# Strategies for Identifying Genes Underlying Drug Abuse Susceptibility

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## INTRODUCTION

It is becoming widely accepted that most, if not all, drug responses are subject to influence by genetic factors. The magnitude, duration, and even the quality or direction of response can differ among individuals with differing genotypes. Most drug responses are not simple genetic traits; rather, multiple genes can be demonstrated to influence a given response. In addition, any gene is likely to affect multiple traits, a condition called pleiotropism. To the extent that two traits are pleiotropically influenced by some of the same genes, they are said to be genetically correlated. Use of genetic animal models has achieved significant progress in the analysis of drug response traits.

The goal of this chapter is to discuss two related methods for tracing the pathways from genes to drug-related behaviors. An example of one method, Quantitative Trait Locus (QTL) gene mapping, will demonstrate that a gene has been identified that appears to affect the severity of withdrawal from multiple drugs. The utility of this method for extrapolating animal model findings to humans will be discussed.

## STUDIES WITH PANELS OF INBRED STRAINS

One of the classic methods of pharmacogenetics depends upon the analysis of inbred strains. In a seminal report, McClearn and Rodgers (1959) reported that inbred mouse strains differed markedly in their consumption of alcohol solutions. Because an inbred strain is created by mating close genetic relatives for many generations, all mice of the same sex within an inbred strain are virtually identical genetically. There are more than 100 inbred strains of mice commercially available. If individuals from several inbred strains are tested for a response in carefully controlled environ-mental conditions, differences among strain mean values are due to genetic differences, while pooled individual differences within strains are by definition

nongenetic or environmental (see figure 1). Therefore, partitioning variance in responses leads to a direct estimate of the proportion of trait variability due to genetic influences, technically known as the heritability (Falconer 1989).

Comparisons of multiple strains on multiple traits can then be used to estimate genetic correlations. Statistically, this is accomplished by partitioning trait covariance into among-strain and within-strain components (Falconer 1989). An experiment testing two strains for two traits is uninformative: multiple strains are required before correlations can be estimated with any degree of confidence. How many strains should be examined depends on the genetic structure of the traits examined, which is usually not known. One estimate of the number suggested in practice as sufficient to detect genetic correlations of modest degree with modest statistical power is 7 to 10 strains (Crabbe et al. 1990).

In one example of this approach, mice from 15 inbred strains were tested for the severity of acute withdrawal following intraperitoneal (IP) injection of 4 grams per kilogram (g/kg) ethanol by assessing the magnitude of their handling-induced convulsions over several hours. The same mice were tested 1 to 2 weeks later for withdrawal severity after 60milligrams per kg (mg/kg) pentobarbital. Strains differed significantly in withdrawal from both drugs. Figure 2 shows the genetic correlation resulting from the analysis of mean strain responses on the two traits. The significant corre-lation seen suggests that about half the genetic variability in each response is shared, and is presumably due to the pleiotropic influence of some genes on both responses (Metten and Crabbe 1994). Significant genetic correlations between ethanol and nitrous oxide withdrawal severity have also been reported in a similar analysis of a set of recombinant inbred strains (Belknap et al. 1993a). Studies with mice selectively bred to express severe (withdrawal seizure prone, WSP) or mild (withdrawal seizure resistant, WSR) ethanol withdrawal handling-induced convulsions have revealed that they differ in severity of withdrawal from diazepam, abecarnil, pentobarbital, phenobarbital, nitrous oxide, several other alcohols, and acetaldehyde. WSP mice have more severe acute or chronic withdrawal than WSR mice from all the above agents (Belknap et al. 1987, 1988, 1989; Crabbe et al. 1991). Together, these results suggest that some genes affect withdrawal from several different central nervous system depressant agents.

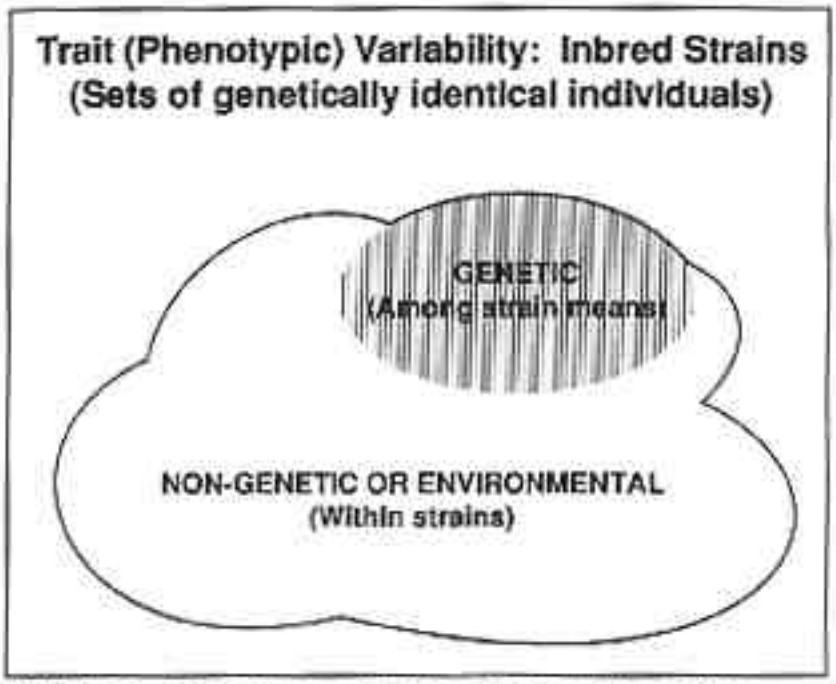


**FIGURE 1.** *Schematic representation of the partition of drug-response variability in a study employing several inbred strains tested under controlled environmental conditions.*

A large literature documenting strain differences in response to virtually all abused drugs has accumulated over the years. A major advantage of inbred strains is their genetic stability, so studies performed 10 years ago can be directly compared with those employing the same strains performed last week. Strain differences in drug responses have been reviewed (Broadhurst 1978; Crabbe and Harris 1991).

A systematic attempt to determine patterns of genetic commonality has recently been undertaken in Portland. Over a 4-year period, 15 inbred strains of mice were studied after administration of saline or 1 of 4 doses of morphine (Belknap et al., submitted), ethanol (Crabbe et al. 1994a), diazepam (Gallaher et al., submitted), or pentobarbital (Crabbe et al., in process). To the extent possible, the same battery of tests was employed. All drugs were tested for their effects on open-field activity and body temperature; in addition, preference drinking of different concentrations of the drugs in tap water, and subsequently in saccharine solutions, was

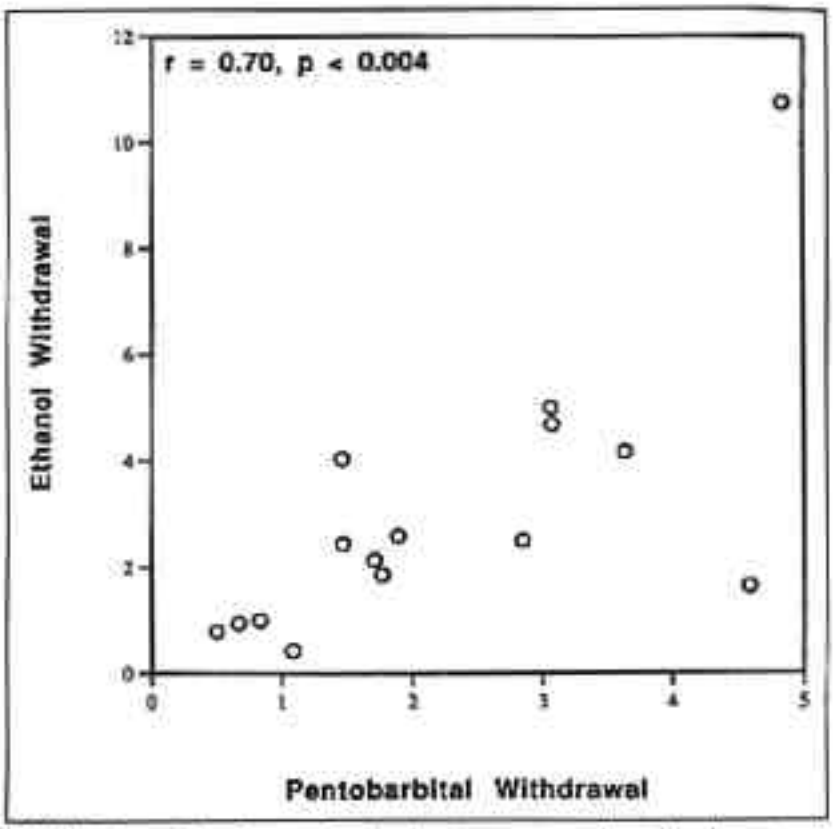


**FIGURE 2.** *Genetic correlation between acute ethanol and acute pentobarbital withdrawal severity in 15 standard inbred strains of mice. Area under the withdrawal curve for each strain on each measure is plotted as a circle.*

SOURCE: From Metten and Crabbe 1994 with permission.

determined (Belknap et al. 1993b, 1993c; Belknap et al., in process). Other drug-specific responses were also examined (e.g.,loss of righting reflex after ethanol and pentobarbital).

An example of results from these studies is given in figure 3. It can be seen that strains differed markedly in the sensitivity to the locomotor stimulant effects of IP morphine. Not only were there quantitative

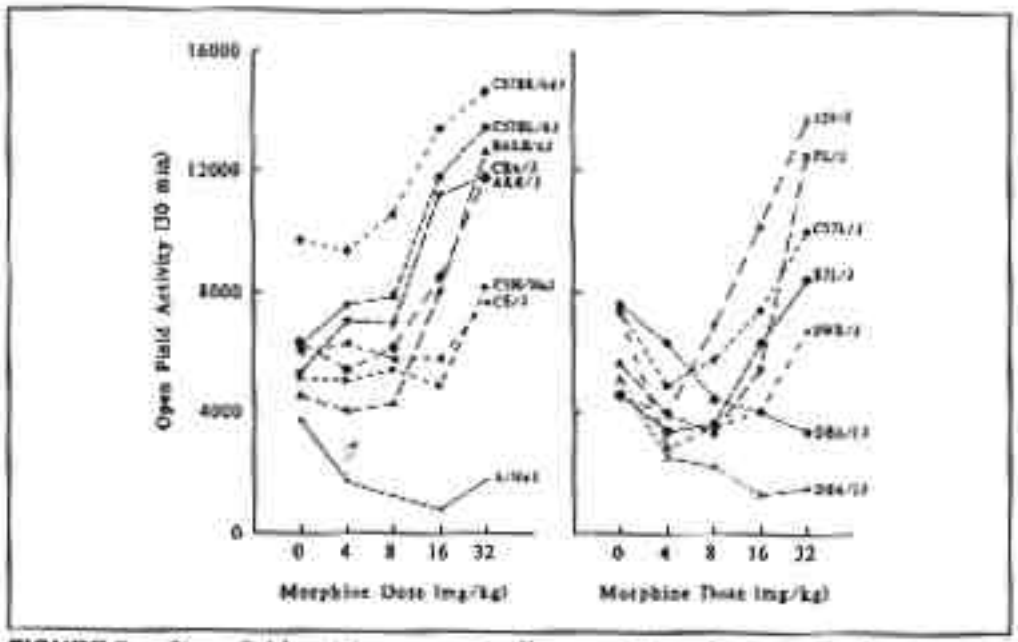


**FIGURE 3.** *Open-field activity automatically recorded as distance traveled (in cm) 30 min following injection of indicated dose of morphine or saline. Strains are divided into two groups for clarity. Each symbol represents the mean value for the indicated dose and strain. N = 24 to 41 mice per strain, approximately equally divided across doses.*

SOURCE: Data from Belknap et al., submitted.

differences in response magnitude, but qualitative differences could be seen: the A/HeJ, DBA/1J, and DBA/2J strains responded with reduced activity at all morphine doses. Similar qualitative strain differences in morphine-stimulated activity have been known for many years (Shuster et al. 1975). Strain differences of the magnitude shown in figure 3 were detected for virtually all responses to all drugs.

Two traits were tested for all four of the drugs examined. To identify common patterns of genetic influence on these traits, a multidimensional scaling (MDS) analysis was performed (Kruskal 1964; Kruskal and Wish 1978). Mean locomotor activity, monitored for 15 minutes, and the mean change in body temperature 30 minutes after injection were calculated for each strain. For each variable, strain mean values for the saline groups were subtracted from the drug-dosage group means. A (genetic) correlation matrix among strain means was calculated and subjected to least squares MDS. The results of this analysis are shown in figure 4. The resulting plot shows the position of each drug dose response relative to all others, mapped by its weighting on the two principal dimensions identified by the MDS analysis. Linear distance represents similarity among the plotted variables in the pattern of inbred strain (genetic) differences. Points close together share a great deal of common genetic influence (i.e., are genetically correlated), while points far apart do not. The numbers near each point identify the dose in milligrams per kilogram or grams per kilogram of EtOH. The two primary dimensions (linear components) mapped accounted for 85 percent of the total genetic variance in this data set.

Several results of this analysis can be identified. Different doses of the same drug on a given response showed a substantial degree of common genetic influence; this was to be expected. It also appeared that both activity and temperature responses to morphine were genetically related at all four doses. This cluster of responses was reasonably distinct from other clusters of variables/doses/drugs. In addition, most doses of diaze-pam and pentobarbital (for both activity and temperature responses), and the activating effects of moderate doses of ethanol, were grouped in a common cluster. All three of these drugs influence gamma aminobutyric acid A (GABAA) receptor-gated chloride ion conductance (Harris and Allan 1989; Macdonald and Olsen 1994). Therefore, modulation of this receptor complex is a possible common mechanism for the mediation of the genetic similarity of this response cluster. It is of interest that ethanol effects on body temperature were genetically distinct from ethanol-induced activity responses. It seems reasonable to suggest that ethanol

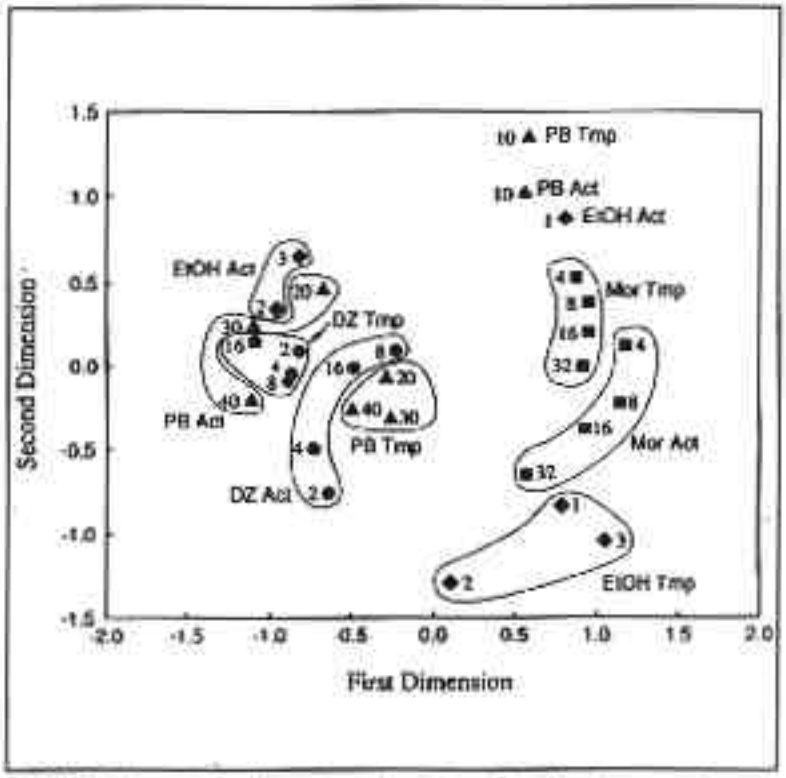


**FIGURE 4.** *Multidimensional scaling plot showing genetic similarity among responses to given doses of ethanol (EtOH), diazepam (DZ), pentobarbital (PB), or morphine (Mor). Perimeters are drawn to emphasize doses from a single drug. Responses displayed are locomotor activity in an open field (Act) or change in body temperature (Tmp).*

KEY: ♦= EtOH; ●= diazepam; ■= morphine; and ▲= pentobarbital.

SOURCE: Reprinted from Crabbe et al. 1994*b*, with permission. Copyright 1994 American Association for the Advancement of Science.

hypothermia is not likely to be influenced to a major degree by the genetically determined mechanisms underlying pentobarbital and diaze-pam hypothermia. Finally, the effects of the lowest doses of ethanol and pentobarbital on activity appeared to be genetically distinct from the higher doses, and are similar to the effects of the lowest dose of pentobarbital on body temperature.

This is only one example of the application of multivariate methods to the analysis of genetic similarity among drugs and responses. There is an accumulating wealth of knowledge about drug responses in a few specified genotypes. It would be of interest to know, for example, whether the several drug responses hypothesized to index drug reward, intravenous (IV) self-administration, preference drinking, place-conditioned and taste-conditioned responses, and forward locomotion and its sensitization with repeated administration of drug shared a common genetic influence. Extension of multiple-strain studies to include neurochemical and neuro-pharmacological responses would be of utility in identifying common mechanisms of drug action.

## QTL GENE MAPPING

Traditional analyses of drug sensitivity using genetic animal models such as those described above are able to estimate degree of genetic influence, and to identify response clusters of genetic codetermination. Many studies not reviewed above have also employed genetic animal models in cases where identified candidate genes are implicated in a given drug effect. (See Crabbe and Harris 1991 and Crabbe et al. 1994b for reviews.) For most drug responses, no clear candidate gene is indicated, and several questions remain to be addressed. It would be useful to determine how many genes influence the trait, what their function is, and where they are located in the genome. Traits affected by multiple genes usually display a continuous degree of response (rather than all-or-none responses), and the genes responsible are referred to as QTLs. Although each QTL may have only a modest effect on the drug response, collectively, the QTLs can determine a major proportion of drug responsiveness.

The recently developed technique of QTL gene mapping (Paterson et al. 1988) can be employed to identify the chromosomal location of QTLs. QTL mapping depends upon linkage, or the tendency of genes physically close to one another, to be inherited as a unit.

Unlinked genes, in con-trast, tend to be inherited independently according to the principles of segregation and independent assortment. Discussions of this method have recently been published (CrabbeandBelknap 1992; Lander and Botstein 1989; Plomin and McClearn 1993; Tanksley 1993).

Most pharmacogenetic research with gene mapping has employed recombinant inbred (RI) strains derived from the cross of C57BL/6J (B6) and DBA/2J (D2) inbred mouse strains. This panel of strains was developed by inbreeding from the F2 population derived from B6XD2 hybrid mice. More than 20 BXD RI strains exist; each represents the more or less random shuffling of the genetic deck for all genes where B6 and D2 mice possess different alleles. Each RI strain is like any other inbred strain in that all its members are homozygous for each gene and are identical to each other. Furthermore, each RI strain can only have inherited either the B6 or the D2 allele (for any gene where the B6 and D2 progenitor strains had the same allele, all BXD RI strains also have only that allele).

The use of RI strains for gene mapping was first recognized by Taylor (1978) and Bailey (1981). The BXD RI panel of strains is powerful for gene mapping because it comprises a substantial number of strains that have been genotyped for more than 1,500 marker loci that are poly-morphic in the B6 and D2 progenitors. Once the utility of restriction fragment length polymorphisms (RFLPs) for gene mapping was clearly recognized (Lander and Botstein 1989), many RFLPs were identified in the BXD RI strains. More recently, markers based on polymerase chain reaction (PCR) amplification of deoxyribonucleic acid (DNA) micro-satellite simple sequence repeats (SSRs) have been typed in great numbers in the BXD and other RI strains (Deitrich et al. 1992). Plomin and colleagues (1991) first applied QTL analysis to behavioral responses in BXD RI mice.

One method for identifying and mapping QTLs is to adopt a two-stage procedure (Belknap et al. 1993a). An example of a QTL analysis for alcohol withdrawal severity will be given to illustrate the procedure. For the first stage, a database was established comprising the allelic status of each RI strain at each mapped marker locus. For a given marker, any RI strain possessing B6 alleles was given a value of 0, and any RI strain possessing D2 alleles a score of 1. The RI strains were then tested for acute ethanol withdrawal and each strain's mean response determined. Ethanol withdrawal severity (the quantitative trait) was then correlated with allelic status for each marker gene in the database.

Six groups of markers on four chromosomes were significantly associated with ethanol withdrawal severity (Belknap et al. 1993a). A similar analysis was performed for nitrous oxide withdrawal severity. The locations of the putative QTLs identified in these analyses are given in figure 5. Each such associated chromosomal region suggests the presence of a QTL affecting withdrawal severity. In this first stage of the analysis, it is undesirable to reject any potential QTL as unassociated; therefore, a relatively weak criterion of significance ($p < 0.05$) was adopted. However, because of the large number of correlations calculated, some QTLs detected during this initial stage will represent false-positive (chance) associations (Belknap 1992; see also other articles in the same issue for methodological considerations and alternative strategies). Therefore, it was necessary to verify the putative QTLs detected with further analyses.

In the second stage of testing, a QTL associated with both ethanol and nitrous oxide withdrawal severity was selected for further study. This apparent QTL appeared to account for as much as 48percent of the genetic variability in ethanol withdrawal severity; the markers most highly associated ($r = 0.69$, $p < 0.001$) between allelic status and withdrawal severity for 19 BXD RI strains were D2Mit9 and Scn2a, which mapped to a position 37 centiMorgans (cM) from the centromere of chromosome 2 (Silver et al. 1993). If a QTL in this region were actually associated with withdrawal severity, then allelic status of individual mice at markers in this region should also predict withdrawal severity. To verify this, approxi-mately 150 F2 mice derived from crosses of C57BL/6J and DBA/2J mice were tested for ethanol withdrawal severity. They were then genotyped for six nearby SSR markers using PCR.

Figure 6 shows how individual F2 mice display segregation and independent assortment at a polymorphic marker, and the codominant inheritance of heterozygotes. For informative markers, about one-half of the F2 mice were homozygous for the B6 (or D2) allele and one-half were heterozygotes. Ethanol withdrawal severity was significantly associated with allelic status only at the marker D2Mit9, but not at other more distant markers (Buck et al., in process). Figure 7 shows that the pattern of the withdrawal response in F2 mice that were B6B6 homozygotes at D2Mit9 was significantly less pronounced and of shorter duration than that in F2 mice that were D2D2 homozygotes (Buck et al., in process).

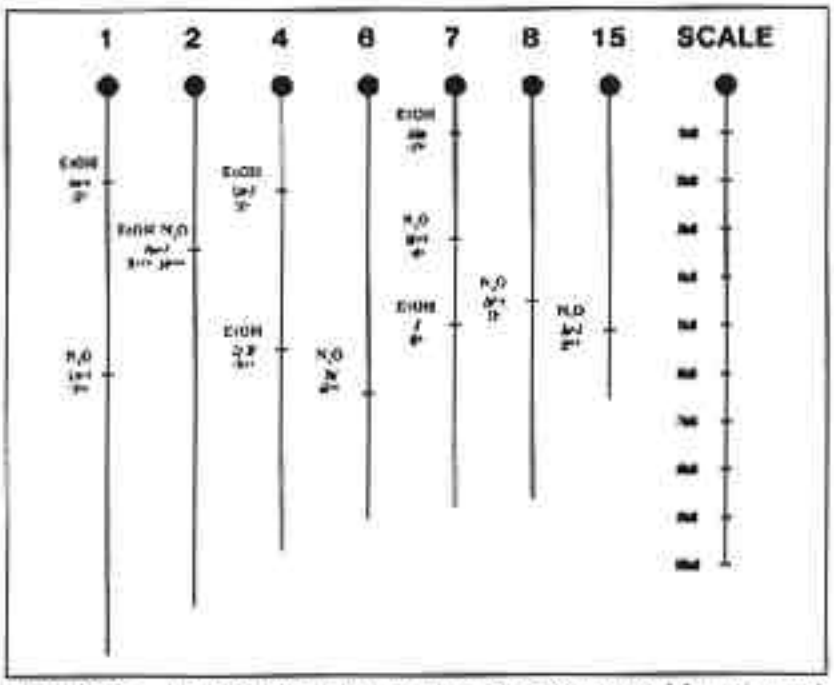


**FIGURE 5.** *Schematic representation of chromosomal locations of putative QTLs influencing acute ethanol (EtOH) or nitrous oxide ($N_2O$) withdrawal severity, determined by analysis of BXD RI strains. The most highly significant marker in each cluster of associated markers is given, along with the correlation coefficient and significance level.*

KEY: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

SOURCE: Data are from Belknap et al. 1993a. Markers are shown in their approximate location according to Silver et al. 1993, and chromosomes are approximately to scale.

This region of mouse chromosome 2 is syntenic with human 2q24-q37, which suggests that there is likely to be an analogous gene in humans. The great degree of homology between mouse and human chromosome maps (Copeland et al. 1993) is one of the most attractive features of QTL

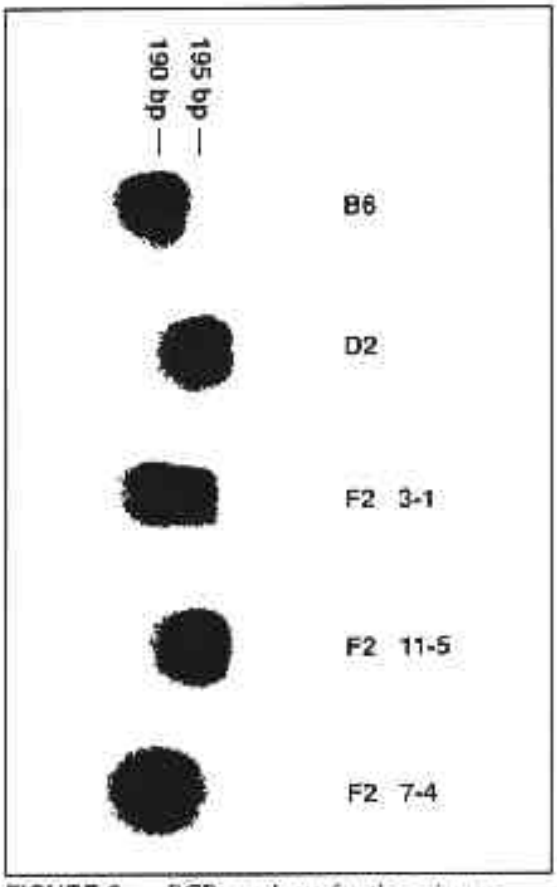


**FIGURE 6.** *PCR products for the primers amplifying the polymorphism at the marker D2Mit9. Inbred B6 mice have a 190 base pair (bp) PCR product, while inbred D2 mice have a 195 bp product. Three F2 mice from B6XD2 F1 crosses are shown. Of the 150 mice genotyped for D2Mit9, one quarter resembled F2 7-4 and were homozygous B6 at this locus; one quarter resembled F2 11-5 and were D2 homozygotes; about one half resembled F2 3-1 and were heterozygotes that displayed both products.*

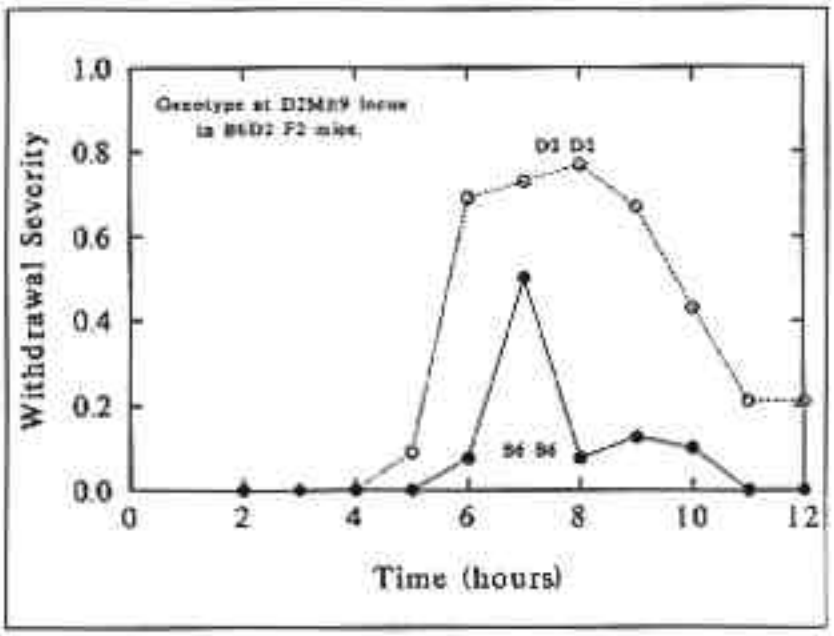


**FIGURE 7.** *Timecourse for ethanol withdrawal severity in B6D2 $F_2$ mice. Handling-induced convulsion scores during ethanol withdrawal in mice homozygous for the D2 allele at D2Mit9 (open circles) were markedly increased in magnitude and duration versus mice homozygous for the B6 allele (closed circles). Scores shown as increases over preinjection baseline differed significantly in the two groups ($p < 0.01$, two-tailed). In the first 4 hr after ethanol administration, convulsions were suppressed in both groups. As alcohol was metabolized and eliminated, withdrawal scores increased above baseline, reaching a peak after 6-7 hr.*

SOURCE: Data from Buck et al., in process.

mapping in mice: any map site that can be verified in mice can likely be extrapolated to a human map site. In addition, there are plausible candidate genes near D2Mit9. Gad-1 codes for glutamic acid decarboxy-lase, an enzyme catalyzing synthesis of GABA, and maps to very nearly the same location as D2Mit9. A cluster of genes coding for a-subunits of brain voltage-dependent sodium channels (Scn1a, Scn2a, and Scn3a), which influence the action potential in

excitable cells including neurons, also map nearby. Experiments are underway to determine whether functional differences in these candidate genes influence drug withdrawal severity.

## FUTURE DIRECTIONS

Several interesting approaches are made possible by the QTL mapping approach. QTL maps for one drug response trait can obviously be compared with those for another (see figure 5). A recent example was able to identify several QTLs influencing both acute ethanol hypothermia and the development of tolerance to this effect (Crabbe et al. 1994c). In addition, a growing number of potential candidate genes are now mapped in mouse (e.g., neurotransmitter receptors and transporters, synthetic and metabolic enzymes, ion channels). A composite map for multiple drug traits can reveal genomic regions where presumed QTLs affecting multiple responses are located. Combining QTL maps for drug response traits and potential candidate genes can reveal associations across biological levels of analysis (e.g., behavior with candidate gene). A recent preliminary attempt to construct such a composite map compared QTLs from more than 20 responses to ethanol and multiple responses to methamphetamine, morphine, nitrous oxide, and other drugs (Crabbe et al. 1994b). In that analysis, several responses to alcohol, morphine, methamphetamine, and haloperidol all were significantly associated with a region spanning 27 to 31 cM on chromosome 9. This outcome is interesting because the gene encoding the dopamine D2 receptor gene (Drd2) has been mapped to mouse chromosome 9, 27 cM from the telomere. Another region of interest was on chromosome 6, where a putative QTL influencing six methamphetamine responses was identified.

At this Technical Review, the existence of a transgenic mouse absent 5-hydroxytryptamine 1B (5-HT1B) receptors was demonstrated (Hen, this volume). The gene encoding this receptor (Htr1b) has been mapped to chromosome 9 at 40cM, near the dilute coat color locus at 42 cM. The composite QTL map mentioned above detected associations of two methamphetamine, three morphine, and three ethanol responses with markers in this region. One of these traits was methamphetamine-induced hypophagia (Angeli-Gade, unpublished data). Hypophagia is known to be induced by stimulation of 5-HT1B receptors (Kitchener and Dourish 1994). It would be of interest to see whether the 5-HT1B knockout mice differed in the drug responses suggested by the QTL associations. It should be noted that nearly all

the QTL analyses collected on the composite map described are preliminary and represent only first-stage (BXD RI) associations; each will need to be verified in a segregating genetic population. Nonetheless, the rapid advances in gene mapping in the area of pharmacogenetics make the prospect of such studies exciting.

Other avenues are also opened by the QTL approach. Using the infor-mation derived from PCR genotyping of individual mice for markers flanking a QTL of interest, it will be possible to breed mice selectively for the genotype of interest (Plomin and McClearn 1993). This approach in distinct from the usual practice of selective breeding based on phenotypes, where the underlying responsible genes remain anonymous. With selection based on genotype, individuals possessing several risk-promoting QTLs could be generated and studied for their drug responses. A similar approach would be to produce congenic lines by backcrossing individuals possessing selected genotypes to an appropriate background strain (Bailey 1981). For example, F1 mice possessing DBA/2J alleles promoting high pentobarbital withdrawal could be repeatedly backcrossed with low-risk C57BL/6J mice. After many generations of selection and backcrossing, a congenic strain will be produced, which possesses C57BL/6J genotype at all genes except for a very small genomic region surrounding the QTL of interest. This approach is already proving feasible using phenotypic markers for selection of alcohol drinking (Dudek and Underwood 1993).

The potential advantage of congenic selected lines is in their utility for subsequent applications with molecular biological methods. Even once a QTL of interest has been identified, it remains a daunting task to find the (relatively) nearby candidate gene that is actually influencing the trait. It has recently been demonstrated that the technique of representational difference analysis (RDA) can be used to identify polymorphisms that can be amplified by PCR, through comparison of two closely related DNA samples (Lisitsyn et al. 1994). Genetically directed RDA has revealed numerous previously unidentified polymorphisms closely targeted to the region of interest. If such a region were defined by the difference between a normal C57BL/6J and a selected congenic C57BL/6J strain possessing only a small segment of DNA containing DBA/2J alleles leading to high pentobarbital withdrawal, genetically directed RDA might eventually increase the QTL map precision to about 200 kilobases (0.2 cM), or about 0.05 percent of the mouse genome. This might make positional cloning of the nearby gene feasible.

## CONCLUSIONS

Pharmacogenetic analyses of drug sensitivity have been fruitful in identifying the important influence of often anonymous genes on virtually all drug response traits. Genetic animal models are now being employed using procedures designed to detect the influence of QTLs on drug responses and to identify their chromosomal location. The rapid identification of such QTLs, and the emerging pattern of influence of drug-related QTLs, show promise as a strategy for linking mouse and human genetic determinants of susceptibility to abuse drugs.

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