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Congenic dissection of a major QTL for methamphetamine sensitivity implicates epistasis

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We previously used the C57BL/6J (B6) × A/J mouse chromosome substitution strain (CSS) panel to identify a major quantitative trait locus (QTL) on chromosome 11 influencing methamphetamine (MA)-induced locomotor activity. We then made an F_2 cross between CSS-11 and B6 and narrowed the locus (Bayes credible interval: 79–109 Mb) which was inherited dominantly and accounted for 14% of the phenotypic variance in the CSS panel. In the present study, we created congenic and subcongenic lines possessing heterozygous portions of this QTL to narrow the interval. We identified one line (84–96 Mb) that recapitulated the QTL, thus narrowing the region to 12 Mb. This interval also produced a small decrease in locomotor activity following prior saline treatment. When we generated subcongenic lines spanning the entire 12-Mb region, the phenotypic difference in MA sensitivity abruptly disappeared, suggesting an epistatic mechanism. We also evaluated the rewarding properties of MA (2 mg/kg, i.p.) in the 84- to 96-Mb congenic line using the conditioned place preference (CPP) test. We replicated the locomotor difference in the MA-paired CPP chamber yet observed no effect of genotype on MA-CPP, supporting the specificity of this QTL for MA-induced locomotor activity under these conditions. Lastly, to aid in prioritizing candidate genes responsible for this QTL, we used the Affymetrix GeneChip® Mouse Gene 1.0ST Array to identify genes containing expression QTLs (eQTL) in the striatum of drug-naïve, congenic mice. These findings highlight the difficulty of using congenic lines to fine map QTLs and illustrate how epistasis may thwart such efforts.

Keywords: Addiction, congenic, genetic, locomotor, microarray, psychostimulant, QTL, reward, striatum, transcriptome

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Sensitivity to the behavioral effects of amphetamines is heritable in mice (Phillips *et al*. 2008) and humans (Crabbe *et al*. 1983; Nurnberger *et al*. 1982) and can predict future drug abuse (Haertzen *et al*. 1983). These observations are consistent with the hypothesis that there are shared alleles between the initial drug response and the motivational behaviors that support abuse of amphetamines (Hart *et al*. in press). In rodents, the mesolimbic reward pathway contributes to the locomotor stimulant response, and thus, the study of this behavior may aid in our understanding of drug reward (Di Chiara & Imperato 1988; Wise & Bozarth 1987). A second major pathway relevant to the locomotor response is the nigrostriatal pathway, which has its own distinct signaling machinery (Sager & Torres 2011).

Methamphetamine (MA) is a substrate for the plasmalemmal dopamine (DA) transporter (DAT) (Fleckenstein *et al*. 2007) and causes an efflux of synaptic DA. A secondary target is the vesicular monoamine transporter-2 (VMAT-2), where MA can increase the level of cytosolic and synaptic DA (Fleckenstein *et al*. 2007). Molecular events that could affect the behavioral response to MA include DAT phosphorylation, trafficking and signaling (Schmitt & Reith 2010); DA receptor signaling (McGinty *et al*. 2008) and binding to VMAT-2, which could affect vesicular sequestration and exocytosis (Fleckenstein *et al*. 2007). MA also targets norepinephrine and serotonin plasmalemmal transporters (Schmitt & Reith 2010), adding further complexity to the potential molecular mechanisms linking genes with behavior.

In an initial step toward identifying the genetic basis of MA-induced locomotor activity, we screened the C57BL/6J × A/J chromosome substitution strain (CSS) panel (Nadeau *et al*. 2000) and identified a quantitative trait locus (QTL) in CSS-11 that accounted for 14% of the variance (Bryant *et al*. 2009). For a CSS-11 × C57BL/6J F_2 cross, we used interval mapping to localize a region spanning 79–109 Mb (Bayes credible interval).

Given the effect size of this QTL, determining the quantitative trait genes (QTGs) underlying the behavior could contribute significantly to the understanding of the neurobiological basis of psychostimulant-induced locomotor activity. Because this QTL spanned 30 Mb and contained many genes, we narrowed this region using interval-specific congenic lines. Here, chromosomal regions spanning a QTL from a donor inbred strain (A/J) were bred onto a second, recipient (background) inbred strain (C57BL/6J; 'B6'). A phenotypic difference in a congenic line relative to B6 mice was assumed to be caused by A/J alleles within the congenic region. This strategy was used to identify quantitative trait genes underlying behavioral models of alcohol dependence and depression (Tomida *et al*. 2009; Shirley *et al*. 2004). We also tested one of the congenic lines for its role in MA reward using the conditioned place preference test (Tzschentke 2007). Lastly, based on the evidence that QTLs for complex traits are mediated by variants that affect gene expression (expression

QTLs, eQTLs) (Nicolae *et al*. 2010), we identified differentially expressed genes caused by genetic variation in the congenic interval to aid in identifying the QTG(s) underlying the QTL.

Materials and methods

Environment and housing

We performed all the experiments in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, which were all approved by the Institutional Animal Care and Use Committee at the University of Chicago. We maintained the mouse colony rooms on a 12/12 light/dark cycle with lights on at 0600 h. We housed two to five same-sex littermates in clear plastic cages with standard corncob-type bedding and provided unlimited access to food and water to the mice, except during testing, which we conducted between 0800 and 1600 h. We transported the mice from the vivarium next door into the test room and allowed them to habituate for at least 30 min before testing. Testing lasted 30 min, after which we returned the mice to the vivarium.

Congenic mice for behavioral testing

Because the CSS-11 strain has already been backcrossed to the B6 recipient strain and is essentially a large congenic carrying chromosome 11 from the A/J strain ('consomic'), we chose a single founder from our F_2 study (Bryant *et al*. 2009) that was heterozygous for our QTL interval and performed two rounds of backcrossing to B6 to create a founder mouse that harbored a 58- to 96-Mb introgressed region on chromosome 11. We further backcrossed this mouse to produce Line 1 spanning 84–96 Mb. We used mice heterozygous for the 84- to 96-Mb region and their wild-type littermates for simultaneous phenotyping. We also backcrossed Line 1 to create smaller, subcongenic lines spanning the entire interval of Line 1 (Lines 2–4). We bred these lines with B6 mice to generate mice heterozygous for their congenic intervals and wild-type littermates for simultaneous phenotyping. We used commercially available fluorescent single nucleotide polymorphism (SNP) markers (Applied Biosystems, Inc., Foster City, CA, USA) as well as custom-designed SNP-targeting primers combined with DNA sequencing to monitor recombinations and to determine the recombination breakpoints (boundaries) within approximately 1- to 2-Mb resolution. We used both female and male mice for each genotype, and we list the group sizes for each sex and genotype in the *Results* section. All mice were 7–10 weeks old at the time of behavioral testing, and we always age-matched across genotype for phenotyping.

DNA sequencing

Briefly, DNA was isolated from tail snips using the salting out method. We used the National Center for Biotechnology Information (NCBI) DNA sequences for each SNP to choose primers for polymerase chain reaction (PCR) using the Primer3 program (http://frodo.wi.mit.edu/primer3/). We used 50 ng/μl DNA in the PCR reaction and passed it through a 1.5% agarose gel via electrophoresis. We observed a single band for each of the primer sets, excised it from the gel and extracted the DNA using the QIAquick® gel extraction kit (Qiagen, Valencia, CA, USA). We then sent the samples to the core facility at the University of Chicago for DNA sequencing using the traditional Sanger sequencing method (http://cancer-seqbase.uchicago.edu/). Personnel from the core facility conducted the cycle sequencing reactions with fluorescent dye terminators, ran the capillaries, collected the data and provided us with the sequences (text files) and chromatograms. We then aligned the sequences of the samples to the NCBI sequences containing the SNPs using Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA).

Locomotor activity

Just prior to testing, we removed the mice from their home cages, placed them in clean holding cages with bedding for approximately 5 min. and then administered an intraperitoneal (i.p.) injection of saline (10 ml/kg) on Days 1 and 2 and an i.p. injection of MA (2 mg/kg) on Day 3. We then placed the mice into the center of the open field and recorded the total distance traveled over 30 min using the automated Versamax activity chambers (AccuScan, Columbus, OH, USA) as previously described (Bryant *et al*. 2009). We used the total distance traveled in 5 min bins over 30 min on Days 1–3 as our primary measures of locomotor activity.

Conditioned place preference

Conditioned place preference (CPP) is an associative learning task in rodents that measures the motivational properties of a drug and the ability to associate these effects with a distinct environment (Tzschentke 2007). Mice must learn to discriminate an environment paired with drug administration from an environment paired with saline administration. Following repeated drug/saline pairings, mice are 'asked' which side they prefer. An increase in time spent on the drug-paired side indicates CPP or, i.e. drug reward.

We divided the open-field activity chambers into two equally sized, distinct sides (37.5 × 18.75 cm) using a 30-cm tall black opaque divider with a 5 × 5 cm mouse entryway excised from the bottom, middle part of the divider. The left and right sides were distinguished by visual cues (vertical black and white stripes vs. horizontal stripes, 2 cm thick) and tactile cues (different floor textures). For confinement during drug and saline trials, the divider was turned upside down so that the entryway was no longer accessible.

We used an unbiased counterbalanced design whereby we randomly assigned half of the mice to either side of the apparatus for drug administration. We tested the mice for initial preference for each side by injecting them with saline (10 ml/kg, i.p.), immediately placing them in the saline-paired side facing the entryway to the drug-paired side and providing them with free access for 15 min to both sides (Day 1). We recorded the time spent on each side using the automated Versamax conditioned place preference program (.DPP files) and the distance traveled using the activity program (.ACT files). Twenty-four hours later, we began conducting the conditioning trials whereby we administered two injections of MA (2 mg/kg, i.p.) separated by 48 h (Days 2 and 4) and two alternating injections of saline (i.p.) separated by 48 h (Days 3 and 5). We confined the mice to the drug- or saline-paired side for 30 min after which time they were returned to their home cages and transported back to the vivarium. Following the four days of training, we left the mice undisturbed for 72 h. On Day 8, we administered a saline injection to the mice (i.p.), placed them into the saline-paired side facing the entryway to the drug-paired side and measured methamphetamine-induced CPP (MA-CPP) over 15 min. We used the change in time spent on the drug-paired side between initial and final preference as the primary measure of MA-CPP.

Behavioral analysis

Using genotype as a factor, we employed repeated measures analysis of variance (ANOVA) of the six 5 min time bins for each day in the open field. For main effects of genotype and interactions with time, we conducted unpaired *t*-tests for the individual time-points to determine the source of the interactions. We analyzed MA-CPP using repeated measures ANOVA (genotype and days as a factors) followed by paired *t*-tests for comparing days and unpaired *t*-tests for comparing genotypes.

Power analysis

In order to ensure that any negative results could not be explained by our experiments being underpowered, we conducted *post hoc* power analyses of the significant results obtained from CSS and congenic mice to determine the sample size required to detect the effect. We first calculated the genotype effect size *r* based on the mean and standard deviation of the two genotypes. We then converted these values to Cohen's *d* and determined the required sample size with an alpha level of 0.05 and 95% power.

Microarray study

We used a total of 14 B6 wild-type mice (7 females and 7 males) and 14 congenic mice (7 females and 7 males) for analysis of gene expression in the striatum. While there are multiple brain areas and cell types that are responsible for genetic differences in MA-induced locomotor activity, we chose to examine the striatum because of its characterization in locomotor activity and cell signaling following psychostimulant administration (McGinty *et al*. 2008). In the first batch of mice, we harvested the striatum from six B6 wild-type and six heterozygous congenic littermates on the same day (6–9 weeks old, age-matched across genotypes, equal sexes for both genotypes). In the second batch of mice, we harvested the striatum from eight B6 mice (bred in house) and eight homozygous congenic mice (offspring of homozygote breeders; bred in house) on the same day (7–9 weeks old, age-matched across genotypes, equal sexes for both genotypes).

We quickly dissected the brains, removed the olfactory bulbs, placed the brains into an ice-cold brain matrix and inserted two razor blades into the second and fifth slots. We transferred the brain sections to the top of an ice-cold petri dish. The anterior commissure served as the dorsal landmark, and the cortex served as the lateral and ventral landmarks. We harvested left and right 2-mm punches at a right angle relative to the tissue/petri dish and pooled them for each sample. We placed the punches in 500 ml of RNAlater® (Qiagen), stored them in 4°C overnight and transferred them to −20°C until we further processed the tissue. We extracted the RNA using Trizol® (Invitrogen, Carlsbad, CA, USA) and chloroform and then precipitated and resuspended the samples in ethanol. We then passed the samples through RNeasy® columns (Qiagen), quality checked them using the Agilent 2100 Bioanalyzer (Santa Clara, CA, USA), packaged them on dry ice and sent them to Precision Biomarker Resources (Evanston, IL, USA) where they once again quality checked the samples [RNA integrity number (RIN) values were all between 8 and 9; 260/280 ratios were all >2.0] and processed and scanned the samples using the Affymetrix GeneChip® Mouse Gene 1.0ST Array (Affymetrix, Santa Clara, CA, USA). We processed and ran the samples in two separate batches (described above).

We used the latest available next-generation DNA sequencing data from the Sanger Institute to identify SNPs between B6 and A/J within the 84- to 96-Mb interval (Line 1) that could potentially be targeted by the gene expression microarray probes (Build 37; http://www.sanger.ac.uk/cgi-bin/modelorgs/mousegenomes/snps.pl; search conducted on 15 September 2011). The only type of SNPs that we did not include in the search query were those located in noncoding, intergenic and intronic regions. We used the UCSC genome browser (http://genome.ucsc.edu/) to convert the individual probe coordinates (www.affymetrix.com) from Build 36 (mm8) to Build 37 (mm9) before we could identify the probes containing Sanger SNPs whose coordinates are based on Build 37. Following probe identification, we then masked these probes during robust multichip average (RMA) analysis using Affymetrix Power Tools 'kill list' option. Next, we used the ComBat.R script to remove batch effects from the RMA values between the two microarray experiments (Johnson *et al*. 2007). Following this correction, neither the two B6 control batches nor the two congenic batches showed any differences in gene expression from each other ($q > 0.99$ for all genes), while at the same time, both the heterozygous congenic batch and the homozygous congenic batch showed differential expression of exactly the same gene list relative to a collapsed, common B6 control group ($q < 0.05$). Therefore, we collapsed across congenic groups as well ('CON') and conducted *t*-tests of B6 and CON mice for each transcript cluster ID. We used the *P* value distribution of the genes within the congenic region (196 total) to generate a *q* value distribution to reveal those genes regulated by *cis*-eQTLs (variants within the congenic region). We also used the genome-wide *P* values to generate a *q* value distribution to reveal genes outside of the congenic region whose expression was regulated by *trans*-eQTLs within the congenic region. We used the Q-VALUE program (http://genomics.princeton.edu/storeylab/qvalue/) to generate *q* values. *q* value is a measure of the false discovery rate (FDR), which helps to correct for multiple comparisons (Storey & Tibshirani 2003). In presenting the gene expression results, we employed a typically used cutoff of $q < 0.05$ (5% FDR) to indicate differential expression.

WebQTL (http://genenetwork.org/webqtl/main.py)

Using the only publicly available cDNA microarray data set available on WebQTL for B6 and A/J alleles, which were derived from AXB BXA recombinant inbred strains (*Eye AXBXA Illumina V6.2 (Oct08) RankInv Beta*) (Chesler *et al*. 2004), we first identified all the probe sets targeting any of the differentially expressed genes reported from our data set. We then mapped the expression values of these probe sets (27 total) in WebQTL and employed an arbitrary cutoff of LOD (limit of detection) >4 for reporting whether or not there was a *cis*- or *trans*-eQTL for these genes.

Results

Congenic lines

The SNPs defining the congenic boundaries for the congenic lines are listed in Table 1 and illustrated in Figure 1. Some of these SNPs were genotyped using DNA sequencing (Table 1; 'SEQ') – in these cases, the primer sequences used to generate the amplicons for SNP identification are provided in Table S1, Supporting Information.

When assessing locomotor activity and similar to our previous study (Bryant *et al*. 2009), sex generally did not interact with genotype with regard to locomotor activity. One exception was Line 3 whereby we observed a weak sex × genotype interaction for Day 3 activity ($P = 0.034$; data not shown); this interaction was not significant when correcting for the 15 comparisons across congenic lines (cut-off for significance: $P = 0.0033$). Therefore, we combined the sexes for all repeated measures ANOVAs using genotype as a factor and time as the repeated measure (six time bins total).

CSS-11 mice (14 females and 23 males) vs. B6 wild-type mice (23 females and 30 males)

For CSS-11, the locomotor results for Days 1–3 were previously reported as total distance traveled summed over 30 min (Bryant *et al*. 2009). There was no effect of genotype on Day 1 or Day 2 ($F_{1,88} = 0.18, 0.66$; $P = 0.67, 0.42$; Fig. 2a,b). However, for Day 2, there was a genotype × time interaction ($F_{5,440} = 2.36$; $P = 0.040$) that was explained by CSS-11 mice showing less activity than B6 mice during the first 5 min bin ($t_{88} = 2.34$; $P = 0.022$; Fig. 2b). For Day 3, there was an effect of genotype ($F_{1,88} = 27.98$; $P < 0.0001$) and a genotype × time interaction ($F_{5,440} = 6.11$; $P < 0.0001$) that was explained by CSS-11 mice showing significantly less activity than B6 mice at all six time bins ($t_{88} = 4.70, 4.53, 4.62, 4.50, 4.80, 4.91$; $P < 0.0001$; Fig. 2c). The effect size of CSS-11 for the total distance traveled on Day 3 in response to MA was $r = 0.5$. Thus, in order to achieve 95% power to detect this difference, a sample size of $N = 17$ was required.

Line 1 (14 females and 20 males) vs. B6 wild-type littermates (12 females and 18 males)

Line 1 (84–96 Mb) was defined by SNPs rs13481129 (83.73 Mb) and rs27056693 (96.49 Mb; Table 1, Fig. 1). For Day 1, there was an effect of genotype ($F_{1,62} = 4.32$; $P = 0.042$) that was explained by Line 1 showing significantly less activity at 5 and 15 min ($t_{62} = 2.64, 2.11$; $P = 0.011, 0.039$; Fig. 2d). For Day 2 activity, there was neither an effect of genotype ($F_{1,62} = 0.38$; P = 0.54) nor an interaction with time

Table 1: SNPs that define the boundaries of the congenic and subcongenic lines

Marker	Mb	cM	Geno	Line 1	Line 2	Line 3	Line 4
rs13480888	16.85	9.41	ABI	B6	B6	B6	B6
rs13480921	25.67	15.53	ABI	B6	B6	B6	B6
rs6280308	32.62	19.06	ABI	B6	B6	B6	B6
rs13481015	48.07	28.47	ABI	B6	B6	B6	B6
rs13481044	57.64	35.27	ABI	B6	B6	B6	B6
rs13481076	66.53	40.59	ABI	B6	B6	B6	B6
rs13481105	75.46	45.30	ABI	B6	B6	B6	B6
rs13481117	79.07	46.74	ABI	B6	B6	B6	B6
rs13481125	82.70	50.30	ABI	B6	B6	B6	B6
rs13481129	83.73	51.23	ABI	B6	B6	B6	B6
rs27031679	85.79	51.55	SEQ	**H**	**H**	**H**	B6
rs13481139	86.82	51.85	ABI	**H**	**H**	**H**	B6
rs29397485	87.81	52.36	SEQ	**H**	**H**	**H**	B6
rs27099962	88.80	54.34	SEQ	**H**	**H**	**H**	**H**
rs29392997	89.80	54.65	SEQ	**H**	**H**	**H**	**H**
rs13481156	90.66	56.10	ABI	**H**	**H**	B6	**H**
rs29420084	91.67	56.10	ABI	**H**	**H**	B6	**H**
rs27090049	93.41	58.81	SEQ	**H**	B6	B6	**H**
rs28237884	93.91	58.90	SEQ	**H**	B6	B6	**H**
rs27059622	94.40	58.90	SEQ	**H**	B6	B6	**H**
rs27086824	94.92	59.01	SEQ	**H**	B6	B6	**H**
rs13481170	95.49	59.00	ABI	**H**	B6	B6	**H**
rs27056693	96.49	60.10	SEQ	B6	B6	B6	B6
rs13460821	97.07	60.95	SEQ	B6	B6	B6	B6
rs29387377	98.03	61.75	SEQ	B6	B6	B6	B6
rs27102250	98.87	62.93	SEQ	B6	B6	B6	B6
rs3023315	99.37	62.93	ABI	B6	B6	B6	B6
rs13481220	108.38	71.83	ABI	B6	B6	B6	B6
rs13481224	109.28	71.88	ABI	B6	B6	B6	B6
rs13481256	118.01	82.96	ABI	B6	B6	B6	B6

The rs number and megabase (Mb) position of each genotyped marker are listed. The centimorgan (cM) position was estimated using a recently published genetic map (Cox *et al*. 2009).

ABI, genotype was obtained using commercially available primers from Applied Biosystems, Inc.; B6, homozygous for B6 at the indicated SNP; Geno, genotyping method used; H, heterozygous for the B6 and A/J alleles at the indicated SNP (bolded H represents congenic region); SEQ, genotype was obtained using PCR amplification of a genomic region flanking the SNP followed by DNA sequencing of the amplicon (see *Materials and Methods* section and Table S1 for details).

($F_{5,310} = 0.86$; $P = 0.51$; Fig. 2e). For Day 3 activity, there was an effect of genotype ($F_{1,62} = 18.47$; $P < 0.0001$) and a genotype × time interaction ($F_{5,310} = 7.65$; $P < 0.0001$) that was explained by Line 1 showing less MA-induced activity at all six time bins ($t_{62} = 2.74, 3.47, 4.10, 4.74, 4.06, 3.49$; $P = 0.0080, 0.0010, 0.0001, <0.0001, 0.0001, 0.0009$; Fig. 2f). The effect size of Line 1 for the total distance traveled over 30 min in response to MA on Day 3 was $r = 0.47$. Thus, in order to achieve 95% power to detect this QTL, the required sample size per genotype was $N = 20$. Because the sample sizes used for Lines 2–4 were greater than $N = 20$, we were more than 95% powered to detect this QTL for each of these subcongenic lines.

Line 2 (12 females and 10 males) vs. B6 wild-type littermates (9 females and 21 males)

Line 2 (84–93 Mb) was defined by SNPs rs13481129 (83.73 Mb) and rs27090049 (93.41 Mb; Table 1, Fig. 1). For Day 1 and Day 2 activity, there was neither an effect of genotype ($F_{1,50} = 0.12, 0.40$; $P = 0.73, 0.53$) nor an interaction with time ($F_{5,250} = 0.64, 0.99$; $P = 0.67, 0.42$; Fig. 2g,h). For Day 3, there was a significant genotype × time interaction ($F_{5,250} = 4.34$; $P = 0.0008$); however, this interaction was not explained by genotype for any of the six time bins ($t_{50} = 0.88, 1.31, 1.20, 0.65, 0.78, 1.06$; $P = 0.38, 0.20, 0.24, 0.52, 0.44, 0.29$; Fig. 2i).

Line 3 (23 females and 15 males) vs. B6 wild-type littermates (26 females and 17 males)

Line 3 (84–91 Mb) was defined by rs13481129 (83.73 Mb) and rs13481156 (90.66 Mb; Table 1, Fig. 1). For Days 1–3, there was neither an effect of genotype ($F_{1,79} = 0.22, 0.16, 0.26$; $P = 0.64, 0.69, 0.61$) nor a genotype × time interaction ($F_{5,395} = 1.21, 0.70, 0.66$; $P = 0.30, 0.62, 0.65$; Fig. 2j–l).

Line 4 (17 females and 11 males) vs. B6 wild-type littermates (15 females and 12 males)

Line 4 (88–96 Mb) was defined by rs29397485 (87.81 Mb) and rs27056693 (96.49 Mb; Table 1, Fig. 1). For Days 1–3,

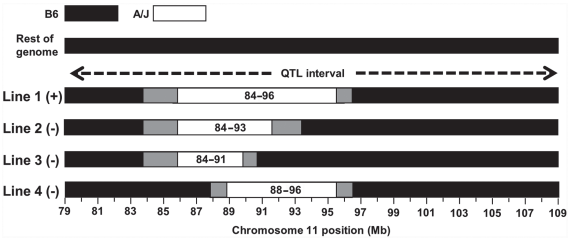

Figure 1: Congenic intervals on chromosome 11. Each bar represents a congenic line that denotes the approximate megabase (Mb) position of the congenic region (*x*-axis). The black portions of each bar represent the regions homozygous for the B6 allele (the background strain), the white portions represent the congenic regions heterozygous for the B6 and A/J alleles (donor alleles) and the gray portions represent the transitional regions that were not genotyped. Each bar spans the length of the original QTL on chromosome 11 (79–109 Mb). (+), the congenic line contained a QTL that decreased MA-induced locomotor activity (Fig. 2f). (−), the congenic line did not show evidence for a QTL (Fig. 2i,l,o).

there was neither an effect of genotype ($F_{1,53} = 0.50, 0.69, 0.2$; $P = 0.48, 0.41, 0.66$) nor any genotype × time interaction ($F_{5,265} = 1.13, 0.18, 0.095$; $P = 0.34, 0.97, 0.99$; Fig. 2 m-o).

Comparing Lines 1–4 to a single B6 control group.

Even though our power analysis indicated that an *N* of 20 was 95% powered to detect the QTL in Line 1 and our samples sizes for Lines 2–4 were sufficiently powered, in an effort to increase our power to detect the QTL in Lines 2–4, we first determined if we could combine B6 wild-type control mice from different lines into a single group that could be compared across lines. In comparing B6 control groups, there was neither an effect of group ($F_{3,118} = 0.19$; $P = 0.91$) nor a group × time interaction ($F_{15,590} = 0.92$; $P = 0.54$). Therefore, we combined B6 wild-type control groups ($N = 122$) and then re-examined the effect of each line on Day 3 activity. As expected for Line 1, the effect of genotype remained significant ($F_{1,154} = 23.25$; $P < 0.0001$). In contrast, there was still no effect of genotype compared to a single B6 control group for Line 2 ($F_{1,150} = 0.00$; $P = 0.99$), Line 3 ($F_{1,158} = 0.057$; $P = 0.81$) or Line 4 ($F_{1,148} = 0.95$; $P = 0.33$; data not shown). These observations provide further support for the negative results obtained in Lines 2–4.

MA-CPP and locomotor activity in Line 1 (23 females and 15 males) vs. B6 wild-type littermates (18 females and 13 males)

In examining MA-CPP, there was a main effect of day on time spent on the drug-paired side between Day 1 and Day 8 ($F_{1,66} = 73.57$; $P < 0.0001$), indicating an overall significant preference for MA. This was confirmed when comparing the time spent on the drug-paired side between Day 1 and Day 8 for each genotype (B6: $t_{29} = 5.24$; $P < 0.0001$; Line 1: $t_{37} = 7.01$; $P < 0.0001$; Fig. 3a). There was no effect of genotype ($F_{1,66} = 0.43$; $P = 0.51$) and no genotype × day interaction ($F_{1,66} = 0.056$; $P = 0.81$), indicating equal time spent on the drug-paired side between B6 and Line 1 on Day 1 and Day 8, which was confirmed when comparing the two genotypes for each day ($t_{66} = 0.67, 0.38$; $P = 0.50, 0.70$; Fig. 3a). Thus, MA-CPP did not differ between the two genotypes.

In examining locomotor activity on the days following saline treatment (Days 1, 3, 5 and 8), there was no effect of genotype ($F_{1,66} = 2.38, 0.46, 0.08, 0.16$; $P = 0.13, 0.5, 0.78, 0.69$) and no interaction with time (Day 1, Day 8: $F_{2,132} = 1.38, 0.99$; $P = 0.26, 0.38$; Day 3, Day 5: $F_{5,330} = 0.34, 1.0$; $P = 0.89, 0.42$; Fig. 3b,d, f, g). In contrast, for Day 2 following MA treatment, there was an effect of genotype

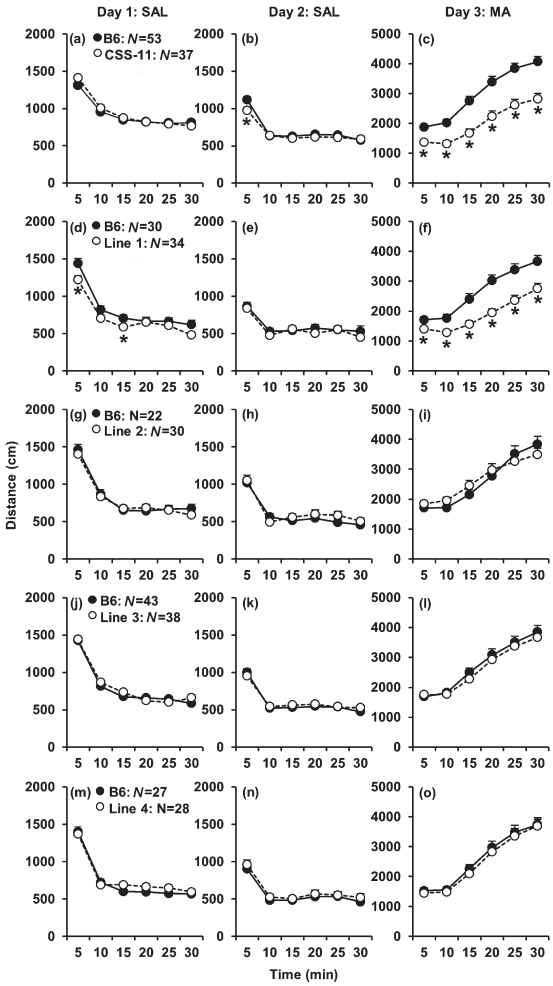

Figure 2: Locomotor activity on Days 1–3 in congenic lines. Total distance traveled in 5 min bins over 30 min is shown following saline treatment (i.p.) on Day 1 [panels (a, d, g, j and m)] and Day 2 [panels (b, e, h, k and n)] and following MA treatment (2 mg/kg, i.p.) on Day 3 [panels (c, f, i, l and o)] in B6 control mice (closed circles, solid lines) and CSS-11 or heterozygous congenic mice (open circles, dashed lines). The *N* for each genotype for each line is listed in the panels on the left hand side of the figure. SAL, saline treatment; MA, methamphetamine treatment. Data for each genotype are presented as the average ±SEM (standad error of the mean). *, $P < 0.05$.

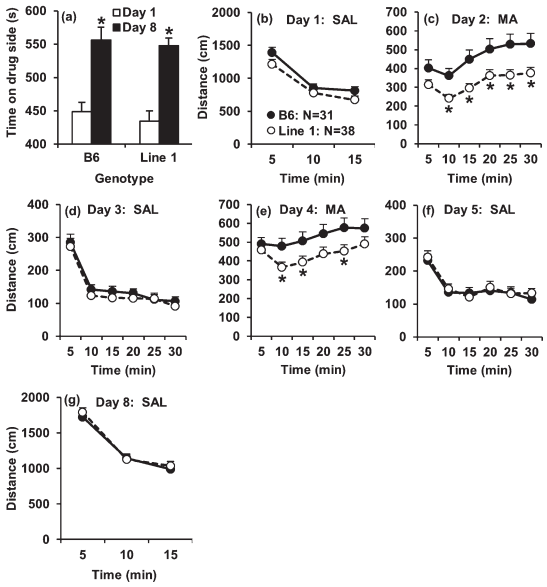

Figure 3: MA-CPP and locomotor activity in Line 1. Panel a: Time spent on the MA-paired side is shown for Day 1 (before training) and Day 8 (after training). Panels (b–g): total distance traveled in 5 min bins is shown before training (Day 1; b), during training (Days 2–5; c, d, e and f, respectively) and after training (Day 8; g). SAL, saline treatment (i.p.); MA, methamphetamine treatment (2 mg/kg, i.p.); B6, B6 wild-type littermates (closed circles, solid lines). Line 1 represents the congenic line spanning the 84- to 96-Mb interval (open circles, dashed lines). Data for each genotype are presented as the average ±SEM. *, $P < 0.05$ for time spent on the drug-paired side from Day 1 to Day 8 for both genotypes (a) or for B6 vs. Line 1 at a particular time bin (c, e).

($F_{1,66} = 7.62$; $P = 0.0075$) that was explained by Line 1 showing significantly less MA-induced locomotor activity at 10, 15, 20, 25 and 30 min ($t_{66} = 2.94$, 2.95, 2.29, 2.80, 2.63; $P = 0.0046$, 0.0044, 0.025, 0.0068, 0.011; Fig. 3c). Similarly, following MA treatment on Day 4, there was a genotype × time interaction ($F_{5,330} = 2.39$; $P = 0.038$) that was explained by Line 1 showing less MA-induced locomotor activity at 10, 15 and 25 min ($t_{66} = 2.35$, 2.05, 2.12; $P = 0.022$, 0.044, 0.037; Fig. 3e). Thus, similar to the locomotor results obtained in the open field, Line 1 demonstrated a robust reduction MA-induced locomotor activity in the CPP chamber on Day 2 and Day 4.

Microarray study

Prior to analysis of the microarray data, we excluded probes that contained SNPs that were polymorphic between B6 and A/J and were located in the 84- to 96-Mb interval of Line 1. At the time of conducting the SNP query (http://www.sanger.ac.uk/cgi-bin/modelorgs/mousegenomes/snps.pl), we identified 1590 SNPs that could potentially be targeted by probes (Table S2). We subsequently determined that approximately 10% of these SNPs (145 total) were located within probe sequences (Build 37 coordinates), and thus, we masked the probes containing these SNPs prior to RMA analysis using Affymetrix Power Tools 'kill list' option (Table S3). Because the behavioral data indicated that there was no effect of sex on MA-induced locomotor activity ($F_{1,60} = 0.05$; $P = 0.82$) and no sex × genotype interaction ($F_{1,60} = 0.075$; $P = 0.79$; data not shown), we combined sexes for examining differences in gene expression. Of the 196 transcript cluster IDs examined within the 84- to 96-Mb interval, 15 reached the 5% FDR for differential expression, the top hit being *Phb*, the gene encoding prohibitin (Table 2). Regarding genome-wide microarray analysis, we identified two additional differentially expressed genes, one located just outside of the congenic interval (*Pnpo*) and the other on chromosome 15 (*Prkag1*; Table 2). Using WebQTL (Chesler *et al*. 2004), we observed eQTLs for some but not all the differentially expressed genes from an AXB BXA recombinant inbred strain data set (Table 2, last column; Table S5).

Discussion

Using interval-specific congenic mice, we narrowed a major QTL influencing MA-induced locomotor activity to an interval on chromosome 11 spanning 84–96 Mb. When we divided this interval into three subcongenic lines spanning the 12-Mb interval, we observed no phenotypic differences for any of the lines (Lines 2–4; Table 1, Fig. 1, 2). This observation is consistent with a model whereby one or more epistatic interactions between donor alleles produce the phenotypic differences. This locus had no effect on MA-CPP while still producing a robust decrease in MA-induced locomotor activity in the CPP chamber (Fig. 3). This could indicate that this QTL is selective for MA-induced locomotor activity or it could be explained by the specific dose or CPP parameters employed. Finally, we identified differentially expressed genes (i.e. eQTLs) in the region that may cause the decrease in MA-induced locomotor activity (Table 2). *Phb* is one plausible candidate gene given that it was the most differentially expressed.

The dissolution of a large-effect QTL into multiple small-effect loci is common in studies using congenic mice. However, the complete and sudden loss of the QTL signal when we divided this interval into three smaller segments was unexpected, although there are similar examples (Garrett & Rapp 2002). We observed this QTL in four separate contexts, including the original finding in CSS-11 (Bryant *et al*. 2009) (Fig. 2c), the CSS-11 × B6 F_2 cross (Bryant *et al*. 2009), Line 1 in the open field (Fig. 2f) and Line 1 in the MA-paired side of the conditioned place preference test (Fig. 3c,e). In all cases, both the direction and effect size of this robust QTL were virtually identical. However, when we split the 84–96 Mb locus into three segments spanning the entire interval, we did not observe any evidence for this QTL (Lines 2–4: Figs. 1 and 2i,l,o). On the basis of our sample sizes, we had greater than 95% power to detect this QTL in these subcongenic lines. Thus, although perplexing, this negative result is quite definitive. Our interpretation is that two or more alleles interact epistatically to produce the QTL. An alternative possibility is that the loss of the QTL signal in

Table 2: Differentially expressed genes in the striatum from B6 control mice vs. Line 1

TC ID	Gene name	Symbol	Chr	Mb	B6	CON	$2^{(CON-B6)}$	P	Q	WebQTL
(a) *cis*-eQTLs										
10380551	Prohibitin	*Phb*	11	95.5	9.8	10.0	1.2	2.5E-10	3.5E-08	Yes, *cis*
10380226	CUE domain containing 1	*Cuedc 1*	11	87.9	8.0	8.3	1.2	1.1E-07	7.8E-06	Yes, *cis*
10380381	Transducer of ErbB-2.1	*Tob 1*	11	94.1	9.4	9.1	0.8	2.0E-07	9.5E-06	No
10379953	RIKEN cDNA 4632419122 gene	*4632419122Rik*	11	86.0	7.2	7.0	0.9	7.3E-07	2.5E-05	—
10379968	Tubulin, delta 1	*Tubd1*	11	86.4	7.3	7.4	1.1	3.7E-06	1.0E-04	Yes, *cis*
10380411	Mitochondrial ribosomal protein L27	*Mrpl27*	11	94.5	6.4	6.2	0.9	1.8E-05	4.2E-04	Yes, *cis*
10389797	Syntaxin binding protein 4	*Stxbp4*	11	90.3	7.9	8.2	1.2	6.3E-05	1.2E-03	Yes, *trans* (Chr 17)
10390022	Radical *S*-adenosyl methionine domain-containing 1	*Rsad1*	11	94.4	8.2	8.4	1.1	1.8E-04	2.9E-03	No
10389858	Nonmetastatic cells 2, protein (NM23B) expressed in	*Nme2*	11	93.8	9.0	8.9	0.9	1.9E-04	2.9E-03	No
10380549	RIKEN clone: F930115102	–	11	95.4	6.9	6.8	0.9	3.2E-04	4.5E-03	—
10390184	Predicted gene 9796	*Gm9796*	11	95.6	7.4	7.2	0.9	3.9E-04	5.0E-03	—
10380238	Mitochondrial ribosomal protein S23	Mrps23	11	88.0	9.1	9.0	0.9	7.8E-04	9.0E-03	Yes, *trans* (Chr 17)
10389738	Diacylglycerol kinase, epsilon	*Dgke*	11	88.9	8.7	8.5	0.9	1.8E-03	1.9E-02	No
10380289	Monocyte to macrophage differentiation associated	*Mmd*	11	90.1	10.3	10.4	1.1	2.1E-03	2.1E-02	Yes, *cis*
10390209	Insulin-like growth factor 2 mRNA binding protein 1	*Igf2bp1*	11	95.8	4.8	4.9	1.1	2.5E-03	2.3E-02	No
(b) *trans*-eQTLS										
10390299	Pyridoxine 5′-phosphate oxidase	*Pnpo*	11	96.8	9.1	8.8	0.8	7.4E-07	2.9E-03	Yes, *cis*
10432281	Protein kinase, AMP-activated, gamma 1 noncatalytic subunit	*Prkag1*	15	98.6	10.3	10.2	0.9	3.9E-06	1.2E-02	No
10591186	—	—	9	19.0	5.3	5.1	0.9	1.2E-05	3.3E-02	—

(a) lists the differentially expressed genes regulated by *cis*-eQTLs that were identified following congenic analysis of the 197 transcript cluster IDs (TC ID) on the array located within the 12-Mb interval.

(b) lists the genes containing *trans*-eQTLs identified following genome-wide analysis.

The following items are listed in the columns: transcript cluster (TC) ID, gene name, gene symbol, chromosome (Chr), Mb position, RMA expression values for B6 mice and congenic mice (CON), fold-change (derived from $\log_2$ RMA values and thus, expressed as $2^{(CON-B6)}$), *P* value, *q* value ($q < 0.05$) and whether or not (yes/no) we observed *cis*- or *trans*-eQTLs (LOD > 4) in a published data set derived from B6 and A/J alleles on WebQTL (*Eye AXBXA Illumina V6.2 [Oct08] RankInv Beta*) (Chesler *et al*. 2004). See Table S5 for more details.

Lines 2–4 can be explained by hidden recombination events that occurred within the proximal or distal intervals that were not genotyped (gray areas of the bars; Fig. 1) or, even less likely, between the 1-Mb-spaced markers. On the basis of a recently published genetic map (Cox *et al*. 2009), there is a 0.3% chance of a recombination event occurring at the proximal boundary of Lines 2 and 3 and a 1.1% chance for the distal boundary of Line 4 (Table 1), making this explanation very unlikely. While not all relevant DNA was available, we were unable to identify such a recombination in the subset of samples that we examined (data not shown).

Human genetic studies of complex traits are limited in their ability to detect epistasis but its influence on heritability is likely greater than what is currently appreciated. Mouse genetic studies using populations such as CSS and congenic mice paint a consistent picture for complex traits: There are massive, nonadditive effects of genomic regions on phenotypic variance that greatly exceed the variance necessary to explain the heritability of the population (Gale *et al*. 2009; Shao *et al*. 2008; Yazbek *et al*. 2011). These populations are (perhaps inadvertently) ideal for isolating epistatic interactions that by chance happen to reside on the same chromosome, sometimes in relatively close proximity. We unambiguously mapped a QTL to a 12-Mb region on chromosome 11 that explained virtually all the phenotypic variance initially observed in CSS-11. On the basis of the subcongenic results, we conclude that the genetic basis of this QTL involves an interaction between two or more alleles within the 84- to 96-Mb region.

Because QTLs for complex traits are often mediated by eQTLs (Nicolae *et al*. 2010), we first sought to identify differentially expressed genes caused by *cis*-eQTLs between drug-naïve B6 and congenic mice in the striatum, a brain area critical for psychostimulant-induced locomotor activity. *Phb* (prohibitin) was the most significant differentially expressed gene (Table 2) and interacts with the phosphoinositide-3 kinase/Akt pathway (Mishra *et al*. 2010), which could affect cell surface expression of DAT and DA release (Schmitt & Reith 2010). *Phb* is also a substrate for the epidermal growth factor (EGF) receptor, which signals through extracellular signal-related kinase 1/2 (Mishra *et al*. 2010) and neonatal administration of EGF affects the locomotor response to cocaine during adulthood (Mizuno *et al*. 2004). *Phb* also interacts with transforming growth factor-β (Mishra *et al*. 2010) whose signaling is important for the induction, survival and neuroprotection of mesencephalic DAergic neurons (Roussa *et al*. 2009).

A second differentially expressed gene was *Stxbp4* (syntaxin binding protein 4; Table 2), which we previously implicated based on gene expression results from haplotype association mapping (Bryant *et al*. 2009). *Stxbp4* contains at least one nonsynonymous coding SNP that could affect the function of the protein (Table S4). Syntaxin 1A binds to DAT (Torres 2006), regulates DAT phosphorylation, surface expression and reuptake (Cervinski *et al*. 2010) and mediates DA efflux in response to amphetamine (Binda *et al*. 2008). Thus, differential function or expression of a syntaxin binding protein such as *Stxbp4* could affect vesicular trafficking of DA and exocytosis.

Dgke (diacylglycerol kinase epsilon) was a third interesting candidate gene (Table 2) and contains at least one nonsynonymous coding SNP (Table S4). Several diacylglycerol kinase (DGK) isoforms, including *Dgke*, contribute to synaptic activity and neurotransmitter release (Tu-Sekine & Raben 2011). DGKs can negatively regulate synaptic transmission by phosphorylating diacylglycerol (DAG) and catalyzing its conversion to phosphatidic acid (PA) (Merida *et al*. 2008). DAG interacts with protein kinase C (PKC) and Munc proteins which in turn bind syntaxin and primes synaptic vesicles for fusion (Palfreyman & Jorgensen 2007). Because DAG can modulate PKC signaling, DGKs could also affect DAT trafficking and regulate DA release (Schmitt & Reith 2010).

In addition to identifying differentially expressed genes mediated by *cis*-eQTLs, we also identified two annotated, differentially expressed genes outside of the congenic region (mediated by *trans*-eQTLs; Table 2). *Pnpo* (pyridoxine 5′-phosphate oxidase), a gene encoding an enzyme involved in vitamin B_6 metabolism (di Salvo *et al*., 2011), was located just outside the congenic region at 96.8 Mb and could be regulated by a nearby genetic variant within the congenic region (95.5–96.5 Mb). The second gene, *Prkag1* (protein kinase, AMP-activated, gamma 1 noncatalytic subunit) is located on chromosome 9 and encodes a subunit of AMP-activated protein kinase (AMPK). AMPK is involved in energy homeostasis and metabolism, mitochondrial homeostasis, autophagy, cell polarity, growth and proliferation (Hardie 2011).

An important consideration in microarray analysis is that the probe intensity values are confounded by SNPs that decreased hybridization of the non-B6 alleles. In order to address this issue, we used next-generation sequencing data from the Sanger Institute (Keane *et al*. 2011) to first identify the SNPs within the congenic region and to then mask probes containing these SNPs from the analysis (Walter *et al*. 2007). Although this slightly changed the data and gene ranking, the same genes reached an FDR rate of less than 5% (with *Phb* remaining at the top of the gene list), indicating that probe bias did not significantly impact our results. However, as new sequencing data become available, it is possible that new SNPs will be identified and that subsequent masking may change the current results.

The microarray results indicate that congenic mice exhibited only modest changes in gene expression compared to the large change in the behavioral phenotype. One limitation of our study was that we only examined one candidate brain area, the striatum, and it is possible that other areas might show more robust differences in gene expression. A second limitation is that we only examined gene expression at one time-point; thus, it is possible that the relevant differences in gene expression occur at any time during development. Lastly, it is important to consider that the QTL we have localized is presumably caused by at least two loci; thus, differential expression or function of multiple genes within the same pathway could have a potentiating effect at the cellular and behavioral levels.

In sum, we narrowed a 12-Mb region on mouse chromosome 11 likely composed of multiple interacting loci that accounts for a large proportion of the phenotypic variance in MA sensitivity. Gene expression analysis in the

striatum of drug-naïve mice revealed candidate genes that could potentially interact to produce differences in MA sensitivity. This locus appears to be selective for the locomotor stimulant response and does not affect the rewarding properties of MA, at least under these conditions. Determining the genetic mechanism of epistasis that drives this QTL will be important because this locus accounts for a substantial portion of the heritability for MA sensitivity in the CSS panel. Future studies utilizing reverse genetic techniques to examine candidate genes in isolation and combination might clarify the nature of these genetic interactions.

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Supporting Information

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

Table S1: Primer sequences used for genotyping SNPs. The SNP ID, megabase (Mb) position, forward (F) and reverse (R) primer sequences and the amplicon size in base pairs (bp) are listed for the PCR reactions used for DNA sequencing and genotyping of the congenic lines at the indicated Mb position on chromosome 11.

Table S2: SNPs between B6 and A/J in the 84- to 96-Mb interval (Line 1). Using the Sanger Institute's mouse SNP query (http://www.sanger.ac.uk/cgi-bin/modelorgs/mousegenomes/snps.pl) and the indicated search coordinates for chromosome 11 ('Location'), we identified 1590 SNPs that could potentially be targeted by probes. The types of SNPs included in the query are listed in the cell following 'Consequence.'

Table S3: SNP 'kill list' used in generation of RMA values. In considering the 1590 SNPs we identified within the 84- to 96-Mb congenic region (Line 1), a total of 145 probes on the array contained SNPs and thus, were removed from RMA analysis. We list the probe ID, the probe set as well as the ID indicated on the PGF file (Affymetrix Mouse Gene 1.0 ST array). We used the PGF file for implementing the kill list command in Affymetrix Powertools, and thus, the text file used for this analysis contained only the probe ID and the PGF ID.

Table S4: Nonsynonymous coding SNPs in the 84- to 96-Mb interval (Line 1). Using the Sanger Institute's mouse SNP query (http://www.sanger.ac.uk/cgi-bin/modelorgs/mousegenomes/snps.pl), we identified a total of 148 nonsynonymous coding SNPs in 65 genes between B6 and A/J.

Table S5: Expression QTLs from our data set (Table 2) that were also identified on WebQTL (GeneNetwork). Using the only available cDNA microarray data set available on WebQTL for B6 and A/J alleles, which were derived from AXB BXA recombinant inbred strains (*Eye AXBXA Illumina V6.2 (Oct08) RankInv Beta*), we first identified all the probe sets targeting any of the genes from our data set (Table 2). We then mapped the expression values of these probesets (27 total) in WebQTL and employed an arbitrary cutoff of LOD $>$ 4 (bolded rows) for reporting whether or not there was a *cis*- or *trans*-eQTL (see also Table 2, last column).

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