Animal Genetic Studies on Alcoholism

Vulnerability to alcohol dependence and abuse is partly determined by genes. Numerous studies of twins and their families and of different racial/ethnic groups have confirmed this link (Ferguson and Goldberg 1997), but much work remains to be done to identify these genes and understand their role in alcohol abuse and dependence.

Some diseases, such as cystic fibrosis and Huntington's disease, are the result of a change in a single gene. No single gene is responsible for alcohol abuse and dependence, however. Many genes that play roles in a variety of normal human behaviors and sensory perception are involved. Research has not yet pinpointed specific genes that "predispose" a person to alcohol abuse or dependence. Once researchers know the genes and the proteins these genes encode, they will have potent targets for the exploration of the biochemical processes that underlie the response to alcohol.

Identifying all the genes involved is a project of enormous magnitude and difficulty, because of the size of the human genome and the complexity of the behaviors involved in abusive drinking and dependence. The human genome (the sum total of genes carried by each person) consists of approximately 100,000 genes located on 23 pairs of chromosomes. Each gene produces a different protein, and each protein has a specific role in chemical processes in the body that shape how people look, think, feel, and behave. The Human Genome Project (HGP) (supported in the United States by the National Institutes of Health and the U.S. Department of Energy) has been an important impetus to the search for genes related to alcohol behavior. HGP researchers are working toward the goal of identifying every gene and the protein it encodes and mapping each gene to a precise location (locus) on one of the chromosomes. This research is providing the tools with which scientists can investigate the genetic underpinnings of a range of human disorders and conditions, including alcohol abuse and dependence. For example, accurate locations have been determined for thousands of the 80,000 to 100,000 genes in both the human and mouse genomes. As gene mapping progresses, investigators use the knowledge about the locations of these "marker" genes to localize other genes in relation to them. All genetic mapping using rodents, for example, relies heavily on research that has identified genetic markers spanning the entire genome of the mouse and rat (Bihoreau et al. 1997; Dietrich et al. 1994).

Quantitative Trait Loci

If alcohol preference were a single-gene trait, the identity of the gene would conceivably be known by now. Researchers would have discovered that alcohol-dependent mice consistently share a limited (though large) number of marker genes. Because the genetic maps of the mouse and rat are densely covered with known markers, it would then be a relatively simple experimental problem to systematically narrow the search to a single chromosomal region. At that point, the region would be small enough that all the genes in this region could be individually examined for alterations that cause different strains of mice or rats to have differences in alcohol preference.

However, vulnerability to alcohol dependence in humans and alcohol preference in animals (along with many other behavioral responses to alcohol) are complex behaviors that are determined by multiple genes. Such traits are known as multigenic or quantitative traits. Rather than being simply present or absent, such traits are expressed along a spectrum from high to low. Moreover, many genes play a role in contributing to such traits. A technique developed in recent years for conducting the search for genes influencing such traits is called quantitative trait locus (QTL) mapping (Lander and Botstein 1989; Tanksley 1993).

QTL mapping analysis provides a means of locating and measuring the effects of a single QTL on a trait, or phenotype. The markers allow identification of probable locations of genes that influence alcohol-related behaviors. These locations can then be verified using other tests, and specific genes can be sought there (Grisel and Crabbe 1995).

Mapping of a gene—assigning it a position relative to existing markers on a chromosome—is based on the concept of linkage: genes that are close together on the chromosome are more likely to be inherited together than are two genes farther apart. Linkage reflects the fact that when the deoxyribonucleic acid (DNA) strands that constitute paternal and maternal chromosomes recombine after fertilization, a piece of DNA on one chromosome is exchanged for its counterpart on the paired chromosome. The result is a chromosome that contains some maternal genes and some paternal genes. The greater the distance between two genes on the chromosome, the less likely that both genes are from one parent. (Genes that are located on different chromosomes are inherited independently of each other.)

It is important to note that genetic effects related to alcohol that have been shown in animal studies, while certainly detectable and significant, are nonetheless relatively small in magnitude. The variation in an alcohol-related behavior or trait that can be accounted for by the underlying gene or genes (heritability) is almost always less than 40 percent. Thus, even in studies with animal models, in which the environment can be rigidly controlled, a large part of the variation in the behavior is apparently not controlled by genes. Genetic differences between human individuals are so extensive that the genes involved in alcoholism may vary from individual to individual. These factors emphasize the need to view alcohol abuse and dependence as both biologically and environmentally determined.

Creating Rodent Models

Animal genetics researchers use a variety of approaches to selectively breed mice and rats that display alcohol-related traits or behaviors (phenotypes) similar to those of humans. Examples of these phenotypes are alcohol preference, sensitivity to alcohol's hypnotic (sleep-inducing) effects, hypothermia (lowered body temperature) after alcohol ingestion, and behavioral activation (mice that become highly active after drinking are believed to model alcohol's euphoric effects) (Crabbe 1989; Crabbe et al. 1994*a*,*b*). Finding specific genes associated with drinking in animals should provide clues to the genetic underpinnings of alcohol's reinforcing properties, a key to its addictive potential, and insight into individual differences in sensitivity to alcohol's effects. It is known from studies in humans that abnormally low sensitivity to alcohol's effects predicts greater risk for alcoholism later in life (Schuckit 1994).

Because humans and rodents share most of their genes and because these genes produce proteins involved in identical physical processes in both species, the results of animal genetic studies can provide insights into human genetics. Studies of animal genetics are useful because of fundamental limitations in human genetic studies. Researchers cannot manipulate the genomes of human subjects by breeding them in a laboratory or causing mutations in or otherwise manipulating their genes. Neither can they control all the variables in a person's environment. The genetic blueprint of each human subject—except for those of identical twins—is unique, as are each person's background and experiences.

In contrast, laboratory researchers can control the mating of mice and rats over many generations and thereby produce strains of animals in which individuals in each strain are genetically identical. Furthermore, researchers can control the environments of the animals: what they eat, their lifetime access to alcohol, the amount of light they receive, the number of other animals they interact with. Because of the high degree of the animals' genetic similarity and the extent of environmental control, researchers can attribute the differences in an alcohol-related behavior between two genetically dissimilar animal strains to differences in their genetic makeup.

Many researchers use mice from recombinant-inbred (RI) strains, especially mice from the BXD series, which contains 25 different strains. The series was created by crossing two "parental" mouse strains (C57BL/6J and DBA/2J) that are genetically distinct and differ from each other phenotypically in many ways, including many traits related to alcohol action. Next the researchers "inbred" many different pairs of offspring (brother-sister mating), which resulted in different strains of mice. Each mouse within a strain is genetically identical to every other mouse in that strain, but between any two of these strains only 50 percent of their genes are shared—the same amount that human siblings share. Thus, the different alcohol-related traits observed in the parents were "sorted" into individual animals and then "fixed" genetically.

Much of the research described below has narrowed the search for genes responsible for observed phenotypic differences in rodents to possible regions on the chromosomes. Only a few studies have used techniques that allow researchers to say with a high degree of certainty that these regions are the actual sites of the genes and not "red herrings" created by imperfections in their mapping methods. In still fewer studies have researchers concluded that they are very likely at the site of the gene. The researchers in these studies worked largely with BXD mice and with other inbred mice known as LS x SS (LS, or "long-sleep," crossed with SS, or "short-sleep") strains. (LS mice are much more sensitive to the sedative effect of alcohol than SS mice are.) In some cases, RI mice from different strains differ in their preferences for alcohol. When offered two bottles of water, one bottle with plain water and one with alcohol mixed with water, a mouse from one RI strain will display preference for the alcohol-water mix, while a mouse from another RI strain will strongly avoid it, and a mouse from yet another strain will have an intermediate preference. The task for researchers then becomes to look for differences in the genetic makeup of these RI strains that might account for some of the differences in their alcohol preferences.

Some researchers work with types of mice other than RI mice. As will be explained below, doing so allows them be more certain about the locations of the alcohol-related genes they map, but at a much greater investment of effort. One type of mice they use is F_2 mice, which is the "grandchild" of a cross between two parents whose offspring are then crossed (sibling mating). The F_2 share only 50 percent of their genes with each other or with either parent. Like the RI mice, individual F_2 mice vary along the spectrum of alcohol seeking or avoidance, for example. However, each F_2 mouse has its own individual genetic profile. Researchers who work with mice other than those from RI strains have the extra task of genotyping each mouse. That is, they must sample the DNA of each mouse to generate a genetic profile.

Quantitative Trait Loci Mapping

Statistical Methods

Statistical methods play a large role in QTL mapping. They are used to measure the degree of association between a marker and the phenotype to determine the magnitude of the effect (effect size) of the QTL on the phenotype and to assess the statistical significance of the observed association between the marker(s) and the QTL (that is, to estimate the probability that the association is real and has not occurred by chance). If the QTL is close to the marker and has a large effect, then detection and mapping can be performed easily and accurately using simple methods such as regression analysis (McClearn et al. 1991). If the QTL is not close to the marker gene, the simplest statistical tests will result in a lower effect size being attributed to the QTL. A variety of methods exists for assessing the statistical significance of the observed associations. A more complex and statistically optimal method than regression analysis is "interval mapping" (Haley and Knott 1992; Lander and Botstein 1989; Markel et al. 1996). Interval mapping uses two adjacent markers, rather than a single one. The two markers block out an interval on the chromosome, and interval mapping estimates the most

probable location of the QTL in the interval between markers. More recently, still more sophisticated methods have been developed, which result in more accurate QTL mapping (Jansen and Stam 1994; Zeng 1994).

A major concern in QTL mapping is that in any given attempt to assign a QTL to a location on the chromosome, researchers use many independent statistical tests because, as noted above, they are assessing so many individual associations and effects. Under these conditions, statistical principles require researchers to make appropriate corrections to their results to avoid mistaking a random association between a chromosomal region and a trait for a biologically real one. That is, each independent test has a margin of error, and when many tests are conducted, the cumulative effect of the errors must be accounted for (Lander and Kruglyak 1995; Lander and Schork 1994). Such corrections affect the significance of results—that is, they reduce the level of certainty about whether a QTL is really located at the point on the chromosome indicated by the experiment (Belknap 1992, 1998; Belknap et al. 1996; Lander and Kruglyak 1995; Lander and Schork 1994; Neumann 1992).

Recent Studies of Alcohol-Related QTL's

Since the publication of the *Ninth Report to the U.S. Congress on Alcohol and Health* (National Institute on Alcohol Abuse and Alcoholism 1997), researchers have used the techniques described in the previous sections to identify provisional QTL's for genes involved in a number of alcohol-related phenotypes exhibited by mice. Alcohol preference is a phenotype of particular interest—it is thought to reflect the rewarding properties that are closely related to alcohol's addictive potential. Several studies have mapped provisional QTL's for alcohol preference in RI mice. In one series of experiments, mice were given a simple two-bottle choice of drinking water (one with alcohol and one without) (Rodriguez et al. 1995). Another study used a more sophisticated two-bottle-choice method, varying the amount of alcohol and adding saccharin to the water and to the alcohol-water mix (Phillips et al. 1994). Another way to measure alcohol preference is to train mice to expect to receive a shot of alcohol when they go to a certain location in their cage and to observe whether they seek out that location when placed in the cage (conditioned place preference) (Cunningham 1995).

QTL mapping in rodents uses all of the techniques described above: (1) rodents that differ in genes involved in alcohol-related traits and behaviors are crossed (either RI mice or other types), (2) a number of individual mice are tested for the extent to which they display the phenotype, (3) the pattern of genetic markers in each of these mice is determined, (4) statistical tests are conducted to determine whether any of the variation in the phenotype is significantly associated with any marker, and (5) further statistical tests are performed to determine the extent to which the marker affects the expression, or predicts the variation, of the quantitative trait.

Several studies in RI mice have mapped provisional QTL's for sensitivity to alcohol's effects. Mice that become highly active after ingesting alcohol are thought to model alcohol's euphoric effects, and investigators have examined behavioral activation with low doses of alcohol in a one-time (acute) administration and repeated administration (Phillips et al. 1995, 1996). Another indicator of sensitivity to alcohol is loss of righting reflex, a measure of how long it takes for a mouse to right itself after being placed on its back (Markel et al. 1996; Rodriguez et al. 1995). Other research has looked at rapid development of tolerance to alcohol's effects (Gallaher et al. 1996).

The studies in RI mice show that genes have a significant effect on alcohol-related traits and behaviors. Although many of these provisional QTL's will be subsequently confirmed by more refined studies, an unknown number—probably more than half—will likely be found on further examination to be false positives. Researchers are concerned about a second problem with use of the RI strains in gene mapping—that of false negatives, or missing QTL's that are really there. For these reasons, several researchers have

emphasized that subsequent confirmation of provisional QTL's is a statistical necessity (Gora-Maslak et al. 1991; Johnson et al. 1992). Investigators now combine RI mapping and other approaches to provide the necessary confirmation (Belknap et al. 1997; Bennett et al. 1997; Buck et al. 1997; Dudek and Tritto 1995).

However, few studies using the newer methods have been undertaken, largely because they are labor-intensive. These studies are not conducted with RI mice, because of the low statistical power inherent in their use. The studies rely instead on the analysis of various kinds of offspring from matings between inbred parental strains. These offspring (typically, the F_2 generation) vary extensively from one another, both in their alcohol-related behaviors and in their genetic patterns. Any two mice in such an experiment are as related to each other as two human siblings—in other words, they share 50 percent of their genes on average. Use of such mice involves phenotyping and genotyping each individual mouse. Because some experiments have used more than 1,000 mice (Markel and Corley 1994; Markel et al. 1997), performing the necessary assessment of the individual mice for about 100 marker genes throughout their genomes involves 100,000 individual assays. These procedures are time-consuming and costly. It has been estimated that verification of a QTL using this approach represents 2 to 5 person-years of work, depending on the method, the extent of automation, and other factors. The benefit is that the statistical tests used to detect associations between marker genes and phenotypes and to examine the effect size of the QTL on the phenotype produce results that are considerably more reliable because of the extensive variation in the sample examined (in effect, a sample of 1,000 or more versus a sample of 25 using the BXD RI mice).

One study screened the entire genome for major QTL's that might be involved in alcohol preference, and two were identified (Melo et al. 1996). The two QTL's are gender specific, with *Alcp1* being specific to males and *Alcp2* being specific to females. An important series of studies focused on sensitivity to alcohol's sedative-hypnotic effects as measured by loss of righting reflex (Markel and Corley 1994; Markel et al. 1996, 1997). It is important to note that of 12 provisional QTL's previously found in LS x SS RI mice, only 2 or 3 (*Lore1* and *Lore2* and possibly *Lore5*) were confirmed to be real. However, two of the five major QTL's were detected, which suggests that use of RI's may be a more powerful method for mapping QTL's than pure statistical methods suggest (also see Belknap et al. 1997). Three QTL's for withdrawal have also recently been confirmed (Buck et al. 1997).

Shared Gene Actions

In other QTL mapping applications, investigators are interested in whether two distinct phenomena, such as sensitivity to alcohol's effects and alcohol tolerance, result from the same underlying suite of genes rather than entirely separate QTL's. Because of the large number of diverse alcohol-related behaviors currently being investigated, finding whether some gene actions are shared is an important area for further work.

One study concluded that sensitivity and tolerance are not mediated by common genetic factors (Phillips et al. 1996). In contrast, other researchers have presented evidence suggesting commonality in function between genes for sedative-hypnotic sensitivity to alcohol and genes that specify the distribution and levels of a chemical in the brain, neurotensin, that plays a role in addiction (Erwin et al. 1997). Another study evaluated the relationship between sensitivity and tolerance by using three traits: (1) alcohol-induced hypothermia (lowering of body temperature after alcohol ingestion), (2) ataxia (incoordination) as reflected in the animals' ability to remain balanced on a revolving rod (rotarod), and (3) ataxia as indicated by their ability to negotiate a grid on the floor of their cage without stepping through its holes (Crabbe et al. 1996*b*). These investigators were surprised to learn that most measures were not correlated, which indicated that the traits had different genetic determinants. In general, there appear to be many more cases of different genes determining different measures of alcohol action, with relatively little commonality.

Identification of Genes Underlying a QTL

No one has identified the individual gene responsible for differential alcohol sensitivity in rodent models. A number of candidate genes have been proposed (for example, *Htr1b* [Crabbe et al. 1996*b*]) in alcohol action (Crabbe et al. 1996*a*), but whether any candidate is actually the gene underlying the QTL remains to be demonstrated. It seems almost certain that within the next several years the genes underlying several QTL's for diverse alcohol-related phenotypes will be identified. A variety of tools for fine-scale genetic mapping are now available (see review by Darvasi 1998), and these are already being applied to alcohol-related behaviors in an effort to narrow the region of interest.

Investigating Gene Function

QTL analysis provides a means of locating and measuring the effects of a single QTL on a trait, or phenotype (Grisel and Crabbe 1995). Another goal of genetic research on alcohol is to determine the biochemical mechanisms that underlie the actions of specific genes involved and how genetic variations manifest themselves in the behavior of a living organism. The section "Genetic Studies of Alcohol's Actions on the Brain" in the previous chapter discusses research approaches using genetic engineering techniques in animals.

Studies in Invertebrates

Studies of invertebrate species have shown clearly that alterations in single genes can lead to differential alcohol sensitivity. Both *Drosophila melanogaster* (fruit fly) and *Caenorhabditis elegans* (a nematode, a kind of worm) offer considerable promise for identifying individual genes that are involved in alcohol-related behaviors and traits (reviewed in Diamond and Gordon 1997). A number of mutations that alter sensitivity to anesthesia have been shown to affect alcohol sensitivity in the nematode (Morgan and Sedensky 1995). In a recent study, researchers reported that they had discovered a strain of fruit fly that they labeled "cheap date," because, like some humans, these fruit flies were affected by much lower doses of alcohol than others (Moore et al. 1998). To conduct the study, the researchers created thousands of fruit flies in which genes were randomly "knocked out," or disabled, so that the genetically altered strains were unable to produce proteins encoded by the disabled genes. The fruit flies were put inside a large glass column, and alcohol vapor was pumped in to see which ones were more sensitive to its effects. Fruit flies like to stay near the top of the column, which has mesh landings at different levels. As they became inebriated, the fruit flies in the study fell from landing to landing, most reaching the bottom in 20 minutes. Individuals from the "cheap date" strain, which tumbled to the bottom in 15 minutes, were found to be defective in a gene that is known as "amnesiac," so called because fruit flies without this gene have been shown in other studies to have very poor memories. The amnesiac gene stimulates production of a chemical messenger called cyclic adenosine monophosphate (cAMP), which is involved in many key processes in both fruit flies and humans, including memory and responses to some hormones. The study showed that fruit flies with low levels of cAMP are more sensitive to alcohol (Moore et al. 1998). The results suggest that individual differences in the production of cAMP in certain brain cells may contribute to alcohol sensitivity in humans. Results like these provide valuable knowledge to other researchers looking for ways to prevent and treat alcoholism.

In Closing

The ultimate use of QTL studies is the identification of the genes underlying the QTL's. The cloning of these genes would allow a rapid exploration of the biochemical underpinnings of alcohol action and would link behavioral change to underlying genetic predisposition and biochemical action. Although human alcoholism is likely to result from genetic variations different than those found in rodents, the genes identified in mice are almost certain to have human homologues that are also involved in alcohol action and that may predispose to human alcoholism. Such genes and the proteins they encode are potent targets for intervention, both diagnostic and pharmacologic. It seems certain

that these results will be exploited dramatically in the next century to provide a variety of "designer drugs," perhaps targeted to individual problems associated with particular forms of alcohol abuse. Genetic diagnosis in humans could also be used to suggest particular forms of behavioral intervention well before the manifestation of any alcoholic behavior.

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