

Gene expression differences in mice divergently selected for methamphetamine sensitivity

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Abstract

In an effort to identify genes that may be important for drug-abuse liability, we mapped behavioral quantitative trait loci (bQTL) for sensitivity to the locomotor stimulant effect of methamphetamine (MA) using two mouse lines that were selectively bred for high MA-induced activity (HMACT) or low MA-induced activity (LMACT). We then examined gene expression differences between these lines in the nucleus accumbens, using 20 U74Av2 Affymetrix microarrays and quantitative polymerase chain reaction (qPCR). Expression differences were detected for several genes, including Casein Kinase 1 Epsilon (*Csnkle*), glutamate receptor, ionotropic, AMPA1 (*GluR1*), GABA B1 receptor (*Gabbr1*), and dopamine- and cAMP-regulated phosphoprotein of 32 kDa (*Darpp-32*). We used the www.WebQTL.org database to identify QTL that regulate the expression of the genes identified by the microarrays (expression QTL; eQTL). This approach identified an eQTL for *Csnkle* on Chromosome 15 (LOD = 3.8) that comapped with a bQTL for the MA stimulation phenotype (LOD = 4.5), suggesting that a single allele may cause both traits. The chromosomal region containing this QTL has previously been associated with sensitivity to the stimulant effects of cocaine. These results suggest that selection was associated with (and likely caused) altered gene expression that is partially attributable to different frequencies of gene expression polymorphisms. Combining classi-

cal genetics with analysis of whole-genome gene expression and bioinformatic resources provides a powerful method for provisionally identifying genes that influence complex traits. The identified genes provide excellent candidates for future hypothesis-driven studies, translational genetic studies, and pharmacological interventions.

Psychostimulants, opiates, sedatives, and nicotine all share the ability to cause addiction, to increase dopamine concentrations in the nucleus accumbens, and to stimulate locomotor behavior (Di Chiara and Imperato 1988; Balfour et al. 2000; Di Chiara 2002; Boileau et al. 2003). Locomotor behavior is more readily measured than self-administration behavior and appears to be regulated by some of the same neurochemical substrates (e.g., dopamine in the nucleus accumbens), although there are also differences (e.g., Sellings and Clarke 2003). Locomotor behavior has been systematically studied in an effort to identify neurochemical mechanisms and genes that might also influence susceptibility to drug abuse. Drug abuse in humans and increased tendency toward voluntary drug intake in animals are known to be heritable traits (Li et al. 1987; Uhl et al. 1995; Vetulani 2001; Kendler et al. 2003; Agrawal et al. 2004).

Genes that control drug-abuse-related traits have been sought using quantitative trait locus (QTL) methodologies. Initial stages of analysis have identified chromosomal regions that contain trait-relevant alleles. However, this field has reached

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a more advanced stage where attention has increasingly shifted from mapping to gene identification (Fehr et al. 2002; Glazier et al. 2002; Complex Trait Consortium 2003; Liang et al. 2003; Shirley et al. 2004). It is not yet clear to what extent mapped QTL are caused by differences in the regulation of gene expression versus differences in the coding of amino acid sequence; however, it has been suggested that regulatory differences will be more common (Mitchison 1997; Mackay 2001; Glazier et al. 2002). Technical advances have made it possible to perform genome-wide mapping of QTL that control the expression of particular transcripts (eQTL; Wayne and McIntyre 2002; Schadt et al. 2003; Chesler et al. 2004). If a QTL for a behavioral trait (bQTL) is caused by a difference in the regulation of a gene, then identification of the bQTL-causing gene is possible by identification of the corresponding eQTL. In practice, this can be accomplished by examining the relatively small number of eQTL that comap with the bQTL. Comapping does not prove a relationship between the gene and the trait, but it does suggest a specific and testable hypothesis.

Short-term selected lines are a particularly advantageous but so far unutilized genetic tool for the comapping of bQTL and eQTL. Because of extreme phenotypic differences caused by selective breeding, selected lines provide a powerful means of mapping bQTL (Belknap et al. 1997), which may then be compared with gene expression differences in relevant tissues from the lines. We have created short-term selected lines for extremely high and low sensitivity to MA-induced locomotor stimulation and have shown that these lines also differ in stimulant response to cocaine, magnitude of behavioral sensitization induced by repeated MA treatment, and in voluntary consumption of MA- and cocaine-containing solutions (Kamens et al. 2005). These data suggest that common genes influence the locomotor response to MA and cocaine, as well as the tendency to self-administer MA and cocaine. However, because the differential response to MA for which these lines were selectively bred is evident within the first 5 minutes following drug administration, gene expression differences that contribute to differential sensitivity must exist prior to drug administration. Therefore, drug-naïve mice from these two lines, rather than drug-treated mice, are the relevant experimental group for the comapping of bQTL and eQTL.

In the present study, we mapped bQTL for stimulant response to MA and then examined gene expression differences between the two lines by examining tissue from the nucleus accumbens, using microarrays and quantitative polymerase

chain reaction (qPCR). The nucleus accumbens was chosen for our analysis because of evidence of its involvement in psychostimulant-induced activation and self-administration (Kim et al. 2003; Brebner et al. 2004; Lecca et al. 2004; Suto et al. 2004). Differentially expressed genes were identified according to stringent statistical criteria and were then assessed to determine whether they had known eQTL that comapped with the bQTL for MA response.

Materials and methods

Subjects. The short-term selected lines were created from a foundation population of C57BL/6J (B6) × DBA/2J (D2) F₂ mice (this population is called B6D2F₂). Details of selection procedures, age of testing, and other information pertinent to these lines have been published (Kamens et al. 2005). Briefly, mice were bidirectionally selected for four generations for high (HMACT) or low (LMACT) acute locomotor stimulant response to 2.0 mg/kg MA. Stimulant response was defined as the difference in total distance traveled during a 15-min test beginning immediately after drug administration, when compared with distance traveled by the same mouse, during the same time period on the prior day, when vehicle had been administered. Selection produced a statistically significant difference in response that was detectable in the first selection generation and increased over the course of the subsequent selection generations. The HMACT line had an average acute MA response of 8146 ± 646 cm in 15 min versus 1799 ± 271 cm in the LMACT line in the third selection generation (S₃), a 4.5-fold difference. DNA from mice of this generation was used for bQTL analysis. In the fourth selection generation (S₄), the mean MA locomotor scores of the two lines differed by 5.3-fold (HMACT = 11030 ± 510; LMACT = 2093 ± 265). Nucleus accumbens tissue was obtained from male mice of this generation for microarray analysis. There was no significant difference in baseline activity between the HMACT and LMACT lines in any of the selection generations (Kamens et al. 2005). S₃ and S₄ generations are first-degree relatives of one another and, therefore, have a high degree of allele sharing and can thus be used to make genetic inferences about the changes associated with selection. Mice from the S₃ generation were used for bQTL analysis to minimize the contribution of genetic drift. Mice from which tissue was obtained for microarray analysis were never treated with MA but were from the same families as those tested for acute stimulant response to MA. All mice were housed in isosexual littermate groups of

2–5 per cage after weaning at 21–23 days of age. Mice used for bQTL mapping and expression work were maintained on a standard 12:12-h light cycle (lights on at 0600 h) at $21 \pm 2^\circ\text{C}$ with constant access to mouse chow (Purina Rodent Chow #5001; Purina Mills, St. Louis, MO) and water. All procedures were conducted in the Veterinary Medical Unit of the Portland Veterans Affairs Medical Center and were approved by the Institutional Animal Care and Use Committee in compliance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Short-term Selected Line Genotyping and bQTL Analysis Using Interval Mapping. Spleens were obtained from 82 HMACT and 62 LMACT mice from generation S_3 several days after phenotypic data collection for stimulant response to MA. Genomic DNA for genotyping of microsatellite markers was obtained by using a standard salting-out protocol (Palmer et al. 2003). Microsatellite markers were genotyped using primers from the MIT series obtained commercially (Research Genetics, Huntsville, AL). Genotyping procedures have been described elsewhere (Bergeson et al. 2003).

Initially, we created two pools of DNA, one for the HMACT line ($n = 82$) and one for the LMACT line ($n = 62$), that had an equal concentration of genomic DNA from each mouse. We used a genome-wide panel of 80 microsatellite markers that are polymorphic between the B6 and the D2 progenitor strains to identify genomic regions that showed a non-1:1 ratio of the two possible marker alleles, which would be indicative of the presence of a bQTL. Quantification of the intensity of the bands on an agarose gel was normalized by using standards that were created by mixing genomic DNA from the two inbred strains in known ratios from 3:1 to 1:3 plus pure B6 and D2 samples. For those markers that showed a deviation in estimated allele frequency from a 1:1 ratio at a single marker $p < 0.05$ (binomial test), half of the S_3 mice (odd-numbered cases) were genotyped individually (not as pooled samples) to verify the difference, and the data were then analyzed by the QTL detection method of Belknap et al. (1997) based on allele frequency differences between the two lines. When a difference was confirmed, individual genotypes and individual behavioral responses to MA were used to estimate the position of the bQTL using the interval mapping methods as implemented in R/qtl (Broman et al. 2003). The latter method takes into account both phenotypic and genetic variation within the lines as well as between them, while the allele frequency analysis method (Belknap et al. 1997) considers only between-line

genetic variation. However, the R/qtl analysis does not take into account the effects of random drift, whereas the allele frequency analysis does consider this source of genetic variation.

For the B6 \times D2 (BXD) recombinant inbred strain data, the position of each marker was defined by a map generated in Map Manager QTX (<http://www.mapmanager.org>) from the strain distribution pattern obtained from www.WebQTL.org using the Morgan mapping option. There were small disagreements between this map and the consensus map for the mouse genome (<http://www.informatics.jax.org/>). We favored the map generated in Map Manager over the consensus map because it allowed us to directly compare our results with interval mapping results for eQTL using data obtained from WebQTL, thus avoiding incompatibilities and ambiguity associated with combining map locations based on different linkage maps.

Short-Term Selected Line Genotyping and bQTL Analysis Using the Allele Frequency Method. The methods of Belknap et al. (1997) were explicitly designed for the detection of QTL using short-term selected lines. This method relies on analysis of differences in marker allele frequency between the high and low selected lines, and it compares these differences to the probability distribution for genetic drift and allele frequency estimation error under the null hypothesis that there are no bQTL. We will refer to the D2 allele frequency as q and the B6 allele frequency as p . In the F_2 , $p = q = 0.5$, which is to say that all micro satellite marker alleles begin with a ratio of about 1:1. Markers close to QTL that influence the trait under selection will diverge from 0.5 with each generation of selection, with the relative frequency of q approaching 1 in one line and 0 in the oppositely selected line. In contrast, markers in those portions of the genome without trait-relevant QTL will not change in allele frequency as a function of selective breeding (within the limits of sampling error, e.g., drift; Belknap et al. 1997). Evidence for the presence of a bQTL was obtained based on the difference in relative allele frequencies between the high and low lines at a marker that significantly exceeded the difference expected from random drift and sampling error (Belknap et al. 1997).

Processing of Tissue, and RNA Isolation and Amplification for Hybridization to Microarrays. To determine whether there were changes in gene expression associated with selection for differential acute locomotor response to MA, we measured transcript abundance in the nucleus accumbens

using U74Av2 Affymetrix microarrays. A total of 80 drug-naïve male mice (40 per line) from the final selection generation (S_4) were used in these studies. The average age of the mice was 88 ± 1 days for the HMACT line and 89 ± 1 days for the LMACT line; ages ranged from 77 to 99 days old. On the day that tissue samples were collected, mice were removed from their home cages and killed by decapitation. Their brains were rapidly removed and a coronal section of 1-mm thickness was obtained using razor blades and a 4°C brain dissection block (Roboz Surgical Instrument Company, Gaithersburg, MD). The section was then placed on a 4°C dissecting stage and the region corresponding to the nucleus accumbens was excised using an RNase-free, blunt 18-gauge needle. The nucleus accumbens was isolated bilaterally from each animal in this manner and rapidly transferred to a clean tube that contained RNAlater (Ambion, Austin, TX). Tissue was subsequently stored at -20°C and shipped to Columbia University for further processing.

Samples from four animals were pooled after transfer to the Columbia University Genome Center to obtain enough RNA for successful processing. Sample pooling was also used because it has been shown to decrease within-group variance, and thus increase the power to detect between-group variance (Peng et al. 2003). Pools were designed so that subjects from the same family were in the same pool whenever possible. This is an important consideration because gene expression is likely to be more similar among siblings due to a higher degree of allele sharing (because they have the same parents), as well as exposure to some of the same environmental variables (because they lived in the same cage). Therefore, distributing subjects from a single family over multiple arrays would tend to artificially depress within-group variability, thus biasing statistical tests in favor of finding a difference between the two groups (each family can belong to only one of the lines and therefore family is a nested variable).

Total RNA from each pool was isolated by homogenizing the nucleus accumbens samples in TRIzol reagent (Invitrogen, Carlsbad, CA), followed by precipitation and purification using an RNA Easy Mini Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. Following isolation of total RNA, poly-A RNA (mRNA) was selectively and linearly amplified using two rounds of reverse transcription and *in vitro* transcription using the MessageAmp aRNA kit (Ambion, Austin, TX), in which the first round of reverse transcription used poly-T primers and the second round used random hexamer primers. To obtain biotin-labeled aRNA, we used the ENZO BioArray HighYield RNA Tran-

script Labeling Kit (Affymetrix, Santa Clara, CA) in the second round of *in vitro* transcription. Amplified RNA was then fragmented and hybridized to Affymetrix Murine Genome U74Av2 arrays at the Columbia Genome Center Affymetrix core facility. A total of 20 microarrays with the same lot number were used in this study, 10 for the high line and 10 for the low line. Because all animals were experimentally naive at the time of sacrifice, microarray results reflect gene expression differences in the *absence* of MA administration.

Statistical Analysis of Microarray

Results. Expression values (transcript abundance) corresponding to each of the 12,488 probesets on the U74Av2 chip were estimated using Affymetrix MAS5 software. We also used the dCHIP and RMA software packages to estimate transcript abundance values and thus determine consistency of results across methods. These three probe-level analysis methods all calculate a single expression value based on the signal intensities from the 11–20 probes comprising each probeset. Each probe in a probeset is designed to detect a different target sequence in a transcript (Irizarry et al. 2003). Probe-level analysis yields a single expression value from all probes in a probeset after normalizing the probe signal intensities given by the chip scanner (CEL files) and after background correction. The dCHIP probe-level analysis method (Li and Wong 2003) was implemented using the default settings for the dCHIP software (version 1.2; <http://biosun1.harvard.edu/complab/dchip/>). RMA (Robust Multiarray Average; Irizarry et al. 2003) was implemented using the default settings for the Affymetrix package within Bioconductor (version 11/24/03) running within the R statistical programming environment (<http://www.r-project.org/>). Because these three methods use different algorithms and assumptions about the data structure, they each produce somewhat different estimates of transcript abundance (Irizarry et al. 2003).

Gene expression values for the two lines were initially compared by a two-sample *t*-test (Microsoft Excel, Microsoft Corp., Redmond, WA) and corresponding *q* values were then calculated for all three sets of expression values (i.e., MAS5, RMA, and dCHIP) using *q*-value software (<http://faculty.washington.edu/~jstorey/qvalue>). The *q* value is an estimate of the false discovery rate or the proportion of all *t*-test comparisons declared to be significant that are expected to be false positives (Benjamini and Hochberg 1995). We used a significance threshold of $q < 0.05$ corrected for multiple comparisons (Storey and Tibshirani 2003). This differs from the tradi-

tional p value, which is the proportion of all t -test comparisons that are expected to be false positives and does not correct for multiple comparisons unless a specific correction (e.g., Bonferroni) is applied.

Identification of eQTL Using *www.WebQTL.org*. For genes that showed expression differences between the short-term selected lines, we sought to identify eQTL that governed the expression of these genes using publicly available data from the *www.WebQTL.org* website (Chesler et al. 2004). Data available from WebQTL used whole-brain (minus cerebellum) gene expression measured with Affymetrix U74Av2 microarrays in BXD recombinant inbred strains (see *www.WebQTL.org* for details). Because our measurements were from nucleus accumbens samples rather than whole-brain, there are likely to be some disagreements between our expression data and those available from *www.WebQTL.org*. eQTL were identified using three related datasets from *www.WebQTL.org*: MAS5 (Data Freeze: Dec 03), RMA (Data Freeze: Mar 04), and dCHIP (Data Freeze: PM, Mar 04). Significance was determined using the empirical permutation significance threshold calculated for the likelihood ratio chi squared score as implemented at that website (<http://www.WebQTL.org>) according to the method of Churchill and Doerge (1994). A number of genes showed suggestive rather than significant eQTL results, which we have not reported. In order to create the plots shown in Fig. 1, in which the bQTL for MA stimulation are plotted alongside the eQTL for specific genes, we mapped the eQTL using the interval mapping function of Map Manager QTX, since only graphical (but not quantitative) interval mapping results may be obtained from *www.WebQTL.org*. We used the "retrieve trait data" feature to obtain expression values for each individual transcript of interest; then we calculated a 1-cM density interval map using the strain distribution patterns from *www.WebQTL.org* and Map Manager QTX. Likelihood ratio scores were converted to LOD scores by dividing by 4.6 (Lander and Botstein 1989).

Verification of Results with Quantitative PCR (qPCR). qPCR was used to verify the results of the microarray experiment for certain genes that were of particular interest. cDNA was produced from the pooled samples (total RNA) used in the microarray analysis, using a modified version of the Invitrogen SUPERScript Choice kit protocol (Invitrogen). RNase inhibitor obtained from Ambion was used, and an RNase-free DNase (Ambion) step was included prior to addition of primers

and the SUPERScript enzyme to remove possible genomic DNA contamination, which would be expected to confound qPCR results. A poly-T primer was used to selectively amplify mRNA. Primers for each gene were developed using Applied Bio systems, Inc. (ABI, Foster City, CA) Primer Express software and were tested to determine whether they produced a single band on an agarose gel. Primers were further evaluated to assure that the melting curve of the PCR product had a single major peak at a temperature compatible with the amplicon size and base composition, using the "melting curve" function for an ABI 7000 real-time PCR machine. Primers were initially designed to flank or include the Affymetrix probeset sequence, although in some cases different primers were substituted. Sequences not based on Affymetrix probesets (such as dopamine- and cAMP-regulated phosphoprotein of 32 kDa; *Darpp-32*) were subjected to BLAT analysis (<http://genome.ucsd.edu:mouse genome build 30 or 32>) to establish that they identified unique sequences (primer sequences are available on request). All samples were run in quadruplicate, and beta-actin was amplified simultaneously from each sample in different wells as a reference to control for differences in the concentration of cDNA as well as variability in PCR conditions unique to each specific reaction. Expression levels for beta-actin showed no significant differences between groups in the microarray analysis. Thus, the average difference in cycle time (Δ CT) between the experimental sample and the beta-actin control was the dependent variable for all analyses. ABI SYBR Green Mastermix was used with the cycling conditions recommended by the Primer Express software. One-tailed t -tests were used to determine significance levels ($\alpha = 0.05$) since we assumed *a priori* that the direction of the difference would be the same in both the microarray and the qPCR studies (this was true in all cases except for *D9Wsu18e*, which showed a statistically significant difference in the *wrong* direction). In addition to using qPCR as a verification step, preliminary data suggested a possible role for *Darpp-32*, which was not represented on the U74Av2 microarray. Expression of this gene was assessed in the same manner except that a two-tailed t -test was used to evaluate significance.

Results

bQTL Analysis. We found evidence of bQTL for MA stimulant response on Chromosomes 5, 9, 11, 12, and 15 (Fig. 1A–E) using the interval mapping method. We obtained largely similar results, albeit at

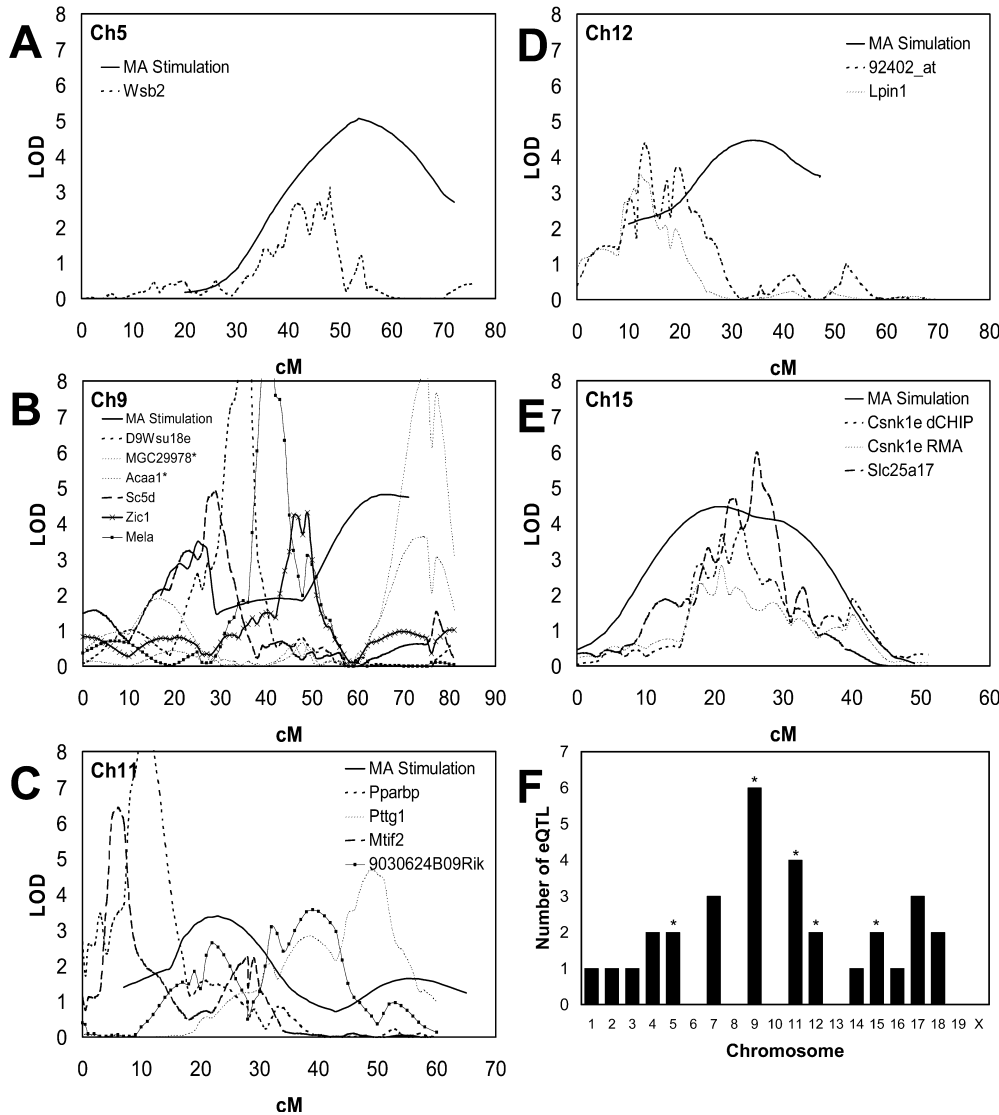


Fig. 1. Interval mapping results for the stimulant response to a 2-mg/kg dose of MA (MA Stimulation) in the short-term selected lines, and the expression of certain genes as measured in the BXD recombinant inbred strains from the WebQTL database. Chromosomes 5, 9, and 15 show a good correspondence between the bQTL and one or more eQTL, suggesting that a single polymorphism may account for both QTL. The y-axis shows LOD scores, while the x-axis shows the position on the chromosome in centimorgan (cM) units as calculated by Map Manager using a strain distribution pattern from www.WebQTL.org. Gene expression data are based on wholebrain (minus cerebellum) BXD recombinant inbred strain gene expression data obtained from www.WebQTL (all MAS5 DecO3, except for *Csnkle*, as noted in the figure legend). All chromosomes where there were suggestive or better results from the short-term selected lines are shown (A–E), and all genes that had eQTL that were significant, according to www.WebQTL.org, are plotted in this figure. All the genes represent *cis*-acting eQTL, with the exception of *Mela*, which appears to be a *trans*-acting eQTL. The short-term selected line results represent LOD scores with two degrees of freedom, whereas the BXD recombinant inbred strains have only one degree of freedom because only homozygous genotypes are possible in a recombinant inbred strain. The results from the short-term selected lines are not statistically corrected for genetic drift, which is a relatively small factor after only three generations. Panel F shows the chromosomal location of all significant eQTL for genes identified in Table 2. Note that while the microarray data indicated that the genes *D9Wsu18e* and *Sc5d* were differently expressed in the two lines, these differences were not confirmed with the qPCR assay. *in panel B indicates that the probesets for *Acaal* and *MGC29978* on Chromosome 9 appear to identify the same transcript. *in panel F indicates chromosomes where bQTL were also identified.

lower significance levels (as expected), using the allele frequency methods of Belknap et al. (1997; Table 1).

Microarray Gene Expression Analysis. Expression values for each gene were estimated with MAS5, RMA, and dCHIP, using the probe-level

Table 1. Allele frequency analysis results for the bidirectionally selected lines

<i>Chr</i>	<i>Marker</i>	<i>cM</i>	<i>qH</i>	<i>qL</i>	<i>p</i>
5	<i>D5Mit197</i>	20	0.63	0.5	0.23
5	<i>D5Mit95</i>	53	0.78	0.24	0.002
5	<i>D5Mit222</i>	71	0.72	0.32	0.015
9	<i>D9Mit90</i>	17	0.57	0.23	0.03
9	<i>D9Mit4</i>	29	0.73	0.27	0.007
9	<i>D9Mit274</i>	48	0.8	0.38	0.01
9	<i>D9Mit18</i>	71	0.88	0.32	0.001
11	<i>D11Mit229</i>	7	0.57	0.82	0.07
11	<i>D11Mit41</i>	41	0.46	0.6	0.19
11	<i>D11Mit338</i>	65	0.4	0.71	0.05
12	<i>D12Mit219</i>	10	0.7	0.27	0.011
12	<i>D12Mit157</i>	47	0.83	0.34	0.0035
15	<i>D15Mit252</i>	2	0.62	0.55	0.26
15	<i>D15Mit72</i>	29	0.66	0.31	0.026
15	<i>D15Mit15</i>	47	0.66	0.48	0.14

The difference in D2 allele frequencies (q) between the HMACT and LMACT lines at markers that were first implicated by the DNA pooling study as likely to be linked to bQTL influencing MA-induced stimulation. The p values are for the line differences in allele frequencies ($N = 41$ S₃ HMACT mice and 31 S₃ LMACT mice) tested against the null hypothesis that the observed differences were due solely to random drift and allele frequency estimation error.

information (CEL files) from the Affymetrix microarrays. Of the 12,488 probesets, we identified 15 transcripts that were significantly different at a $q < 0.05$. Of these 15, one (*Csnk1e*) was identified by all three methods, five were identified by two of the three methods, and nine were identified by only one method (Table 2). However, even in the latter case, the other two methods typically provided supporting evidence, although short of our arbitrarily selected q value. We identified an additional 39 transcripts that had q values between 0.05 and 0.5 by one or more of these methods. Of these 39, only 2 were identified with $q < 0.5$ by two of the methods, and none were identified by all three of the methods at this relaxed q value threshold. Three genes (*D9Wsu18e*, *Pccb*, and *Rpia*) that showed strong disagreements between the three analytical methods were not confirmed by qPCR analysis, which suggests that the use of multiple probe-level analysis methods may be useful for the detection of false-positive results.

It is notable that some of the expression differences that we identified with the microarrays, and verified with qPCR, are much higher than differences reported between the B6 and D2 mice in the WebQTL database. One likely possibility is that there are differences between the whole-brain (minus cerebellum) samples used to generate the WebQTL database and the nucleus accumbens samples used in the present study. Another possibility is that multiple alleles regulate the abundance of these transcripts and that selection has "sorted out" these alleles in the two short-term selected lines such that the observed phenotype is more extreme than in either of the two progenitor strains. This phenomenon is analogous to selection for a

behavioral phenotype leading to extremes of behavior that are not observed in the inbred progenitor strains.

Quantitative PCR. To verify results from the microarray experiment, we used qPCR to measure relative mRNA abundance for 21 of the transcripts listed in Table 2. Of those 21, 17 were verified, suggesting that the q value was a fairly accurate indicator of significance. Nevertheless, 3 of the 15 transcripts that had $q < 0.05$ were not confirmed, which may be a result of lack of sensitivity of the qPCR assay or false positives resulting from either statistical or technical problems with the microarray analysis. Two of these transcripts showed relatively low abundance according to the microarray data, which is consistent with a false-negative result from the qPCR assay resulting from poor sensitivity. The third transcript that was not verified was *D9Wsu18e* for which we obtained a statistically significant result from the qPCR analysis that indicated an expression difference in the direction opposite that predicted by the microarray data. While MAS5 identified *D9Wsu18e* as being differentially expressed with a q value of 0.021, both the RMA and dCHIP analyses yielded q values greater than 0.5 for this gene.

We used qPCR to examine *Darpp-32*, which was not represented on the microarrays, based on an *a priori* interest in this gene because of its known role in determining locomotor sensitivity to cocaine and amphetamine (Greengard 2001). Ogden et al. (2004) recently reported differential expression of *Darpp-32* 24 hours after administration of MA in mice. In addition, *Csnk1e*, whose gene product is known to

Table 2. Results from microarray analysis of gene expression differences in the nucleus accumbens of HMACT and LMACT short-term selected lines

Gene name	Abbr.	ProbeSet ID	Chr:MB	eQTL	Mean LMACT Mean HMACT FC		MAS5	RMA	dCHIP	qPCR	q value
					(MAS5)	(MAS5)					
Casain kinase 1, epsilon	Csnk1e	97925_at	15:80.8	cis (15)	34 ± 11.5	329 ± 32.7	9.82	0.00099	0.039	0.012	7.37E-08
Melanoma antigen	Mela	97282_at	8:125.3	trans (9)	126 ± 24.4	515 ± 41.7	4.1	0.0011	0.059	0.064	0.002077
Peroxisome proliferator activated receptor binding protein	Pparbp	160603_at	11:97.8	cis (11)	1009 ± 54	274 ± 77.9	3.68	0.0012	0.045	0.064	0.000488
Sterol-C5-desaturase (fungal ERG 3, delta-5-desaturase) homolog (<i>S. cerevisiae</i>)	Sc5d	102769_f_at	9:44	cis (9)	24 ± 1.6	7 ± 1.5	3.28	0.0012	0.025	0.91	0.157557
Mouse intracisternal A-particle-related retroviral elements and envelope pseudogene	—	92402_at	—	cis (12)	81 ± 17.4	598 ± 185.8	7.34	0.69	0.025	0.012	
FXYD domain containing ion transport regulator 2	Fxyd2	94827_at	9:47.2		97 ± 11.8	43 ± 6.5	2.25	0.25	0.21	0.015	
Gamma-aminobutyric acid (GABA-B) receptor 1	Gabbr1	98011_at	17:36.8		9424 ± 467.1	11322 ± 658.7	1.2	0.72	0.025	0.019	
Chemokine (C-C motif) receptor 5	Ccr5	161968_f_at	9:126.5		86 ± 14.2	15 ± 5.8	5.7	0.17	0.025	0.019	0.00055
DNA segment, Chr 7, Roswell Park 2 complex, expressed	D7Rp2e	96629_at	7:29.5	cis (7)	1488 ± 115.7	916 ± 73.5	1.62	0.2	0.33	0.019	
Annexin A3	Anxa3	101393_at	5:95.9		63 ± 6.4	48 ± 5.8	1.3	0.72	0.056	0.02	
DNA segment, Chr 9, Wayne State University 18, expressed	D9Wsu18e	95430_at	9:67.9	cis (9)	853 ± 90.5	250 ± 43.7	3.42	0.021	0.6	0.91	0.003881
Propionyl coenzyme A carboxylase, beta polypeptide	Pccb	160128_at	9:103.4		134 ± 11.3	62 ± 4.2	2.15	0.021	0.36	0.91	0.499088
Dual specificity phosphatase 1	Dusp1	104598_at	17:26.1		287 ± 28.3	410 ± 28.5	1.43	0.55	0.55	0.027	
Histocompatibility 47	H47	94245_at	7:58.5		368 ± 48	388 ± 21.2	1.05	0.75	0.03	0.35	
Keratin complex 1, acidic, gene 17	Krt1-17	92861_i_at	11:99.8		9 ± 4.1	7 ± 2	1.32	0.74	0.93	0.047	
Histidyl-tRNA synthetase	Hars	92580_at	18:36.9	cis (18)	319 ± 48.2	852 ± 87.8	2.67	0.061	0.17	0.052	0.000664
RIKEN cDNA 6330575P11 gene	6330575P11Rik	94426_at	7:111.7	cis (7)	131 ± 21.7	383 ± 76.4	2.91	0.53	0.096	0.052	
Ribonucleotide reductase M2	Rrm2	102001_at	2:19.1		25 ± 11.4	24 ± 4.2	1.04	0.79	0.056	0.2	
Ribose 5-phosphate isomerase A	Rpia	103322_at	6:71.7		553 ± 19.5	717 ± 24.2	1.3	0.061	0.64	0.91	0.112139
Acetyl-coenzyme A acyltransferase 1	Acaa1	160482_at	9:121.7	cis (9)	1041 ± 51.6	597 ± 69.6	1.74	0.078	0.17	0.13	0.041274
Glutamate receptor, ionotropic, AMPA1 (alpha 1)	Gria1	92943_at	11:56.6		2341 ± 211.1	1127 ± 175.9	2.08	0.19	0.17	0.078	4.46E-05
RIKEN cDNA 3300001G02 gene	3300001G02Rik	97412_at	11:32.1		1014 ± 53.2	783 ± 52.9	1.29	0.55	0.18	0.081	
Formin binding protein 2	Fbnp2	96516_at	1:134.2		31 ± 6.9	25 ± 2.1	1.21	0.72	0.9	0.084	
Pituitary tumor-transforming 1	Pttg1	101027_s_at	11:43	cis (11)	176 ± 38.6	349 ± 25.4	1.98	0.32	0.091	0.22	
RIKEN cDNA 1190005106 gene	1190005106Rik	93143_at	8:122.4		143 ± 15.9	103 ± 6.8	1.39	0.72	0.33	0.094	
Phosphatidylinositol-4-phosphate 5-kinase, type II, alpha	Pip5k2a	95358_at	2:19		451 ± 20.8	544 ± 19.3	1.21	0.49	0.64	0.12	
RIKEN cDNA 2310047E01 gene	2310047E01Rik	103905_at	9:69.2		95 ± 8.1	165 ± 12	1.74	0.13	0.14	0.2	0.016605
3-Ketoacyl-CoA thiolase B	MGC29978	99571_at	9:121.5	cis (9)	270 ± 19	160 ± 14.2	1.68	0.17	0.32	0.86	0.0375
UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 1	Galnt1	97934_at	18:24.8		482 ± 21	722 ± 48.3	1.5	0.17	0.47	0.74	0.002947

(Continued)

Table 2. Continued

Gene name	Abbr.	ProbeSet ID	Chr:MBcQTL	q value						
				Mean LMACTMean (MAS5)	HMACTFC (MAS5)	MAS5MAS5RMA dCHIP qPCR				
RIKEN cDNA E130012P22 gene	E130012P2	2Rik 97365_at	9:64.8	235 ± 15.8	339 ± 16.2	1.44	0.17	0.33	0.44	0.000659
RIKEN cDNA 1110033I19 gene	1110033I19	Rik 98149_s_at	6:149.9	28 ± 1.8	14 ± 2.6	2.05	0.17	0.9	0.91	0.045672
Phospholipase D2	Plid2	94371_at	11:70.1 trans (14)	84 ± 9.4	145 ± 9.9	1.73	0.17	0.94	0.84	0.023432
Mitochondrial translational initiation factor 2	Mtif2	93859_at	11:29.4 cis (11)	86 ± 11.7	175 ± 17.7	2.03	0.2	0.24	0.17	
Lipin 1	Lpin1	98892_at	12:16.9 cis (12)	193 ± 15.6	113 ± 9.2	1.71	0.19	0.31	0.92	0.015672
CCAAT/enhancer binding protein alpha (C/EBP), related sequence 1	Cebpa-rs1	94467_at	17:79.6	25 ± 3.2	9 ± 1.6	2.72	0.2	0.69	0.2	0.000923
Aldehyde dehydrogenase family 7, member A1	Aldh7a1	97450_s_at	18: cis (18)	745 ± 43.1	530 ± 31.4	1.41	0.25	0.31	0.2	
Solute carrier family 25 (mitochondrial carrier; peroxisomal membrane protein, 34 kDa), member 17	Slc25a17	97472_at	15:82.8 cis (15)	510 ± 31.9	314 ± 32.7	1.63	0.2	0.32	0.7	
Microsomal glutathione S-transferase 3	Mgst3	96258_at	1:171.1 cis (1); trans (3)	250 ± 15.6	158 ± 15.3	1.59	0.2	0.22	0.22	
H2-K region expressed gene 6	H2-Ke6	102991_s_at	17:33.7 cis (17)	266 ± 18.3	167 ± 17.1	1.59	0.3	0.31	0.86	
Antigen identified by monoclonal antibody MRC OX-2	Mox2	101851_at	16:45.5 cis (16)	152 ± 19.9	297 ± 31.5	1.96	0.3	0.53	0.91	
Cyclic AMP phosphoprotein	Arpp19	97260_at	9:77.5	321 ± 26.9	471 ± 28.3	1.47	0.31	0.47	0.44	0.050343
RIKEN cDNA 9030624B09 gene	9030624B09	9Rik 94983_at	11:57.1 cis (11)	69 ± 4.9	101 ± 6.8	1.46	0.31	0.48	0.91	
Expressed sequence A1854265	A1854265	97752_at	2:159 cis (2)	908 ± 88.3	1466 ± 137	1.61	0.44	0.85	0.31	
ADP-ribosylation-like factor 6 interacting protein	Arl6ip	160371_at	7:110 cis (7)	1144 ± 95.1	1612 ± 87.7	1.41	0.38	0.31	0.91	
RIKEN cDNA 1700030C10 gene	1700030C10	1700030C1	0Rik 102120_f_at —	425 ± 34	647 ± 55.6	1.52	0.44	0.33	0.91	
WD-40-repeat-containing protein with a SOCS box 2	Wsb2	160296_at	5:116.4 cis (5)	920 ± 67.4	638 ± 35.7	1.44	0.34	0.89	0.91	
Zinc finger protein of the cerebellum 1	Zic1	104169_at	9:93.8 cis (9)	1410 ± 123.6	899 ± 86.5	1.57	0.44	0.73	0.34	
DNA segment, Chr 4, Wayne State University 24, expressed	D4Wsu24e	104263_at	4:126 cis (4)	206 ± 18.3	339 ± 32.2	1.65	0.4	0.55	0.91	
Histocompatibility 2, K region	H2-K	93120_f_at	17:33.6 cis (17)	238 ± 14.8	313 ± 15	1.31	0.42	0.79	0.91	
Crystallin, gamma F	Crygf	97579_f_at	1:66.7	60 ± 6.8	29 ± 5.5	2.09	0.42	0.91	0.91	
Synapsin I	Syn1	93730_at	X:19.4	549 ± 35	370 ± 37.1	1.48	0.43	0.5	0.86	0.249723
Complexin 1	Cplx1	101198_at	5:107.5	38 ± 3.8	74 ± 9.6	1.97	0.43	0.93	0.91	
Aminolevulinatase, delta, dehydratase	Alad	101044_at	4:61.1 cis (4)	182 ± 17.9	111 ± 12.4	1.63	0.52	0.47	0.67	
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7 [B14.5a]	Ndufa7	95652_at	17:33.5 cis (17)	525 ± 35.9	684 ± 32.6	1.3	0.49	0.94	0.92	

The full name for the gene identified by each probeset, as assigned by Affymetrix, listed in the first column, followed by its abbreviation, Affymetrix probeset ID, chromosome location, and estimated position (in Mb). Next, whether or not an eQTL was identified using WebQTL.org (the chromosomal location of the eQTL is in parentheses; MAS5, Dec03 freeze), and the average expression level as estimated by MAS5 is listed. FC MAS5 is the fold change between the HMACT and LMACT lines for the gene as calculated with the expression values obtained from MAS5. The *q*-values obtained with MAS5, RMA, and dCHIP are listed, and the *p* value for a *t*-test based on qPCR results listed (when available) in the last column (because we used a 1-tailed *t*-test, the result for the gene D9Wsu18e is considered negative since the expression difference was in the opposite direction to the microarray results). Probesets 99571_at and 160482_at appear to identify the same gene based on BLAT analysis.

phosphorylate DARPP-32 (Desdouits et al. 1995), was differentially expressed in the two oppositely selected lines, suggesting that alterations in this pathway may have contributed to the response to selection. We observed a significant difference of 0.42 cycle for *Darpp-32* ($p < 0.01$; Fig. 2), which corresponds to about 35% more *Darpp-32* mRNA in the samples from the nucleus accumbens of the HMACT line. Additional verification of genes identified by the microarrays was not possible because we exhausted our supply of several of the primary samples.

eQTL Analysis. To integrate the bQTL data with the expression data, we determined whether there were detectable eQTL for any of the transcripts found to be differentially expressed in our microarray study using www.WebQTL.org. Six of the 15 genes identified in Table 2 with $q < 0.05$ had apparent *cis*-acting eQTL, defined as those that map to the chromosomal region where the gene being modulated is located. One of these genes had a *trans*-acting eQTL, i.e., an eQTL that maps to a region or chromosome other than the one where the gene is located. All three analytical methods agreed on three of these eQTL (*Pparbp*, *Mela*, *D7Rp2e*), three were identified by only two methods, and one gene (probeset 92402_at) was identified only by MAS5. MAS5 and RMA identified all but one of these seven eQTL, while dCHIP identified only four of them.

The distribution of eQTL across the genome is shown in Fig. 1F. The pattern was not random; eQTL clustered together on particular chromosomes where bQTL were also observed. One possible explanation for this clustering is that certain chromosomes have a greater density of polymorphisms between the two lines being examined. If this were the case, then the identification of bQTL and eQTL on the same chromosomes might simply indicate which chromosomes had a higher density of polymorphisms. To address this possibility, we compared the number of genes that were differentially expressed between the two selected lines that also had known eQTL (as shown in Fig. 1F) with the number of SNPs per chromosome (<http://www.jax.org/phenome>) divided by the length of each chromosome in MB (<http://genome.ucsc.edu>). We found a nonsignificant trend ($p < 0.1$) toward a weak positive relationship ($r^2 = 0.16$) between these two variables (data not shown). In particular, Chromosomes 7 and 11 had a high SNP density and showed high numbers of differentially expressed genes that had known eQTL as well however, note that Chromosome 7 did not contain a bQTL for MA response). This calculation is potentially flawed for several reasons:(1) The genes

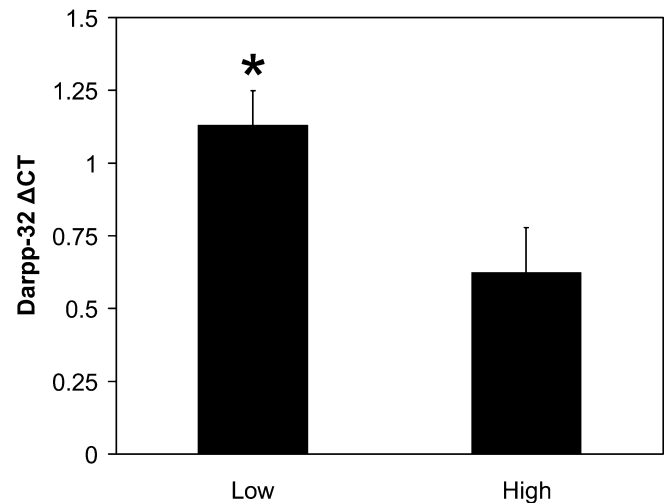


Fig. 2. Because *Csnk1e* levels were very different between the HMACT and LMACT lines and because *Csnk1e* is known to regulate the activity of *Darpp-32*, a critical mediator of the response to dopaminergic agonists, we measured *Darpp-32* using qPCR (there is no probeset for this gene in the U74Av2 array). The results show that the *Darpp-32* transcript is more abundant in the HMACT line relative to the LMACT line (transcript abundance is inversely related to Delta CT). * indicates a $p < 0.01$.

represented on the microarrays may be a nonrandom sample of the genome;(2) known SNP polymorphisms may also be overrepresented on certain chromosomes (to partly address this issue we excluded the 50,000 + SNP polymorphisms from Celera at <http://www.jax.org/phenome> since they all reside on Chr 16);(3) there may be differences in the density of genes per MB for certain chromosomes. However, based on this crude analysis there appears to be only a weak relationship between the location of genes that were differentially expressed in our selected lines that also had known eQTL from the WebQTL database and the density of between-strain polymorphisms as measured by known SNPs.

We suspect that the observed clustering was instead the result of differences between the HMACT and LMACT lines in the frequency of alleles that control expression of these genes. To further illustrate the comapping of the bQTL and eQTL, we plotted them on the same figures (Fig. 1A–E). In some instances, the bQTL and eQTL comap to the same chromosome but do not appear to map to the same region of that chromosome (e.g., *Pparbp* on Chromosome 11). There are several possible explanations for these results: The eQTL are linked to alleles that cause the bQTL but are not themselves functionally related to the bQTL; the eQTL or bQTL findings are false positives; or the differential expression of these genes is determined by a different mechanism (different alleles) in the whole-brain

samples used to map the eQTL and the nucleus accumbens samples from which our data were derived. Such results are less interesting than those for which the bQTL and the eQTL map to the same region of the chromosome in question (e.g., *Csnk1e* on Chromosome 15). In these latter cases, we hypothesize that the eQTL may comap with the bQTL because they stem from the same polymorphism—a highly informative and testable hypothesis that is the result of our integration of bQTL and eQTL data.

Discussion

Selective breeding from an F₂ cross derived from two inbred strains causes the alleles of the loci influencing the trait under selection (bQTL) to diverge from their initial frequency of 0.5, with the frequency of one allele increasing in one line and decreasing in the oppositely selected line (Belknap et al. 1997). In contrast, allele frequencies in portions of the genome that do not contain trait-relevant bQTL show comparatively small and random changes due to genetic drift. When the effect of the bQTL on the selected trait is a result of a difference in gene expression (an eQTL), then selection will be associated with differential transcript abundance since the eQTL and the bQTL are actually caused by the same locus. In the present study, we identified a number of genes that were differentially expressed in nucleus accumbens tissue from mice that had undergone selection for differential stimulant response to MA. eQTL were identified that comap with bQTL for MA response, suggesting that the identified eQTL are logical candidates to cause the bQTL (i.e., there is a single allele causing both QTL).

Gene expression differences were observed in drug-naïve mice, thus they were the result of changes in allele frequencies due to selection rather than a response to drug treatment. These differences exist prior to MA administration and are hypothesized to alter the acute response to MA, which occurs too rapidly to depend on drug-induced changes in gene expression *per se*. Because expression is the hypothetical mechanism of action for the bQTL-causing allele, these candidates provide readily testable hypotheses. Identification of the genetic basis for bQTL has been an elusive and challenging goal (Belknap et al. 2001; Phillips et al. 2002). In these experiments we have progressed rapidly from broad regions to specific candidate genes through an integration of traditional mapping techniques, gene expression microarrays, and bioinformatics. This success builds on existing efforts to integrate gene mapping with gene expression within a single organism (e.g., Wayne and McIntyre 2002; Hitze-

mann et al. 2003, 2004; Schadt et al. 2003) and across species (e.g., Niculescu et al. 2000; Tabakoff et al. 2003; Ogden et al. 2004).

The chromosomes shown in Fig. 1A–E contain more than half of the eQTL for genes that we found to be differentially expressed (Table 2, Fig. 1F). While the LOD curves for these eQTL are relatively broad, in some cases there is good correspondence between the bQTL for MA response and the eQTL for these genes. In other cases it seems likely that the eQTL do not colocalize with the bQTL. In these cases, there is little reason to believe that the eQTL are causally related to the bQTL.

Several genes identified by our analysis have already been implicated in the response to MA. The gene product of *Csnk1e* phosphorylates and thus increases the activity of the *Darpp-32* protein, which is known to be a critical regulator of the locomotor response to dopamine-releasing drugs such as cocaine and MA (Greengard 2001). Higher expression of both *Csnk1e* and *Darpp-32* was observed in the HMACT line and would be expected to lead to a stronger locomotor-stimulant response to MA (see Fig. 3). The LMACT and HMACT lines show a genetically correlated difference in locomotor stimulant response to cocaine and in cocaine consumption, suggesting that some common genes may influence MA- and cocaine-related traits in these lines (Kamens et al. 2005). Differences in *Csnk1e* and *Darpp-32* expression may underlie a portion of this shared sensitivity. Intriguingly, a putative bQTL for the locomotor response to cocaine (5 mg/kg) and for binding of several dopaminergic ligands in the nucleus accumbens has been identified that comaps with the eQTL for *Csnk1e* and the bQTL for MA response (Jones et al. 1999). In addition, a study of home cage activity in response to MA indicated that there were suggestive bQTL for that phenotype on Chromosomes 5, 9, 12, and 15, all of which were implicated in this study; however, the bQTL on Chromosome 15 does not colocalize particularly well with the location of *Csnk1e* (Grisel et al. 1997). *Darpp-32* has recently been suggested as a candidate gene for bipolar disorder, based in part on changes in its expression 24 hours following administration of 10 mg/kg MA and on the ability of the mood-stabilizing drug valproate to block MA-induced behaviors and increases in *Darpp-32* expression (Ogden et al. 2004).

The gene *Gria1* codes for an AMPA glutamate receptor subunit that was more highly expressed in the LMACT line and that has been implicated in amphetamine- and cocaine-induced changes in synaptic plasticity that may be related to drug abuse (Wolf et al. 2003). Furthermore, AMPA receptor ag-

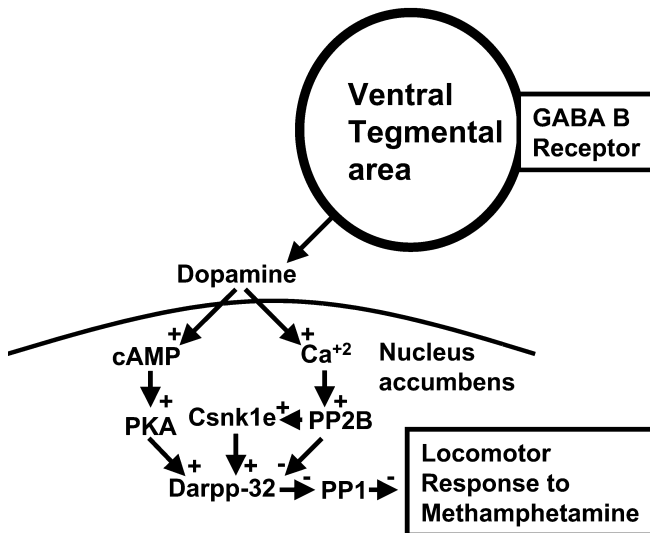


Fig. 3. Proposed model demonstrating how our results can be integrated with the current understanding of signaling mechanisms associated with the locomotor stimulant response to MA. Dopamine stimulates the release of intracellular Ca^{2+} , which stimulates protein phosphatase 2B (PP2B; aka calcineurin), which activates *Csnk1e* by dephosphorylation, which in turn phosphorylates the ser-137 site on *Darpp-32*, which inhibits dephosphorylation of Thr-34 on *Darpp-32* by PP2B, thus preserving the protein phosphatase activity of *Darpp-32*, which is inhibitory toward protein phosphatase 1 (PP1), which is itself a critical inhibitor of locomotor stimulation (for details and primary references, see Greengard 2001). Dopamine also causes increases in cAMP, which leads to activation protein kinase A (PKA) and subsequent activation of *Darpp-32*. The gene *Gabbr1* codes for the GABA B1 receptor, which promotes dopamine release in the nucleus accumbens by neurons that project from the ventral tegmental area to the nucleus accumbens (Brebner et al. 2002; Cousins et al. 2002). We detected an almost tenfold increase in *Csnk1e* (Table 2) as well as smaller increases in *Darpp-32* (Fig. 2) and *Gabbr1* (Table 2) expression in the HMACT line, which also showed a greatly enhanced locomotor stimulant response to MA.

onists and antagonists have been shown to modulate the locomotor response to amphetamine when both drugs are microinjected into the nucleus accumbens (David et al. 2004). Ogden et al. (2004) identified a decrease in *Gria1* expression 24 hours after administration of 10 mg/kg MA. Given these results, examining gene expression in our selected lines after saline versus MA treatment to determine whether the MA sensitivity phenotype is associated with differential expression in response to MA would be of interest.

The *Gabbr1* gene codes for the GABA B1 receptor. Agonists of this receptor have shown promising results for the treatment of drug addiction (Cousins et al. 2002). In addition, they have been shown to attenuate amphetamine and cocaine self-adminis-

tration or seeking in the rat (Di Ciano and Everitt 2003; Brebner et al. 2004), to attenuate the acute and sensitized locomotor responses to amphetamine (Phillis et al. 2001; Bartoletti et al. 2004), and to reduce stimulant-induced increases in dopamine in the nucleus accumbens (Fadda et al. 2003; Brebner et al. 2004). The effect on dopamine release appears to depend on GABA B receptors in the ventral tegmental area, which is the source of dopamine-projecting neurons to the nucleus accumbens. While we measured *Gabbr1* expression in the nucleus accumbens rather than in the ventral tegmental area, genetically determined gene expression differences in specific brain regions are frequently also observed in other brain regions (Pavlidis and Noble 2001; unpublished observations). In light of these data, which suggest that the GABA B receptor inhibits the response to amphetamine and related compounds, higher expression of *Gabbr1* in the HMACT line appears to be contradictory. This difference may reflect a compensatory response to excessive activity of the ventral tegmental area \rightarrow nucleus accumbens circuit in the HMACT line, or heightened expression of *Gabbr1* because of a low resting tone of the ventral tegmental area GABAergic system. Alternatively, the difference in *Gabbr1* expression may not extend to the ventral tegmental area. While there is significant evidence that administration of GABA B agonists decreases the response to MA, we do not know what effect such agonists would have had on the response to MA in the HMACT and LMACT lines.

A major goal of QTL mapping in model organisms is the identification of novel genes. The most significant bQTL for MA response are on Chromosomes 5 and 9, which appear to harbor at least three bQTL and which contain several eQTL for genes that are not currently known to play a role in MA response. One or more of these genes may represent novel candidates, a possibility that we are currently pursuing. Alternatively, the gene(s) causing the bQTL on Chromosome 9 may not be represented on our microarray, may not be expressed in the nucleus accumbens, may be differentially expressed in development rather than adulthood (similar to Gross et al. 2002), or may be due to a coding sequence difference rather than an expression difference. Clearly, future studies should attempt to examine other brain regions and address these issues; our results must be labeled "provisional" until such data are available.

The statistical power of short-term selected lines for mapping bQTL is similar to that of a standard F_2 population on a per-mouse basis (Belknap et al. 1997). A significant advantage of short-term selected

lines over F_2 populations is their usefulness for examining putatively genetically correlated traits (Palmer and Phillips 2002; Kamens et al. 2005). We examined expression of 12,488 transcripts, each of which is a putatively correlated trait. Correlated traits can also be examined using an F_2 population. However, since each F_2 mouse is genetically unique, the correlation that one finds is not purely genetic (Palmer and Phillips 2002). Environmental factors unique to a particular F_2 mouse (such as rough handling by the vivarium staff or fighting with cage mates) might alter both of the putatively correlated traits, but there would be no way to determine what proportion of the apparent correlation was a result of genetic versus environmental differences among individuals. Furthermore, identification of a correlation in an F_2 population requires measurement of both traits in each individual. Measuring the first phenotype (e.g., MA response) may itself alter the second phenotype (e.g., gene expression). Correlations obtained using selected lines are relatively free of these problems because each trait can be measured in separate individuals. Such correlations are more purely genetic because the test of a correlated trait is based on a comparison of the high and low groups; independent sets of experimentally naïve animals from each selected line are tested for each trait, and "noise" from environmental factors will be equally distributed across both lines.

Selected lines also allow us to identify a second class of genes, namely, those whose expression is governed by multiple small-effect eQTL. Selection will change the frequency of each eQTL-causing allele for genes that are important for the selection trait, leading to gene expression differences that may be due to multiple (and thus less easily mapped) eQTL. Such genes are important to identify in order to better understand the genetic determinants of the trait under selection. Nevertheless, these genes would be difficult to identify with nonselection-based approaches since multiple small alleles will only rarely be grouped together in randomly bred (e.g., F_2) populations. In the present study, we identified *Gabbr1*, *Gria1*, and *Darpp-32*, none of which have significant eQTL (based on currently available data), possibly because their expression is governed by multiple small-effect eQTL. Both *Gria1* and *Darpp-32* are located on Chromosome 11, which contains a bQTL for MA response, suggesting that one of these small-effect eQTL may be *cis*-acting. Indeed, www.WebQTL.org provided suggestive (but not quite significant) evidence of a *cis*-acting eQTL for *Gria1*, and this eQTL becomes significant when considering preliminary data obtained from an F_2

cross between B6 and D2 strains (www.WebQTL.org; data not shown), suggesting that an eQTL for *Gria1* may be responsible for the bQTL on Chromosome 11.

When using selected lines (e.g., Tabakoff et al. 2003) for which eQTL data are not available, identification of gene expression differences is still possible; however, it is difficult to determine whether gene expression differences are a result of one or more *cis*- or *trans*-acting eQTL. It is also more difficult to map bQTL using such populations. Therefore, short-term selected lines produced from progenitor strains for which eQTL data are also available are uniquely powerful because evidence of the bQTL and eQTL is obtained from the same population. At present, only crosses between the B6 and D2 strains have a corresponding eQTL database available.

Our approach is related to an approach that has been termed "convergent functional genomics" by Niculescu et al. (2000; also see Ogden et al. 2004). In those studies, gene expression in response to administration of MA and the mood-stabilizing drug valproate was examined in mice or rats to identify genes that might be involved in the behavioral responses to these drugs. The results of these studies were combined with human linkage results from bipolar and schizophrenia studies in order to identify candidate genes within those linkage regions. mRNA and protein levels in postmortem human brain samples were also examined, and bioinformatic tools were used to further parse among the possible candidate genes. Both their strategy and ours are designed to generate candidate genes for testing in human subjects. Our approach is distinct because it focuses on gene expression differences between two groups of mice that differ in their genetic response to MA rather than on the analysis of gene expression associated with MA treatment. Thus, our fundamental manipulation is genetic, whereas theirs is pharmacologic.

We have brought classical genetics and modern genomic and computational resources together in a unique configuration that can serve as a blueprint for subsequent investigations of other phenotypes. Taken together, the present data suggest that *Csnk1e*, *Gabbr1*, *Gria1*, and *Darpp-32* are logical candidates for future studies in mice and for translational genetic studies of drug-abuse susceptibility in human subjects. Additionally, these data demonstrate the potential of short-term selective breeding studies in combination with microarrays to identify genes that are differentially regulated in a manner that affects a complex behavioral phenotype.

Acknowledgments

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References

1. Agrawal A, Jacobson KC, Prescott CA, Kendler KS (2004) A twin study of personality and illicit drug use and abuse/dependence. *Twin Res* 7, 72–81
2. Balfour DJ, Wright AE, Benwell ME, Birrell CE (2000) The putative role of extra-synaptic mesolimbic dopamine in the neurobiology of nicotine dependence. *Behav Brain Res* 113, 73–83
3. Bartoletti M, Gubellini C, Ricci F, Gaiardi M (2004) The GABAB agonist baclofen blocks the expression of sensitisation to the locomotor stimulant effect of amphetamine. *Behav Pharmacol* 15, 397–401
4. Belknap JK, Richards SP, O'Toole LA, Helms ML, Phillips TJ (1997) Short-term selective breeding as a tool for QTL mapping: ethanol preference drinking in mice. *Behav Genet* 27, 55–66
5. Belknap JK, Hitzemann R, Crabbe JC, Phillips TJ, Buck KJ, et al. (2001) QTL analysis and genomewide mutagenesis in mice: complementary genetic approaches to the dissection of complex traits. *Behav Genet* 31, 5–15
6. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J Stat Soc* 57, 289–300
7. Bergeson SE, Warren RK, Crabbe JC, Metten P, Erwin VG, et al. (2003) Chromosomal loci influencing chronic alcohol withdrawal severity. *Mammal Genome* 14, 454–463
8. Boileau I, Assaad JM, Pihl RO, Benkelfat C, Leyton M, et al. (2003) Alcohol promotes dopamine release in the human nucleus accumbens. *Synapse* 49, 226–231
9. Brebner K, Ahn S, Phillips AG (2004) Attenuation of d-amphetamine self-administration by baclofen in the rat: behavioral and neurochemical correlates. *Psychopharmacology (Berl)* 177, 409–417
10. Broman KW, Wu H, Sen S, Churchill GA (2003) R/qtl: QTL mapping in experimental crosses. *Bioinformatics* 19, 889–890
11. Chesler EJ, Lu L, Wang J, Williams RW, Manly KF (2004) WebQTL: rapid exploratory analysis of gene expression and genetic networks for brain and behavior. *Nat Neurosci* 7, 485–486
12. Churchill GA, Doerge RW (1994) Empirical threshold values for quantitative trait mapping. *Genetics* 138, 963–971
13. Complex Trait Consortium (2003) The nature and identification of quantitative trait loci: a community's view. *Nat Rev Genet* 4, 911–916
14. Cousins MS, Roberts DC, de Wit H (2002) GABA(B) receptor agonists for the treatment of drug addiction: a review of recent findings. *Drug Alcohol Depend* 65, 209–220
15. David HN, Sissaoui K, Abirami JH (2004) Modulation of the locomotor responses induced by D(1)-like and D(2)-like dopamine receptor agonists and D-amphetamine by NMDA and non-NMDA glutamate receptor agonists and antagonists in the core of the rat nucleus accumbens. *Neuropharmacology* 46, 179–191
16. Desdouits F, Cohen D, Nairn AC, Greengard P, Girault JA (1995) Phosphorylation of DARPP-32 a dopamine- and cAMP-regulated phosphoprotein, by casein kinase I in vitro and in vivo. *J Biol Chem* 270, 8772–8778
17. Di Chiara G (2002) Nucleus accumbens shell and core dopamine: differential role in behavior and addiction. *Behav Brain Res* 137, 75–114
18. Di Chiara G, Imperato A (1988) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc Natl Acad Sci USA* 85, 5274–5278
19. Di Ciano P, Everitt BJ (2003) The GABA(B) receptor agonist baclofen attenuates cocaine- and heroin-seeking behavior by rats. *Neuropsychopharmacology* 28, 510–518
20. Fadda P, Scherma M, Fresu A, Collu M, Fratta W (2003) Baclofen antagonizes nicotine-, cocaine-, and morphine-induced dopamine release in the nucleus accumbens of rat. *Synapse* 50, 1–6
21. Fehr C, Shirley RL, Belknap JK, Crabbe JC, Buck KJ (2002) Congenic mapping of alcohol and pentobarbital withdrawal liability loci to a < 1 centimorgan interval of murine chromosome 4: identification of Mpdz as a candidate gene. *J Neurosci* 22, 3730–3738
22. Glazier AM, Nadeau JH, Airman TJ (2002) Finding genes that underlie complex traits. *Science* 298, 2345–2349
23. Greengard P (2001) The neurobiology of slow synaptic transmission. *Science* 294, 1024–1030
24. Grisel JE, Belknap JK, O'Toole LA, Helms ML, Charlotte WD, et al. (1997) Quantitative trait loci affecting methamphetamine responses in BxD recombinant inbred strains. *J Neurosci* 17, 745–754
25. Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, et al. (2002) Serotonin 1A receptor acts during development to establish normal anxiety-like behaviour in the adult. *Nature* 416, 396–400
26. Hitzemann R, Malmanger B, Reed C, Lawler M, Hitzemann B, et al. (2003) A strategy for the integration of QTL, gene expression, and sequence analyses. *Mamm Genome* 14, 733–747
27. Hitzemann R, Reed C, Malmanger B, Lawler M, Hitzemann B, et al. (2004) On the integration of alcohol-related quantitative trait loci and gene expression analyses. *Alcohol Clin Exp Res* 28, 1437–1448
28. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, et al. (2003) Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* 31, e15
29. Jones BC, Tarantino LM, Rodriguez LA, Reed CL, McClearn GE, et al. (1999) Quantitative-trait loci analysis of cocaine-related behaviours and

- neurochemistry. *Pharmacogenetics* 9, 607–617
30. Kamens HM, Burkhart-Kasch S, McKinnon CS, Li N, Reed C, Phillips TJ (2005) Sensitivity to psychostimulants in mice bred for high and low stimulation to methamphetamine. *Genes Brain Behav* 4, 110–125
 31. Kendler KS, Jacobson KC, Prescott CA, Neale MC (2003) Specificity of genetic and environmental risk factors for use and abuse/dependence of cannabis, cocaine, hallucinogens, sedatives, stimulants, and opiates in male twins. *Am J Psychiatry* 160, 687–695
 32. Kim JH, Creekmore E, Vezina P (2003) Microinjection of CART peptide 55-102 into the nucleus accumbens blocks amphetamine-induced locomotion. *Neuropeptides* 37, 369–373
 33. Lander ES, Botstein D (1989) Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics* 121, 185–199
 34. Lecca D, Piras G, Driscoll P, Giorgi O, Corda MG (2004) A differential activation of dopamine output in the shell and core of the nucleus accumbens is associated with the motor responses to addictive drugs: a brain dialysis study in Roman high- and low-avoidance rats. *Neuropharmacology* 46, 688–699
 35. Li C, Wong WH (2003) DNA-Chip analyzer (dChip). In *The Analysis of Gene Expression Data: Methods and Software*, Parmigiani ES, Garrett EA, Irizarry RA, Seeger SL (eds.) New York: Springer, pp 120–141
 36. Li T-K, Lumeng L, McBride WJ, Murphy JM (1987) Rodent lines selected for factors affecting alcohol consumption. *Alcohol Alcohol Suppl* 1, 91–96
 37. Liang T, Spence J, Liu L, Strother WN, Chang HW, et al. (2003) alpha-Synuclein maps to a quantitative trait locus for alcohol preference and is differentially expressed in alcohol-preferring and -nonpreferring rats. *Proc Natl Acad Sci USA* 100, 4690–4695
 38. Mackay TF (2001) The genetic architecture of quantitative traits. *Annu Rev Genet* 35, 303–339
 39. Mitchison A (1997) Partitioning of genetic variation between regulatory and coding gene segments: the predominance of software variation in genes encoding introvert proteins. *Immunogenetics* 46, 46–52
 40. Niculescu AB 3rd, Segal DS, Kuczynski R, Barrett T, Hauger RL, et al. (2000) Identifying a series of candidate genes for mania and psychosis: a convergent functional genomics approach. *Physiol Genomics* 4, 83–91
 41. Ogden CA, Rich ME, Schork NJ, Paulus MP, Geyer MA, et al. (2004) Candidate genes, pathways and mechanisms for bipolar (manic-depressive) and related disorders: an expanded convergent functional genomics approach. *Mol Psychiatry* 9, 1007–1029
 42. Palmer AA, Phillips TJ (2002) Quantitative trait loci (QTL) mapping in mice. In *Methods in alcohol-related neuroscience research*, Liu Y, Lovinger DM, (eds.) (Boca Raton, FL: CRC Press), pp 1–30
 43. Palmer AA, Low MJ, Grandy DK, Phillips TJ (2003) Effects of a Drd2 deletion mutation on ethanol-induced locomotor stimulation and sensitization suggest a role for epistasis. *Behav Genet* 33, 311–324
 44. Pavlidis P, Noble WS (2001) Analysis of strain and regional variation in gene expression in mouse brain. *Genome Biol* 2, RESEARCH0042
 45. Peng X, Wood CL, Blalock EM, Chen KG, Landfield PW, et al. (2003) Statistical implications of pooling RNA samples for microarray experiments. *BMC Bioinformatics* 4, 26
 46. Phillips TJ, Belknap JK, Hitzemann RJ, Buck KJ, Cunningham CL, et al. (2002) Harnessing the mouse to unravel the genetics of human disease. *Genes Brain Behav* 1, 14–26
 47. Phillis BD, Ong J, White JM, Bonnielle C (2001) Modification of d-amphetamine-induced responses by baclofen in rats. *Psychopharmacology* 153, 277–284
 48. Schadt EE, Monks SA, Drake TA, Luskis AJ, Che N, et al. (2003) Genetics of gene expression surveyed in maize, mouse and man. *Nature* 422, 297–302
 49. Sellings LH, Clarke PB (2003) Segregation of amphetamine reward and locomotor stimulation between nucleus accumbens medial shell and core. *J Neurosci* 23, 6295–6303
 50. Shirley RL, Walter NA, Reilly MT, Fehr C, Buck KJ (2004) Mpdz is a quantitative trait gene for drug withdrawal seizures. *Nat Neurosci* 7, 699–700
 51. Storey JD, Tibshirani R (2003) Statistical significance for genome-wide experiments. *Proc Natl Acad Sci USA* 100, 9440–9445
 52. Suto N, Tanabe LM, Austin JD, Creekmore E, Pham CT, Vezina P (2004) Previous exposure to psychostimulants enhances the reinstatement of cocaine seeking by nucleus accumbens AMPA. *Neuropsychopharmacology* 29, 2149–2159
 53. Tabakoff B, Bhave SV, Hoffman PL (2003) Selective breeding, quantitative trait locus analysis, and gene arrays identify candidate genes for complex drug-related behaviors. *J Neurosci* 23, 4491–4498
 54. Uhl GR, Elmer G, Labuda M, Pickens RW (1995) Genetic influences in drug abuse. In *Psychopharmacology: The Fourth Generation of Progress*, Bloom FE, Kupfer F (eds.) (New York: Raven Press), pp 1793–1806
 55. Vetulani J (2001) Drug addiction. Part II. Neurobiology of addiction. *Pol J Pharmacol* 53, 303–317
 56. Wayne ML, McIntyre LM (2002) Combining mapping and arraying: An approach to candidate gene identification. *Proc Natl Acad Sci USA* 99, 14903–14906
 57. Wolf ME, Mangiavacchi S, Sun X (2003) Mechanisms by which dopamine receptors may influence synaptic plasticity. *Ann NY Acad Sci* 1003, 241–249