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Genome-wide association for methamphetamine sensitivity in an advanced intercross mouse line

C. C. Parker[†], R. Cheng[†], G. Sokoloff[†] and A. A. Palmer[*,†,‡]

[†]Department of Human Genetics, and [‡]Department of Psychiatry and Behavioral Neuroscience, The University of Chicago, IL 60637, USA
**Corresponding author: A. A. Palmer, PhD, Department of Human Genetics, University of Chicago, 920 E 58th Street, CLSC-507D, Chicago, IL 60637, USA. E-mail: aap@uchicago.edu*

Sensitivity to the locomotor stimulant effects of methamphetamine (MA) is a heritable trait that utilizes neurocircuitry also associated with the rewarding effects of drugs. We used the power of a C57BL/6J × DBA/2J F_2 intercross ($n = 676$) and the precision of a C57BL/6J × DBA/2J F_8 advanced intercross line (Aap: B6, D2–G8; or F_8 AIL; $n = 552$) to identify and narrow quantitative trait loci (QTLs) associated with sensitivity to the locomotor stimulant effects of MA. We used the program QTLREL to simultaneously map QTL in the F_2 and F_8 AIL mice. We identified six genome-wide significant QTLs associated with locomotor activity at baseline and seven genome-wide significant QTLs associated with MA-induced locomotor activation. The average per cent decrease in QTL width between the F_2 and the integrated analysis was 65%. Additionally, these QTLs showed a distinct temporal specificity within each session that allowed us to further refine their locations, and identify one QTL with a 1.8-LOD support interval of 1.47 Mb. Next, we utilized publicly available bioinformatics resources to exploit strain-specific sequence data and strain- and region-specific expression data to identify candidate genes. These results illustrate the power of AILs in conjunction with sequence and gene expression data to investigate the genetic underpinnings of behavioral and other traits.

Keywords: activity, addiction, amphetamine, drug abuse, genetic, GWAS, psychostimulant, quantitative trait loci

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Among humans, there is dramatic individual variability in both the positive and negative subjective effects of numerous drugs, including stimulants (Hart *et al*. in press). This variability is known to be partially genetic in origin (Crabbe *et al*. 1983; Nurnberger *et al*. 1982), and has been associated with subsequent development of drug use disorders (Fergusson *et al*. 2003; Haertzen *et al*. 1983; King *et al*. 2011; Schuckit & Smith 2011).

Mouse models are complementary to human genetic studies and offer unique advantages and opportunities (Parker & Palmer 2011). Numerous classes of drugs increase locomotor activity in mice and this response is mediated through the same neurocircuitry that is implicated in drug reward in animals and drug-induced euphoria in humans (Di Chiara & Imperato 1988; Koshikawa *et al*. 1989; Wise & Bozarth 1987). Therefore, much attention has been focused on examining genetic factors that influence the locomotor response to various drugs, including methamphetamine (MA) (Bryant *et al*. 2009a,b; Downing *et al*. 2006; Grisel *et al*. 1997; Kamens *et al*. 2005; Palmer *et al*. 2005). In mice, differences in the sensitivity to the locomotor activating effects of MA are heritable (Phillips *et al*. 2008), and predictive of later drug self-administration (Kamens *et al*. 2005).

Genetic mapping studies in mice have traditionally used recombinant inbred (RI) lines, backcrosses (BCs), F_2 intercrosses (F_2), consomic and congenic mice to identify quantitative trait loci (QTLs) for traits such as sensitivity to the locomotor stimulating effects of MA. Due to a lack of recombination, these techniques are only able to identify large genomic regions and are therefore suboptimal for identifying the genes that underlie QTLs (Flint 2011; Parker & Palmer 2011; Peters *et al*. 2007). We have recently begun to address this limitation by using populations with greater numbers of accumulated recombinations such as advanced intercross lines (AILs; Cheng *et al*. 2010; Lionikas *et al*. 2010; Parker *et al*. 2011; Samocha *et al*. 2010). Advanced intercross lines are created by successive generations of pseudo-random mating after the F_2 generation. Each additional generation leads to the accumulation of new recombinations, which allows for more precise mapping due to a breakdown in linkage disequilibrium. Because AILs are derived from two inbred founders, they maintain the simplicity of more traditional crosses, possess no rare alleles and every marker that differs between the parental strains is perfectly informative in terms of identifying which inbred strain the allele is inherited from.

Here, we created an F_2 intercross and an F_8 AIL derived from C57BL/6J (B6) × DBA/2J (D2) mice (Aap:B6,D2–G8, hereafter referred to as the F_8 AIL). We chose B6 and D2 inbred mice as our progenitor strains in order to take advantage of the vast amount of bioinformatic data that already exists for these specific strains, including genomic sequence and expression QTL (eQTL) data. By combining genome-wide association (GWAS) with complementary bioinformatic resources available for B6 and D2 mice, we utilized sequence data to identify single nucleotide

polymorphisms (SNPs) that may alter proteins directly, and eQTL data to identify putatively causal expression polymorphisms.

Materials and methods

Animals and housing

All procedures were approved by the University of Chicago Institutional Animal Care and Use Committee in accordance with National Institute of Health guidelines for the care and use of laboratory animals. Inbred female B6 and male D2 mice were obtained from Jackson Labs (Bar Harbor, ME, USA) and mated to produce the B6 × D2 F_1 mice. Thereafter, mice were pseudo-randomly bred to minimize relatedness and avoid brother–sister mating. The subsequent F_2 intercross (319 males and 357 females) was created from 60 different breeding pairs and the F_8 AIL (276 males and 276 females) was created from 106 different breeder pairs. The average number of breeder pairs across all seven generations was 58. Colony rooms were maintained on a 12:12-h light–dark cycle (lights on at 0630 h) in same-sex groups of two to five mice with standard laboratory chow and water available *ad libitum*. Mice were approximately 2 months of age at the start of testing (F_2 mean age = 76 days, SD = 7.3, range = 60–92; F_8 AIL mean age = 61.2 days, SD = 5.8, range = 46–93).

Activity chambers

Locomotor activity was measured using automated Versamax activity chambers (AccuScan, Colombus, OH, USA) as described previously (Bryant *et al*. 2009a). Briefly, each chamber consisted of a clear acrylic arena (40 × 40 × 30 cm) placed inside a frame containing evenly spaced infrared photobeams from the front to the back and from the left to the right of the arena. Beam breaks were recorded on a computer and converted into distance traveled (cm). Each activity chamber was encased within a sound attenuating polyvinyl chloride/lexan environmental chamber (AccuScan). In each chamber, overhead lighting provided dim illumination (~80 lx) and a fan provided both ventilation and masking of background noise.

MA-induced locomotor activity

Testing was conducted over three consecutive days during the light phase, between 0800 and 1700 h, as described previously (Bryant *et al*. 2009a). Mice were transported from the adjacent vivarium and allowed to habituate to the procedure room for 30 min in their home cages. On the first and second days of testing, mice were removed from their home cages, weighed and placed in individual holding cages filled with clean bedding. Mice then received an intraperitoneal (i.p.) injection of physiological saline and were then immediately placed in individual activity chambers where locomotor activity was recorded for 30 min. On the third day of testing, mice received an i.p. injection of 2 mg/kg MA and were then immediately placed in the activity chambers to measure locomotor activity for 30 min. Methamphetamine response was defined as the total distance traveled on day three during the 30-min test beginning immediately after drug administration. All systemic injections were administered in a volume of 0.01 ml/g body weight. On all the three days, mice were returned to their home cages immediately after the 30-min test. Activity chambers were cleaned with 10% isopropanol between tests. Mice were returned to the vivarium at the end of each day.

Data analysis

First, independent samples *t*-tests were calculated to measure generational effects (F_2 vs. F_8 AIL) on total distance traveled for each day. Next, a one-way analysis of variance (ANOVA) was used to determine the effects of day on distance traveled in the F_2 and in the F_8 AIL mice. Finally, two-way ANOVAs were performed to assess the influence of sex and time on distance traveled in the F_2 and in the F_8 AIL mice on each day. Analyses were conducted in Microsoft Excel 2010 and PASW Statistics 18 (SPSS Inc., Chicago, IL, USA).

Genotyping

DNA from the F_2 generation was extracted and genotyped by KBiosciences (Hoddesdon, Hertfordshire, UK) using KASPar, a fluorescence-based PCR assay. Markers consisted of 164 evenly spaced, informative SNPs selected from Petkov *et al*. (2004). In the F_8 AIL, DNA was extracted using a salting-out protocol and genotyped using the Illumina Mouse Medium Density Linkage Panel (Illumina, San Diego, CA, USA) at the Genomics Core Facility at Northwestern University (http://web.cgm.northwestern.edu/cgm/Core-Facilities/Genomics-Core). The SNP panel consists of 1449 loci, 1060 of which are polymorphic between B6 and D2 mice.

QTL mapping

Genome-wide association analysis was performed in the combined population of the F_2 intercross and the F_8 AIL using the R package QTLRel V.0.2.9 (http://cran.r-project.org/web/packages/QTLRel/index.html). This software accounted for the complex relationships (e.g. sibling, half-sibling and cousins) among the F_8 AIL mice by using a mixed model as described previously (Cheng *et al*. 2010). For each analysis, $P < 0.05$ significance thresholds were estimated using 1000 permutations. Sex was included as an interactive covariate.

Bioinformatic analyses

The GeneNetwork mapping module (www.genenetwork.org; Chesler *et al*. 2004; Wang *et al*. 2003) was used to identify eQTLs in striatum and whole-brain mRNA from untreated B6 × D2 F_2 mice [accessed on 15 September 2011; OHSU/VA B6D2F2 Striatum M430v2 (September 2005) RMA; OHSU/VA B6D2F2 Brain mRNA M430 (August 2005) RMA; Hitzemann *et al*. 2004; Hofstetter *et al*. 2008], as well as nucleus accumbens and prefrontal cortex from saline-injected BXD RI mice [accessed on 15 September 2011; VCU BXD NA Sal M430 2.0 (October 2007) RMA; VCU BXD PFC Sal M430 2.0 (December 2006) RMA; Wolen, Putnam & Miles unpublished data) that co-mapped with our behavioral QTLs. We focused on these regions because of their well-known role in drug-induced locomotor activity and reward (Di Chiara & Imperato 1988; Swerdlow *et al*. 1986); however, examining gene expression in additional brain areas would also be informative. Next, in order to narrow the list of candidate genes within the QTL intervals, we used high density sequence data provided by the Wellcome Trust Sanger Institute (accessed on 19 September 2011; http://www.sanger.ac.uk/cgi-bin/modelorgs/mousegenomes/snps.pl; Keane *et al*. 2011; Yalcin *et al*. 2011) to compare genomic regions between B6 and D2 mice. These strains were sequenced to an average of 25× coverage on the Illumina GAII platform (Illumina) with a mixture of 54p, 76 and 108 bp paired reads. We used this data to search for genes within the QTL intervals that possessed 'consequential' polymorphisms between B6 and D2 mice (such as nonsynonymous coding SNPs, stop-gain SNPs, stop-loss SNPs, SNPs resulting in frameshifts and SNPs located in essential splice sites).

Results

Phenotypic analysis

Figure 1 displays the distance traveled across all three sessions in male and female F_2 and F_8 AIL mice. For both F_2 and F_8 AIL mice, there was a significant effect of treatment on distance traveled over 30 min (F_2: $F_{2,2006} = 2859.02$, $P < 0.0001$; F_8: $F_{2,1649} = 1499.28$, $P < 0.0001$). Scheffe's *post hoc* analysis showed that all sessions were significantly different from one another in both the F_2 and F_8 AIL mice

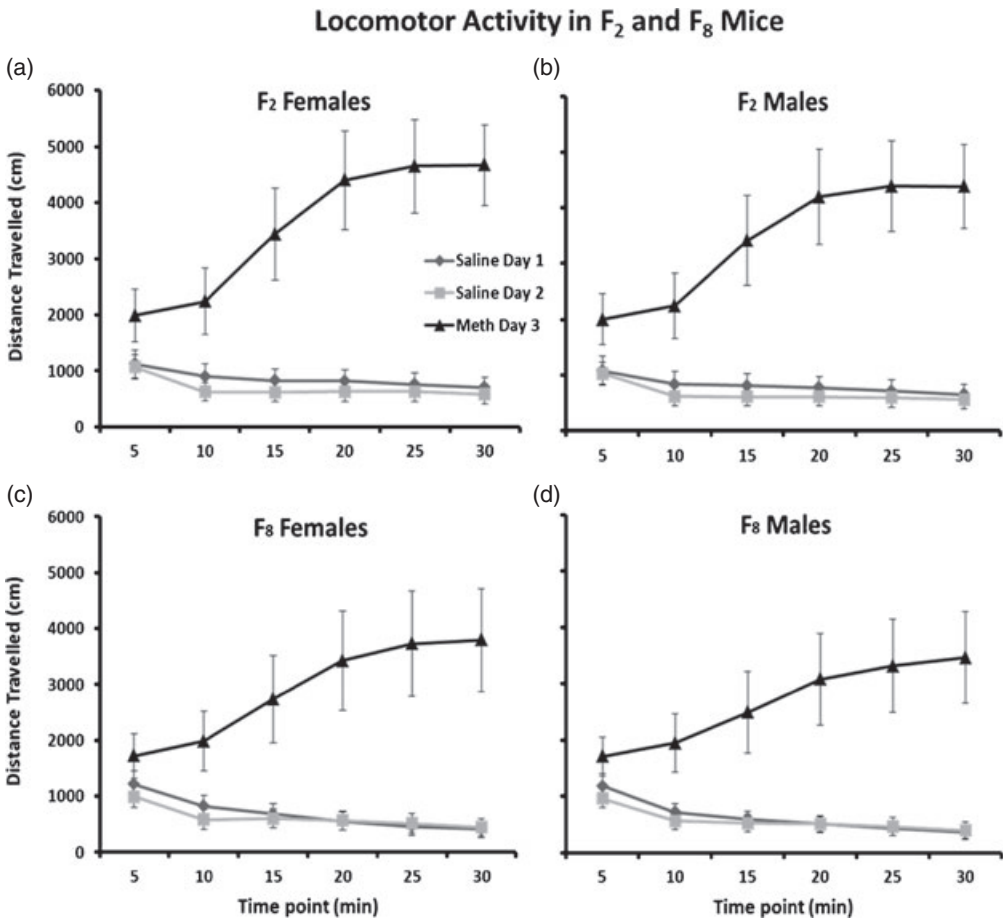

Figure 1: Locomotor activity in F2 and F8 mice. Distance traveled on saline day1 (♦), saline day 2 (■) and meth day 3 (▲) in the F2 female (a) and male (b) mice; as well as in F8 female (c) and male (d) mice. For both the F_2 and F_8 mice, distance traveled on day 1 was slightly greater than on day 2 ($P < 0.005$) and distance traveled on day 3 was dramatically greater than on either day 1 or 2 ($P < 0.0001$). The F_2 and F_8 generations also differed from each other on day 1 ($P < 0.0001$) and day 3 ($P = 0.014$), but not on day 2. Additionally, F_2 and F_8 AIL mice displayed sex and time differences for all the three sessions. In the F_2 mice, a two-way ANOVA reported varying effects of sex and time-point on distance traveled for saline day 1 (sex: $F_{1,676} = 56.2$, $P = 0.0007$; time: $F_{5,676} = 375.3$, $P < 0.0001$), saline day 2 (sex: $F_{1,676} = 18.6$, $P = 0.008$; time: $F_{5,676} = 674.1$, $P < 0.0001$) and MA day 3 (sex: $F_{1,676} = 5.0$, $P = 0.08$; time: $F_{5,676} = 273.2$, $P < 0.0001$). A two-way ANOVA also reported significant effects of sex and time-point on distance traveled in the F_8 AIL mice for saline day 1 (sex: $F_{2,552} = 17.2$, $P = 0.009$; time: $F_{5,552} = 336.4$, $P < 0.0001$), saline day 2 (sex: $F_{2,552} = 30.9$, $P = 0.003$; time: $F_{5,552} = 335.9$, $P < 0.0001$) and MA day 3 (sex: $F_{2,552} = 11.4$, $P = 0.02$; time: $F_{5,552} = 95.7$, $P < 0.0001$).

($P < 0.005$). The F_2 and F_8 AIL mice also differed from each other on saline day 1 ($F_{1,1219} = 169.76$, $P < 0.0001$) and MA day 3 ($F_{1,1218} = 6.04$, $P = 0.014$), but not on saline day 2 (Fig. 1). Additionally, F_2 and F_8 AIL mice displayed sex and time differences for all three sessions. In the F_2 mice, a two-way ANOVA reported varying effects of sex and time-point on distance traveled for saline day 1 (sex: $F_{1,676} = 56.2$, $P = 0.0007$; time: $F_{5,676} = 375.3$, $P < 0.0001$), saline day 2 (sex: $F_{1,676} = 18.6$, $P = 0.008$; time: $F_{5,676} = 674.1$, $P < 0.0001$) and MA day 3 (sex: $F_{1,676} = 5.0$, $P = 0.08$; time: $F_{5,676} = 273.2$, $P < 0.0001$). A two-way ANOVA also reported significant effects of sex and time-point on distance traveled in the F_8 AIL mice for saline day 1 (sex: $F_{2,552} = 17.2$, $P = 0.009$; time: $F_{5,552} = 336.4$, $P < 0.0001$), saline day 2 (sex: $F_{2,552} = 30.9$, $P = 0.003$; time: $F_{5,552} = 335.9$, $P < 0.0001$) and MA day 3 (sex: $F_{2,552} = 11.4$, $P = 0.02$; time: $F_{5,552} = 95.7$, $P < 0.0001$). While the disparity between F_2 and F_8 AIL populations may be due to the segregation of alleles associated with high locomotor activity during the creation of the F_8 AIL mice, we suspect it is more likely a result of handling effects of different testers across the 2-year period between the F_2 and F_8 generations. The F_2 mice were an average of about 15 days older than the F_8 AIL mice when tested, we have not seen much impact of similar differences in age on these behaviors in the past. As a result of these differences, both the F_2 and the F_8 AIL data were converted to *z*-scores prior to genome-wide analysis.

QTL mapping

We performed genome-wide analysis on the integrated F_2 and F_8 AIL populations for distance traveled during the first 15 min (0–15 min) as well as distance traveled from 15 to 30 min, and distance traveled during the entire 30-min testing period (0–30 min) across all treatments (Fig. 2). However, we focused our subsequent analyses on the 0–15- and 0–30-min time periods given the fact that previous studies in our laboratory have identified QTLs for MA-induced locomotor activity specific to these time-points (Bryant *et al*. 2009a,b; Cheng *et al*. 2010; Palmer *et al*. 2005; Sokoloff *et al*. 2011). Some of the QTLs we identified were present only in the first 15 min, whereas others were only present for the total 30-min time period. For example, we identified the same six QTLs associated with distance traveled on day 1 from 0 to 15 min and 0 to 30 min (on chromosomes 1, 4, 6, 9, 11 and X; Fig. 2a,c). For day 2, we identified five QTLs associated with distance traveled from 0 to 15 min (on chromosomes 4, 5, 11, 15 and X; Fig. 2d). All of these QTLs were also significant for the 0–30-min time period, in addition to a

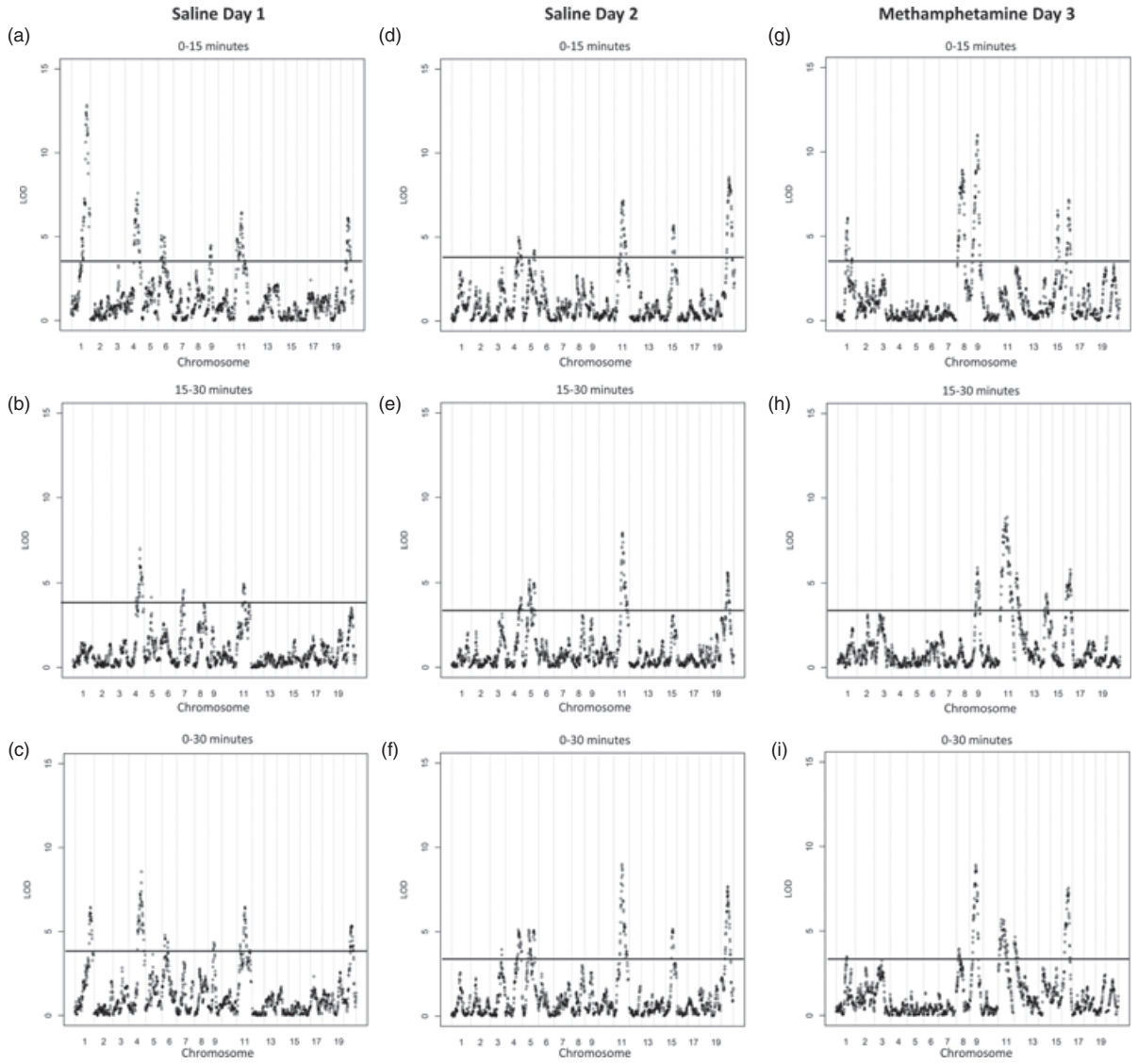

Figure 2: Integrated genome-wide results for distance traveled. Integrated genome-wide results for distance traveled on (a) saline day 1 for 0–15 min ($P < 0.05$ significance threshold LOD = 3.93), (b) saline day 1 for 15–30 min ($P < 0.05$ significance threshold LOD = 4.00), (c) saline day 1 for 0–30 min ($P < 0.05$ significance threshold LOD = 4.03), (d) saline day 2 for 0–15 min ($P < 0.05$ significance threshold LOD = 4.04), (e) saline day 2 for 15–30 min ($P < 0.05$ significance threshold LOD = 3.92), (f) saline day 2 for 0–30 min ($P < 0.05$ significance threshold LOD = 3.87), (g) meth day 3 for 0–15 min ($P < 0.05$ significance threshold LOD = 3.98), (h) meth day 3 for 15–30 min ($P < 0.05$ significance threshold LOD = 4.01) and (i) meth day 3 for 0–30 min ($P < 0.05$ significance threshold LOD = 3.84).

QTL on chromosome 3 (Fig. 2f). However, on day 3, we identified five QTLs associated with distance traveled from 0 to 15 min (on chromosomes 1, 8, 9, 15 and 16; Fig. 2g). Only four of these QTLs were significant for distanced traveled from 0 to 30 min (on chromosomes 1, 8, 9 and 16), and three additional QTLs were identified (on chromosomes 3, 11 and 12; Fig. 2i). Using 1000 permutations, significance thresholds were determined to range from 3.84 to 4.04 LOD. We also mapped QTLs for the difference between activity on day 3 and day 2, which we and others have sometimes used as a way to distinguish between differences that are specific to drug treatment vs. those that are secondary to differences in basal locomotor activity and occur even in the absence of drug exposure. The QTLs identified were similar to those of day 3 (data not shown). We were most interested in locomotor activity in a novel environment and MA-induced locomotor activity; thus, the remainder of our analyses focused on the day 1 and day 3 results. Because the use of F_2 and F_8 AIL mice is most interpretable when there is concurrence between the QTL locations in both generations, we further constrained our analyses to QTLs that were evident (although not necessarily significant) in both the F_2 and the F_8 cohorts. This left three QTLs for activity on day 1 (in a novel environment: *Act1*, *Act4* and *ActX*) and three QTLs for activity on day 3 (MA response: *Meth9*, *Meth15* and *Meth16*).

Time-dependent nature of QTLs

Next, we split the 30-min testing period into six consecutive 5-min bins in order to determine if a particular time-point was driving each QTL. In both *Act1* (Fig. 3a,b) and *ActX* (Fig. 3e,f), the 0–5-min time bin was largely responsible for the QTLs. In contrast, *Act4* (Fig. 3c,d) reached its peak during the 5–10-min bin. For MA response, *Meth15* (Fig. 4c,d) peaked during the 0–5-min bin and *Meth9* (Fig. 4a,b) and *Meth16* (Fig. 4e,f) both showed the strongest effect at the 10–15-min bin. The 1.8-LOD intervals for these six QTLs ranged from 1.5 to 50.0 Mb, with a median width of 15.6 Mb. Table 1 displays the LOD scores, peak SNP, Mb location and width for each QTL interval for the peak time-point.

Bioinformatic analyses

Numerous eQTLs across multiple brain regions were identified that co-mapped with these six behavioral QTLs (Table S1). The majority of these eQTLs were *cis*-eQTLs, meaning that the eQTL was coincident with the location of the gene. Additionally, we confirmed the results of previous studies in our laboratory with the identification of casein kinase 1, epsilon (*Csnk1e*; Bryant *et al*. 2009a,b; Palmer *et al*. 2005) and identified other genes that had been implicated in amphetamine sensitivity (epha receptor A3, *Epha3*, Sieber *et al*. 2004; epha receptor A6, *Epha6*, Sieber *et al*. 2004; galanin receptor 3, *Galr3,* Kuteeva *et al*. 2005). We then examined our 1.8-LOD support interval for the presence of SNPs that had the potential to directly alter proteins (i.e. nonsynonymous coding, stop-gain, stop-loss, frameshift and splice sites; Table S2).

Discussion

We performed genome-wide mapping of QTL affecting locomotor activity in a novel environment, as well as QTL associated with MA-induced locomotor activity in an F_2 and F_8 AIL population of mice. We identified a total of six QTLs (on chromosomes 1, 4, 6, 9, 11 and X) associated with locomotor activity in a novel environment and eight QTLs (on chromosomes 1, 3, 8, 9, 11, 12, 15 and 16) associated with MA-induced locomotor activation. Four of the QTLs associated with MA sensitivity (*Meth9, Meth11, Meth12* and *Meth15*) replicated the results of previous studies in STSL derived from B6 × D2 F_2 mice (chromosomes 9, 11, 12 and 15; Palmer *et al*. 2005), and one (*Meth11*) overlapped with a QTL region identified in a LG/J × SM/J F_{34} AIL (chromosome 11; Cheng *et al*. 2010). In addition, five agreed with results from a B6 × AJ consomic panel (chromosomes 8, 9, 11, 12 and 16; Bryant *et al*. 2009b), and one agreed with results from BXD RI strains (chromosome 15; Grisel *et al*. 1997). Importantly, our AIL provided greater resolution and narrower support intervals as compared to the STSL, CSS and the BXD RI panel. Additionally, some of the QTLs we identified for MA sensitivity (*Meth1, Meth11* and *Meth12*) also overlapped with QTLs underlying ethanol (chromosomes 1 and 11; Bennett *et al*. 2006; Downing *et al*. 2006), opioid (chromosome 11; Bryant *et al*. 2009b) and etomidate (chromosome 12; Downing *et al*. 2003) sensitivity, suggesting that the genes underlying these QTL regions may not be drug specific.

Interestingly, one of the QTLs we identified, which was associated with locomotor activity in a novel environment (*Act1*, 1.8-LOD interval = 172.36–173.83 Mb), mapped to the proximal region of known QTL hotspot called *Qrr1*. *Qrr1* extends from 172.5 to 177.5 Mb on chromosome 1, and is highly enriched in QTL that controls neural and behavioral phenotypes including basal locomotor behavior, escape latency, emotionality, ethanol-induced locomotor activity and responses to caffeine, pentobarbital and haloperidol (Mozhui *et al*. 2008). *Qrr1* contains 164 known genes and is thought to contain a highly complex gene expression regulatory interval composed of multiple loci modulating the expression of functionally similar sets of genes. In addition to *Act1*, a chromosome 1 QTL associated with MA sensitivity (*Meth1*, 1.8-LOD interval = 111.83–184.52 Mb) also maps to *Qrr1*. Because *Qrr1* consists of multiple regions (each associated with the expression of distinct subsets of genes and QTLs), it is possible that *Act1* and *Meth1* represent either the same or distinct loci.

Traditionally, F_2 intercrosses are used to identify QTLs underlying phenotypic variation, and fine-mapping is carried out as second step, often in congenic strains. Efforts at subsequent dissection and gene identification are often impeded by the existence of multiple causative loci of small effect located in the same chromosomal region (Mott *et al*. 2000; Shao *et al*. 2010). An AIL is an improvement over these traditional methods because of the additional recombinations it accumulates over successive generations. The accumulated recombinations allow identification and fine-mapping to be merged into a single step, which can

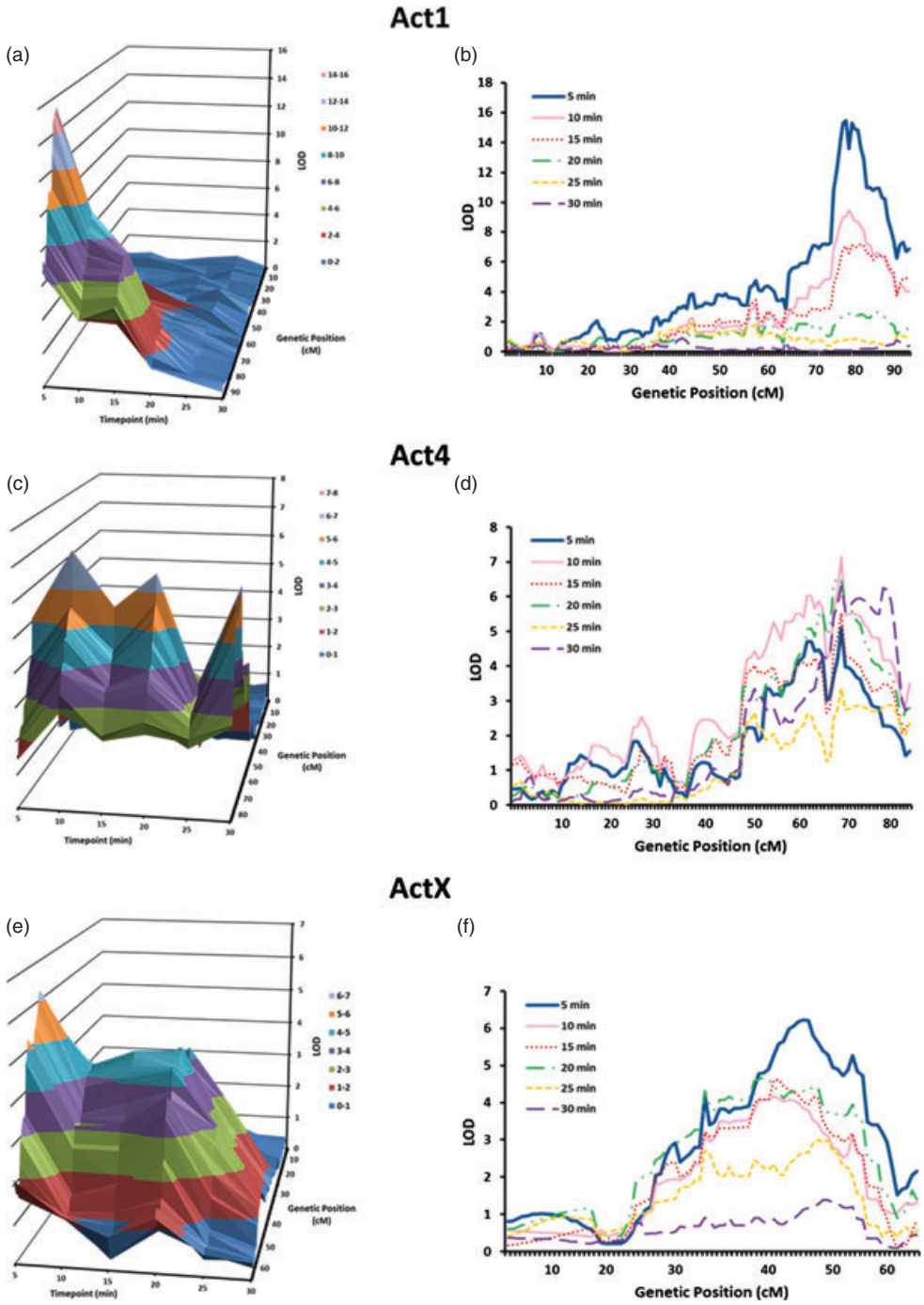

Figure 3: Time-dependent nature of baseline locomotor activity QTLs. (a) Three-dimensional plot of the integrated QTL results for the *Act1* QTL across time-point (depth axis), genetic distance (horizontal axis) and LOD score (vertical axis). (b) Plot of six LOD curves, separated by time-point (0–5, 5–10, 10–15, 15–20, 20–25 and 25–30) for the *Act1* QTL. (c) Three-dimensional plot of the integrated QTL results for the *Act4* QTL across time-point (depth axis), genetic distance (horizontal axis) and LOD score (vertical axis). (d) Plot of six LOD curves, separated by time-point (0–5, 5–10, 10–15, 15–20, 20–25 and 25–30) for the *Act4* QTL. (e) Three-dimensional plot of the integrated QTL results for the *ActX* QTL across time-point (depth axis), genetic distance (horizontal axis) and LOD score (vertical axis). (f) Plot of six LOD curves, separated by time-point (0–5, 5–10, 10–15, 15–20, 20–25 and 25–30) for the *ActX* QTL.

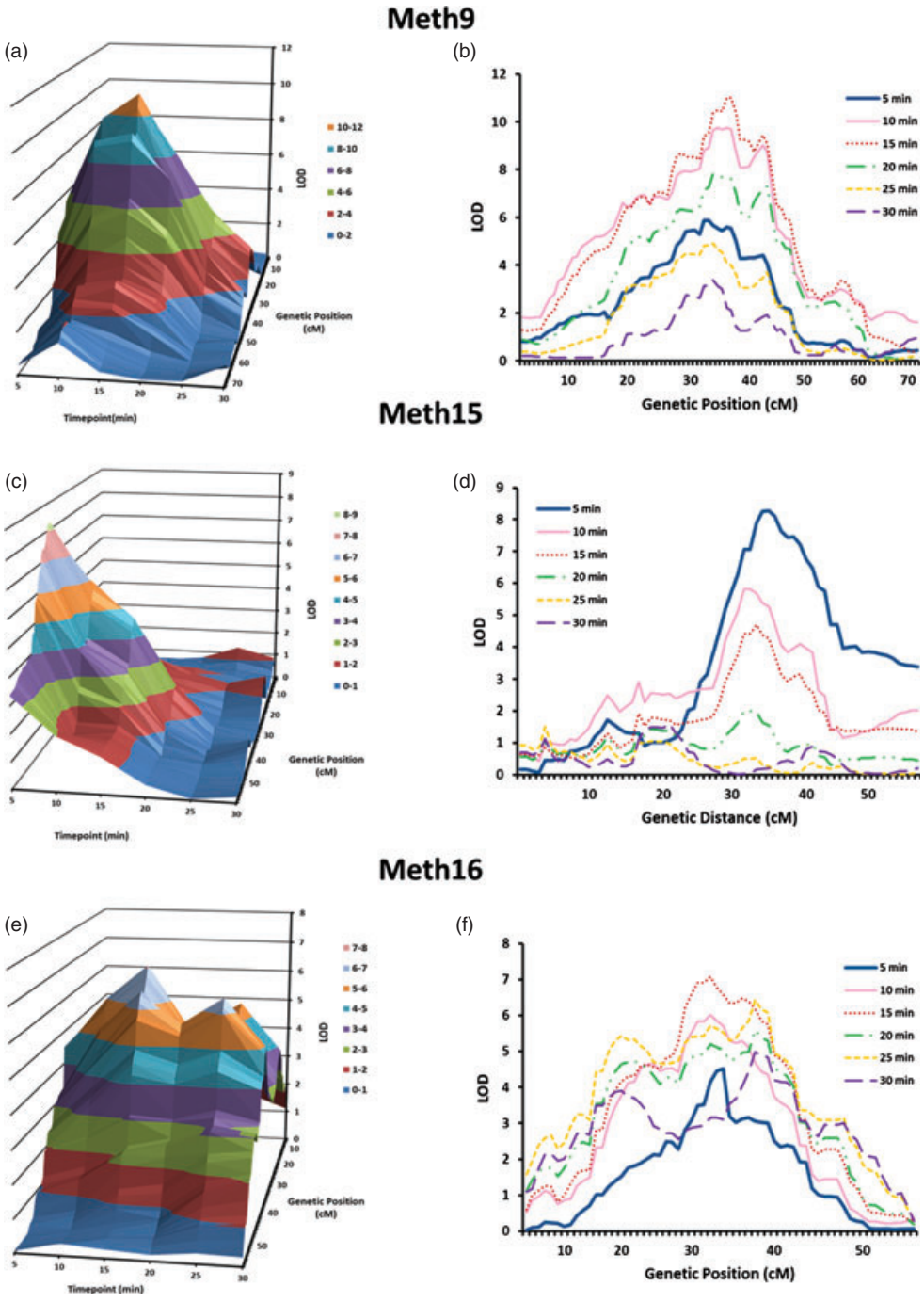

Figure 4: Time-dependent nature of MA-induced locomotor activity QTLs. (a) Three-dimensional plot of the integrated QTL results for the *Meth9* QTL across time-point (depth axis), genetic distance (horizontal axis) and LOD score (vertical axis). (b) Plot of six LOD curves, separated by time-point (0–5, 5–10, 10–15, 15–20, 20–25 and 25–30) for the *Meth9* QTL. (c) Three-dimensional plot of the integrated QTL results for the *Meth15* QTL across time-point (depth axis), genetic distance (horizontal axis) and LOD score (vertical axis). (d) Plot of six LOD curves, separated by time-point (0–5, 5–10, 10–15, 15–20, 20–25 and 25–30) for the *Meth15* QTL. (e) Three-dimensional plot of the integrated QTL results for the *Meth16* QTL across time-point (depth axis), genetic distance (horizontal axis) and LOD score (vertical axis). (f) Plot of six LOD curves, separated by time-point (0–5, 5–10, 10–15, 15–20, 20–25 and 25–30) for the *Meth16* QTL.

Table 1: QTLs selected for fine-mapping

	Chromosome	Peak time-point (min)	LOD	Peak SNP	1.8-LOD start Mb	1.8-LOD end Mb	Peak Mb position	Width (Mb)	Genes with coding SNPs
Saline day 1: total distance traveled QTLs	1	0–5	15.4	rs8245216	172.357	173.832	173.174	1.475	20
	4	5–10	7.1	rs13478002	133.275	141.023	136.412	7.749	64
	X	0–5	6.2	gnfX.086.039	81.842	131.953	99.159	50.111	12
Meth day 3: total distance traveled QTLs	9	10–15	10.7	rs3655717	57.888	72.491	68.204	14.603	24
	15	0–5	9.3	rs13482642	68.959	85.617	75.523	16.659	52
	16	10–15	7.6	rs4186744	42.090	70.632	54.759	28.542	42

Peak time-point, LOD at peak SNP, 1.8-LOD support interval and the width of the support interval are shown.

often discriminate between loci that are due to single vs. multiple alleles (Parker *et al*. 2011). The integration of the F_2 and F_8 AIL population combines the detection power of the F_2 with the precision of the F_8 AIL. In the integrated analysis, we reduced the 1.8-LOD support intervals by approximately 65% over the F_2 analysis alone (Fig. S1). In several instances, significant QTLs identified in the F_2 population were not supported by the F_8 AIL data. These regions are difficult to interpret, as they may be caused by either a false positive result in the F_2 mice (this is very unlikely when the LOD score vastly exceeds the threshold for significance) or a false negative in the F_8 AIL mice, which has less power than a similarly sized F_2 (resulting from the reduced association between genotypes at markers). Alternatively, lack of a significant peak in the F_8 AIL may be due to the presence of multiple loci of small effect located in the same chromosomal region, which segregate as a unit in the F_2 but segregate independently in the F_8 AIL. Because of the ambiguity of QTLs not replicated in the F_8 AIL, we chose to focus our fine-mapping efforts on regions where the F_2 and F_8 AIL QTLs were in agreement.

Most studies of drug response traits identify QTLs based on summary measures that collapse out the within-subjects factor time. This approach implies that the QTLs are expected to have a uniform effect over the testing period. To better determine if a QTL was driven by a particular time-point, we split the 30-min testing period into six bins of 5-min each. This indicated a temporal nature to our QTLs, although a formal test examining the QTL-by-time interaction would be necessary to definitively state that differences across time bins are statistically significant. We plotted these results in three dimensions (time × position × LOD score). Quantitative trait loci for initial locomotor activity in a novel environment as well as QTLs for MA-induced locomotor activity displayed peak LOD scores in the first half of the testing period. By considering the time–course in greater detail, we were able to observe that in some situations, the peak LOD scores were primarily driven by a particular time-point, as was the case with *Act1*, *ActX*, *Meth9* and *Meth15*. In other instances, the LOD scores for the QTLs were consistently high across all time-points; this was the case for *Act4* and *Meth16*.

To further narrow our QTLs and to attempt to identify the underlying genes, we used a series of bioinformatic approaches. First, we identified eQTLs that co-mapped with our QTLs. eQTLs are believed to underlie many QTLs for more complex traits (Li & Deng 2010; Nicolae *et al*. 2010). We used an existing database (www.GeneNetwork.org) of eQTLs from whole brain and striatum of untreated B6 × D2 F_2 mice and from the nucleus accumbens and prefrontal cortex of saline-injected BXD RI mice. In many cases, this identified a smaller number of genes that co-mapped within the 1.8-LOD intervals of our QTLs (Table S1). Some of the genes we identified have been implicated in other studies examining the stimulant properties of drugs of abuse, and may be promising candidates for follow-up studies. In the case of *Meth15* we replicated our previous finding regarding the gene *Csnk1e*, which has been shown to influence the locomotor stimulant response to MA (Bryant *et al*. 2009a; Palmer *et al*. 2005). Others have found associations between *Csnk1e* and MA dependence as well as heroin addiction (Levran *et al*. 2008; Veenstra-VanderWeele *et al*. 2006). In addition, expression of the gene for the galanin 3 receptor (*Galr3*) also mapped to the *Meth15* QTL. Transgenic mice overexpressing galanin were reported to have attenuated amphetamine-induced locomotor activity, as compared to controls (Kuteeva *et al*. 2005). We also found that the expression of *Epha3* and *Epha5* mapped to the *Meth16* QTL. Sieber *et al*. (2004) investigated the functional role of Epha signaling by overexpressing a broad-range Epha receptor antagonist in the central nervous system of transgenic mice. Transgenic mice displayed a 40–50% reduction of dopaminergic neurons in the striatum, as well as insensitivity to the locomotor activating effects of amphetamine. While co-mapping of a QTL and an eQTL does not constitute proof that the latter causes the former, it does suggests a clear and testable hypothesis – the candidate gene can be directly manipulated using a variety of molecular or pharmacological approaches (Bryant *et al*. 2009a). In addition, gene expression differences in other brain regions, across multiple developmental time-points, and in a variety of cell types may further aid in the identification of genes underlying these QTLs. Finally, we identified between 12 and 64 genes with 'consequential' SNPs within each of our constrained QTL intervals (Table S2). A subset of these resulted in premature stop codons, which are especially likely to alter the function of the gene. Follow-up studies will determine if any of these SNPs result in non-conservative amino acid changes, or if they occur in evolutionarily conserved amino acids, as these SNPs are most likely to cause phenotypic differences. Taken together, these bioinformatic approaches

allowed us to narrow both the size of the QTLs and to identify a smaller subset of genes that we believe are likely to cause these QTLs.

In conclusion, we have mapped a large number of QTLs associated with novel locomotor activity and MA sensitivity in an AIL. Some of the QTLs correspond to regions identified by other researchers, and in the majority of cases we have narrowed the confidence intervals quite significantly as compared to previous studies. While it is clear that the integrated analysis of the F_2 and F_8 AIL offers vast improvement over only using F_2 mice, it is still insufficient for obtaining single gene resolution. However, the combination of high resolution mapping with sequence and expression data offers a powerful approach and permitted identification of several candidate genes that may underlie differences in these phenotypes. In summary, AILs allow GWAS to be performed in a situation where all alleles are common, and where uniform environmental conditions can be maintained, which limits the interactions between genes and environment. These advantages allowed us to map QTL with a modest sample size and identify small regions that warrant further molecular evaluation.

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Supporting Information

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

Figure S1: Narrowing of QTL intervals in integrated analyses. (a) Integrated, F_2 and F_8 *Act1* QTL results during 0–5 min. (b) Integrated, F_2 and F_8 day 1 QTL results for *Act4* QTL during 5–10 min. (c) Integrated, F_2 and F_8 *ActX* QTL results during 0–5 min. (d) Integrated, F_2 and F_8 *Meth9* QTL results during 10–15 min. (e) Integrated, F_2 and F_8 *Meth15* QTL results during 0–5 min. (f) Integrated, F_2 and F_8 *Meth9* QTL results during 10–15 min.

Table S1: eQTLs for *Act1, Act4, ActX, Meth9, Meth15* and *Meth16*. Table includes gene symbol and name, as well as Mb location for each gene, mean expression, maximum LOD, and eQTL chromosome and Mb location

Table S2: Genes with nonsynonymous coding SNPs for *Act1, Act4, ActX, Meth9, Meth15* and *Meth16*. Gene symbol and the number of nonsynonymous SNPs are shown. SNPs resulting in a frameshift mutation, stop-gain, stop-loss and SNPs located in essential splice sites are indicated with an asterisk

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