Identification of sex-specific quantitative trait loci controlling alcohol preference in C57BL/6 mice

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Mice from various inbred strains consume alcoholic beverages at highly reproducible and strain-specific levels. While most mice consume alcohol in moderate amounts, C57BL/6J animals exhibit sustained oral ingestion of high levels of alcohol in the presence of competing water and food. We now report a genetic investigation of this phenotype as one potential model for alcoholism. An intercross-backcross breeding protocol was used to identify two recessive alcohol preference quantitative trait loci (QTLs) that are both sex-restricted in expression. A comparison of our results with those of an earlier morphine preference study argues against the hypothesis of a single unified phenotype defined by a preference for all euphoria-producing drugs.

Hundreds of studies carried out over the past 80 years or so have demonstrated conclusively that the genetic constitution of an individual plays an important role in determining whether he or she will become an alcoholic[1,2]. However, little progress has been made toward the identification of loci that predispose individuals to this common disease. Alcoholism is a complex multifactorial trait whose genetic dissection is confounded by genetic heterogeneity, polygenic inheritance, incomplete penetrance, and strong environmental influences on both expression and non-expression[2].

Experimental animal models for complex human traits provide a means for circumventing two factors — heterogeneity and environment — that complicate human studies of complex traits; and crosses with inbred strains greatly simplify the analysis and interpretation of genotypic and phenotypic data. But, while it is relatively easy to ascertain the suitability of a particular animal model for a physiological disease — such as cancer susceptibility[3–7], hypertension[8,9], or asthma[10] — models of human behaviour are more problematic. In the case of alcoholism, a perfect model may not actually exist, given the wide variety of very different subtypes proposed for the human condition[11]. Nevertheless, various animal models have been employed with assays for neurological sensitivity, dependence, withdrawal and alcohol consumption with or without the availability of competing fluids[2]. With an appreciation for its limitations, we have chosen a model based on the consistent and sustained oral ingestion of high levels of alcohol in the presence of competing water and food[12,13]. With the use of an analogous model for morphine preference[14], three potential predisposition QTLs were found for this trait.

Over 35 years ago, McClearn and Rodgers[12] first showed that mice from various inbred strains consume alcohol at highly reproducible and strain-specific levels when given a choice between 10% ethanol and water. The combined data from this original study, and others that have followed, show that most inbred strains — including 129/ReJ, C3H/HeSnJ, BALB/cJ, and an A/J-related strain — consume 10% ethanol in moderate amounts that range from 0.15 to 0.25 as a fraction of total liquid intake[12,15–17]. However, several strains exhibit extreme levels of alcohol consumption that are significantly above or below this moderate range. In particular, C57BL/6 (B6) mice exhibit extreme alcohol preference with intake fractions that may be 0.75 or more of total liquid consumed over long periods of time. In contrast, mice from the DBA/2J strain exhibit extreme alcohol avoidance with measured intake fractions of 0.07 or less. These data provide evidence of a strong genetic influence on the proportion of alcohol that is consumed by individual mice as a fraction of total liquid intake. Furthermore, the large differences in mean intake values observed between B6, in particular, and other inbred strains suggests that this behavioral system might be amenable to QTL analysis.

All earlier attempts to map alcohol preference QTLs made use of the BXD set of 26 recombinant inbred (RI) strains[18–20]. A major advantage of the RI approach is that quantitative trait expression values for each RI genotype can be replicated in an unlimited number of animals. The major disadvantage is the

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Table 1 Alcohol consumption in parental strains

Genotype	Both sexes[a] (*n*)	Males (*n*)	Females (*n*)	Possible sex difference[b]
DBA	0.07 ± 0.03 (40)	0.06 ± 0.02 (16)	0.07 ± 0.03 (24)	n.s
(B6 × DBA) F1	0.23 ± 0.17 (126)	0.21 ± 0.16 (60)	0.25 ± 0.18 (66)	n.s.
B6	0.77 ± 0.14 (68)	0.72 ± 0.18 (32)[c]	0.81 ± 0.10 (36)[c]	$P = 0.02$

[a]Results are expressed as mean values ± S.D. (with number of trials in each group). The outcome of each trial was treated as a separate value in the calculation of population means and standard deviations.
[b]The *t*-test was used to test for significance between mean values observed for subpopulations separated according to sex.
[c]Based on the ascertainment of normality, extreme outlying values (5 from the male distribution and 3 from the female distribution) were excluded from the calculations of mean and variance for the B6 subpopulations only.

small number of genotypes available for analysis. Unfortunately, with the analysis of complex traits controlled by two or more loci, the BXD RI set is unlikely to provide sufficient statistical power for mapping QTLs[21]. So far, numerous provisional alcohol preference QTLs have been suggested by RI analysis[18–20,22], but none are statistically significant according to a newly recommended set of QTL threshold values[23].

In our study, we used a two generation outcross-backcross breeding protocol to generate several hundred second generation animals that we tested for alcohol consumption. Phenotypic analysis of the whole N2 population was followed by selective genotyping of extreme alcohol preferring animals. Markers from candidate regions identified by this approach were typed across the whole N2 population. Our results have led to the mapping of two B6 alcohol preference QTLs symbolized *Alcp1* and *Alcp2*.

Alcohol consumption

The first step in our analysis was a determination of alcohol consumption values for the parental populations of B6, DBA, and (B6 × DBA)F1 mice (by convention we place the maternal strain to the left of the cross sign, and the paternal strain to the right). Our experimental assay was based on a two-bottle choice test with three separate 3-day trials performed over a nine day period. Alcohol consumption values (*a*) are expressed as the proportion of 10% ethanol consumed as a fraction of total liquid intake[24]. With all three parental populations as well as subpopulations segregated according to sex, no significant or large differences (>0.06) in mean consumption values were observed in any within-population trial-to-trial comparison. On the basis of this result, we chose to treat each trial value as a separate and independent sample for the purposes of further analysis. The normality of each distribution was examined by the method of ranked normal deviates.

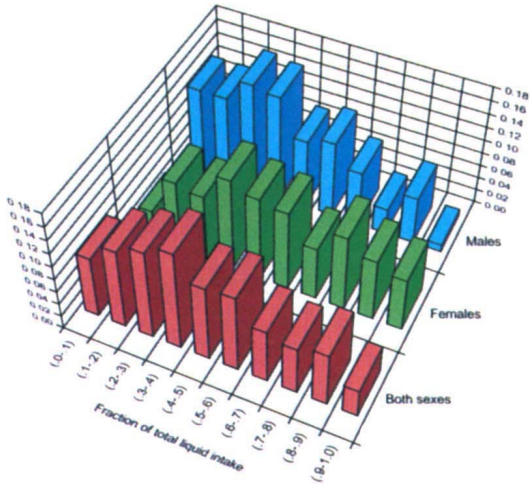

Fig. 1 Relative distributions of alcohol consumption values for the complete N2 population (335 animals), and the separate female and male N2 subpopulations (with 151 and 184 animals respectively).

As anticipated from results obtained by other investigators, DBA mice uniformly avoided alcohol consumption (Table 1). Both male and female subpopulations exhibited approximately normal distributions with very low levels of variance. The low levels of alcohol consumption measured with these animals could be accounted for by initial tasting at each drinking phase or evaporation from the opening of each sipper tube.

Also as anticipated, B6 mice exhibited a high degree of alcohol preference. The distributions obtained with both male and female B6 subpopulations are approximately normal with a higher degree of variance (Table 1). The third population — (B6 × DBA)F1 animals — exhibited a mean level of alcohol consumption that is intermediate to that observed with the two parental inbred strains. The F1 consumption level is significantly different from that observed with either the DBA or B6 populations ($P < 0.001$ in both cases), but is within the range observed previously for most other inbred strains of mice that drink 'in moderation'[12,15,16]. Unlike the inbred distributions, both male and female F1 distributions are clearly non-normal with a strong skewing toward low-end values and long upper-end tail.

The simplest interpretation of these results is that the extreme alcohol avoidance expressed by DBA and the extreme alcohol preference expressed by B6 are independent traits controlled by different genetic pathways that both act in a recessive manner to wild type. According to this interpretation, F1 animals would carry wild-type B6 alleles that are dominant to the alcohol avoidance DBA genes, as well as wild-type DBA alleles that are dominant to alcohol preference B6 genes. This interpretation is supported by evidence from human studies where it has been shown that at least some forms of alcohol avoidance are due to abnormal levels of enzymes that metabolize alcohol or its breakdown products, such as alcohol dehydrogenase or aldehyde dehydrogenase[25,26], whereas alcohol abuse shows no associations with different levels of these enzymes[27]. Nevertheless, one cannot rule out more complicated models of genetic transmission for either alcohol preference or avoidance in mice.

As reported previously by others[24,28], a significant sex-specific difference in alcohol consumption is observed with B6 animals ($P = 0.02$, Table 1) but not with DBA or F1 animals. This result suggests that a preference for alcohol, rather than consumption levels *per se*, may be controlled by different genetic mechanisms in males and females. The data provide estimates for the broad heritability of alcohol preference in males and females as 62% and 79% respectively.

Table 2 Alcohol consumption of N2 animals grouped by sex and cross

Cross	Both sexes[a] (*n*)	Males (*n*)	Females (*n*)	Significance[b]
Both crosses	0.43 ± 0.25 (335)	0.38 ± 0.24 (184)	0.50 ± 0.26 (151)	$P = 0.00004$
(B6 × DBA) × B6	0.43 ± 0.26 (262)	0.38 ± 0.24 (147)	0.49 ± 0.27 (115)	$P = 0.0008$
B6 × (B6 × DBA)	0.46 ± 0.24 (73)	0.39 ± 0.23 (37)	0.53 ± 0.24 (36)	$P = 0.017$
Significance[c]	n.s.	n.s.	n.s.	

[a]Mean values are shown for each population or subpopulation ± S.D., (with the number of animals in each group).
[b]The *t*-test was used to test for significance between mean values observed for subpopulations separated according to sex.
[c]The *t*-test was used to test for significance between mean values observed for subpopulations separated according to cross type.

Alcohol consumption in backcross mice

If the expression of a polygenic trait is controlled entirely by recessive alleles that are all present in one of the two parental strains chosen for analysis, the outcross-backcross breeding protocol is more efficient than the outcross-intercross protocol for mapping the trait loci[21]. As indicated above, we began our mapping studies with a working hypothesis that the extreme alcohol preference trait expressed by B6 mice is fully recessive to the moderate alcohol consumption phenotype expressed by (B6 x DBA)F1 hybrids. Accordingly, we set up a backcross between F1 and B6 parents to obtain an N2 population of animals for QTL analysis. This mapping approach has the further advantage of eliminating any potential interference in trait expression that might be caused by recessive DBA alleles responsible for alcohol avoidance. The only disadvantage to the backcross approach is that it would fail to elicit map positions for any B6 QTLs that act in a strictly dominant manner, if such loci exist.

A serious problem that is often encountered in the analysis of complex behavioral traits is the lack of replicability in measurements of trait expression for some individual animals[18]. Non-replicability in alcohol consumption measurements could be caused by errors in measurement (such as dripping bottles) or experimental setup (for example, unintended retention of washing residues in one bottle but not the other). But, it is also possible that some animals may simply express different preference levels within different trials due to subtle differences in environmental cues or to the effects of prior exposure to alcohol in an earlier trial. Although we did not find significant effects of prior alcohol exposure in our parental populations, we can not rule out the possibility that such effects might occur in a genotype-specific manner within a subset of the animals from the heterogenous N2 population. With the RI approach, non-replicability due to experimental error, environmental variation, or trial-to-trial variation can be measured and minimized by combining expression values obtained from large numbers of animals within each strain to obtain a single mean value for each genotype[29,19,28]. Unfortunately, with a two-generation breeding protocol, each animal must be treated as a separate sample.

Since our goal was to uncover genetic, rather than environmental, factors that affect alcohol consumption, we made a decision at the outset to eliminate measurements that were clearly non-replicable irrespective of their root cause. We assessed replicability by subjecting each animal to three consecutive 3-day trials. The B6-defined standard deviation (see Methods) was used as a tool for measuring within-animal replicability. Aberrant single trial measurements were identified and eliminated from the calculation of individual animal mean values. A small number (3) of N2 animals showed no replicability among all three trials, and these animals were eliminated entirely from the analysis. This method of adjustment is validated by its implementation in all experiments prior to knowledge of genotypic information.

Reciprocal crosses between B6 and (B6 × DBA) F1 parents were set up and 6–12 week old N2 animals were assayed individually for alcohol consumption. After the elimination of animals classified as inconsistent in trait expression, a total of 335 animals (262 from the [(B6 × DBA) × B6] cross and 73 from the [B6 × (B6 × DBA)] cross) were retained for further studies. The distribution of alcohol consumption values (a) for the N2 population is abnormal, as expected for a polygenic trait controlled by a small number of genes. The full distribution shows a clustering of values at the low end of the range and a long tail through the high end (Fig. 1).

When male and female subpopulations are considered separately, it can be seen that the low-end clustering is due primarily to male samples. In contrast, the female distribution is more symmetric about its mean value of 0.50 but is platykurtic rather than normal in shape. The difference between male and female N2 consumption levels is highly significant ($P = 0.00004$). This sex-specific difference is maintained at a significant level even when each backcross population is considered independently (Table 2). In both N2 subpopulations, as well as the parental B6 population, females consume more alcohol, on average, than males. The observed paucity of N2 males with high levels of alcohol preference, considered together with the sex-specific differences observed in both mean values and trait distributions, suggest the possibility that alcohol preference in male and female animals might be controlled by non-identical sets of QTLs. This suggestion has been made previously based on a significant departure from complete covariance in alcohol consumption levels measured by sex in the BXD RI strain set[28]. Further support comes from studies of alcoholism in humans where there is strong evidence for a difference in the contribution of genetic factors to trait expression in men and women[30].

Genotypic analysis of backcross mice

Our approach to mapping alcohol preference (ALCP) QTLs was divided into two phases. In the first phase, we employed a selective genotyping strategy in which only highly preferring animals ($a_i > 0.70$) were typed at microsatellite loci distributed across the genome at intervals of approximately 30 cM. Selected groups of male and female animals were considered separately based on the strong *a priori* evidence for sex specificity in genetic mechanisms. The χ^2 test was used to identify marker loci that show a significant departure from the 1:1 ratio expected for B/B and B/D genotypes in the absence of linkage. Five unlinked chromosomal regions with potential ALCP QTLs ($P < 0.05$) were uncovered by this approach.

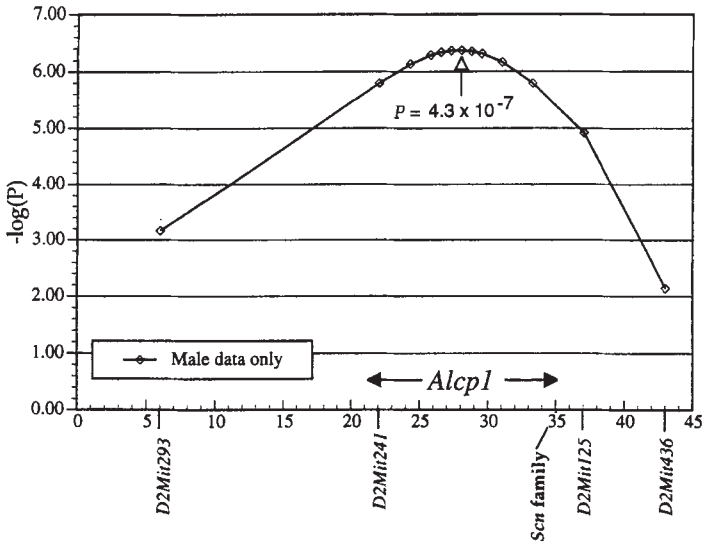

Fig. 2 Localization of the *Alcp1* locus on chromosome 2. *P* values associated with individual microsatellite loci (*D2Mit293*, *D2Mit241*, *D2Mit125*, *D2Mit436*) were determined by the *t*-test. *P* values for chromosomal points between marker loci were determined by simple regression. The results across 37 cM of chromosome 2 are presented in $-(\log_{10})$ converted form. The 90% confidence interval for the location of *Alcp1* is indicated with the double arrowed line. *Scn* family refers to the cluster of seven sodium channel alpha-subunit genes that are expressed in the brain. The cM scale along the X axis starts at the centromere; distances between marker loci are based on the results of this study.

In the second phase of our mapping strategy, marker loci from each of these chromosomal regions were genotyped across the complete set of N2 animals. Further statistical tests for linkage were confined to subpopulations segregated by sex. This analysis led to the identification of two sex-restricted ALCP QTLs. A male-specific locus (*Alcp1*) was identified on chromosome 2, and a female-specific locus (*Alcp2*) was identified on chromosome 11.

A male-specific alcohol preference QTL

Selective genotyping results obtained with the male cohort indicated the likely presence of an ALCP QTL linked to the marker locus *D2Mit241* on chromosome 2. The *t*-test was used to compare alcohol consumption levels between all N2 males typed directly with B/B or B/D genotypes at this locus. The mean consumption value obtained for the B/D genotype is 0.29 and for the B/B genotype, it is 0.45. Nearly identical mean values were obtained for each of these genotypes in male N2 subpopulations divided according to type of cross (data not shown). The difference between these means is highly significant with a *P* value of 1.1×10^{-5}. This result demonstrates the presence of a B6-associated alcohol preference locus — which we name Alcohol Preference-1 (*Alcp1*) — linked to *D2Mit241*.

The *t*-test was also used to determine whether *Alcp1* influences alcohol consumption in female mice. The mean consumption value of female mice with a B/D genotype, 0.52, is actually higher than the consumption value of females with a B/B genotype, 0.48, but this is not a significant difference ($P = 0.33$). This result demonstrates that the effect of *Alcp1* on alcohol preference is restricted to male animals.

Interval mapping of *Alcp1* was performed by simple regression (Fig. 2). The most likely position for *Alcp1* is at fractional distance of 0.4 between the DNA markers *D2Mit241* and *D2Mit125*. At this location, the *P* value obtained for significance by linear regression is 4.3×10^{-7}. The allelic difference at the *Alcp1* locus accounts for 14% of the total variance, and 22.5% of the genetic component of variance in the male N2 subpopulation (based on a male heritability factor of 62%).

A female-specific alcohol preference QTL

Selective genotyping results obtained with the female cohort indicated the likely presence of an ALCP QTL linked to the marker locus *D11Mit195* on chromosome 11. A *t*-test was used to compare alcohol consumption levels between all N2 females typed directly with B/B or B/D genotypes at this locus. The mean consumption value obtained for the B/D genotype is 0.40 and for the B/B genotype, it is 0.56. The *P* value obtained for a difference between these means is 2.3×10^{-4}, which falls just above the newly recommended threshold value of 1.0×10^{-4} for significant evidence of linkage based on the backcross protocol[23]. However, when N2 female subpopulations are segregated according to cross type and considered separately, a *P* value of 4.1×10^{-5} is obtained for animals with a B6 father, while the *P* value for animals with a B6 mother is 0.91 (Table 3). For a *post hoc* division of data in this manner, the threshold of significance must be re-adjusted downward by a factor of two to $P = 5 \times 10^{-5}$.

Table 3 *Alcp2* effects on alcohol consumption by N2 animals grouped by cross

Cross	*Alcp2* genotype (mat./pat.)[a]	Alcohol consumption	Significance within cross	Significance between crosses[b]	X chromosome genotype[c]
B6 × (B6 × DBA)	B/B	0.54 ± 0.20 (11)	$P = 0.94$	$P = 0.73$	B/B
B6 × (B6 × DBA)	B/D	0.54 ± 0.31 (20)		$P = 0.74$	B/B
(B6 × DBA) × B6	B/B	0.56 ± 0.26 (67)	$P = 8.5 \times 10^{-6}$		B/B or D/B
(B6 × DBA) × B6	D/B	0.33 ± 0.21 (37)			B/B or D/B

[a]Only animals that are non-recombinant between *D11Mit219* and *D11Mit195* are included in this analysis. Genotype: (maternally derived allele (mat.)/paternally derived allele (pat.)).
[b]The *t*-test was used to test for a significant difference between mean values observed for each marker genotype from the B6 × (B6 × DBA) cross and the mean value obtained for the B/B marker genotype from the (B6 × DBA) × B6 cross.
[c]All female offspring of the [B6 × (B6 × DBA)] cross are obligatory B6 homozygous for the entire X chromosome. In contrast, B6 and DBA alleles of X-chromosome genes will segregate equally into female offspring from the [(B6 × DBA) × B6] cross. Thus, one could explain the observed cross-specific difference by postulating the existence of an X-linked ALCP locus with complementary action to *Alcp2*. According to this hypothesis, dominant DBA alleles at *Alcp2* and the postulated X-linked ALCP locus are both required to suppress alcohol preference; females that are homozygous B/B at either or both loci will exhibit the same degree of alcohol preference. Therefore, suppression of the alcohol preference trait would only be expected in the subset of N2 females with a B6 father and a D/B genotype at *Alcp1* that also carried a D/B genotype at the postulated X-linked ALCP locus. A genomic scan of the X chromosome in this class of N2 animals rules out the existence of such a hypothetical locus with a high degree of confidence.

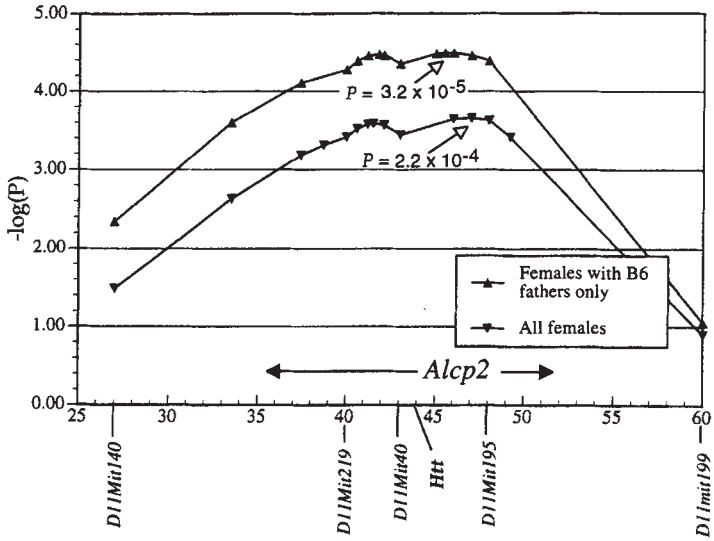

Fig. 3 Localization of the *Alcp2* locus on chromosome 11. *P* value calculations and data display format are the same as described in the Fig. 2 legend. Two separate curves are shown for the complete female data set and for the subset of females with B6 fathers only. The *Htt* locus encodes the serotonin transporter.

Based on this threshold level, we demonstrated the existence of a second alcohol preference QTL, which we name Alcohol Preference-2 (*Alcp2*).

A *t*-test was also used to determine whether *Alcp2* influences alcohol consumption in male mice. The mean consumption value for male mice with a B/D genotype at *D11Mit195* is 0.36, and for a B/B genotype, it is 0.40; the difference between these means is not significant ($P = 0.37$). Together, the results obtained with *D11Mit195* suggest that the *Alcp2* locus operates only on female animals, and only on those with a B6 father. A possible explanation for this unusual finding is discussed below.

Interval mapping of *Alcp2* by simple regression was performed (Fig. 3) for both the whole female population and the subpopulation with a B6 father. The lowest *P* value obtained for the whole female population is 2.2×10^{-4}, and for the subpopulation with a B6 father, it is 3.2×10^{-5}. Both curves show nearly identical peaks on either side of the marker locus *D11Mit40*. Thus, for the purposes of determining the contribution of *Alcp1* to variance in trait expression, we considered only animals that were non-recombinant across the entire region between *D11Mit219* and *D11Mit195*. This analysis shows that the allelic difference at *Alcp2* accounts for 18% of the total variance and 23% of the genetic variance observed in the female subpopulation with a B6 father (based on a female heritability factor of 79%).

Discussion

We have identified two loci with significant effects on the consumption of alcohol by mice in the presence of non-limiting amounts of water and food. The *Alcp1* locus maps to chromosome 2 in a region that shows conserved synteny with the human chromosomal regions 9q32–q34 and 2q14–q37. The *Alcp2* locus maps to chromosome 11 in a region that shows conserved synteny with the human chromosomal region 17q21. *Alcp1* may, or may not, represent one of the numerous provisional QTLs suggested by analysis of alcohol preference in the BXD RI strain set, however, *Alcp2* has not been detected previously[22,28,20]. Each of the two loci identified in this study affects alcohol consumption in only one sex. The *Alcp1* locus acts only on males and the *Alcp2* locus acts only on females. Each of these loci accounts for approximately 23% of the total genetic variance observed between B6 and (B6 × DBA)F1 hybrid animals. Although we have not identified any other major ALCP QTLs, such loci might be detected with a higher resolution genomic scan. Evidence in support of separate genetic mechanisms that incur a predisposition to alcohol abuse by males and females was obtained by Cloninger and colleagues in a large Swedish study of adopted men and women[31]. The results of this 1981 study demonstrate patterns of patrilineal inheritance in men and matrilineal inheritance in women as one would predict if separate genes were responsible for this predisposition in each sex.

We have observed parent-of-origin effects for the *Alcp2* locus. Our linkage analysis was based on a backcross between the (B6 × DBA)F1 hybrid and the B6 parental strain. However, one set of backcross animals was obtained by mating a B6 mother with an F1 father, and the second set of animals was obtained with the reciprocal mating between an F1 mother and a B6 father. While *Alcp1* appears to act upon all N2 males without regard to the sex of the B6 backcross parent, *Alcp2* only exerts an effect on females with a B6 father (Table 3). Furthermore, when one examines the mean values of alcohol consumption exhibited by females with each *Alcp2* genotype — B/D and B/B — from each cross, it can be seen that three of the four groups of animals are indistinguishable from each (Table 3). Only animals with a B/D genotype and a B6 father exhibit reduced levels of consumption. This observation suggests that animals from this class alone are missing a genetic factor for alcohol preference that is present in the other three classes.

A current understanding of mammalian genetics allows two possible explanations for this result. The first is based on the cross-specific difference in X chromosome genotypes inherited by female N2 animals (Table 3). This difference can be used to postulate the existence of an X-linked ALCP locus with complementary activity to *Alcp2* (Table 3). Results obtained from a marker scan of the X chromosome allow us to rule out this possibility with a high degree of confidence (data not shown).

The second explanation is based on genomic imprinting of the *Alcp2* locus. To account for the data obtained, one can postulate the existence of imprinting at the paternal copy of *Alcp2*, with the only functional copy of *Alcp2* being the one inherited from the mother. The *Alcp2* locus maps to a region that overlaps with one previously shown to contain one or more imprinted loci[32]. A prediction of the imprinting hypothesis is that the genotype of the *Alcp2* locus should have no effect on alcohol consumption by N2 females with a B6 mother, as observed (Table 3). Furthermore, the mean consumption level of all females from this cross should be indistinguishable from that of females with a B6 father and a B/B genotype at *Alcp2*, as observed (Table 3). The only animals without

a maternally derived B6 allele at *Alcp2* are offspring from the (B6 × DBA) × B6 cross with a D/B genotype, and it is only these animals that exhibit a reduced level of alcohol consumption.

We might also expect to observe higher alcohol consumption levels for F1 females from a (B6 × DBA) cross than from a (DBA × B6) cross because only the former would have a maternally derived B6 allele at *Alcp2*. However, the relatively low level of alcohol consumption actually exhibited by (B6 × DBA)F1 females (Table 1) would appear to indicate that the activity of *Alcp2* alone is not sufficient to induce alcohol preference, and that additional recessive B6 alleles at one or another *ALCP* loci are required to facilitate the action of *Alcp2* observed in the N2 population. Additional genetic studies will be necessary to confirm or refute the hypothesis of genomic imprinting at the *Alcp2* locus. However, it is interesting to note that recent evidence has been obtained for the involvement of genomic imprinting in the transmission of another behavioral trait — biopolar affective disorder — through human pedigrees[33].

A family of brain-expressed sodium channel alpha-subunit genes (*Scn1a–Scn7a*) maps to the furthest limit of the region containing *Alcp1* (Fig. 2) and is therefore a potential candidate for this QTL. A more suggestive candidate gene, *Htt*, is found directly in the center of the region defined by *Alcp2* (Fig. 3)[34]. The *Htt* gene encodes the serotonin transporter responsible for the re-uptake of the neurotransmitter into presynaptic terminals. A role for serotonin in the aetiology of alcoholism is suggested by studies in humans and animals[35,17]. These studies showed that higher levels of serotonin are associated with lower levels of alcohol consumption and, conversely, lower levels of serotonin are associated with higher levels of alcohol consumption. Most interestingly, Rausch *et al.*[36] have shown that a higher maximum velocity for the uptake of serotonin may act to reduce its availability to receptors on postsynaptic neurons, which could have the same effect as reducing the level of the neurotransmitter directly. In this way, alternative alleles at *Htt* could clearly have an impact on alcohol preference, however, direct functional studies will be required to test this hypothesis.

Various investigators have noted the fact that B6 mice prefer multiple euphoria-producing drugs, including opiates and cocaine as well as alcohol, to a much greater extent than most other inbred strains including DBA[37]. This observation has led to the hypothesis that B6 mice have a genetic predisposition to drug-mediated euphoria[14]. If this hypothesis were correct, one would expect to identify the same QTLs in (B6 × DBA) crosses that were analysed for preferences for different euphoria-producing drugs. In an analysis of a (B6 × DBA) intercross[14] a B6-dominant or semi-dominant morphine preference QTL was identified, with a lod score of 20, on chromosome 10 near the marker *D10Mit28*; no QTLs were found on either chromosome 2 or 11. In contrast, we tested *D10Mit28* directly in our studies and found no evidence of any effect on alcohol consumption levels in either male or female animals (data not shown). This negative result has been confirmed by Berrettini and colleagues (personal communication). Taken together, the results of our study and those of Berrettini and colleagues suggest that alcohol preference and morphine preference are genetically distinct traits expressed by B6 mice and not by DBA or most other inbred mouse strains. Why the B6 mouse has acquired independent preferences for multiple addictive and euphoria-producing substances remains a mystery.

Methods

Measurement of alcohol consumption. C57BL/6J (B6), DBA/2J (DBA) and (B6 × DBA)F1 (BXD) animals were purchased from the Jackson Laboratory. N2 animals were bred at Princeton University. Alcohol consumption was measured for individually housed animals over a nine-day period, with three sequential three-day trials. In all trials, animals were provided with a choice of two outwardly identical bottles — one containing tap water and the other containing a 10% ethanol solution (v/v) in tap water. The relative positions of the bottles on each cage were reversed after each trial to control for potential position effects; no such effects were observed. The 'bottle' consisted of a 30 ml Corex tube topped by a #3 one hole stopper implanted with a 2 inch ball-less sipper tube. Bottles and drippings were weighed at the beginning and end of each three-day trial. Alcohol consumption was recorded individually for each trial as the fraction of total liquid intake derived from the bottle containing 10% ethanol.

Phenotypic analysis and tests for consistency. The heritability of alcohol preference was calculated independently for male and female animals based on the complete, uncorrected data sets obtained with B6 and F1 animals. Heritability was calculated as (1–U/V), where U is the pooled variance measured within each of the two parental populations, and V represents the variance between the mean values obtained for each parental population.

A consistency test was performed on each set of three values obtained for each N2 animal. We chose a metric of two-standard-deviations — which represents 95% of a normal distribution — to evaluate consistency. When all three values were located within two sex-specific B6-standard deviations (SD_{B6f} or SD_{B6m}) of each other, they were averaged together for a single animal value; this classification accounted for 54% of the N2 females and 83% of the N2 males. When the three values were not within $2 \times SD_{B6}$ of each other, the two values closest to each other were used to calculate the animal mean, so long as both of these values were within $2 \times SD_{B6}$ of each other. Of the 338 N2 animals originally tested, three (all females) were eliminated entirely from the analysis because no single value was within $2 \times SD_{B6}$ of the other two values. This method of adjustment was implemented in all analyses prior to knowledge of genotypic information.

Genotypic analysis. Genomic DNA was prepared from animal tissues according to standard protocols. Microsatellite markers[38] spaced at distances of approximately 30 cM across the genome were chosen for genotypic analysis. Primers were purchased from Research Genetics and PCR was performed as indicated by the manufacturer[38]. When possible, markers were selected with large differences in size between B6 and DBA products so that typing could be performed by ethidium bromide staining. When necessary, markers with product size differences of less than eight basepairs were analysed with ^{32}P-labelled primers and product was separated by electrophoresis on denaturing gels.

Linkage analysis. All data input and analysis was performed with the Microsoft Excel software package on the Macintosh computer. In the first phase of the analysis, the number of highly preferring animals with B/D versus B/B genotypes at each marker were compared and tested for a significant departure from a 1:1 ratio with the $\chi 2$ test. This analysis allowed the identification of markers linked to potential QTLs. In the second phase of the analysis, these and neighboring markers were typed across the entire N2 population, and alcohol consump-

tion means associated with alternative marker genotypes were compared by *t*-test[39]. Interval mapping was performed by linear regression[40]. The linear regression approach has the same statistical power as the more commonly used maximum likelihood estimation approach for identifying and localizing QTLs, but offers a great advantage in computational speed for simple crosses between inbred animals. The contribution to total variance exerted by individual QTLs was measured as the proportion of the total variance in linear regression that is not accounted for by the residual variance; the values obtained are likely to underestimate the true contributions of each QTL[41].

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