Genetic Studies of Alcohol's Actions on the Brain

Evidence from twin, family, and adoption studies has firmly established that genetic factors play a major role in the development of alcohol abuse and alcoholism. It is clear that individuals inherit specific genes that increase or decrease their risk for alcoholism, but the identity and location of those genes remain elusive (National Institute on Alcohol Abuse and Alcoholism 1995).

Researchers involved in the Human Genome Project, a massive effort to map the entire human genome, have developed technologies enabling scientists to identify specific chromosomal regions that are likely to contain genes affecting sensitivity to alcohol (Carr et al. 1998; Crabbe et al. 1994). Research focused on these regions, called quantitative trait loci (QTL), is also discussed elsewhere in the section "Animal Genetic Studies on Alcoholism" in the chapter on genetic and psychosocial influences.

Once the QTL for a trait has been provisionally established, it is possible to search the genome or the location of candidate genes of known function that are likely to influence the alcohol-related trait in question. With use of recently developed molecular biological techniques, several of these candidate genes are currently under investigation. Some of these were nominated by mapping studies (the serotonin 1B gene); others were investigated because historical data implicated them in the alcohol-related response of interest (the gene encoding the γ_{2L} subunit of the subtype A gamma-aminobutyric acid [$GABA_A$] receptor). In one case, both pharmacologic data and mapping data converged to implicate a particular gene, that encoding the α_6 subunit of the $GABA_A$ receptor. This report describes the development of animal models for these genetic investigations, reviews recent research on several candidate genes for alcohol-related traits, and describes studies using immediate early genes (IEG's), a class of genes that can be used to identify which brain structures are first activated by a particular stimulus, including the administration of alcohol.

Development of Animal Models

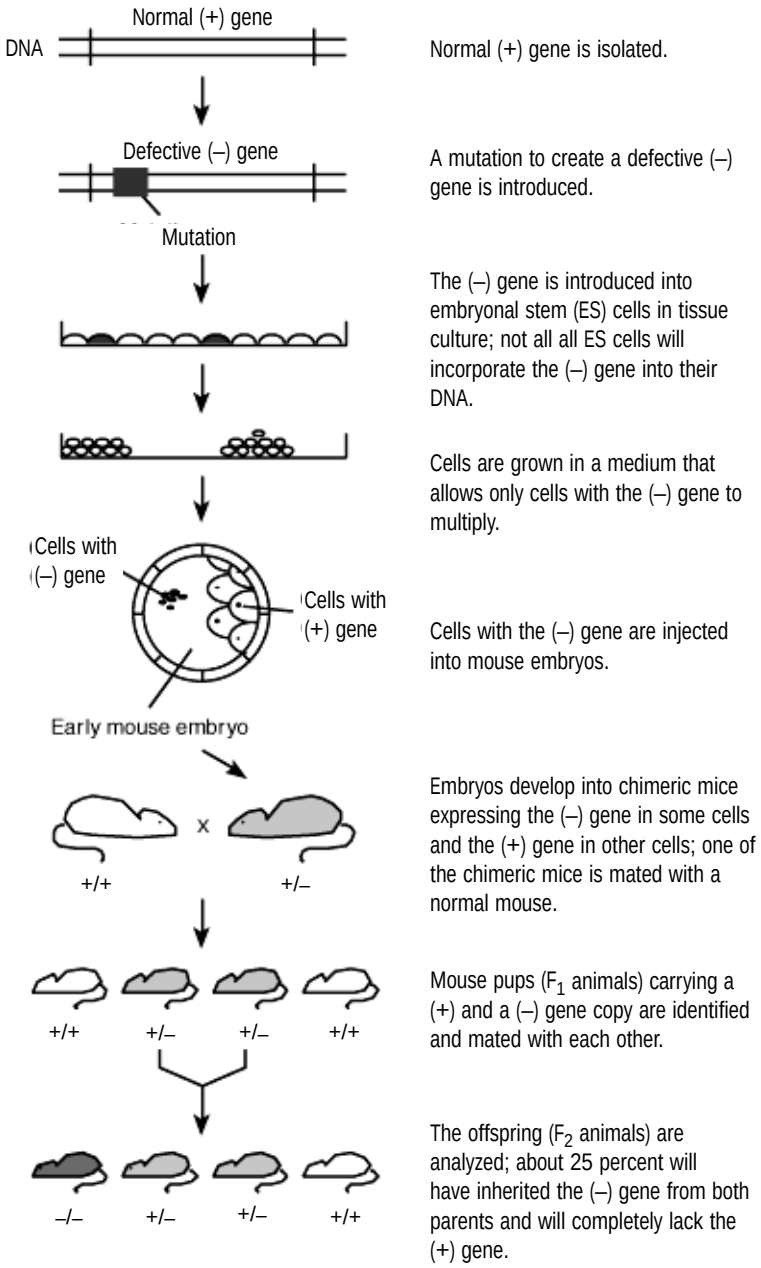
Because the genetic makeup of humans and rodents is similar and their biochemical processes are virtually the same, results of genetic studies with rodents may generally be extrapolated to humans. Investigators have produced many paired strains of rats and mice that are selectively bred to express specific responses to alcohol, such as those that prefer or do not prefer alcohol and those that differ in their susceptibility to alcohol-induced incoordination or loss of righting reflex. (The development of recombinant inbred strains is discussed in the chapter in this report on the etiology of alcoholism.) Recently developed genetic engineering techniques have made it possible to inactivate (knock out) specific genes or to create transgenic mice by inserting foreign genes into the genome. This approach permits investigators to observe whether and how an animal's response to alcohol is altered depending on whether or not the gene is present. Most of the studies described here use strains of knockout or transgenic mice.

Knockout Mice

A powerful approach to analyzing the role of a specific gene in alcohol-related disorders is to inactivate that gene and study the impact of the inactivation on later development and behavior. Through inbreeding and selection, a knockout strain of animals can be produced in which that gene is nonfunctional in every individual animal.

Because two copies of each gene are inherited, one from each parent, it is necessary to inactivate both copies of a given gene. The process of creating a knockout strain requires multiple steps (figure 1) (Homanics and Hiller-Sturmhöfel 1997). Researchers first isolate gene X and transfer it into a short piece of deoxyribonucleic acid (DNA) that is used as a vector. They inactivate the gene, usually by inserting a marker gene that disrupts gene X and also confers a resistance to certain antibiotics. Then they

Figure 1: General procedure for generating knockout mice



Source: Homanics and Hiller-Sturmhöfel 1997.

transfer the vector with the defective gene into embryonic stem cells. These are undifferentiated early-stage embryo cells that can eventually develop into every type of cell in the organism. In some stem cells, the defective gene changes places with its normal counterpart in a process called homologous recombination. The altered stem cells are grown in a medium containing antibiotic, in which only the cells that have successfully incorporated the defective gene can survive. These surviving stem cells are then injected into mouse embryos at an early stage of development, and the embryos are implanted into surrogate mothers. Some of the resulting pups are chimeras; some of their cells contain the mutated gene X, while other cells, derived from the embryo's normal stem cells, contain the normal gene X. It is possible, through easily

identifiable phenotypic characteristics of the parents, such as coat color, to ascertain which pups are the chimeras. Although chimeras do not contain the mutated gene in all of their cells, researchers are able to identify those that carry the altered gene in their reproductive cells by mating the chimeras with normal mice and determining which of their offspring carry the mutated gene. Further inbreeding and selection eventually produce a strain of mice that carry the mutated gene in every cell. These are the knockout mice. In experimental work with knockouts, the normal, or wild-type, mice from which they were derived are used as controls. The two strains differ only in the presence or absence of the knockout gene and are otherwise much like identical twins.

Transgenic Mice

In transgenic mice, a foreign gene is permanently integrated into the animal's DNA. Investigators insert the foreign gene into a vector and then inject the vector into a newly fertilized mouse egg. The fertilized egg contains two pronuclei, one from each parent, and the vector is injected into the larger male pronucleus. The pronuclei fuse to form a single nucleus, carrying chromosomes from both parents, and the fertilized egg then develops into an embryo. In a small percentage of embryos, the foreign gene integrates into one of the embryo's chromosomes. The researchers then implant the embryos into surrogate mothers, identify the pups that are positive for the foreign gene, and mate them with normal mice. As with knockouts, these are mice from the same strain and are identical to the transgenic mice except for the altered gene. Subsequent inbreeding and selection eventually produce a strain of mice that contain the foreign gene in all of its cells.

Investigation of Candidate Genes

As more candidate genes are identified, researchers have begun to use knockout or transgenic strains of mice to determine how these genes affect the development of alcohol-related disorders. This report describes current research using knockout or transgenic technology on five candidate genes for alcohol-related traits.

Serotonin Receptor Genes

Studies of alcohol consumption in animals have implicated the neurotransmitter serotonin (5-HT) (LeMarquand et al. 1994*b*). (See the first section of this chapter, "Setting the Stage: The Structure and Function of Neurons and the Brain," for background on neurotransmitters and cell signaling processes discussed here.) Experiments with rats and mice have suggested that neuronal systems using 5-HT may modulate the degree of development of sensitivity and/or tolerance to alcohol-induced ataxia (physical incoordination) and hypothermia (reduced body temperature) (Lê et al. 1980). Clinical studies have reported lowered brain 5-HT activity in a subgroup of aggressive alcoholics (LeMarquand et al. 1994*a*).

There are more than 15 receptors for 5-HT, and experiments so far have been unable to specify which subtype or subtypes of 5-HT receptors mediate the effects of alcohol. One type of receptor, 5-HT_{1B} in rodents, or 5-HT_{1D} in humans, appears to be particularly interesting. In rodents, receptors generally are presynaptic auto- and heteroreceptors. This means that when a nerve terminal containing these receptors is stimulated to release its own neurotransmitter, 5-HT_{1B} autoreceptors inhibit further release of 5-HT from the terminal; in the case of terminals that release other neurotransmitters, such as gamma-aminobutyric acid (GABA) or dopamine-5-HT heteroreceptors prevent further release of these neurotransmitters. In this way, these receptors reestablish brain homeostasis. Interference with 5-HT_{1B} receptor levels might thus exert a cascade of influences on multiple brain systems through the effects on a number of neurotransmitters. Further, it is also potentially important that 5-HT_{1B} heteroreceptors are located on nerve terminals containing the inhibitory neurotransmitter GABA. These terminals project from the nucleus accumbens to the ventral tegmental area, two brain structures which, with their connecting pathways, are thought to be important in drug reward (Koob 1992).

Researchers have developed a strain of knockout mice lacking the 5-HT_{1B} receptor gene. These mice are highly aggressive; the investigators suggest that 5-HT_{1B} receptors play a role in aggressive behavior (Ramboz et al. 1996; Saudou et al. 1994). Brain slice preparations from some brain areas show changes in 5-HT release, suggesting that serotonergic function may be altered and that other 5-HT receptors besides the 5-HT_{1B} may help regulate 5-HT release (Piñeyro et al. 1995).

QTL mapping studies suggested the presence of a gene influencing alcohol consumption in the midportion of mouse chromosome 9 (Crabbe et al. 1994; Phillips et al. 1994; Rodriguez et al. 1995). Because this chromosome region contains the 5-HT_{1B} receptor gene, researchers characterized 5-HT_{1B} knockout mice and their wild-type controls for several alcohol-related traits. For example, alcohol preference drinking is frequently taken as an index of alcohol's reinforcing, or rewarding, properties. When given a choice between solutions of alcohol or tap water, the knockout animals voluntarily drank twice as much alcohol as the wild-type controls, at concentrations of up to 20 percent (Crabbe et al. 1996). After many days of drinking, the knockouts with the strongest preference for alcohol drank between 16 and 28 grams of alcohol per kilogram of body weight during the last 24 hours of the experiment, a very high level of intake, even though water was freely available. (It should be noted that a subsequent study did not replicate this result in knockouts [Crabbe 1999].)

Two other behavioral assays of reinforcement depend upon mice learning to associate a behavioral response with the presence of alcohol as a stimulus. The 5-HT_{1B} knockout mice were tested for conditioned place preference, an experimental design that pairs one environment with alcohol injections, and another environment with saline injections (Risinger et al. 1996). When these animals were subsequently given a choice between the alcohol- and the saline-paired environments, the knockout mice showed no preference for the alcohol-paired environment while the wild-type controls showed the expected alcohol-conditioned place preference. This finding suggests that the knockouts were less sensitive to the reinforcing effects of alcohol in this test.

A conditioned taste aversion test paired daily drinking of a novel-flavored solution with a subsequent alcohol injection, and the animals gradually developed an aversion for the novel solution. (Although drugs like alcohol have rewarding properties that underlie the behavior of animals in conditioned place preference tests, they also can elicit aversion, as observed in this experiment. The level of sensitivity to both the rewarding and aversive effects of alcohol is thought to be involved in the development of dependence.) However, in this test of alcohol's aversive effects, 5-HT_{1B} knockouts and wild-types were equally sensitive, and both developed a dose-dependent aversion (Risinger et al. 1996).

Results of these experiments highlight the need to cautiously interpret the results of studies with genetically altered animals using behavioral assays. Another study comparing 5-HT_{1B} knockout and wild-type mice used a grid test designed to measure alcohol-induced ataxia. In this test, investigators place mice on a wire mesh floor after an injection of alcohol. Intoxicated mice occasionally slip through the grid floor and a foot makes contact with a metal plate. The 5-HT_{1B} knockout mice were half as sensitive as wild-types to the intoxicating effects of alcohol, and they developed tolerance to a lesser extent after repeated testing (Crabbe et al. 1996). However, in consideration of the variations in response to the reinforcement tests, it would be premature to assume that this apparent behavioral insensitivity of knockouts can be extrapolated to all measures of intoxication, even to other tests designed to measure ataxia.

Additional tests with 5-HT_{1B} knockout mice showed that these mice were more sensitive than wild-types to the locomotor stimulant effects of alcohol (Risinger et al. 1996). Tests measuring the severity of acute and chronic withdrawal showed that the knockouts did not differ significantly from wild-types in locomotion,

indicating that they had acquired the same level of physical dependence (Crabbe et al. 1996).

The studies described above illustrate the wide-ranging effects of manipulating a single gene. The results tend to confirm the role of 5-HT in several different responses to alcohol that are important in alcoholism research. These studies suggest new avenues for future research. For example, the entire course of development of these mice occurred after deletion of the 5-HT_{1B} gene, and the brain must have struggled in unknown ways to compensate for the deletion. The compensations are largely successful, as the knockout mice appear normal, generally behave within normal limits, grow at a normal rate, and breed fairly successfully. Future research should aim at identifying the nature of these compensations, as well as determining the nature of the relationship between the genetic deficit and the proclivity for drinking alcohol.

There is currently a high interest in investigating serotonergic systems because recent studies suggest that a class of drugs known as selective serotonin reuptake inhibitors (SSRI's) may be effective in treating alcoholism (Pettinati 1996). The prototypic SSRI is fluoxetine (Prozac). These drugs act to increase binding of 5-HT by prolonging its availability at the site of its receptors after its release from nerve cells.

GABA Receptor Genes

GABA is the principal inhibitory neurotransmitter in the brain. The GABA receptor, which is embedded in the neuronal cell membrane, is composed of a tightly linked set of five protein subunits arranged to form a channel which, when the receptor is activated, opens to allow the passage of chloride ions into the cell. The influx of negatively charged ions decreases the excitability of the cell. The GABA receptor subtype that appears to be the most sensitive to alcohol is $GABA_A$. Many of alcohol's acute and chronic effects appear to involve its actions on $GABA_A$ receptors (Buck 1996; NIAAA 1997).

The effects of alcohol on $GABA_A$ receptors involve the action of other proteins, such as protein kinase C (PKC). This is an enzyme that phosphorylates (adds phosphate groups to) other proteins, thereby altering their function. Phosphorylation by PKC appears to enhance the sensitivity of the $GABA_A$ receptors to alcohol. The gamma subtype of PKC (PKC-γ) has been implicated in alcohol's effect on GABAergic neurons and on subsequent behavior (Harris et al. 1995). To study this relationship further, researchers created a strain of knockout mice lacking the gene coding for PKC-γ. The knockouts were less sensitive than wild-type controls to alcohol-induced hypothermia and to alcohol-induced loss of righting reflex, both measures of alcohol's sedative effects. However, the knockouts and wild-types were equally sensitive to two other sedative drugs that affect the $GABA_A$ receptor (Harris et al. 1995). These findings suggest that PKC-γ may play an important role in mediating the effects of alcohol on the $GABA_A$ receptor, but that other sedatives appear to affect the receptor through a different mechanism.

There is some evidence that one of the subunits of the $GABA_A$ receptor, α_6, may mediate some of the behavioral effects of alcohol. One QTL mapping study sought to identify chromosomal stretches associated with severity of withdrawal symptoms, that is, regions that were inherited with high frequency along with a tendency toward severe withdrawal symptoms. The investigators found such an association (or linkage) on mouse chromosome 11, near genes coding the α_1, α_6, and γ_2 subunits of $GABA_A$ receptors (Buck et al. 1997). However, this is a large chromosomal region containing other genes, so any conclusions regarding the actions of the α_6 subunit gene would be premature. Researchers developed a strain of mice lacking the α_6 subunit and compared these animals with wild-type controls on several measures of behavioral sensitivity to alcohol, pentobarbital, and general anesthetics (Homanics et al. 1997). They found no significant difference between the two strains,

demonstrating that the α_6 subunit is not a requirement for sensitivity to alcohol's sedative-hypnotic effects. However, as with the 5-HT studies discussed above, interpretation of studies with knockout mice must consider the fact that the genes were absent during neurodevelopment, so functional adaptations in the GABA or other neural systems could have compensated for the knockout. (It should also be noted that in a recent study mice in which the α_1 subunit gene was point mutated, no change was observed in alcohol's potentiating effects [Rudolph et al. 1999].)

Dopamine Receptor Genes

Dopamine, another neurotransmitter, has an important role in locomotor response to rewarding drugs (Koob 1992). There are five known dopamine receptors. Researchers have developed a knockout strain of mice lacking one of them, the D4 receptor, and have tested the response of these mice to a number of psychoactive drugs (Rubenstein et al. 1997). The knockout mice are hypersensitive to the acute locomotor stimulant effects of alcohol, cocaine, and methamphetamine. These animals also had enhanced dopamine function in the dorsal striatum, a brain area associated with locomotion. The authors suggest that this receptor modulates drug-stimulated locomotor behaviors.

There are genetic differences in human D4 receptor types, and certain alleles (alternate forms of a gene) may be associated with risk for alcoholism (Geijer et al. 1997; George et al. 1993; Muramatsu et al. 1996) and risk-taking behavior (Benjamin et al. 1996; Ebstein et al. 1996). The human studies are still controversial, and the association with both alcoholism and novelty seeking has been questioned (Malhotra et al. 1996). Further research with D4 knockout mice may prove important in understanding risk for alcoholism and some of the personality factors that are often associated with alcoholism.

Insulin-Like Growth Factor Genes

Insulin-like growth factor I (IGF-I) plays a critical role during development in the proliferation and differentiation of new cells, including brain cells. Using transgenic technology, researchers recently have developed strains of mice characterized by overexpression of IGF-I or its binding protein, IGF-binding protein 1 (one of several such proteins that bind and modulate the action of IGF-1) (Pucilowski et al. 1996). In tests of alcohol-induced loss of righting reflex, IGF-I transgenics were less sensitive than their wild-type controls, and IGF-binding protein 1 transgenics were more sensitive than their controls. There were no significant differences among the strains in sensitivity to alcohol-induced hypothermia or in alcohol-induced ataxia assessed by performance on a rotating drum. After repeated alcohol administration for 8 days, the control animals developed tolerance to both alcohol-induced hypothermia and loss of righting reflex. The IGF-I transgenic mice did not develop tolerance to either effect, but the IGF-binding protein 1 transgenics developed greater tolerance to both effects than did the controls.

Because IGF-I plays a role in the homeostatic regulation of calcium ions in brain tissue, these investigators have speculated that calcium may play a role in the behavioral differences observed in these experiments. Therefore, specific studies of calcium function in these transgenic strains are needed.

Fyn Tyrosine Kinase Genes

Glutamate is the brain's major excitatory neurotransmitter. Alcohol can inhibit this excitatory action by acting on the glutamate receptors. The *N*-methyl-D-aspartate (NMDA) glutamate receptor, an ion channel receptor, is particularly sensitive to alcohol's effects. Fyn tyrosine kinase is a protein kinase that phosphorylates the NMDA receptors, thereby affecting their function. Researchers have developed knockout mice lacking the *fyn* gene to study the effects of Fyn tyrosine kinase on sensitivity to alcohol (Miyakawa et al. 1996, 1997). This work is discussed in detail in the section "From Cell Membrane to Nucleus: The Effects of Alcohol on Brain Neurons" earlier in this chapter.

Knockout mice offer researchers the opportunity to observe the influence of single genes on aspects of alcohol sensitivity that play a role in the incentives that lead people to drink, in the development of dependence, and in the physiologic effects of alcohol, such as withdrawal. Among the avenues that investigators are pursuing that will further enhance the insights possible from this work are the development of multigene knockouts that will allow observation of the mutual influence of multiple genes, creation of knockouts in which the gene deletion is restricted to a certain tissue region, and development of methods to introduce genes that could compensate for the function lost with a knockout gene.

Immediate Early Genes

Gene Expression

Recent research is increasingly making use of knowledge of gene expression to identify the areas of the brain that are acted upon by alcohol (see the box "From DNA to Protein: How Genetic Information Is Realized" in the first section of this chapter for background on gene expression). These techniques depend on an understanding of the steps of gene expression, the first of which is transcription. This is the process of transferring genetic information within the cell from DNA to ribonucleic acid (RNA), specifically messenger RNA (mRNA). Within the cell nucleus, the genetic code is copied, or transcribed, from one strand of DNA to a complementary strand of mRNA. The mRNA then moves from the nucleus into the cytoplasm, where it first binds to structures called ribosomes and then directs the synthesis of a particular protein in a process called translation. The amino acid sequence that has been encoded in the mRNA determines the structural and functional characteristics of the protein. Gene transcription is regulated by transcription factors, proteins that bind to specific regulatory regions within genes and that control the rate at which DNA is copied into mRNA. Promoter sites are regions of the DNA strand where transcription of a particular gene is initiated.

Alcohol consumption results in the nearly immediate response of neurons in certain critical brain areas. As a consequence of this initial response, many brain processes "downstream" are likely to be affected. IEG's are genes that can be used to identify which brain areas are the first to be affected by a given stimulus, such as alcohol. One such gene, c-*fos*, codes for a component of a transcriptional regulatory complex, activator protein 1 (AP-1). AP-1 binds to promoter sites and thus regulates many other genes. In experimental animals, the expression of c-*fos* is increased by many second messengers, such as PKC, in response to a wide variety of stimuli, such as handling or exposure to novel situations. Because c-*fos* induction is both rapid and transient, it can be used as an indicator of which brain regions are most immediately affected by alcohol.

IEG's and Mapping of Brain Regions

Early studies on IEG expression established that acute and chronic alcohol administration could exert effects on IEG expression that were specific to certain brain regions (Davidson et al. 1996). A recent review concludes that areas of the hippocampus, a brain center involved in the consolidation of new memories, are preferentially sensitive to alcohol (Ryabinin 1998). Some studies support the suggestion that alcohol also preferentially affects some behavioral responses, such as certain forms of learning, that are believed to be mediated by the hippocampus.

Reviews of early research (Crabbe 1997; Ryabinin 1998) point out a major difficulty in interpreting IEG expression data. Because so many behavioral endpoints can themselves induce IEG expression, studies must be designed with rigorous behavioral controls. In one study using extremely rigorous control procedures, researchers compared the effects on 38 specific brain areas of two doses of alcohol given acutely or chronically (Ryabinin et al. 1997). Acute alcohol administration induced IEG expression in most brain areas, a pattern of results resembling those from an earlier study (Chang et al. 1995). However, the research group that performed the 1997 study had previously reported a different result following

administration of a slightly higher dose of alcohol when fewer habituation sessions were used (Ryabinin et al. 1995). Other investigators had earlier reported that a single alcohol injection induced c-*fos* expression in the periventricular nucleus of the hypothalamus, but decreased expression of c-*jun*, another IEG, both there and in the hippocampus (Zoeller and Fletcher 1994). The variation in results following relatively small changes in procedures suggests that results cannot necessarily be extrapolated to other situations.

An interesting finding of the 1997 study (Ryabinin et al. 1997) was that acute alcohol administration blocked the novelty-induced increase in Fos protein levels in several hippocampal subregions, while repeated alcohol injections lost their effectiveness. The alcohol response also seemed to be enhanced in some brain areas with chronic administration. This progressive increase, or sensitization, of alcohol's effects on IEG expression resembles the "kindling" of withdrawal responses, in which successive detoxification episodes lead to progressively more severe symptoms. Some researchers have suggested that withdrawal kindling may be a basis for some of the pathologic effects of long-term alcohol abuse on the brain. If proven to be true, this finding would suggest that aggressive treatment of any and all withdrawal episodes in human alcoholics might be beneficial (Becker 1996).

One IEG study used fear conditioning by exposing rats to a novel environment, then subjecting some of these rats to a foot shock paired with the sound of a tone (Melia et al. 1996). After 48 hours, the rats were returned to the novel environment. Reexposure to the environment alone, or to the environment plus the shock-tone pairing, induced c-*fos* expression in both the cortex and the hippocampus. Administration of alcohol before each exposure eliminated the c-*fos* response to novelty and fear conditioning in the hippocampus and attenuated it in the cortex. Other experimenters have also reported that fear-conditioned stimuli induce c-*fos* in both these brain structures, as well as in nearly all of 58 other brain structures studied (Beck and Fibiger 1995).

Investigators have used c-*fos* expression mapping to compare the responses of rodent genotypes known to differ in alcohol sensitivity. One study used two commonly studied inbred mouse strains for their differential behavioral sensitivity to alcohol. The objective of this study was to elucidate differences in areas of the brain that might underlie these differences in sensitivity. The two strains were DBA/2J mice, which are extremely sensitive to alcohol-induced locomotor stimulation, and C57BL/6J mice, which are nonresponsive to this stimulation (Hitzemann and Hitzemann 1997). Several low-to-moderate doses of alcohol increased Fos-like immunoreactivity (an immune-based measure of the presence a protein) in selected limbic areas of the brain in both strains, but the central amygdala in particular was much more responsive at all doses in the DBA/2J strain. There were generally no strain differences in the basal ganglia. The limbic areas are associated with emotion and behavior, while the basal ganglia are associated with motor coordination.

Another IEG study used paired strains of rats genetically selected for alcohol preference or avoidance. Using alcohol-preferring (P) and nonpreferring (NP) rats and Finnish paired strains of alcohol-preferring (AA) and alcohol-avoiding (ANA) rats, investigators studied responses in different areas of the brain to two doses of alcohol compared with saline, using c-*fos* expression as a marker of neuronal activity (Thiele et al. 1997). Several brain areas responded with increases in Fos-like immunoreactivity, and increases in some brain areas were dose dependent. The principal difference characterizing the P and AA rats (vs. NP and ANA rats) was a greatly attenuated response to the high alcohol dose (3 grams per kilogram of body weight) in the locus ceruleus, suggesting that this brain region may play a role in mediating the differences in alcohol preference in these paired strains.

The use of IEG expression to map brain areas for response to alcohol is clearly a growing field of interest, even though these studies of IEG are technically demanding and immediate interpretation may be difficult. Recent experimental results using this approach suggest that alcohol

does indeed preferentially affect specific brain areas, and this hypothesis can now be tested in a variety of ways that will further our understanding of the genetic bases for individual differences in susceptibility to the development of alcoholism.

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