

# Sensitivity to the locomotor-stimulant effects of ethanol and allopregnanolone: a quantitative trait locus study of common genetic influence

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Previous studies have suggested that common genetic mechanisms influence sensitivity to the locomotor-stimulant effects of ethanol and allopregnanolone. We conducted two quantitative trait locus (QTL) studies to identify chromosomal regions that harbor genes that influence locomotor response to ethanol (2 g/kg) and allopregnanolone (17 mg/kg) using F<sub>2</sub> crosses between C57BL/6J and DBA/2J mice. Because our previous data from the BXD recombinant inbred strains had indicated that chromosome 2 contained QTLs for sensitivity to the locomotor-stimulant effects of both ethanol and allopregnanolone, we also tested reciprocal chromosome 2 congenic strains for sensitivity to the locomotor-stimulant effects of both drugs. The F<sub>2</sub> analysis for ethanol sensitivity identified significant QTLs on chromosomes 1 and 2 and suggestive QTLs on chromosomes 5 and 9. The analysis of the allopregnanolone F<sub>2</sub> study identified suggestive QTLs on chromosomes 3, 5 and 12. Suggestive evidence for a female-specific QTL on chromosome 2 was also found. The studies of congenic mouse strains indicated that both the congenic strains captured one or more QTLs for sensitivity to the locomotor-stimulant effects of both ethanol (2 g/kg) and allopregnanolone (17 mg/kg). When Fisher's method was used to combine the *P* values for the RI, F<sub>2</sub> and congenic studies of the chromosome 2 QTL, cumulative probability scores of  $9.6 \times 10^{-15}$  for ethanol and  $7.7 \times 10^{-7}$  for allopregnanolone were obtained. These results confirm the presence of QTLs for ethanol and allopregnanolone sensitivity in a common region of chromosome 2 and suggest possible pleiotropic genetic influence on sensitivity to these drugs.

Keywords: 3 $\alpha$ -Hydroxy-5 $\alpha$ -pregnan-20-one, 3 $\alpha$ ,5 $\alpha$ -tetrahydroprogesterone, alcohol, allopregnanolone, ethanol, GABA, genetic, locomotor, mouse, neurosteroid, QTL

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Initial sensitivity to certain effects of ethanol, such as its stimulant or euphoric effects or its effect on motor co-ordination, may be markers for genetic susceptibility to ethanol abuse (Heath *et al.* 2001; Holdstock *et al.* 2000; Newlin & Thomson 1999; Poikolainen 2000; Schuckit & Smith 2000, 2001). In rodents, ethanol produces an increase in locomotor behavior in a dose- and strain-dependent manner (Crabbe *et al.* 1994; Deitrich 1993; Dudek *et al.* 1991; Erwin *et al.* 1990; Phillips *et al.* 1995; Spuhler & Deitrich 1984). Sensitivity to the locomotor-stimulant effect of ethanol has been manipulated by selective breeding, and the heritability of this trait has been estimated to be as high as 0.49 using inbred strain panels and selected lines (Phillips *et al.* 1991, 1995; Shen *et al.* 1995). It has been suggested that acute sensitivity to the locomotor-stimulant effects of ethanol and other drugs may be related to reward in part, because both the responses share some common neurochemical pathways (Di Chiara & Imperato 1988; Katner & Weiss 2001). Therefore, identification of the genes that mediate initial sensitivity to ethanol may advance our understanding of the processes involved in risk for the development of alcoholism and could indicate strategies for treatment and prevention.

Previous studies in our laboratory have focused on the common genetic regulation of acute sensitivity to the effects of ethanol and allopregnanolone. We identified a positive genetic correlation between acute sensitivity to the effects of ethanol and allopregnanolone on the locomotor behavior of a panel of C57BL/6J  $\times$  DBA/2J recombinant inbred (BXD RI) strains (Palmer *et al.* 2002b). We observed a similar genetic correlation in mice selected for high or low acute locomotor (Palmer *et al.* 2002a, 2002b) or hypothermic (Palmer *et al.* 2002c) response to ethanol. On the basis of these data, we hypothesized that there would be one or more common genes that would affect sensitivity to both ethanol and allopregnanolone on locomotor behavior.

To investigate this possibility, we sought to identify regions of the genome that make quantitative contributions to this phenotype [quantitative trait loci (QTL)]. The dopaminergic system is clearly important for ethanol-induced locomotor stimulation (Carboni *et al.* 2000; Di Chiara & Imperato 1988; Palmer *et al.* 2003; Phillips & Shen 1996); however, there is substantial evidence that  $\gamma$ -aminobutyric acid (GABA) systems are also involved in this response (Boehm *et al.* 2004; Phillips & Shen 1996). Allopregnanolone is a neuroactive steroid known to modulate GABA-A receptor function. It dose-dependently induces stimulant, anxiolytic, ataxic and depressant effects – all reminiscent of ethanol intoxication (Purdy & Paul 1999). Their similar behavioral effects and the identified genetic correlations suggest regulation by common alleles. The goals of the current work were to confirm QTL locations and test the hypothesis that common genes influence sensitivity to the locomotor effects of ethanol and allopregnanolone. To accomplish this, we followed up initial QTL analyses that had been performed using BXD RI stains (Palmer *et al.* 2002b; Phillips *et al.* 1995) using an F<sub>2</sub> population. In addition, for a QTL on chromosome 2 that appeared to influence response to both ethanol and allopregnanolone, reciprocal congenic strains for this chromosomal region were studied.

## Materials and methods

### Subjects

All mice were born in the vivarium at the Portland VA Medical Center. They were group-housed 2–5 per cage with same-sex littermates when possible or rarely with non-littermates of the same sex, genotype and similar age ( $\pm 5$  days) to avoid single housing. Mice were maintained on a 12:12 h light dark cycle (lights on at 0600 h) at  $21 \pm 2$  °C, with food (Purina Laboratory Rodent Chow 5001; Purina Mills, St. Louis, MO) and water available *ad libitum*, except during behavioral testing. Purina Mouse Breeder Chow (5015) was used in lieu of standard Purina Laboratory Rodent Chow for breeder pairs. To create an F<sub>2</sub> population, we obtained C57BL/6J (B6)  $\times$  DBA2/J (D2) F<sub>1</sub> mice from The Jackson Laboratory and bred them to create F<sub>2</sub> litters (termed B6D2F2). Separate breeders were used for the ethanol and allopregnanolone studies. Congenic mice were a generous gift from Dr Robert Klein at the VA Medical Center and were created in his laboratory by backcrossing mice from B6D2F1 to B6 mice or to D2 mice. Selection of breeders for the initial and subsequent backcrosses was based on their genotypes at several markers on chromosome 2. Mice that carried D2 alleles in this region were chosen for backcrossing to B6 mice to create the B6.D2 congenic and those with B6 alleles in the region were backcrossed to D2 to create the D2.B6 congenic. We obtained the mice after the 10th generation of backcrossing and genotyped them to determine the exact size of the congenic segment. Congenic mice and the

corresponding controls were maintained by homozygous breeding. For all experiments, to obtain adequate group sizes, we tested mice in multiple batches with all doses and genotypes balanced across each batch. All of the experiments were performed in accordance with the Institutional Animal Care and Use Committee and National Institutes of Health guidelines for the care and use of laboratory animals. 3

### Drugs

Ethanol was obtained from Pharmco Products Inc. (Brookfield, CT). Allopregnanolone was synthesized by, and purchased from, Dr Robert Purdy (San Diego, CA). Allopregnanolone was suspended in a 20% (w/v) solution of 2-hydroxypropyl- $\beta$ -cyclodextrin (2HBC; H-107, Sigma Chemical Co., St. Louis, MO) using a stir bar at room temperature followed by brief sonication. Concentrations of allopregnanolone were made such that a volume of 10.0 ml/kg body weight was injected intraperitoneally (i.p.). For i.p. ethanol injections, the concentration was held constant at 20% (v/v) ethanol/saline, and the volume injected was based on the animal's body weight. Vehicle injections were normal saline or 20% 2HBC solution, as appropriate. The doses used were chosen to maintain consistency with our previous BXD RI studies (Palmer *et al.* 2002b; Phillips *et al.* 1995) and were the doses for which we found a correlation between the response to ethanol and allopregnanolone among BXD RI strains (Palmer *et al.* 2002b).

### Testing apparatus

We have previously described the locomotor activity testing apparatus and procedures (Palmer *et al.* 2002b; Phillips *et al.* 1995). Briefly, all mice were tested using a set of eight automated locomotor activity monitors (40  $\times$  40  $\times$  30 cm, Accuscan Instruments Inc., Columbus, OH) that were housed in light-proof, sound-attenuating cabinets. A fan was used to provide ventilation and to produce a constant low level of background noise. The inside of each cabinet was lit by a 15-W fluorescent light. Two arrays of eight infrared beams and detectors were arranged along the two horizontal axes just outside the testing cage at a height of 2 cm above the chamber floor. Interruption of these beams was used by the automated system to track the movement of the mice within the chambers. Total distance traveled in centimeters over the course of the test session served as the dependent variable for our studies.

### Testing procedure

Our testing procedure has been described previously (Palmer *et al.* 2002b; Phillips *et al.* 1995). On days 1 and 2, all mice were weighed, injected with vehicle and immediately placed in the center of an activity monitor to permit habituation to the testing apparatus and procedures (day 1) and to obtain baseline activity data (day 2). On the third test day, mice from

the F<sub>2</sub> studies were injected with either ethanol (2 g/kg) or allopregnanolone (17 mg/kg), whereas mice from the congenic studies were injected with either ethanol (2 g/kg) or allopregnanolone (vehicle, 10 or 17 mg/kg) on the third day. The length of each test session was 15 min for the ethanol studies and 30 min for the allopregnanolone studies. These times are based on our previous work with these drugs. The difference between the baseline and drug day (day 3 – day 2) was used as the dependent measure for all analyses. This procedure provides an estimate of stimulant response that is not confounded by differences in response to a novel environment (day 1) and that is corrected for non-drug-induced differences in locomotor activity based on a within-subject measurement (day 2). All of these parameters are consistent with those used for our previous analyses of sensitivity to the stimulant effects of ethanol (Phillips *et al.* 1995) and allopregnanolone (Palmer *et al.* 2002a) in the BXD RI strains. Following testing on days 1 and 2, mice were immediately returned to their home cages. After testing on day 3, microcapillary pipettes (Fisher Scientific, Allentown, PA) were used to obtain 20 µl of blood from the peri-orbital sinus of all mice treated with ethanol to obtain a sample for analysis of blood ethanol concentration (BEC), and mice were then euthanized by CO<sub>2</sub> asphyxiation. The spleen was then harvested from the F<sub>2</sub> mice as a source of DNA for genotyping and kept at –80 °C until processed.

#### Analysis of BEC

Each blood sample was placed into a 1.5-ml microcentrifuge tube (Fisher Scientific) containing 50 µl of ice cold ZnSO<sub>4</sub> (5%; Sigma) and further processed as previously described (Boehm *et al.* 2000). Blood ethanol concentrations were determined using gas chromatography (Hewlett-Packard 5890, Corvallis, OR) with flame ionization detection.

#### Reanalysis of BXD RI data

To take advantage of improvements in the genetic maps of the BXD RI strains, we used the most current map available from the WebQTL ([www.nervenet.org](http://www.nervenet.org)) website (August 4, 2005) to identify QTL for the locomotor-stimulant response to ethanol for the 15-min test following administration of 2 g/kg ethanol (Phillips *et al.* 1995) and for the 30-min test following administration of 17 mg/kg allopregnanolone (Palmer *et al.* 2002b). In our previous publication (Phillips *et al.* 1995), only the data from the first 5 min of the ethanol test were presented, whereas all analyses in this article focus on the total 15-min test period, because it was most strongly correlated with stimulant response to allopregnanolone. These phenotype data are available in the WebQTL phenotype database ([www.nervenet.org](http://www.nervenet.org)). Figures were generated using the interval-mapping function available at WebQTL, with the default options selected. All BXD phenotype data for both ethanol and allopregnanolone are from

female mice, because only female BXD mice were tested in our previous studies (Palmer *et al.* 2002b; Phillips *et al.* 1995).

#### B6D2F2 studies

For ethanol, 439 B6D2F2 mice (220 female and 219 male) between the ages of 54 and 90 days old (mean = 73 ± 0.45 days) were used. We selected the 27.2% of mice with the most extreme phenotypes, 13.6% from each end of the distribution, split evenly between the two sexes and genotyped them at a total of 186 markers evenly spaced across all chromosomes. We had intended to genotype 25% of the population, because this fraction of the population provides the majority (approximately 73%) of the power from the total population (Lander & Botstein 1989). The exact number was dictated by technical considerations related to the genotyping procedure. 4

For the 17 mg/kg allopregnanolone dose, 403 B6D2F2 mice (228 female and 175 male) between the ages of 45 and 74 (mean = 58.7 ± 0.3 days) were used. We selected 22.4% of mice with the most extreme phenotypes, 11.2% from each end of the distribution, split evenly between the two sexes and genotyped them at a total of 172 markers evenly spaced across all chromosomes. We had intended to genotype 25% of the population, because this fraction of the population provides the majority (approximately 73%) of the power from the total population (Lander & Botstein 1989). The exact number was dictated by technical considerations related to the genotyping procedure. This approach of selectively genotyping the phenotypic extremes of an F<sub>2</sub> population has been successfully used to provide evidence for QTL (Buck *et al.* 2002; Phillips *et al.* 1998). 5

#### Congenic studies

Reciprocal D2.B6 (D2 genetic background, B6 introgressed segment) and B6.D2 (B6 background, D2 introgressed segment) congenic mice were compared to the appropriate pure background strains. For ethanol, 247 mice were used in the D2.B6 study (114 D2 and 133 D2.B6 congenic mice; average age 76.2 ± 0.9 days, range 50–103 days), and 213 mice were used in the B6.D2 study (113 D2 and 100 B6.D2 congenic mice; average age 84.1 ± 1.34 days, range 50–139 days). Both the sexes were represented in approximately equal numbers for all genotypes. For the ethanol studies, all mice were treated with 2 g/kg ethanol on day 3 (no vehicle control group was required because each animal behavior on day 2 served as the control). For allopregnanolone, 203 mice were used in the D2.B6 study (90 D2 mice and 113 D2.B6 congenic mice; average age 93 ± 1.6 days, range 52–173 days), and 247 were used in the B6.D2 study (127 D2 mice and 120 B6.D2 congenic mice; average age 80.3 ± 0.35 days, range 50–136 days). Both the sexes were represented in approximately equal numbers for all

genotypes. For the allopregnanolone studies, mice were treated with vehicle or allopregnanolone (10 or 17 mg/kg) on day 3.

### DNA isolation

DNA was isolated from frozen spleens after overnight Proteinase K digestion using a standard salting-out protocol, followed by precipitation of DNA using two volumes of ethanol (Palmer *et al.* 2003).

### Genotyping

Genotyping was performed by Kbiosciences ([www.kbioscience.co.uk](http://www.kbioscience.co.uk)) using single nucleotide polymorphism (SNP) markers from the set of 1638 SNPs reported in Petkov *et al.* (2004). Most of the 172 SNP markers were spaced evenly across all autosomes and the X chromosome for mapping purposes; however, we included seven more closely spaced markers on medial chromosome 2, because this region was a special focus of our project. In addition to these SNP markers, we also used 14 microsatellite markers from the MIT series to genotype mice from the ethanol F<sub>2</sub> study. Genotyping of these markers was performed using standard polymerase chain reaction methodology (Palmer *et al.* 2005). Amplicons were resolved on a 2% agarose gel. A total of four genotypes with error logarithm of odds (LOD) scores >3 were changed to unknown.

### Statistical analyses for congenic experiments

Day 3 – Day 2 distance traveled data were initially analyzed by two- or three-way analysis of variance (ANOVA) for the between-subject variables sex and strain as well as dose for the allopregnanolone experiments (0, 10 or 17 mg/kg). Interactions and main effects were then analyzed as appropriate using two-factor ANOVA and simple main effect analysis.

### QTL analysis

Genotype and phenotype data were analyzed using R/qtl (Broman *et al.* 2003). We used the genotype data from both of the F<sub>2</sub> populations to define a single empirical genetic map, which generally showed good agreement with existing maps. We used the 'scanone' command to identify QTLs for

the phenotypes using the EM algorithm. In addition, we analyzed the dataset broken down by sex (males only or females only) in an effort to identify potentially sex-specific QTLs. We generated permutation-derived significance levels for each analysis (10 000 permutations).

We also ran analyses for each datasets, in which sex was treated as an additive or an interactive covariate (data not shown), similar to the approach of Solberg *et al.* (2004). Treating sex as an additive covariate had little effect on the outcome of our analysis. The results were somewhat weaker, and hence, we ultimately rejected this model. Treating sex as an interactive covariate increased the LOD scores dramatically at many loci throughout the genome; however, it also added two degrees of freedom to the model. We determined that this approach was not justified for two reasons. First, we determined that the largest increase in LOD score when sex was treated as an interactive vs. an additive covariate was less than the average increase obtained using 1000 permutations of the two models. Second, we ran permutation tests of both the models to determine the empirical significance threshold. The threshold for the interactive models were substantially higher (>2 LOD units higher). When the results of the interactive model were normalized using the ratio of the two empirically derived significance thresholds, the interactive model appeared to be less powerful than the additive model. On the basis of these analyses, we determined that there was no advantage to treating sex as a covariate.

### Combining P values using Fisher's method

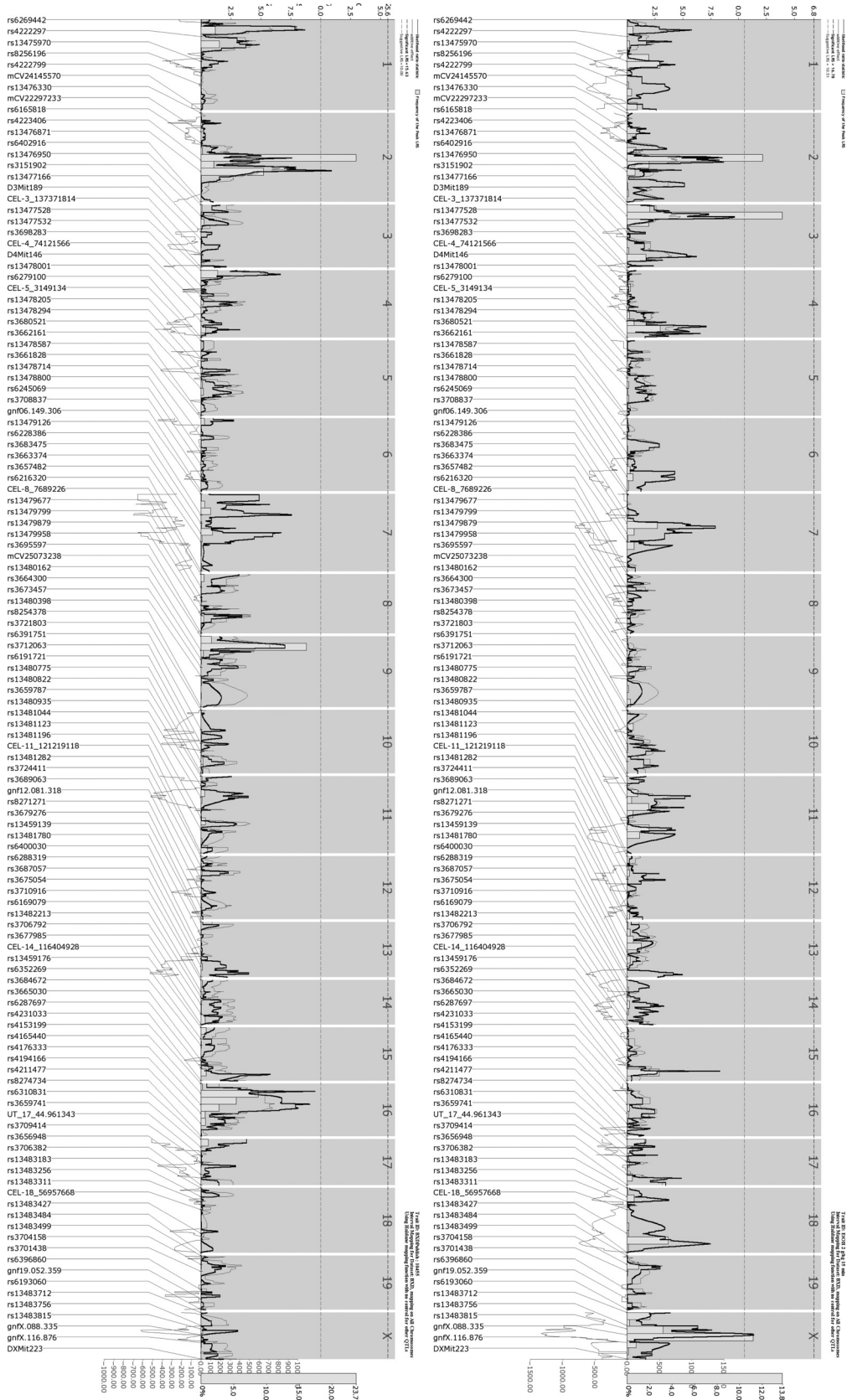
We calculated the joint probability for our three independent tests of the hypothesis that there is a significant QTL for ethanol and for allopregnanolone sensitivity on chromosome 2 by converting each probability estimate to a P value and then using Fisher's method to calculate a single probability statistic for each drug (Rohlf & Sokal 1995).

## Results

### Reanalysis of BXD RI data

Figure 1 shows the results of QTL analyses of previously collected data from female members of the BXD RI strains for the locomotor response to 2 g/kg ethanol (Phillips *et al.*

**Figure 1: Plots generated by WebQTL (<http://www.nervenet.org>) showing quantitative trait locus for the response to ethanol (above; 2 g/kg; 15 min) or allopregnanolone (below; 17 mg/kg; 30 min) based on strain means from 21 and 24 BXD RI strains, respectively.** These strain means for allopregnanolone have been published previously (Palmer *et al.* 2002b) while the strain means for ethanol are for a 15-min test period rather than the 5-min period published in Phillips *et al.* (1995). The horizontal axis represents the genome and is broken into chromosomes. A representative sample of the single nucleotide polymorphism of microsatellite markers used for the mapping is shown along the horizontal axis. The vertical axis shows the probability score in terms of likelihood ratio statistic (LRS) units. The heavy line reflects the LRS score at each point along the genome, while the histograms reflect the frequency of the peak LRS score based on a bootstrapping method. The upper line reflects a permutation-derived significance threshold, while the lower dotted line represents a suggestive level of evidence. Further details of this output are described at the WebQTL website (<http://www.nervenet.org>).



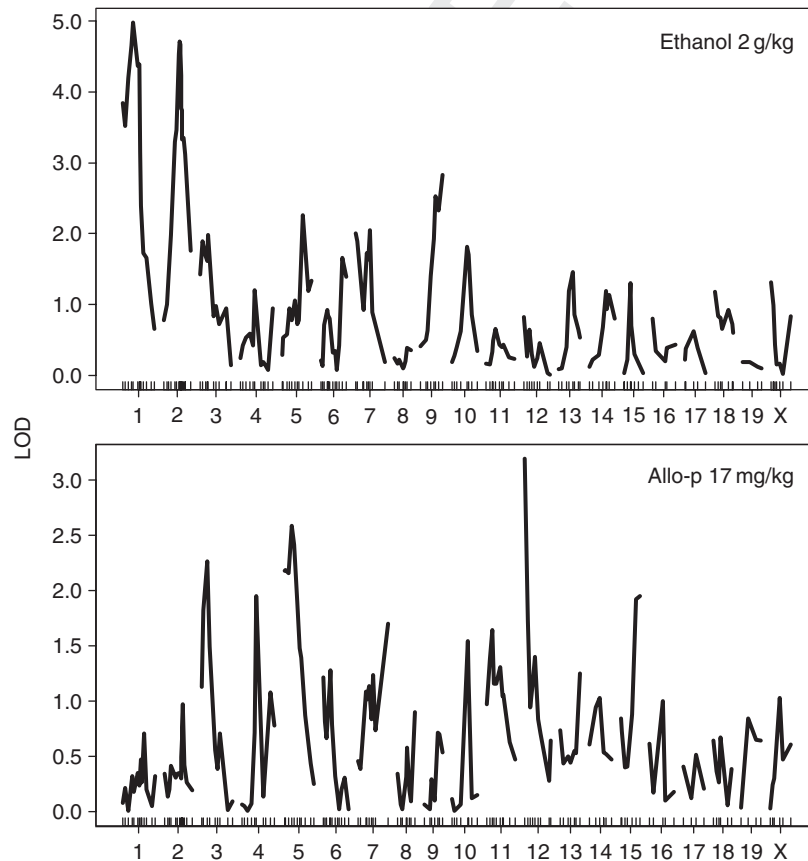
1995) and 17 mg/kg allopregnanolone (Palmer *et al.* 2002b). None of the QTLs can be considered significant from the BXD analysis alone; however, there were suggestive peaks in approximately the same region of chromosome 2 for both the traits, which is consistent with our earlier analyses of these data (Palmer *et al.* 2002b; Phillips *et al.* 1995). The peak LOD scores for the chromosome 2 QTL were 1.9 at 118 MB and 1.06 at 149 MB for ethanol and 1.65 at 106 MB and 2.37 at 149 MB for allopregnanolone. The D2 allele was associated with greater sensitivity to the stimulant effects of both drugs. We did not reanalyze the 10 mg/kg allopregnanolone data, because there was no correlation between the locomotor response to 10 mg/kg allopregnanolone and 2 g/kg ethanol in the BXD data (see Palmer *et al.* 2002b) and because our  $F_2$  study only used the 17 mg/kg allopregnanolone dose.

### QTL mapping for ethanol $F_2$ study

The results of the QTL analysis are shown in Fig. 2, and the exact LOD scores are summarized in Table 1. We identified

two QTLs with significant LOD scores, which were located on chromosomes 1 (62.9 MB) and 2 (107.8 MB). We also identified suggestive QTLs on chromosomes 5 (124.2 MB) and 9 (112.0 MB). All QTLs reflected a greater locomotor response associated with the D2 allele, except for the chromosome 5 QTL, which showed a greater response associated with the B6 allele. D2 mice are well known for their extreme sensitivity to ethanol stimulation and B6 mice for their insensitivity to this effect of ethanol (Phillips *et al.* 1995). All QTLs showed an additive pattern of inheritance. Permutation indicated that a LOD score above 3.60 should be considered significant ( $P < 0.05$ ) and that a LOD score above 2.08 should be considered suggestive ( $P < 0.63$ ).

Analysis of the female only subset of these data identified a significant QTL on chromosome 2 (107.8 MB) and also identified suggestive QTLs on chromosomes 1 (79.6 MB), 5 (124.2 MB), 7 (85.3 MB), 10 (93.8 MB), 13 (81.2 MB) and 18 (25.1 MB). The chromosome 1, 2 and 13 QTLs all reflected greater response when the D2 allele was present. The chromosome 5, 7 and 18 QTLs reflected greater



**Figure 2: Quantitative trait locus (QTL) plots from the  $F_2$  studies of the locomotor-stimulant response to ethanol (2 g/kg) and allopregnanolone (17 mg/kg).** The horizontal axis represents the genome and is broken into chromosomes. Each tick mark along the horizontal axis represents the location of a genotyped single nucleotide polymorphism or microsatellite marker. The vertical axis shows the probability [logarithm of odds (LOD)] that a QTL is located at that point on a chromosome. Table 1 distills these data to only those markers with peak LOD scores that provided suggestive or significant evidence for a QTL.

**Table 1:** Table showing peak logarithm of odds (LOD) scores for each chromosome for locomotor stimulation (day 3–day 2) in response to ethanol (2 g/kg; 15 min) or Allo-p (17 mg/kg; 30 min)

Chromosome	Ethanol (male + female)	Ethanol (female only)	Ethanol (male only)	Allo-p (male + female)	Allo-p (female only)	Allo-p (male only)
1	rs3683410 (62.9) 4.98*	rs3677697 (79.6) 3.08	rs3683410 (62.9) 2.52			
2	D2Mit58 (107.8) 4.71*	D2Mit58 (107.8) 3.72*			rs3022898 (126.6) 2.41	
3			rs3678814 (18.0) 2.17	rs3659585 (32.0) 2.23	rs3659585 (32.0) 2.17	
4						rs3726519 (82.8) 2.64
5	rs3664741 (124.2) 2.26	rs3664741 (124.2) 2.95		rs3708877 (48.3) 2.54		
6			rs3024195 (36.7) 2.36			
7		rs3686613 (85.3) 2.42			rs4226997 (139.3) 2.48	rs3720603 (45.6) 3.30
9	D9Mit20 (112?) 2.83					
10		rs3704566 (93.8) 2.32			rs3704566 (93.8) 2.36	
12				rs4140017 (10.5) 3.22		rs4140017 (10.5) 2.85
13		rs3716022 (81.2) 2.63				
16			rs3090912 (5.8) 2.45			
18		rs3660676 (25.1) 2.35				

Each cell contains the NCBI refSNP ID for the marker that was nearest to the peak followed by the location of that marker in megabase units (MB) based on the current assembly of the mouse genome (March 2005; mm<sup>6</sup>, NCBI build 34) in parenthesis, with the LOD score on the next line. Chromosomes are only listed if there was a suggestive or significant association detected.

\*Indicates that this LOD score should be considered significant based on our permutation analysis.

response when the B6 allele was present. These female-specific QTLs all reflected additive inheritance patterns with the exception of the chromosome 10 QTL, which showed the greatest response in heterozygous animals.

Analysis of the male only subset of these data identified suggestive QTLs on chromosomes 1 (62.9 MB), 3 (18.0 MB), 6 (36.7 MB) and 16 (5.8 MB). The chromosome 1, 3 and 16 QTLs all reflected that greater locomotor response was associated with the D2 allele, whereas the chromosome 6 QTL indicated a greater response when the B6 allele was present. All male-specific QTLs showed an additive pattern of inheritance.

#### QTL mapping for allopregnanolone F<sub>2</sub> study

The results of the QTL analysis are shown in Fig. 2 and the exact LOD scores, and positions in megabase units are summarised in Table 1. Permutation indicated that a LOD score above 3.63 should be considered significant

( $P < 0.05$ ) and that a LOD score above 2.12 should be considered suggestive ( $P < 0.63$ ). Using these criteria, we did not identify any significant QTLs; however, we did find suggestive QTLs on chromosomes 3 (32.0 MB), 5 (48.3 MB) and 12 (10.5 MB). The chromosome 3 and 12 QTLs indicated that a greater response to allopregnanolone was associated with the D2 allele, whereas the chromosome 5 QTL was associated with a greater response when the B6 allele was present. Locomotor activity data collected under the same test conditions have shown greater stimulation in the D2 mice, relative to B6 mice, following administration of 17 mg/kg allopregnanolone (Palmer *et al.* 2002b). The highest recorded LOD score on chromosome 2 was 0.97 at 126.6 MB. Like the peak at 107.8 MB for ethanol sensitivity, this QTL was associated with greater stimulation when the D2 allele was present. All QTLs showed an additive pattern of inheritance.

Analysis of only the female subjects identified suggestive QTLs on chromosomes 2 (126.6 MB), 3 (32.0 MB),

7 (139.3 MB) and 10 (93.8 MB). Female-specific QTLs indicated that the D2 alleles imparted greater locomotor-stimulant response to allopregnanolone with the exception of the chromosome 7 QTL, which indicated that a greater response was associated with the B6 allele. These relationships were all additive.

When only the male subjects were analyzed, suggestive QTLs on chromosomes 4 (82.8 MB), 7 (45.6 MB) and 12 (10.5 MB) were identified. The chromosome 4 QTL showed the highest locomotor response in the heterozygous mice and lower responses in either homozygous state. The chromosome 7 QTL showed higher response with the B6 allele, and the chromosome 12 QTLs showed greater response for the D2 allele, and the chromosome 7 and 12 QTLs showed additive patterns of inheritance.

### Genotyping of D2.B6 congenic strains

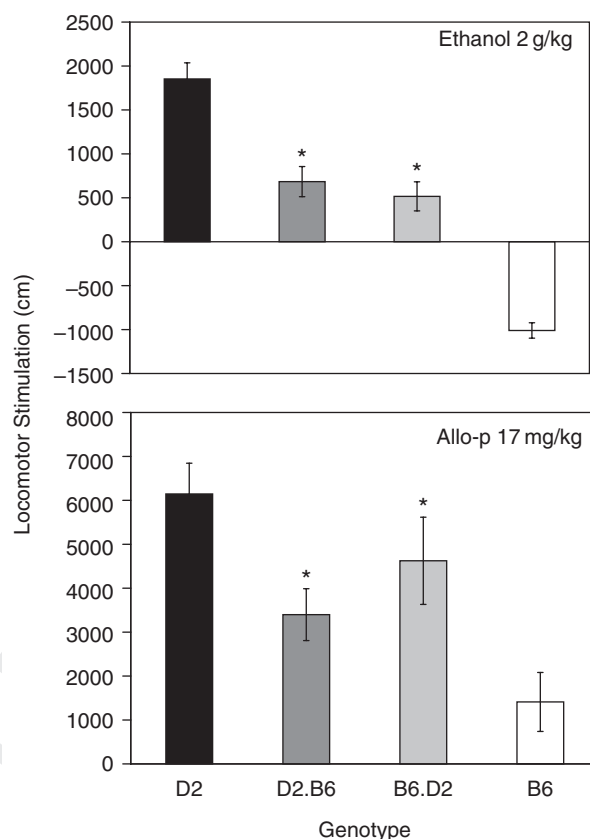
Genotyping of DNA obtained from these mice revealed that there was a segment of B6 origin that started between 20.2 and 37.6 MB (markers rs3677975 and rs3709811) and ended between 130.6 and 153.0 MB (markers rs3674906 and rs4223578), with the exception of a region flanked by markers at 50.6 and 75.8 MB where a single marker at 62.5 MB was of D2 origin (markers rs3710476, rs3699089 and rs3022886, respectively). Thus, a segment of B6 origin minimally between 37.6 and 130.6 MB (but with a D2 segment possibly as large as 50.6–75.8 MB) was contained on a background that was of uniformly D2 origin (all 157 markers examined on the other chromosomes were homozygous D2).

### Genotyping of B6.D2 congenic strains

Genotyping of DNA obtained from these mice revealed that there was a segment of D2 origin that started before 20.2 MB (marker rs3677975) and ended between 153.0 and 168.1 MB (markers rs4223578 and rs3024096), and a few markers at the boundaries of this region were genotyped as heterozygous in individual subjects. Thus, a segment of D2 origin minimally between 20.2 and 153.0 MB was contained on a background that was of uniformly B6 origin (all 157 markers examined on the other chromosomes were homozygous B6).

### Congenic ethanol studies

The results of the two congenic studies examining the effects of ethanol are shown in the top panel of Fig. 3. The appropriate analyses included a single congenic vs. its background strain. An ANOVA for the D2.B6 vs. D2 strain examined the factors genotype and sex and found a significant main effect of genotype ( $F_{1,243} = 20.8$ ;  $P < 0.00001$ ) but no main effect or interaction between sex and genotype. Mice possessing the introgressed B6 region of chromosome 2 had an ethanol-stimulant response that was lower than that



**Figure 3: Results of D2.B6 and B6.D2 congenic studies of the effect of ethanol (2 g/kg) and allopregnanolone (17 mg/kg) on locomotor stimulation.** The height of the bar reflects the locomotor-stimulant response (day 3 – day 2) of the indicated group. Comparisons were not made between inbred strains or between the two reciprocal congenic strains, because each congenic study was conducted separately; therefore, the only relevant comparisons are between the congenic and its matched background strain. Details of the congenic segments as well as the statistical comparisons performed are in the text. Note that we also examined response to 10 mg/kg allopregnanolone, but because neither congenic strain showed a different locomotor-stimulant response when compared with the corresponding background strain, the data are not shown here; means are listed in the text. Likewise, no difference was found between the congenic and the background strains when tested for locomotor behavior after vehicle injection. Error bars reflect the standard error of the mean. \*indicates that the congenic group was significantly different from the corresponding pure inbred strain.

exhibited by the pure D2 strain. There was no significant difference for BEC between the two groups.

An ANOVA that examined the factors genotype and sex for the B6.D2 vs. B6 strain also found a significant main effect of genotype ( $F_{1,209} = 83.8$ ;  $P < 0.000001$ ) and a main effect of sex ( $F_{1,209} = 20.4$ ;  $P < 0.000001$ ) but no interaction between the two. Mice possessing the introgressed D2 region of chromosome 2 had a higher ethanol-stimulant response compared with the pure B6 strain mice, which

exhibited locomotor depression. Female mice had larger day 3 – day 2 activity scores than male mice. There was no significant difference for BEC between the two groups.

### Congenetic allopregnanolone studies

The results of the two congenic studies examining the effects of allopregnanolone (17 mg/kg) are shown in the bottom panel of Fig. 3. An ANOVA that examined the factors genotype, dose and sex for the D2.B6 vs. D2 strain found an interaction between dose and genotype ( $F_{2,197} = 3.37$ ;  $P < 0.05$ ) but no main effect or interactions involving sex; therefore, all further analyses excluded sex as a factor. The D2.B6 congenic strain exhibited significantly reduced stimulation compared with the D2 strain at the 17 mg/kg dose ( $P < 0.01$ ), but the two strains were similar at the 0 (D2:  $315 \pm 290$ ; D2.B6:  $310 \pm 233$ ) and 10 mg/kg doses (D2:  $3349 \pm 722$ ; D2.B6:  $2626 \pm 570$ ). An ANOVA that examined the factors genotype, dose and sex for the B6.D2 vs. B6 strain also found an interaction between dose and genotype ( $F_{2,235} = 4.53$ ;  $P < 0.05$ ) and a main effect of sex ( $F_{1,235} = 16.2$ ;  $P < 0.00001$ ) but no interaction involving sex and genotype; therefore, all further analyses excluded sex as a factor. The B6.D2 congenic strain exhibited significantly enhanced stimulation compared with the B6 strain at the 17 mg/kg dose ( $P < 0.01$ ), but the two strains were similar at the 0 (B6:  $-540 \pm 260$ ; B6.D2:  $-1255 \pm 316$ ) and 10 mg/kg (B6:  $3517 \pm 675$ ; B6.D2:  $3726 \pm 714$ ) doses.

### Combining P values using Fisher's method

We combined the probability scores for the RI,  $F_2$  and congenic studies pertaining to the putative chromosome 2 QTL using Fisher's method. All the three studies indicated that the D2 allele was associated with greater sensitivity to both drugs. We calculated the two-tailed scores for the BXD RI study, because it had been used in the discovery phase, and then used one-tailed  $P$  values for all subsequent studies, because they were considered verification steps. Note that the BXD studies used only female mice, whereas the  $F_2$  and congenic studies used approximately equal numbers of male and female mice. One reason that the LOD scores for allopregnanolone may be lower for the congenic studies is that

only 68 and 81 mice were tested at the 17 mg/kg dose because of reduced availability of these mice at the time these studies were conducted, whereas more than 200 were tested at the 2 g/kg ethanol dose. The combined  $P$  values and corresponding LOD scores are shown in Table 2.

### Discussion

The major result of these studies is the identification of a chromosome 2 QTL that appears to pleiotropically influence sensitivity to the locomotor-stimulant effects of both ethanol and allopregnanolone. This QTL was provisionally identified by examining BXD RI strains (Palmer *et al.* 2002b; Phillips *et al.* 1995) (Fig. 1). On the basis of those results, we conducted the  $F_2$  and congenic studies reported here. The  $F_2$  study of ethanol confirmed the chromosome 2 QTL. The  $F_2$  study of the effects of allopregnanolone found weaker evidence for this QTL; however, there was suggestive evidence when data from only female subjects were examined. All four of the congenic studies provided strong support for the presence of a chromosome 2 QTL for both ethanol (2.0 g/kg) and allopregnanolone (17 mg/kg) sensitivity. To integrate the results of these three studies, we used Fisher's method to combine the  $P$  values.

The failure to obtain a suggestive or significant LOD score, other than in female mice, in the  $F_2$  allopregnanolone study may reflect the limited power of a moderately sized  $F_2$  to detect all QTLs for a given phenotype, a phenomenon that has been demonstrated previously in mice and other model organisms, as well as in simulation studies (Bennett *et al.* in press). Alternatively, there may be a second allele on chromosome 2 that inhibits the locomotor response to allopregnanolone (but presumably not ethanol) that is linked to and thus co-inherited with the allele that positively affects allopregnanolone sensitivity. This linkage would tend to obscure detection of the former QTL but might be avoided in the RI studies (where more crossovers has diminished this linkage) and in the congenic strains, which may not include the linked region. The  $F_2$  female-specific analyses and the congenic studies both provided limited evidence that there may be an interaction between the chromosome 2 QTL and the

**Table 2:** Combination of  $P$  values from multiple tests of the hypothesis that a QTL exists on chromosome 2

	RI (1 df)	$F_2$ (2 df)	D2.B6 (1 df)	B6.D2 (1 df)	Combined $P$ value	Combined LOD (1 df)
Ethanol 2 g/kg	1.9 {0.003}	4.71 {0.000019}	4.5 {0.000006}	5.2 {0.000001}	$9.6 \times 10^{-15}$	12.0
Allo-p 17 mg/kg	1.65 {0.006}	0.97 {0.106}	1.84 {0.0036}	1.5 {0.0086}	$7.7 \times 10^{-7}$	5.3

Logarithm of odds (LOD) scores and their corresponding  $P$  values (in {} brackets) are shown for the RI,  $F_2$  and both congenic studies. In the far right columns, the combined  $P$  values and LOD scores for all studies are shown. The combined scores were calculated using Fisher's method, based on two-tailed scores for the RI data and one-tailed  $P$  values for all other studies (Rohlf & Sokal 1995).

sex of the animal being tested. The BXD studies, which first identified the chromosome 2 QTL, used only female mice. The failure to detect a QTL using the  $F_2$  approach provides an important cautionary note for those seeking to use multiple crosses among inbred strains in order to narrow the location of QTLs (Hitzemann *et al.* 2000; Li *et al.* 2005; Shifman & Darvasi 2005). Had we been using this approach, we would most likely have concluded that the chromosome 2 QTL for allopregnanolone-induced locomotor activity did not exist for this cross; a conclusion that is clearly incorrect based on the results of the congenic studies.

Previous studies from our group and others have sought to investigate QTLs for this phenotype. In a series of articles reporting the results of a large B6xD2  $F_2$  intercross as well as the results of BXD RI studies and studies of a heterogeneous stock (HS), Hitzemann *et al.* (1998, 2000), Demarest *et al.* (1999, 2001) and Xu *et al.* (2002) have identified a QTL for the locomotor response to 1.5 g/kg ethanol on chromosome 2. Their testing paradigm was somewhat different from the one that we used (e.g. 1.5 vs. 2.0 g/kg ethanol dose), but it seems likely that the chromosome 2 QTL that they identified is the same as that of ours. Taken together, these previously published  $F_2$  studies define a QTL between 50 and 148 MB. The HS studies appear to fine map this QTL to a region that includes at least 111.8–127.8 MB, although the proximal side may extend further than 111.8 MB based on the data shown (Demarest *et al.* 2001). This region is included in our congenics and is also supported by the RI and  $F_2$  data presented in this article. Interestingly, BXD data from Demarest *et al.* (1999) suggested that chromosome 2 might also contain a QTL for sensitivity to the benzodiazepine receptor agonist chlordiazepoxide. This finding is similar to our studies of allopregnanolone; in that, it focuses on the GABA-A receptor and suggests that the chromosome 2 QTL may rely on a mechanism that is shared with other GABA-A-acting compounds. It is interesting to note that the chromosome 2 QTL was not detected when a cross between the BALB/cJ and LP/J strains was examined (Hitzemann *et al.* 2000), suggesting that the QTL-causing allele may not differ between those two strains (however, caution should be used when interpreting such negative data, as our results from the allopregnanolone  $F_2$  study demonstrate). Along a similar line, Downing *et al.* (2003) also examined locomotor response to a 1.5 g/kg ethanol dose in crosses between B6  $\times$  C3H/HeJ (C3H) mice and found QTLs on chromosomes 1, 6 and 15 but not 2, suggesting that B6 and C3H share a similar version of the QTL-causing allele on chromosome 2. Finally, Gill *et al.* (2000) use AXB/BXA recombinant inbred strains to map QTLs for the locomotor-stimulant response to a 2 g/kg ethanol dose; neither chromosomes 1 or 2 showed suggestive or significant LOD scores, perhaps indicating that both the B6 and the A/J strains share similar version of the QTL-causing alleles on these chromosomes. Observations of strain contrasts such as these may be useful for identifying the QTL-causing gene on chromosome 2, because one could hypothesize that the allele would be different between B6

and D2 but similar between B6, A/J and C3H and also between BALB/cJ and LP/J.

In addition to the QTL on chromosome 2 that was the focus of our studies, our  $F_2$  studies identified other significant and suggestive QTLs. In particular, a QTL on chromosome 1 was identified with a LOD score of 4.98. This may be the same QTL that was identified by Demarest *et al.* (2001) using B6  $\times$  D2  $F_2$  and HS populations and by Downing *et al.* (2003) using a BALB/cJ  $\times$  IP/J cross. We also found modest support for QTLs on chromosomes 3, 5 and 7 in the BXD RI and/or  $F_2$  studies, suggesting that these regions may harbor alleles that influence sensitivity to one or both drugs.

The mechanism by which ethanol induces locomotor activity has yet to be satisfactorily elucidated; however, there is strong evidence that GABA-A receptors are involved in many aspects of ethanol sensitivity as has been recently reviewed (Boehm *et al.* 2004). The present results suggest that the chromosome 2 QTL may influence sensitivity to GABA-A acting compounds, including both allopregnanolone and perhaps also chlordiazepoxide. This QTL may be one of the many loci that underlie the correlations observed among BXD mice between ethanol and allopregnanolone sensitivity (Palmer *et al.* 2002b).

The present results have several potential limitations. In much of our discussion, we assume that the colocalization of QTLs for sensitivity to both ethanol and allopregnanolone indicates that the causative allele is similar (pleiotropic). This may be a false assumption. It is quite possible that two different alleles are located within the congenic segments on chromosome 2, one of which influences ethanol sensitivity and the other of which influences allopregnanolone sensitivity. It is also possible that the QTL that we have identified is actually made up of multiple smaller QTLs, only some of which affect sensitivity to the two drugs in a pleiotropic manner. Both of these limitations can and will be addressed in future studies by more finely mapping the location of these QTLs.

In summary, these studies support the existence of one or more QTL on chromosome 2 that influences sensitivity to ethanol and allopregnanolone. If there is indeed a single QTL that influences sensitivity to both drugs, it is likely to be involved in GABAergic signaling. In addition, data from other studies have defined certain strain combinations that appear to have different alleles for the QTL and others that appear to have the same allele. This knowledge should be useful in future studies in which the gene(s) causing the QTL will be identified using fine mapping techniques.

## References

- Bennett, *et al.* (in press) Replication of small effect QTLs for behavioral traits: I. Estimation of effect size from independent cohorts. *Genes Brain Behav.*
- Boehm, S.L. 2nd, Crabbe, J.C. & Phillips, T.J. (2000) Sensitivity to ethanol-induced motor incoordination in FAST and SLOW

- selectively bred mice. *Pharmacol Biochem Behav* **66**, 241–247.
- Boehm, S.L. 2nd, Ponomarev, I., Jennings, A.W., Whiting, P.J., Rosahl, T.W., Garrett, E.M., Blednov, Y.A. & Harris, R.A. (2004) gamma-Aminobutyric acid A receptor subunit mutant mice: new perspectives on alcohol actions. *Biochem Pharmacol* **68**, 1581–1602.
- Broman, K.W., Wu, H., Sen, S. & Churchill, G.A. (2003) R/qtl: QTL mapping in experimental crosses. *Bioinformatics* **19**, 889–890.
- Buck, K.J., Rademacher, B.S., Metten, P. & Crabbe, J.C. (2002) Mapping murine loci for physical dependence on ethanol. *Psychopharmacology* **160**, 398–407.
- Carboni, E., Silvagni, A., Rolando, M.T. & Di Chiara, G. (2000) Stimulation of in vivo dopamine transmission in the bed nucleus of stria terminalis by reinforcing drugs. *J Neurosci* **20**, 1–5.
- Deitrich, R.A. (1993) Selective breeding for initial sensitivity to ethanol. *Behav Genet* **23**, 153–162.
- Demarest, K., McCaughran, J. Jr, Mahjubi, E., Cipp, L. & Hitzemann, R. (1999) Identification of an acute ethanol response quantitative trait locus on mouse chromosome 2. *J Neurosci* **19**, 549–561.
- Demarest, K., Koyner, J., McCaughran, J. Jr, Cipp, L. & Hitzemann, R. (2001) Further characterization and high-resolution mapping of quantitative trait loci for ethanol-induced locomotor activity. *Behav Genet* **31**, 79–91.
- 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.
- Downing, C., Rodd-Henricks, K.K., Flaherty, L. & Dudek, B.C. (2003) Genetic analysis of the psychomotor stimulant effect of ethanol. *Genes Brain Behav* **2**, 140–151.
- Dudek, B.C., Phillips, T.J. & Hahn, M.E. (1991) Genetic analyses of the biphasic nature of the alcohol dose–response curve. *Alcohol Clin Exp Res* **15**, 262–269.
- Erwin, V.G., Jones, B.C. & Radcliffe, R. (1990) Further characterization of LSxSS recombinant inbred strains of mice: activating and hypothermic effects of ethanol. *Alcohol Clin Exp Res* **14**, 200–204.
- Gill, K., Boyle, A., Lake, K. & Desaulniers, N. (2000) Alcohol-induced locomotor activation in C57BL/6J, A/J, and AXB/BXA recombinant inbred mice: strain distribution patterns and quantitative trait loci analysis. *Psychopharmacology* **150**, 412–421.
- Heath, A.C., Whitfield, J.B., Madden, P.A., Bucholz, K.K., Dinwiddie, S.H., Slutske, W.S., Bierut, L.J., Statham, D.B. & Martin, N.G. (2001) Towards a molecular epidemiology of alcohol dependence: analysing the interplay of genetic and environmental risk factors. *Br J Psychiatry Suppl* **40**, s33–s40.
- Hitzemann, R., Cipp, L., Demarest, K., Mahjubi, E. & McCaughran, J. Jr (1998) Genetics of ethanol-induced locomotor activation: detection of QTLs in a C57BL/6J × DBA/2J F2 intercross. *Mamm Genome* **9**, 956–962.
- Hitzemann, R., Demarest, K., Koyner, J., Cipp, L., Patel, N., Rasmussen, E. & McCaughran, J. Jr (2000) Effect of genetic cross on the detection of quantitative trait loci and a novel approach to mapping QTLs. *Pharmacol Biochem Behav* **67**, 767–772.
- Holdstock, L., King, A.C. & de Wit, H. (2000) Subjective and objective responses to ethanol in moderate/heavy and light social drinkers. *Alcohol Clin Exp Res* **24**, 789–794.
- Katner, S.N. & Weiss, F. (2001) Neurochemical characteristics associated with ethanol preference in selected alcohol-preferring and -nonpreferring rats: a quantitative microdialysis study. *Alcohol Clin Exp Res* **25**, 198–205.
- Li, R., Lyons, M.A., Wittenburg, H., Paigen, B. & Churchill, G.A. (2005) Combining data from multiple inbred line crosses improves the power and resolution of quantitative trait loci mapping. *Genetics* **169**, 1699–1709.
- Newlin, D.B. & Thomson, J.B. (1999) Chronic tolerance and sensitization to alcohol in sons of alcoholics: II. Replication and reanalysis. *Exp Clin Psychopharmacol* **7**, 234–243.
- Palmer, A.A. & Phillips, T.J. (2002) Effect of forward and reverse selection for ethanol-induced locomotor response on other measures of ethanol sensitivity. *Alcohol Clin Exp Res* **26**, 1322–1329.
- Palmer, A.A., McKinnon, C.S., Bergstrom, H.C. & Phillips, T.J. (2002a) Locomotor activity responses to ethanol, other alcohols, and GABA-A acting compounds in forward- and reverse-selected FAST and SLOW mouse lines. *Behav Neurosci* **116**, 958–967.
- Palmer, A.A., Miller, M.N., McKinnon, C.S. & Phillips, T.J. (2002b) Sensitivity to the locomotor stimulant effects of ethanol and allopregnanolone is influenced by common genes. *Behav Neurosci* **116**, 126–137.
- Palmer, A.A., Moyer, M.R., Crabbe, J.C. & Phillips, T.J. (2002c) Initial sensitivity, tolerance and cross-tolerance to allopregnanolone- and ethanol-induced hypothermia in selected mouse lines. *Psychopharmacology* **162**, 313–322.
- Palmer, A.A., Lov, M.J., Grandy, D.K. & Phillips, T.J. (2003) Effects of a Drd2 deletion mutation on ethanol-induced locomotor stimulation and sensitization suggest a role for epistasis. *Behav Genet* **33**, 311–324.
- Palmer, A.A., Verbitsky, M., Suresh, R., Kamens, H.M., Reed, C.L., Li, N., Burkhart-Kasch, S., McKinnon, C.S., Belknap, J.K., Gilliam, T.C. & Phillips, T.J. (2005) Gene expression differences in mice divergently selected for methamphetamine sensitivity. *Mamm Genome* **16**, 291–305.
- Petkov, P.M., Ding, Y., Cassell, M.A., Zhang, W., Wagner, G., Sargent, E.E., Asquith, S., Crew, V., Johnson, K.A., Robinson, P., Scott, V.E. & Wiles, M.V. (2004) An efficient SNP system for mouse genome scanning and elucidating strain relationships. *Genome Res* **14**, 1806–1811.
- Phillips, T.J. & Shen, E.H. (1996) Neurochemical bases of locomotion and ethanol stimulant effects. *Int Rev Neurobiol* **39**, 243–282.
- Phillips, T.J., Burkhart-Kasch, S., Terdal, E.S. & Crabbe, J.C. (1991) Response to selection for ethanol-induced locomotor activation: genetic analyses and selection response characterization. *Psychopharmacology* **103**, 557–566.
- Phillips, T.J., Huson, M., Gwiazdon, C., Burkhart-Kasch, S. & Shen, E.H. (1995) Effects of acute and repeated ethanol exposures on the locomotor activity of BXD recombinant inbred mice. *Alcohol Clin Exp Res* **19**, 269–278.
- Poikolainen, K. (2000) Risk factors for alcohol dependence: a case-control study. *Alcohol Alcohol* **35**, 190–196.
- Purdy, R.H. & Paul, S.M. (1999) Potentiation of GABAergic neuro-transmission by steroids. In Baulieu, E.-E., Robel, P. & Schumacher, M. (eds), *Contemporary Endocrinology: Neuro-Steroids: A New Regulatory Function in the Nervous System*. Humana Press, Totowa, NJ, pp. 143–153.
- Rohlf, F.J. & Sokal, R.R. (1995) *Biometry*. W.H. Freeman, New York.
- Schuckit, M.A. & Smith, T.L. (2000) The relationships of a family history of alcohol dependence, a low level of response to alcohol and six domains of life functioning to the development of alcohol use disorders. *J Stud Alcohol* **61**, 827–835.
- Schuckit, M.A. & Smith, T.L. (2001) The clinical course of alcohol dependence associated with a low level of response to alcohol. *Addiction* **96**, 903–910.
- Shen, E.H., Harland, R.D., Crabbe, J.C. & Phillips, T.J. (1995) Bidirectional selective breeding for ethanol effects on

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- locomotor activity: characterization of FAST and SLOW mice through selection generation 35. *Alcohol Clin Exp Res* **19**, 1234–1245.
- Shifman, S. & Darvasi, A. (2005) Mouse inbred strain sequence information and yin-yang crosses for quantitative trait locus fine mapping. *Genetics* **169**, 849–854.
- Solberg, L.C., Baum, A.E., Ahmadiyah, N., Shimomura, K., Li, R., Turek, F.W., Churchill, G.A., Takahashi, J.S. & Redei, E.E. (2004) Sex- and lineage-specific inheritance of depression-like behavior in the rat. *Mamm Genome* **15**, 648–662.
- Spuhler, K. & Deitrich, R.A. (1984) Correlative analysis of ethanol-related phenotypes in rat inbred strains. *Alcohol Clin Exp Res* **8**, 480–484.
- Xu, Y., Demarest, K., Hitzemann, R. & Sikela, J.M. (2002) Gene coding variant in Cas1 between the C57BL/6J and

DBA/2J inbred mouse strains: linkage to a QTL for ethanol-induced locomotor activation. *Alcohol Clin Exp Res* **2002**, 1–7.

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