and A/J, homozygous for A/J.

Despite the specificity of CSS-11 for drug-induced locomotor activity, the B6 × CSS-11 F₂ study identified a QTL influencing day 1 activity (Fig. 4a,b) that we did not observe in the parental CSS-11 (Figs. 2a and 3a,b). Nevertheless, the most robust finding of the F₂ study was the confirmation and fine mapping of the QTL for response to MA (Fig. 4e,f). The QTLs for activity on day 1 (Fig. 4a) and day 3 (Fig. 4e) are very likely to be independent for several reasons. First, the 95% confidence intervals for these two QTLs do not overlap (Fig. 4a,e). Second, the A/J allele for the day 1 QTL *increases* locomotor activity (Fig. 4b); whereas the A/J allele for the day 3 QTL *decreases* activity (Fig. 4f). Third, the day 1 QTL shows an additive mode of inheritance (Fig. 4b); whereas the day 3 QTL shows a dominant mode of inheritance (Fig. 4f). Thus, we conclude that the QTL on chromosome 11 for day 3 activity is specific for MA-induced locomotor activity and has no effect on activity in the absence of drug.

Both psychostimulants and opioids increase dopamine release, which is associated with both locomotor stimulation and the subjectively rewarding effects of these drugs in humans (Wise and Bozarth 1987). Thus, if we assume that differences observed in CSS-11 are due to the same QTL, it is possible that genes involved in dopaminergic neurons or their targets underlie this QTL. Darpp-32 (dopamine- and cyclic AMP-regulated phosphoprotein-32) is a potent phosphatase inhibitor that is highly expressed in dopamine-receiving neurons in the NAc (Ouimet *et al.* 1984; Walaas *et al.* 1983) and modulates psychostimulant-induced locomotor activity (Fienberg *et al.* 1998; Greengard 2001; Lindskog *et al.* 2002; Snyder *et al.* 2000; Zachariou *et al.* 2006) and reward (Zachariou *et al.* 2002) and opioid-induced locomotor activity (Borgkvist *et al.* 2007). *Ppp1r1b*, the gene that encodes Darpp-32, is located on chromosome 11 at 98.2 Mb which is between the two markers that showed the highest LOD

Table 4: Expression QTLs derived from haplotype association mapping for the region between 79 and 109 MB

Gene	Probe	Location (Mb)	NAc	Str	PFC	Amyg	HC
Al450353	1440345_at	83.1			4.36		3.58
Taf15	1438130_at	83.3	5.70	4.24	4.68	6.00	3.59
Acaca	1444810_at	84.0			3.57		
4632419I22Rik	1433954_at	86.0			3.80	5.40	4.42
Rps6kb1	1460705_at	86.3	5.40		4.31	6.00	4.15
Cltc	1440457_at	86.5	5.40			4.60	4.89
Gdpd1	1424077_at	86.8	6.00	4.19	6.00	6.00	6.00
Stxbp4	1442267_at	90.3	3.54				
Utp18	1454817_at	93.7	4.74	3.53	6.00	4.48	3.53
Mbtd1	1417241_at	93.7		4.17			
Mbtd1	1417261_at	93.7	4.03		5.52	5.10	
Mbtd1	1441100_at	93.7			4.52	5.05	
Nme2	1448808_a_at	93.8					3.73
C77673	1444677_at	94.2					4.96
Rsad1	1437449_at	94.4				4.72	3.71
Mrpl27	1415690_at	94.5	3.71		4.34		
Pdk2	1448825_at	94.9	4.44		5.05	5.30	4.46
Spop	1458886_at	95.3		4.19	4.92	4.92	3.50
Phb	1448563_at	95.5	3.52			3.78	
Atp5g1	1444874_at	95.9					3.60
Snx11	1424031_at	96.6	4.25				
Cbx1	1436266_x_at	96.7	3.97				
Pnpo	1415793_at	96.8		3.91	4.57		
Mel13	1435017_at	97.5			4.62	4.96	
B230217C12Rik	1428568_at	97.7	6.00		6.00	6.00	6.00
Smarce1	1422675_at	99.1		3.88			3.98
Krt12	1419230_at	99.3		3.55	5.00	4.64	
Mpp3	1419077_at	101.9				5.40	
Gfap	1440142_s_at	102.7				4.18	3.54
Cdc27	1426076_at	104.4	4.64				
Smurf2	1429045_at	106.7	3.96				
Smurf2	1429046_at	106.7	4.77			4.10	
Cacng5	1434785_at	107.8		4.60			
Prkca	1437393_at	107.8			4.40		
Amz2	1417241_at	109.3				3.59	

Genes, expression probes, physical location (build 37) and the $-\log P$ values are listed for each of the five brain regions. NAc, nucleus accumbens; Str, striatum; PFC, prefrontal cortex; Amyg, amygdala and HC, hippocampus. eQTLs are only included if B6 and A/J belong to different haplotypes. Darkened horizontal lines encapsulate one or multiple probe sets for individual genes. Genes in bold indicate those for which eQTLs were identified in the NAc, Str or both.

scores in our F_2 study. Furthermore, we previously found that a mouse line selected for high MA sensitivity exhibited an increase in *Darpp-32* transcript abundance in the NAc (Palmer *et al.* 2005). However, when comparing B6 and A/J mice, a previous study found no difference in *Darpp-32* protein expression (Brodtkin *et al.* 1998) and we found no eQTL for *Ppp1r1b* (Table 4) nor did we find any coding differences in *Ppp1r1b* between B6 and A/J (Table S1) nor did Mouse Phenome Database contain many polymorphic SNPs between B6 and A/J in the vicinity of *Ppp1r1b*, suggesting that B6 and A/J are likely to have inherited a functionally equivalent region from a recent common ancestor. Thus, we do not believe that *Ppp1r1b* underlies this QTL.

The 95% confidence interval for the MA-induced locomotor activity QTL on chromosome 11 is large and contains a

correspondingly large number of genes. It is therefore not possible to implicate a specific gene with high confidence. One gene of particular interest that was in a B6-AJ SNP-dense region was *Cacna1g*, which encodes the alpha 1 G subunit of the t-type calcium channel $Ca_v3.1$. This gene is located within 1 Mb of the marker with the highest LOD score for MA-induced locomotor activity (rs13481170; 95.5 Mb). Although we did not observe an eQTL for *Cacna1g* (Table 4), we identified a single coding mutation rs27076081 (T1078A) in the A/J allele within exon 16 of *Cacna1g* (Table S1). Calcium channel antagonists reduce psychostimulant locomotor activity (Hori *et al.* 1998; Mills *et al.* 1998; Pani *et al.* 1990; Pierce *et al.* 1998), cocaine-induced dopamine release in the caudate (Mills *et al.* 1998) and striatum (Pani *et al.* 1990) and cocaine reward

(Pani *et al.* 1991). Mice lacking the gene *Cacna1e*, which encodes the r-type Ca^{2+} channel $\text{Ca}_v2.3$, show a complete loss of the locomotor response to cocaine (Han *et al.* 2002). Interestingly, calcium channel antagonists reduce the subjectively rewarding effects of MA in healthy human volunteers (Johnson *et al.* 1999). We also identified an eQTL in the striatum for another calcium channel gene, *Cacng5*, which encodes the voltage-dependent calcium channel subunit (Table 3).

Last, in considering genes possessing both eQTLs within the NAc and/or striatum (both regions arguably being the most relevant for our phenotype) and coding SNPs, we identified two genes, syntaxin binding protein 4 (*Stxbp4*) and sorting nexin 11 (*Snx11*). Both of these genes are located in regions that are highly polymorphic between B6 and A/J. However, out of these two genes, *Stxbp4* stood out as a particularly interesting candidate. Syntaxins bind to the dopamine transporter (Torres 2006) and increase dopamine efflux in response to amphetamine (Binda *et al.* 2008). Syntaxin binding proteins bind syntaxins that result in a decrease in vesicular exocytosis (Zhang *et al.* 2000) and thus, a decrease in neurotransmitter release. Thus, differential expression of or coding differences in syntaxin binding proteins could affect methamphetamine-induced neurotransmitter release and contribute to differences in the locomotor response.

In summary, we used the B6.A/J CSS panel to dissect the genetic architecture of locomotor behavior after both saline and MA treatment. We mapped a QTL on chromosome 11 to a small interval and utilized bioinformatic resources to further parse among the candidate genes in the identified region. Because this QTL may influence sensitivity to both stimulants and opioids, identification of the underlying gene or genes could significantly enhance our understanding of sensitivity to drugs of abuse.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1: Non-synonymous coding SNPs between A/J and B6 from 79 and 109 Mb. Genes, SNP I.D., physical location, A/J and B6 nucleotides and the amino acid change are listed for all non-synonymous coding SNPs within the region (Mouse Phenome Database; build 37). Darkened horizontal lines encapsulate either one or multiple SNPs for individual genes. As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

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