COMMON GENETIC DETERMINANTS OF SEVERITY OF ACUTE WITHDRAWAL FROM ETHANOL, PENTOBARBITAL, AND DIAZEPAM IN INBRED MICE

by

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Abstract

Physical dependence upon ethanol, barbiturates, or benzodiazepines produces many signs and symptoms upon withdrawal. Seizures are a potentially life-threatening withdrawal sign common to all species studied, including mouse and human, which can occur following physical dependence on these drugs. Genetic mediation of seizures, or convulsions, has been demonstrated in selectively bred mouse lines following withdrawal from chronic ethanol, pentobarbital, and diazepam. Furthermore, it has been shown that mild to severe withdrawal convulsions can occur following elimination of single, acute administrations of each of these drugs.

Inbred strains can also be used to directly test the degree of genetic relatedness determining severity of withdrawal convulsions produced by these drugs. Previous work has shown that genetic variability exists among inbred mouse strains in response to withdrawal from chronic ethanol. The goals of these studies were to determine whether genetic variability in withdrawal severity could be demonstrated among inbred strains following single injections of ethanol, pentobarbital, and diazepam and whether withdrawal severities from the three drugs were genetically correlated.

The handling-induced convulsion (HIC) was used to index withdrawal. Mice from each of fifteen inbred strains were serially tested with ethanol, pentobarbital, and diazepam. Assessment of baseline HICs preceded drug injections. For ethanol and pentobarbital, HICs were assessed hourly from 2 - 12 and 1 - 12 hours following injection, respectively. For diazepam, HICs were assessed at 30 and 55 minutes following diazepam or vehicle injection, and all animals then were injected with the benzodiazepine antagonist, flumazenil, and assessed for HICs 1, 3, 5, 8, and 12 minutes later.

Withdrawal severity was indexed as the area under the withdrawal curve from a plot of HIC score versus time, corrected for basal differences in HIC. Significant strain differences in withdrawal severity were found for each drug. Two strains showed severe withdrawal from each drug, while 6 strains showed low withdrawal from each drug. The remaining strains showed mixed responses.

Positive genetic correlation of ethanol and pentobarbital withdrawal severity was demonstrated, indicating some commonality of genetic influence underlying withdrawal from these drugs. Strain mean withdrawal responses of pentobarbital and diazepam were also positively genetically correlated. The genetic correlation between diazepam and ethanol withdrawal severities was not significant. When the effect of repeatedly testing the animals was examined, six strains were found to have been affected. Only the ethanol/pentobarbital correlation was significant after omission of these strains.

These studies provide clear evidence that the severities of acute ethanol, pentobarbital, and diazepam withdrawal are genetically mediated phenomena. They also demonstrate that ethanol and pentobarbital withdrawal are genetically correlated, indicating some commonality of genetic involvement. Genetic correlation of diazepam and pentobarbital withdrawal severity was also shown; however, ethanol and diazepam withdrawal severities were not genetically correlated in these studies.

Introduction

Drug Dependence and Withdrawal

Dependence on central nervous system (CNS) depressant drugs, such as ethanol, the barbiturates, and the benzodiazepines, is a widespread human problem. The theoretical bases of dependence have been explored (Kalant et al., 1971). These authors differentiated physical dependence from psychological dependence. Physical dependence occurs when the organism's normal physiological functioning is altered under the influence of a drug, such that continued normal functioning requires the presence of the drug. Cessation of intake and subsequent elimination produces a specific set of physiological disturbances known as the drug's characteristic withdrawal syndrome. For CNS depressant drugs, withdrawal produces rebound neural hyperexcitability, among other signs specific to each drug. This thesis does not attempt to address psychological dependence, which was defined as a state of discomfort, or desire or craving, produced by withdrawal from a drug after a period of chronic exposure, and alleviated by renewed intake of that drug (Kalant et al. 1971). When the term dependence is used here, it is meant only to refer to the physical dependence, and particularly, the dependence of the CNS, on the drug in question.

It is customary for dependence to be measured in terms of the severity of the resulting withdrawal syndrome (Jaffe, 1985, p. 537). Each of the drugs tested in these studies (ethanol, pentobarbital, and diazepam) has its own withdrawal syndrome; therefore, the three drugs discussed here will be treated separately.

History

Dependence is a relatively young medical concept in the historical sense (Victor, 1973). The symptoms which today are recognized as resulting from withdrawal of sedative/hypnotic drugs were, not long ago, seen as occurring as a result of the presence of the drug rather than its absence. This theme is prevalent in many papers of the early- to mid-1900s. As late as the 1950s, there was confusion regarding how alcohol could both produce symptoms such as convulsions and delirium tremens *and* alleviate them (Victor & Adams, 1953). In fact, many early studies with barbiturates provided physicians with information that these drugs were not dependence-producing (e.g., Stanton, 1936).

In 1950, Isbell and colleagues published their report of experimental attempts to induce barbiturate dependence in human subjects (Isbell et al, 1950). This work is historically important from two standpoints. The first is that it gave clear descriptions of withdrawal symptoms and their time-courses. The second is that it is highly unlikely that the study will ever be repeated due to ethical considerations (nor should it). Definitive demonstration of barbiturate dependence in animal models finally occurred in the late 1950s and 1960s, more than 50 years after the first barbiturate was released for clinical use. For example, convulsions were reported in cats following withdrawal from barbital (Essig & Flanary, 1959), whereas the first demonstration of barbiturate dependence in rats was published in 1963 (Crossland & Leonard, 1963).

Isbell and colleagues were also successful in the experimental induction of ethanol intoxication and withdrawal in human subjects (Isbell et al., 1955). Surprisingly, this study was the first to characterize the ethanol withdrawal syndrome in humans under controlled conditions.

Experimental cross-dependence of ethanol and barbiturates in humans was demonstrated two years later (Fraser et al., 1957). Demonstration of ethanol dependence and withdrawal in animal models lagged behind, partially due to methodological difficulties. However, in the late 1960s, dependence was induced in several species. Freund (1969) was first to characterize the ethanol withdrawal syndrome following a period of chronic drinking by ICR-DUB mice. Using gastric intubation, Ellis and Pick (1970) induced ethanol dependence in rhesus monkeys and demonstrated that progression of withdrawal severity was correlated with the decline of blood ethanol concentrations.

The benzodiazepine chlordiazepoxide was released for clinical use in 1961, the same year Hollister et al. (1961) demonstrated its dependence liability in humans. Benzodiazepines of varying durations of action were shown to have similar withdrawal signs in humans, although shorter-acting benzodiazepines effected quicker symptom onsets than longer-acting ones (Busto et al., 1986). Yanagita and Takahashi (1973) showed that diazepam and chlordiazepoxide could produce withdrawal symptoms in rhesus monkeys, including convulsions, and could suppress the withdrawal signs induced by chronic barbital.

It is now generally accepted that dependence on CNS depressants develops after chronic use of substantial amounts of drug, although no universal threshold has been quantified (Kalant et al., 1971).

Withdrawal Signs and Symptoms

Ethanol.

The ethanol withdrawal syndrome has a variety of signs and symptoms, and ranges in severity from very mild to severe. Mild withdrawal in humans produces slight tremulousness, irritability,

gastrointestinal discomfort, anxiety, and insomnia, whereas more severe withdrawal produces sweating, tachycardia, nausea, vomiting, fever, and neural hyperexcitability, which may produce hallucinations, delusions (delirium tremens), and grand mal seizures (Jaffe & Ciraulo, 1985). Many of these symptoms are also displayed in animal models; tremor and convulsions have been shown to be common to all species studied, including humans, primates, dogs, cats, and rodents (Friedman, 1980; Kalant, 1977). In all species, the convulsions produced by ethanol withdrawal can be intense enough to cause death. Importantly, ethanol itself, and not acetaldehyde, has been shown to be responsible for the severity of withdrawal in rodents (Goldstein & Miller, 1977). Although less well-known, other alcohols tend to produce similar withdrawal syndromes (e.g., t-butanol; McComb & Goldstein, 1979).

Barbiturates.

There are over 2500 different barbiturates, of which about 50 have been commercially licensed, mainly for use as sedative-hypnotics, anxiolytics, or anticonvulsants (Harvey, 1985). Pentobarbital (used as a sedative-hypnotic) and phenobarbital (an anticonvulsant and anxiolytic) are historically the barbiturates tested in studies of acute and chronic withdrawal in rodents. As with ethanol dependence, barbiturate dependence typically has been shown to occur following a period of chronic use. The withdrawal syndrome displayed varies in severity and includes anxiety, twitches, tremor, weakness, dizziness, visual deficits, nausea, vomiting, weight loss, orthostatic hypotension, convulsions, and, at times, a psychotic condition resembling delirium tremens. As with ethanol, animals of several species show similar barbiturate withdrawal

symptoms, including convulsions (Boisse & Okamoto, 1980). Longer-acting barbiturates such as barbital have been shown to induce milder symptoms over a more protracted withdrawal period than shorter-acting barbiturates (e.g., pentobarbital; Boisse & Okamoto, 1978).

Benzodiazepines.

In humans, cessation of intake of benzodiazepines after chronic use produces withdrawal symptoms much like those of barbiturates, although following a longer time course (Sellers, 1988). Symptoms generally appear within 3 to 10 days following cessation of chronic intake and include anxiety, irritability, insomnia, dizziness, tremor, excessive sweating, tachycardia, nausea, vomiting, twitching, perceptual disturbances, delirium, hallucinations, and seizures (Edwards et al., 1990). As with ethanol and pentobarbital, animals of several species show similar symptoms upon withdrawal, although for longer-acting benzodiazepines such as diazepam, symptoms such as convulsions are seen primarily after administration of a quick-acting antagonist such as flumazenil. For example, in a study by Gallaher et al. (1986), withdrawal of Swiss-Webster mice from chronic dietary diazepam (without antagonist) produced signs such as piloerection, tremor, pelvic elevation, tail elevation, and changes in body tone, abdominal tone, and pupil size. In contrast, Crabbe et al. (1991) have precipitated diazepam withdrawal with flumazenil in mice selectively bred for ethanol withdrawal convulsion severity and shown that these mice display more severe convulsions after diazepam and flumazenil than mice injected with vehicle and flumazenil.

General Methodology in Rodents

Drug dependence in rodents has been induced using several

methods; however, the major routes of chronic administration are dietary (e.g., drug or ethanol mixed with food), intragastric injection (either via cannulation or intubation), and, in the case of ethanol, vapor inhalation. Severity of dependence, as mentioned earlier, is inferred from severity of withdrawal. Methods for measuring withdrawal also vary; for example, measures of tremor, rigidity, and even stereotypic wall climbing (Becker et al., 1987) have been employed. When convulsions are of primary interest, withdrawal from a CNS depressant drug has been measured by recording increased sensitivity to convulsant treatments, such as picrotoxin or electroconvulsive shock, or to convulsions induced by sensory stimulation, such as loud noise or handling. The latter method is employed in the present research. It should be noted that subjective symptoms of withdrawal (e.g., anxiety) have also been modeled. For example, low doses of the GABA antagonist, PTZ, produce a state which can be trained to serve as a cue in drug discrimination paradigms, and withdrawal from ethanol or benzodiazepines substitutes fully for PTZ (Emmett-Oglesby et al., 1990). This state is thought to model self-reported anxiety in humans.

The sections below review pertinent studies of withdrawal following chronic, then acute, drug administration, followed by studies which may help to elucidate the mechanisms underlying withdrawal. Literature regarding each drug is discussed in turn.

Ethanol

Chronic Withdrawal

In 1958, McQuarrie and Fingl published the results of their work on the effects of ethanol on experimental convulsions in mice. They studied the time courses of the anticonvulsant effects of single dose (next section)

and chronic ethanol administration via intragastric intubation (i.g.) on minimal low-frequency electroshock convulsion threshold. In one experiment, ethanol was administered to Carworth (CF#1) adult male albino mice. Subjects were given 2 g/kg ethanol (i.g.) every 8 hours for 14 days. Control animals received an equivalent volume of water. Minimal electroshock convulsion thresholds were measured daily for 17 days (immediately following the morning dose), and on days 19, 21, 23, and 27, and results were reported as a ratio of threshold for the ethanol group divided by threshold for the control group. During the 14 days of ethanol administration, a nearly three-fold higher convulsion threshold was obtained for the ethanol group versus the control group. Interestingly, the effect of withdrawing ethanol, apparent on days 15 through 21, was to decrease the convulsion threshold of the ethanol group to nearly half that of the control group, indicating substantial rebound hyperexcitability. This withdrawal hyperexcitability effect was no longer apparent by day 23. Thus, although chronic ethanol had anticonvulsant effects during administration, its potential use as a clinical anticonvulsant drug was prohibited by the dependence produced, as evidenced by the persistent rebound hyperexcitability upon its withdrawal (McQuarrie & Fingl, 1958). This work, although seminal in the field of ethanol dependence from today's viewpoint, remained largely unrecognized for ten more years according to a review by Goldstein (1975).

McQuarrie and Fingl (1958) had clearly demonstrated the effects of ethanol withdrawal on convulsions produced by the overtly convulsant agents PTZ and electric shock. The same year that Kalant et al. published their critically important theoretical work on tolerance and dependence, Goldstein and Pal (1971) published their proposal of the handling-induced

convulsion or HIC as a useful and reliable method of quantifying withdrawal convulsions in mice following chronic ethanol vapor inhalation. The HIC thus provided dependence researchers with a method to quantify dependence that was not reliant upon administration of a confounding convulsant treatment or drug (Goldstein and Pal, 1971). The measure was rated on a four point scale (Goldstein, 1972b). The HIC method involves lifting the mouse by the tail, observing for convulsion signs, and if absent, gently spinning the mouse in a 180° arc, and again observing. A score of four was given if picking up the mouse elicited a violent tonic-clonic convulsion which often lasted after the release of the mouse; a score of one was given if spinning was required to elicit a tonic convulsion; while a score of zero was given if spinning elicited no convulsion. Goldstein (1972b) scored convulsions hourly for 14 hours, and then periodically thereafter until scores reached zero. Peak mean scores were attained at approximately 10 hours after withdrawal from the vapor chambers. Importantly, this work also demonstrated a clear dose-intensity relationship for HIC, based on duration of exposure to ethanol vapor. Mice treated for one day in the chamber scored a mean of 0.26 at peak withdrawal, while mice treated for six days scored about 2.1, and nine days treatment resulted in mean scores of about 3.4 (Goldstein, 1972b).

The temporal characteristics of cycles of dependence and withdrawal have been studied (Goldstein, 1974). Consistent with Goldstein (1972b), adult male Swiss-Webster mice exposed to ethanol vapor inhalation for 3 days showed significantly elevated HIC and mice exposed to 6, 9, or 12 days of ethanol vapor showed duration-dependent increases in HIC. The blood ethanol concentrations in the mice in this study were held constant at 1.5 to 2.5 mg/ml by manipulating the vapor

chamber ethanol/air ratio. This study also investigated the effect on HIC of four 3-day vapor inhalation periods separated by varying intervals of withdrawal (0, 3, 8, 12, and 24 hours). Greater HIC scores were shown with 3, 8, or 12 hour intervals, indicating that dependence "carried over" between ethanol exposures. However, if the interval was as long as 24 hours, no further increases in HIC were seen, indicating that there was recovery from dependence (Goldstein, 1974). Since the BECs of the mice were held constant, it is not plausible that HICs were increased due to increased dose in each of the 0 - 12 hour interval groups. Instead, this study implies that the duration of exposure and the length of the interval between exposures are critical to the severity of withdrawal.

Acute Withdrawal.

Evidence is accumulating that rebound hyperexcitability can occur following a single injection of a sedative drug. For example, McQuarrie & Fingl (1958) showed that a single administration of ethanol dose-dependently increased convulsion thresholds in male albino mice, whether the convulsions were induced by minimal low frequency electroshock or intravenous PTZ. Furthermore, they showed that eight hours after a single oral dose of ethanol (4 g/kg), a time when all ethanol was metabolized, experimental animals had lower convulsion thresholds than control animals. In other words, acute ethanol withdrawal had a proconvulsant effect.

Goldstein (1972b) examined in male Swiss-Webster mice the effects on withdrawal HICs of a single injection of ethanol, both with and without pyrazole. Pyrazole (an alcohol dehydrogenase inhibitor) is an important part of the chronic vapor inhalation procedure, as mice subjected

to several days of inhalation without it are much more likely to die (Goldstein, 1972b). However, pyrazole alone has been shown to exacerbate handling-induced convulsions in mice dose-dependently (Crabbe et al., 1981). Goldstein (1972b) reported that an intraperitoneal (i.p.) injection of 5 g/kg ethanol produced enhancement of HIC in male Swiss-Webster mice. When this injection was preceded (24 hours before) by injection of 1 mmole/kg pyrazole, peak withdrawal occurred at 10 to 11 hours after the ethanol injection, and mean HIC score was low (0.4). Without pyrazole, peak withdrawal occurred at about 7 hours after injection, and the mean (±SE) HIC score was 0.7 ± 0.1 (Goldstein, 1972b). Therefore, pyrazole slowed the elimination of ethanol and produced a more protracted and milder withdrawal characteristic of longer-acting drugs. Although a saline control group was not used in these experiments, these results imply that HIC is elevated after metabolism of a single injection of ethanol, with or without prior injection of pyrazole, and that pyrazole may attenuate the increase in HIC. Thus, although pyrazole alone may increase HIC, this effect may not be additive with that of ethanol. Regardless, it is clear that ethanol alone, in a single, sedative dose, can exacerbate HIC at a time when metabolism is complete or nearly complete (Goldstein, 1972b).

Mechanisms of Action.

Unlike the other two classes of drugs under discussion, ethanol does not seem to have a specific receptor to which it binds to produce its pharmacological effects (Tabakoff & Hoffman, 1987). Rather, ethanol has been postulated to have relatively nonspecific effects. Two theories having general relevance to ethanol's action in producing withdrawal convulsions

are the denervation supersensitivity hypothesis (Jaffe & Sharpless, 1968), and the membrane disordering hypothesis (Meyer, 1901; cited in Tabakoff & Hoffman, 1987). The denervation hypothesis states that sedatives such as ethanol and barbiturates depress CNS neuronal function and thereby produce a temporary state of functional denervation of the tissues or neurons served by the affected neurons. This leads to an attempted compensation by the secondary neurons; that is, they adapt to the diminished neurochemical input by becoming supersensitive to stimulation (possibly via receptor up-regulation). In the absence of the drug, which no longer depresses CNS activity, this previously adaptive response becomes maladaptive, and results in the symptoms of withdrawal, most importantly, convulsions.

The second hypothesis is the membrane disordering hypothesis initially formulated by H. H. Meyer (1901). This hypothesis states that ethanol has a fluidizing effect on neuronal membranes, particularly on the lipid membrane structure. Subsequent work has revealed that low concentrations of ethanol added *in vitro* increased fluidity (as determined by spectral analysis) in erythrocyte, as well as brain mitochondrial and synaptosomal brain membranes from adult male DBA/2J mice (Chin & Goldstein, 1977). Further, this fluidizing effect of ethanol was dose-dependent. Other studies have shown that ethanol's effects are exerted at specific areas of neuronal membranes, indicating that ethanol may be affecting various membrane-bound proteins, such as ion channels and receptors, perhaps by exerting a fluidizing effect on the lipids at their immediate locations (Tabakoff & Hoffman, 1987).

Withdrawal Mechanisms.

Two classes of ionophores in particular have been implicated in ethanol withdrawal convulsions. These are calcium channels (both voltage-sensitive and ligand-gated types) and chloride channels.

Calcium.

Research has suggested that ethanol dependence and subsequent withdrawal produces an increase in the number (Harper et al., 1989) and function of dihydropyridine (DHP)-sensitive calcium channels (Dolin et al., 1987), and therefore, possibly in calcium influx. Increases in DHP-sensitive channels have been linked to enhanced catecholamine release (Harper et al., 1989). Some evidence suggests that ethanol disrupts the second messenger system linking calcium influx and catecholamine release: ethanol withdrawal enhanced the BAY K8644 (a DHP channel agonist) -induced increase in inositol phosphates in rat cortex preparations above control levels (Dolin et al., 1987).

The DHP antagonists nimodipine and nitrendipine have been shown to inhibit spontaneous and audiogenically-induced ethanol withdrawal convulsions in rats (Little et al., 1986). Taken together, these studies suggest that the rebound neurohyperexcitability following cessation of ethanol treatment may occur through a calcium-dependent mechanism, producing enhanced excitatory neurotransmitter release.

Evidence is mounting that ethanol also acts acutely to inhibit calcium ion flux into the neural cell through action at the NMDA receptor, possibly by interfering with the binding of the co-agonist, glycine (Hoffman et al., 1991). The NMDA receptor is one of the excitatory amino acid (L-glutamate) receptors, and is coupled with a calcium ionophore that is both

ligand-gated and voltage-sensitive (Buck & Harris, 1991). Ethanol has been shown to inhibit the NMDA-stimulated increase of calcium influx dose-dependently when added prior to NMDA to an *in vitro* preparation of brain cells from newborn rats (Dildy & Leslie, 1989). Furthermore, it is postulated that withdrawal of ethanol produces enhanced calcium flux into the cell, resulting in increased release of catecholamines and therefore, rebound hyperexcitability and convulsions. Using the non-competitive NMDA antagonist, dizocilpine (MK-801), NMDA binding sites have been shown to be increased in hippocampus after chronic ethanol treatment in male C57BL mice (Grant et al., 1990). Dizocilpine is known to bind to a site inside the channel of the NMDA receptor/calcium ionophore, thereby blocking calcium from entering (Hoffman et al., 1991). Chronic ethanol treatment may induce receptor up-regulation, possibly by blocking calcium influx at the NMDA receptor.

The effects of NMDA and dizocilpine on ethanol withdrawal handling-induced convulsions have been examined (Crabbe, Merrill, & Belknap, in press; Grant et al., 1990). Both studies showed that NMDA exacerbated and dizocilpine dose-dependently attenuated ethanol withdrawal convulsions, respectively. Interestingly, it has been shown that lines of mice selectively bred to be prone to ethanol withdrawal convulsions (WSP) have a greater number of dizocilpine binding sites in hippocampus in the absence of ethanol treatment than the resistant lines (WSR; Valverius et al., 1990); however, another group has found no difference in the number of dizocilpine binding sites in hippocampus or cortex of either ethanol-naive or ethanol-treated WSP versus WSR mice (Janowsky et al., 1992). The results of the withdrawal modulation studies are consistent with the hypothesis that ethanol withdrawal induces

hyperexcitability and convulsions via the increased influx of calcium; however, drugs acting at non-glutamate receptors (e.g., picrotoxin, reserpine, diazepam, and abecarnil: Goldstein, 1973a; Crabbe, 1992) have also been shown to modulate ethanol withdrawal convulsions.

Chloride.

Ethanol also has been shown to have effects on the γ-aminobutyric acid (GABA) receptor. The A-subtype of this receptor (denoted $GABA_A$) has many putative specific binding sites, including separate sites for benzodiazepines and barbiturates, and is coupled with a chloride ionophore (see Sieghart, 1992, for a recent discussion). The action of GABA, and GABA agonists such as muscimol, is to enhance chloride ion flux into the neural cell. This influx increases the hyperpolarization state of the cell, decreasing its likelihood of depolarizing and releasing neurotransmitter, and thus inhibiting excitation. Ethanol may prevent binding of endogenous GABA functional antagonists, which decrease chloride flux, thereby enhancing GABA-mediated inhibition. In support of this hypothesis, it has been found that ethanol, at low concentrations, prevents binding of the antagonist TBPS to the picrotoxin binding site inside the chloride channel coupled to the $GABA_A$ receptor (Tabakoff & Hoffman, 1987). Removal of ethanol is hypothesized to produce rebound hyperexcitability and convulsions by decreasing chloride ion flux into the cell, increasing the likelihood of depolarization. It has been shown in rat cerebral cortical synaptoneurosomes that chronic ethanol exposure significantly decreases ethanol enhancement of muscimol-stimulated chloride ion flux (Morrow et al., 1990). The same effect has been shown in cerebellar membrane microsacs from DBA/2J mice treated either

chronically or acutely with ethanol (Allan & Harris, 1987). These studies provide evidence for a biochemical mechanism in ethanol tolerance.

It has been shown that several GABAergic compounds alter the withdrawal reaction produced after chronic ethanol exposure. For example, GABA/chloride channel blockers such as picrotoxin enhance ethanol withdrawal when measured as latency to myoclonic and tonic convulsions and to mortality (Szabó et al., 1984) or as measured by handling-induced convulsions (Goldstein, 1973a). PTZ (30 mg/kg), which blocks picrotoxin binding and decreases chloride flux, also exacerbates ethanol withdrawal convulsions following a single 4 g/kg dose of ethanol (Crabbe, Merrill, & Belknap, in press). Aminooxyacetic acid, which inhibits GABA degradation through inhibition of the enzyme, GABA-transaminase, reduced chronic ethanol withdrawal convulsion severity, as measured by HIC (Goldstein, 1973a). Ethanol itself (2.0 g/kg), pentobarbital (60 mg/kg), and phenobarbital (75 mg/kg) were also effective in reducing withdrawal; meprobamate (200 mg/kg) first decreased, and then increased HIC versus the ethanol-only condition (Goldstein, 1972a).

Modulation of the benzodiazepine binding site coupled to $GABA_A$ receptors in the CNS has been shown following chronic ethanol treatment. Chronic ethanol reduced binding affinities of benzodiazepines to this receptor in rat cerebral cortex, with no effect on the number of receptors (Rottenberg, 1985). However, ethanol has been shown to enhance tritiated diazepam binding *in vitro* in rat brain membrane homogenates (Burch & Ticku, 1980). Also, loss of benzodiazepine receptors as well as muscarinic cholinergic receptors from human hippocampus have been seen in post-mortem brains of alcohol abusers versus control brains (Freund & Ballinger, 1989). Reduction of GABA content and GABAergic

neurons has also been shown in mouse hippocampus after long-term ethanol consumption, using quantitative immunocytochemistry (Lescaudron et al., 1986).

The effects of acute and chronic ethanol on benzodiazepine agonist and inverse agonist actions on chloride flux have been examined (Buck & Harris, 1990a,b). Treatment of mice with chronic ethanol attenuated the flunitrazepam enhancement of muscimol-stimulated chloride flux, while it enhanced the actions of Ro15-4513 and DMCM (benzodiazepine inverse agonists) to further inhibit chloride flux (Buck & Harris, 1990b). Acute ethanol treatment also enhanced the action of DMCM to inhibit chloride flux as stimulated by muscimol. Addition of ethanol (10, 50, or 100 mM) to an *in vitro* preparation from the tissue of naive mice produced dose-dependent sensitization to DMCM. (Buck & Harris, 1990a). These actions are consistent with the hypothesis that chloride flux during ethanol withdrawal is reduced.

Several benzodiazepine agonists have been shown to alter the withdrawal reaction produced after ethanol exposure: diazepam (20 mg/kg) and chlordiazepoxide (50 mg/kg), like the barbiturates, were effective in attenuating withdrawal convulsions as measured by HIC following chronic ethanol vapor inhalation (Goldstein, 1972a). Furthermore, diazepam (0.5 mg/kg) and the β-carboline, abecarnil (0.5 mg/kg) have been shown to attenuate the withdrawal convulsions following a single 4 g/kg injection of ethanol (Crabbe, 1992).

There are conflicting reports as to whether the benzodiazepine antagonist, flumazenil, can antagonize the ethanol withdrawal syndrome in mice. When male C57BL/6J mice were treated chronically with an ethanol liquid diet, and injected with 25 mg/kg flumazenil either immediately, 14, or

24 hours before withdrawal, no effect on HIC was seen in any group versus control animals injected with vehicle (Chan et al., 1991). In contrast, when male ICR mice were treated chronically with a liquid diet containing ethanol, and injected with 10 mg/kg flumazenil 14 hours before withdrawal, HIC was significantly attenuated at hours 6, 8, and 10 post-withdrawal versus control animals injected with vehicle (Buck et al., 1991). Differences between the findings in these studies may be due to the different mouse strains tested or to procedural differences. For example, the studies differed in concentration of ethanol and duration of liquid diet administration (15 days of 3.5% to 8% versus 10 days of 5%, respectively), as well as in the dose of flumazenil (25 versus 10 mg/kg). It seems unlikely that a difference in ethanol consumption can explain the difference in findings since consumption was similar (15 to 22 g/kg/day versus about 18 g/kg/day, respectively). However, the flumazenil dose tested by Chan et al. (1991) is much higher than that required by Crabbe and colleagues to precipitate withdrawal in benzodiazepine-treated mice (2.5 mg/kg, unpublished observations); perhaps 25 mg/kg produces an effect not specific to benzodiazepine receptors due to over-saturation. Finally, there may be differences in the scoring of HIC, although both groups used the criteria of Goldstein. Further studies will have to clarify this issue.

Finally, as mentioned earlier, modulation of ethanol withdrawal with drugs affecting other neurotransmitter systems has also been investigated. The catecholaminergics reserpine, α-methyltyrosine, phentolamine, and propranolol all enhance ethanol withdrawal convulsions, while little or no effect of the cholinergics atropine, dihydro-β-crythoidine, and physostigmine, or the serotonergics p-chlorophenylalanine, tryptophan, and 5-hydroxytryptophan was shown (Goldstein, 1973a).

Barbiturates

Chronic Withdrawal

Methods of measuring withdrawal not based on convulsions are common in studies testing rats; for example, increases in response to startle, vocalization to prodding or to air puff, arched back posturing, piloerection, spontaneous locomotor activity, and struggling or vocalization in response to being grasped and held have been measured following chronic, continuous i.p. infusion of pentobarbital (Yutrzenka, 1989). Furthermore, dependence of severity of these signs on length of administration period (i.e., dose) has been demonstrated (Yutrzenka et al., 1985). Increased startle amplitude has also been demonstrated in rats after withdrawal from phenobarbital mixed with food (Letz & Belknap, 1975).

Various groups have studied the effects of chronic administration of phenobarbital and pentobarbital on withdrawal in mice. Yutrzenka and Kosse (1989) studied the effects of phenobarbital versus pentobarbital administered to CF-1 mice in a drug-adulterated food paradigm. Phenobarbital was administered at a dosage of 2.5 mg/g food for 5 days, and then 3.0 mg/g food for 2 days. Pentobarbital was administered at a dosage of 5 mg/g food, increased by 5 mg/day, for either 7 or 14 days, or a dosage of 10 mg/g food, increased by 10 mg/day, for 7 days. Immediately following substitution of control diet, and every four hours to hour 48 for the phenobarbital group, or hourly for 12 hours, and at 24, 36, 48, and 72 hours for the pentobarbital groups, each mouse was suspended by its tail for 10 seconds, returned to its home cage, and observed for 1 minute. Results showed that only the phenobarbital-treated group showed significant withdrawal, based on a rating scale of presence of signs

including tremor, Straub tail, jumpiness, wild running, and convulsions (Yutrzenka & Kosse, 1989).

The dose-dependence and temporal characteristics of phenobarbital dependence have been measured in adult male DBA/2J mice (Belknap et al., 1977). Results showed that longer duration of continuous exposure to drug-adulterated food dose-dependently increased withdrawal scores, and that discontinuous exposure to drug-adulterated food significantly attenuated the increases in withdrawal scores, indicating at least partial recovery from dependence during control diet administration periods (Belknap et al., 1977).

Acute Withdrawal.

Although many effects of single doses of barbiturates have been studied (e.g., Boisse & Okamoto, 1980; Chan & Siemens, 1979), very few studies have examined the acute dependence which may occur in rodents following single administrations of pentobarbital. The most relevant studies utilize genetic animal models and will be reviewed below in the section on genetic studies of acute withdrawal.

Mechanisms of Action.

Barbiturates putatively bind to a unique site on the GABA/chloride ionophore complex (Harvey, 1985; Sieghart, 1992). Like ethanol and the benzodiazepines, they act to enhance GABA agonist-stimulated chloride influx, although pentobarbital increases the amount of time the channel stays in the open state while diazepam increases the frequency of channel opening without affecting its duration of opening. Furthermore, barbiturates have been found to enhance the binding of benzodiazepines

to their site on the GABA complex, further potentiating chloride flux (Harvey, 1985, p. 354). Pentobarbital and phenobarbital have been shown to enhance GABA-stimulated chloride flux in brain membrane microsacs from male DBA/2N mice, although pentobarbital was about ten-fold more potent. Additionally, pentobarbital prevented the antagonism of GABA-stimulated chloride flux by picrotoxin (Allan & Harris, 1986).

A comprehensive review of the effects of acute and chronic barbiturate treatments on the GABA system, as well as a comparison of these effects with those of ethanol and the benzodiazepines, has been published recently (Yu & Ho, 1990). Even a thumbnail discussion of this review is beyond the scope of this paper; however, one important conclusion borne out by the potency data cited above (Allan & Harris, 1986) is that acute barbiturate studies support a role for enhanced GABAergic transmission in mediating sedative, but not necessarily anticonvulsant, effects of barbiturates. Correlation of anesthetic potencies of several barbiturates with their potencies at increasing GABA-mediated chloride flux was found to be highly significant ($r^2 = 0.99$; Allan & Harris, 1986). Furthermore, convulsant barbiturates have been shown to have only mildly inhibitory effects on GABA-mediated chloride flux (Allan & Harris, 1986).

There is evidence that pentobarbital acts at receptors in addition to the GABA/chloride complex to exert its effects. For example, pentobarbital has been shown to acutely inhibit accumulation of calcium stimulated by potassium but not by glutamate in synaptosomes prepared from male BALB/c mice. This inhibition was shown to occur as a result of enhanced efflux of calcium (Harris & Stokes, 1982).

Withdrawal Mechanisms.

Calcium.

Chronic barbital treatment has been shown to increase the number of dihydropyridine (DHP)-sensitive calcium channels in cerebral cortex of TO mice (Rabbani & Little, 1990). Furthermore, it has been shown that the DHP calcium antagonist, nitrendipine, protects against the barbiturate withdrawal syndrome, although with less efficacy than when used against ethanol withdrawal (Brown et al., 1988; Little, 1991).

Chronic barbital treatment and subsequent withdrawal of male TO mice has been shown to increase the binding of the NMDA antagonist dizocilpine without affecting its binding affinity (Wright et al., 1991). However, it has been shown that neither pentobarbital nor phenobarbital preferentially inhibited NMDA- versus kainate-stimulated calcium uptake *in vitro*, further supporting the notion that, as in the case of ethanol, there are several potential sources of calcium flux involved (Rabe & Tabakoff, 1989). Nevertheless, pentobarbital and dizocilpine have each been shown to protect against tonic convulsions induced by either picrotoxin or maximal electroshock (Kulkarni & Ticku, 1989). Furthermore, pentobarbital and dizocilpine given together also protected against these convulsants in doses that were too low to provide protection when administered alone (Kulkarni & Ticku, 1989). Regardless of its sources, all of these studies implicate calcium flux in barbiturate action.

Chloride.

Barbital, a medium-long acting barbiturate, has been shown to modulate binding of muscimol at the $GABA_A$ receptor, as well as quinuclidinyl benzilate binding at muscarinic cholinergic receptors during

withdrawal in rat cerebral cortex homogenates (Nordberg et al., 1986). In this study, chronic barbital decreased the affinity of muscimol to bind to the low affinity GABA site when measured 3 days into withdrawal. Yu & Ho (1990) reviewed studies indicating that modulation of GABA binding by chronic barbiturate treatment and withdrawal is unclear; some studies have shown no change in either number of binding sites or in affinity, while others show an increase in number or reduction in affinity. However, GABA levels and GAD activity both seem to be decreased following chronic barbiturate treatment and withdrawal (Yu & Ho, 1990).

Chronic pentobarbital has been shown to cause a decrease in cortical binding of tritiated flunitrazepam, indicating that barbiturate dependence and withdrawal down-regulates benzodiazepine receptors (Saunders et al., 1990); however, flunitrazepam binds both to benzodiazepine receptors coupled to the $GABA_A$ receptor/chloride ionophore and non-GABA mitochondrial receptors in brain (see p. 27). Further research will have to clarify which class of receptors is involved. Since withdrawal following chronic pentobarbital treatment decreases the latency to PTZ-induced convulsions (Saunders et al., 1990), it seems likely that at least the GABA-coupled benzodiazepine receptors are involved.

The effects on phenobarbital withdrawal of three neuroleptic (anti-dopaminergic) drugs, the catecholamine synthesis inhibitor, α-methyl-tyrosine (AMT), and diazepam have been studied (Belknap, 1978). C57BL/6J mice were made dependent on chronic phenobarbital admixed in food. Tremor, Straub tail, and convulsions were scored as in Belknap et al. (1977), and HIC was scored as in Goldstein (1975). Results showed that only diazepam treatment decreased phenobarbital withdrawal severity. Haloperidol produced no effects on convulsions;

however, chlorpromazine, reserpine, and AMT significantly worsened convulsions (90% were tonic-clonic). These results indicate that a benzodiazepine can attenuate barbiturate withdrawal, and that dopaminergic antagonists and drugs blocking catecholamine synthesis may exacerbate barbiturate withdrawal in mice (Belknap, 1978).

Benzodiazepines

Chronic Withdrawal

Like many studies of chronic barbiturate withdrawal, studies using chronic benzodiazepine administration to study withdrawal in rodents generally find symptoms like twitches, tremor, arched back, piloerection, and increases in muscle tone (File, 1990). Frequently studied are the effects of chronic dosing using precipitated withdrawal; i.e., the benzodiazepine is administered chronically, and then withdrawal is precipitated by a benzodiazepine antagonist such as flumazenil. This type of paradigm usually produces more severe withdrawal signs in rodents, among which are convulsions. Compared to ethanol, dramatically fewer studies of benzodiazepine withdrawal have been published; very few of these studied diazepam.

An example of non-precipitated withdrawal from diazepam in mice was introduced above in the section on signs and symptoms of diazepam withdrawal. Briefly, Gallaher et al. (1986) used a chronic drug-adulterated food procedure to administer diazepam to adult male Swiss-Webster mice. Upon substitution of control diet, daily composite withdrawal scores were compiled based on tests of piloerection, tremor, pelvic elevation, tail elevation, body tone, abdominal tone, and pupil size and compared to the grand mean of those of control animals given no diazepam. Removal of

the drug-adulterated diet produced a significant increase in food consumption and composite withdrawal scores for several days (Gallaher et al., 1986).

The agent most commonly used to precipitate benzodiazepine withdrawal in rodents is the competitive antagonist flumazenil (previously known as Ro15-1788), although other compounds have been used, such as the inverse agonist Ro15-3505 (Moreau et al., 1990). An example is provided by a study in which male Swiss-Webster mice were treated for 9 days with increasing daily doses of diazepam (final daily dose: 900 mg/kg, divided into two injections) and then injected 24 hours later with flumazenil (2.5, 5, or 10 mg/kg). Clonic convulsions occurred in 60, 66, and 100% of the mice treated with increasing doses of flumazenil, respectively (Patel et al., 1988). Four days of increasing daily doses of diazepam failed to produce convulsions precipitated by 10 mg/kg flumazenil (Patel et al., 1988). While this study provides useful information, exact replication might prove difficult due to discrepancies in reporting of the methods used. Furthermore, administration of flumazenil is usually performed closer in time to administration of the last benzodiazepine dose. Other examples of flumazenil precipitation of benzodiazepine withdrawal in mice include the acute diazepam studies performed in genetic models (see that section) and the following study using chlordiazepoxide.

The effects of chronic administration of a liquid diet containing gradually-increasing amounts of chlordiazepoxide on flumazenil-precipitated and spontaneous withdrawal in male C57BL/6J mice have been tested (Chan et al., 1989). Flumazenil was injected one hour after removal of the diet. This treatment initiated withdrawal signs within 1 minute of injection that persisted for at least 10 minutes. Flumazenil dose-

dependently increased a composite withdrawal score composed of ordinal scales for tremor and tail lift (following Gallaher et al., 1986), HIC (modified from Goldstein, 1972a), and locomotor depression. Spontaneous withdrawal produced no well-defined withdrawal signs.

Acute Withdrawal.

Convulsions following acute benzodiazepine treatment also typically are not seen in animal models without precipitation with flumazenil, even when the half-life of the drug is short. Two studies of acute withdrawal in rats are discussed here. One demonstrated a failure to produce spontaneous acute withdrawal; the other demonstrated both spontaneous and precipitated withdrawal. Neither study demonstrated convulsions. Examples of precipitated withdrawal convulsions in mice following single injections of benzodiazepines are discussed in the section on acute genetic models.

Spontaneous withdrawal from a single dose of midazolam was studied in rats in comparison with withdrawal following chronic administration (Boisse et al., 1990). Male Sprague-Dawley rats were injected (i.g.) with midazolam, 120 mg/kg once only, 120 mg/kg 4 times a day for 3 days, or 120 - 180 mg/kg twice daily for 5 weeks. Withdrawal from midazolam was quantified on the basis of the sums of twenty different motor, autonomic, and behavioral signs from 3 to 4 raters. The maximum possible withdrawal score was 49 when all signs were expressed maximally and all raters concurred. Acute midazolam treatment failed to produce withdrawal signs, although the 3 day treatment produced a score of ~11 and the 5 week treatment a score of ~16 (Boisse et al., 1990).

In a similar study, acute chlordiazepoxide withdrawal was

demonstrated in male Sprague-Dawley rats after precipitation with flumazenil, the nonbenzodiazepine antagonist CGS-8216, and after spontaneous withdrawal (Boisse et al., 1986). Flumazenil (25 mg/kg, i.p.) was administered 4, 28, 52, 76, 100, 124, or 148 hours after a single dose of chlordiazepoxide (450 mg/kg, i.g.) and withdrawal was rated essentially as in Boisse et al. (1990). Maximal withdrawal was seen at 76 hours post-chlordiazepoxide. The time-course of flumazenil action was observed for one hour, with maximal withdrawal occurring at the first (5 minute) time-point. When CGS-8216 (25 mg/kg) was injected 76 hours after chlordiazepoxide, maximal withdrawal occurred 15 and 30 minutes later. Spontaneous withdrawal following chlordiazepoxide was maximal at 4 days, but significantly greater in magnitude than controls from 4 to 8 days after 450 mg/kg (Boisse et al., 1986).

Mechanisms of Action.

As mentioned earlier, diazepam binds to the benzodiazepine binding site on the $GABA_A$ receptor/chloride ionophore complex and acts to augment GABA agonist-stimulated chloride influx (Harvey, 1985). This effect can be blocked by flumazenil *in vitro* and is not affected by pretreatment *in vivo* with a single dose of diazepam (Yu et al., 1988). Tolerance can be demonstrated: four weeks of flurazepam treatment significantly attenuated the ability of three benzodiazepines to augment GABA-stimulated chloride influx (Yu et al., 1988).

Effects on benzodiazepine receptors in various brain areas of male Sprague-Dawley rats as a result of acute diazepam and chronic flurazepam treatments have been reported, using quantitative autoradiography (Tietz et al., 1986). Chronic flurazepam treatment

decreased specific flunitrazepam binding in several brain areas: dentate gyrus, hippocampus (CA1 and CA4), temporal cortex, superior colliculus, lateral amygdaloid nucleus, and lateral hypothalamus. Longer treatment also decreased binding in the substantia nigra pars reticulata. No significant changes in binding 30 minutes after acute diazepam intubation (10 mg/kg) were seen in any of the brain areas studied (Tietz et al., 1986). This suggests that changes in benzodiazepine receptors are unlikely following acute diazepam.

Chronic treatment with diazepam has been shown to attenuate both the inhibition of serotonergic neurons in the dorsal raphe nucleus by GABA and the protective effect of an acute dose of diazepam against bicuculline-induced convulsions (Gonsalves & Gallager, 1988). Interestingly, flumazenil injected 24 hours before testing restored both behaviors to control levels, thus demonstrating a reversal of tolerance (Gonsalves & Gallager, 1988). It has been shown that flumazenil has a half-life of 16 minutes from rat brain following an acute intraperitoneal injection (Lister et al., 1984). This suggests that these actions of flumazenil were not due to competitive antagonism at the benzodiazepine receptor.

Stereo specific binding sites for benzodiazepines that are not linked to the GABA/chloride ionophore complex have been identified in rat brain membrane (Bowling & DeLorenzo, 1982). These binding sites differ from the GABA-linked benzodiazepine receptor in their binding, kinetic, and pharmacologic characteristics. Often referred to as peripheral-type benzodiazepine receptors, these receptors exhibit micromolar affinity for benzodiazepine ligands (rather than nanomolar as do the GABA-linked receptors), and agonists antagonize voltage-dependent calcium channels (Bowling & DeLorenzo, 1982; Taft & DeLorenzo, 1984; Rampe et al.,

1987). These micromolar affinity receptors also appear to be more numerous in rat brain than their nanomolar affinity counterparts (360.4 vs. 0.9 picomoles per mg protein from rat brain synaptosomes; Bowling & DeLorenzo, 1982).

Withdrawal Mechanisms.

Many researchers have shown that the CNS depressant drugs exhibit cross-dependence; for example, it is common for humans undergoing ethanol withdrawal to be treated with anticonvulsant drugs, such as the benzodiazepines, in order to suppress seizures (Jaffe & Ciraulo, 1985). Chan and colleagues have investigated the question of ethanol and chlordiazepoxide (CDP) cross-tolerance and -dependence in several papers (e.g., Chan et al., 1990). Male C57BL/6J mice were made dependent on CDP in a chronic liquid diet paradigm, then injected with either ethanol or saline preceding withdrawal precipitation by flumazenil (Chan et al., 1990). Ethanol (1.5 g/kg) injected 5 minutes before flumazenil (25 mg/kg) almost completely suppressed the enhancement of the composite withdrawal scores shown by the CDP/saline/flumazenil group. Subsequent to CDP withdrawal, and with appropriate control groups, these mice were offered a liquid ethanol diet for 9 days. Mice pretreated with CDP, given chronic ethanol, and then withdrawn from ethanol showed more severe hypothermia during ethanol withdrawal than mice given control diet followed by ethanol diet (Chan et al., 1990). These studies clearly implicate a common mechanism for ethanol and benzodiazepine dependence.

Calcium.

Concentrations of various benzodiazepines in rat brain which

protect against maximal electroshock-induced seizures and against kindling have been found to be in the micromolar range, indicating that the anticonvulsant effects of these drugs may be mediated by micromolar affinity receptors. The action of benzodiazepine agonists at this receptor is to attenuate calcium influx in a concentration-dependent manner. The peripheral benzodiazepine agonist Ro5-4864 and the central agonist medazepam were approximately equally potent in inhibition of calcium uptake; diazepam was only slightly less potent (Taft & DeLorenzo, 1984).

Triggle and colleagues have shown that the effect of benzodiazepines is selective in inhibiting the fast-phase of calcium influx, and not the slow-phase. Interestingly, the peripheral ligands Ro5-4864 and PK11195 (an antagonist) both inhibit calcium influx while flumazenil, which is a selective GABA/benzodiazepine antagonist, has no appreciable effect on calcium uptake (Rampe & Triggle, 1987). Ro5-4864 and diazepam were further shown to attenuate tritiated nitrendipine binding in guinea-pig cortical synaptosomes; PK11195 potentiated nitrendipine binding (Rampe & Triggle, 1987; Triggle et al., 1987). This clearly implicates the dihydropyridine-sensitive calcium channel in these actions.

In agreement with this hypothesis, Dolin, Little and colleagues have shown that nitrendipine decreases the FG7142-induced seizure incidence in TO mice withdrawing from chronic flurazepam treatment (Patch et al., 1989; Dolin et al., 1990). While FG7142 is a partial inverse agonist at the GABA/benzodiazepine complex, this indirect evidence suggests that it may act at DHP calcium channels since nitrendipine does not protect against bicuculline-induced convulsions (Dolin et al., 1990). However, the role of the micromolar affinity benzodiazepine receptor in CNS depressant drug withdrawal remains to be elucidated.

Chloride.

Miller et al. (1988a) have shown that benzodiazepine receptor binding as well as muscimol-stimulated chloride flux are decreased during chronic lorazepam treatment. These effects appear to be a result of binding at GABA-linked benzodiazepine receptors, since nanomolar-range concentrations were achieved in brain and plasma. In another study, these measures were assessed at various periods following withdrawal from seven days of lorazepam treatment (2 mg/kg/day via osmotic minipumps implanted subcutaneously). Benzodiazepine binding, chloride flux, chloride channel ligand (TBPS) binding, and open field activity were assessed at one day and from two to seven days (depending on measurement) after removal of the minipump (Miller et al., 1988b). Results showed that all measures were increased at four days into withdrawal and returned to near baseline on day seven. These studies suggest that the action of GABA-coupled benzodiazepine receptor binding and function are altered following chronic benzodiazepine administration; however, the involvement of these two types of benzodiazepine receptors in the expression of withdrawal convulsions *per se* remains to be established.

Genetic Models of Dependence and Withdrawal in Rodents

Principles of Genetic Animal Models

Researchers have been using animal models of human problems for many years, and the area of drug abuse is no exception. Among the most successful animal models for studying drug effects are genetic models. A genetically heterogeneous group of animals may display a

wide range of responses to a test, and it is extremely difficult to determine whether genetics, different environmental influences, or an interaction of the two led to the large variability of responses. In contrast, experimenters using animals of known genetic makeup have an opportunity to sort out this issue. A measurable characteristic such as the response made to a test is known as the phenotype. The phenotype displayed will reflect the genotype; however, it is possible for more than one genotype to yield a particular phenotype. For example, an individual with blood type A (the phenotype) can have one of two genotypes: either (s)he has inherited type A alleles from both parents (genotype AA) or type A (dominant) from one parent and type O (recessive) from the other (genotype AO). Often, the phenotype displayed is also influenced by the environment. The environment is defined to be everything that is not genetic, including interactions of environment and genotype. For example, in phenylketonuria, the affected individual is born without the enzyme that oxidizes the amino acid, phenylalanine, to tyrosine. When a normal diet containing phenylalanine is given (part of the environment), toxic levels of phenylketones are built up, and brain damage (one of several possible phenotypes) occurs. However, if a diet lacking phenylalanine is given, brain damage can be prevented.

Variability in response to a test (phenotypic variance) can have genotypic or environmental sources. Following Falconer (1989), the phenotypic variance (V_P) can be mathematically compartmentalized into the genotypic variance (V_G) and the variance due to the environment (V_E):

$$V_P = V_G + V_E$$

This implies that when the genotypic variance is very small, or zero, as when the genotypes of the experimental animals are identical, the

variability in response (V_P) is equal to the variability in environment. Therefore, it is possible to attempt control of the environment of a group of animals and reduce the variability in their responses to a test. Complete control of the environment is practically impossible, since sources of V_E include error of measurement, but environmental variability may be reduced through careful experimental design and animal husbandry practices.

Selectively Bred Lines.

Lines of rodents are selectively bred usually to display extremes in the range of responses to a particular test. For example, the WSP and WSR lines of mice were selectively bred to display enhanced or reduced severity of withdrawal convulsions (as measured by HIC), respectively, in response to three days of ethanol vapor inhalation (Crabbe et al., 1983b). Selection began from the genetically defined heterogeneous stock, HS/Ibg mice, and was performed in independent replicates, including independent nonselectively bred control lines (WSC). An earlier example of selection for high and low withdrawal convulsion scores following chronic ethanol vapor inhalation has been reported. Goldstein (1973b) tested male and female Swiss-Webster mice for two 3-day cycles of vapor inhalation, chose two males and two females that had shown similarly high scores in both cycles, and mated them. She also chose low scoring breeding pairs. Twenty offspring from these matings (11 from high, 9 from low) were tested in the same paradigm, and again the two breeding pairs per line were chosen. When these offspring were tested, the high line showed further increases in HIC scoring (Goldstein, 1973b).

Studies in the WSP/WSR mice will be discussed further in the

sections below; however, the procedures and principles of selection will be discussed here. Selective breeding involves mating of animals (or plants) for a particular phenotypic response over many generations so that the particular response is maximized, while other traits (phenotypes) unrelated to the selected trait remain unaffected. Obviously, opposing phenotypes are selected against. If the variability in the phenotypic response of the foundation stock is at all genetically influenced ($V_G > 0$), then mating of individuals displaying a similar degree of responses produces offspring that have greater phenotypic value. For example, the bidirectional WSP/WSR selection would have failed if none of the phenotypic variance in ethanol withdrawal convulsion severity displayed by HS/Ibg mice had been due to genetic variance. Instead, in agreement with Goldstein (1973b), mating of females and males with high HIC scores produced WSP offspring with higher HIC scores than animals bred randomly (WSC lines), and than WSR offspring from parents bred for low HIC scores (Crabbe et al., 1983b). High- and low-scoring offspring were each bred *inter se* over subsequent generations to produce lines increasingly divergent in severity of ethanol withdrawal.

The underlying principle here is that genes influencing chronic ethanol withdrawal convulsion severity as measured by HIC become homozygously fixed (both alleles are identical and V_G becomes zero) while the remaining genes continue to segregate randomly according to Mendelian law. If the selected genes influence other traits (pleiotropy), then the selectively bred lines should also display divergent responses to those traits, but not to traits that are genetically unrelated. For example, naive WSP and WSR mice from selected generations 7 to 16 (S_7 - S_{16}) were tested for their responses to ethanol-induced hypothermia and loss of

righting reflex, as well as tolerance to these effects. No differences in sensitivity between the lines were found for these effects of ethanol, indicating that the selected genes do not influence these responses (Crabbe & Kosobud, 1986). However, WSP mice have been shown to have a higher incidence of both tremor and Straub tail, and a lower incidence of backward walking, than WSR mice following withdrawal from chronic ethanol vapor, indicating that these effects are genetically correlated with ethanol withdrawal convulsion severity (Kosobud & Crabbe, 1986).

Inbred Strains.

Experiments in inbred strains of rodents also take advantage of the concept of fixed genetic material. Inbred strains are produced through systematic inbreeding (mating of family members, usually brother and sister) over 20 or more generations (Falconer, 1989). Inbreeding produces random fixation of alleles; it is done without respect to any phenotype (save reproductive fitness). Inbreeding reduces genetic variability through mating of individuals who are more genetically alike (siblings are 50% genetically identical on average). Offspring of such matings are homozygous at more loci than their parents, and inbreeding over generations therefore eventually produces animals that are homozygous at each gene locus. Therefore, it produces animals that are genetically identical to each other (with the exception of sex-specific loci on the sex chromosomes), because all the alleles passed on to offspring, although segregating according to Mendelian law, are the same in both the male and female parent. As a result, when the genotype of one member of an inbred strain is known, the genotype of every member of that inbred strain

is known, and $V_G = 0$. It follows that the phenotypic variance displayed by the inbred strain is due to environmental variability. Therefore, when two or more inbred strains are compared for their responses to a test under equal environmental conditions, the difference in responses between the strains is due to genotypic variance, while the difference in responses among individual animals within strains is due to environmental variance.

Use of Inbred Strains to Study V_G.

Data bases for mean strain responses to biochemical and behavioral tests have been established for a number of inbred mouse strains. This is possible because the knowledge of the constancy of the genetic makeup of experimental subjects allows generalizability of results across laboratories and time. Therefore, it is possible to test a battery of inbred strains for a particular trait of interest, and compare strain mean responses for that trait with those for another trait, without having to test individual animals twice. Correlational analyses can be performed, allowing researchers to determine whether two or more traits are genetically related (positive correlation) or perhaps inversely related (negative correlation), or genetically unrelated (non-significant correlation). For example, it has been shown that strain means of mice tested for open field activity stimulation by ethanol at 10 minutes following injection are positively correlated with those of mice tested for loss of balance induced by ethanol on the dowel test, while this stimulation is negatively genetically correlated with the ethanol-induced decrease in open-field activity at 30 minutes (Crabbe, 1983).

Because of the fact that genes are fixed randomly during inbreeding, inbred strains differ in many ways for reasons which may be

unrelated to the trait being tested. Therefore, if too few inbred strains are tested for a particular pair of traits, a genetic correlation might be found even though the traits actually are genetically unrelated. Statistically speaking, the fewer the inbred strains compared, and the more traits compared, the more likelihood exists of committing a type-I error (i.e., mistakenly accepting a fortuitous correlation). Therefore, testing a sufficiently large battery of inbred strains is strongly advised for analyzing genetic correlations (Crabbe et al., 1990). Confirmation of the correlations in other genetic models is also recommended.

Genetic Studies of Chronic Withdrawal

Ethanol.

As discussed above, the WSP/WSR selection has resulted in lines of mice that are differentially sensitive to chronic ethanol withdrawal convulsions as measured by HIC. In fact, these lines (in both replicates) exhibited a ten-fold difference in HIC severity by selected generation 11 (Crabbe et al., 1985).

In a comprehensive study, mice from eighteen inbred strains were tested for chronic ethanol withdrawal using ethanol vapor inhalation (Crabbe et al., 1983a). Other mice of the same strains were tested minus the ethanol vapor (pyrazole control group) and minus ethanol and pyrazole (saline control group). Each of the strains was tested for withdrawal convulsion severity hourly for 15 hours and again at 24 and 25 hours post-withdrawal from the chambers. The index of withdrawal severity used was the area under the withdrawal HIC curve (AUC). The strain mean AUC scores for the pyrazole groups were subtracted from the strain mean AUC scores for the ethanol groups in order to control for

differences in strain sensitivity to HIC and pyrazole. Corrected area scores for representative strains showed that DBA/2N mice had the highest scores (mean 36.75), indicating they had the most severe withdrawal reaction; C57BL/6N mice had intermediate scores (mean 11.20); BALB/cAnN scores were very low (5.70); and AKR/J mice had the least severe withdrawal reaction (mean 2.29).

Crabbe and colleagues (1980) have also reported strain sensitivity differences to HIC following a two- to five-week rest after testing for ethanol-induced loss of righting reflex (4 g/kg). After this rest period, it is likely that no residual effects of the acute dose of ethanol on HIC would be present (Goldstein, 1974). Mice were scored for HIC at hours 2, 3, 4, 7, 8, and 9 after lights on (12:12 hour light:dark cycle). Twenty inbred strains were tested according to the scale of Goldstein (1974) and characterized as responsive, unresponsive, or intermediate. To be responsive, all mice of a strain had at least one convulsion (no mouse of any strain ever scored above a 1, a tonic convulsion elicited by a spin). To be unresponsive, all mice of a strain always scored zero. Intermediate strains were those having at least one mouse, but not all mice, showing a convulsion. There were eight responsive strains (among them, BALB/cAnN). C57BL/6N, AKR/J, and three other strains were unresponsive, and DBA/2N and six others were intermediate responders (Crabbe et al., 1980). This study indicates that baseline differences in HIC should be taken into account when testing for strain differences in withdrawal using this measure.

Pentobarbital.

Two genetic studies of chronic barbiturate withdrawal in mice have been reported. These studies both employed dietary phenobarbital; no

such studies were found that used pentobarbital. WSP and WSR mice from S_5 were individually housed and fed a drug-adulterated diet of 0.225% phenobarbital (Belknap et al., 1988). Upon withdrawal of the diet after 6.7 days, withdrawal signs were monitored at three-hour intervals beginning with hour 8 after withdrawal, and continuing to hour 27-30. HIC (following Goldstein, 1972b), tremor, Straub tail, wild running, spontaneous tonic/clonic convulsions, and body temperature were used to assess withdrawal. WSP mice showed significantly higher HIC, and spontaneous convulsions and wild running occurred significantly more often in WSP mice. Furthermore, WSP mice showed significantly greater hypothermia than WSR mice, although the magnitude of the difference was small. No differences in incidence of Straub tail or tremor were seen. These results indicate that the selection for enhanced severity of ethanol withdrawal convulsions in WSP mice has produced enhanced susceptibility to withdrawal convulsion severity from chronic phenobarbital treatment (Belknap et al., 1988).

Two inbred mouse strains have been tested for withdrawal from chronic phenobarbital administration in much the same paradigm (Belknap et al., 1973). This study compared C57BL/6J and DBA/2J mice, and found that withdrawal from chronic phenobarbital (measured as in the above study except without assessment of HIC) was significantly more severe in DBA/2J mice. This strain also had higher incidence of spontaneous convulsions and wild running than C57BL/6J mice (Belknap et al., 1973).

Suzuki and colleagues have reported genetic differences in the withdrawal severities displayed following chronic ethanol, barbital, and diazepam in Fischer 344 and Lewis inbred rat strains (Suzuki et al., 1992a; Suzuki et al., 1992b). All three drugs were administered in the diet (for 26,

36, and 30 days, respectively). Upon withdrawal of the diet, sixteen signs were scored at three hour intervals. Fischer 344 rats showed more pronounced withdrawal from all three drugs compared to Lewis rats.

Diazepam.

One study tested chronic diazepam withdrawal in a genetic mouse model. Similarly to Belknap et al. (1988), WSP and WSR mice were tested for differential sensitivity to diazepam withdrawal in a drug-adulterated food paradigm. Mice from both S_5 and S_{13} were housed singly and fed 1.5 mg diazepam/g diet for 7 days. At one hour after light onset, each mouse was challenged with 20 mg/kg flumazenil (i.p.), and scored for withdrawal using HIC. Results were reported as the area under the withdrawal curve. In generation S_5, WSP mice exhibited approximately two-fold higher AUC scores than WSR mice. When reassessed in generation S_{13}, this difference was more pronounced (about ten-fold); both diazepam studies closely paralleled chronic ethanol withdrawal severity differences in these lines (Belknap et al., 1989). These results indicate that the WSP/WSR selection has fixed genes mediating severity in withdrawal convulsions from phenobarbital and diazepam, as well as from ethanol.

Genetic Studies of Acute Withdrawal

The hypothesis that WSP and WSR mice are differentially sensitive to severity of withdrawal convulsions in response to acute CNS depressant drug treatment has been tested (Crabbe et al., 1991). In confirmation of Kosobud and Crabbe (1986), acute injection of 4 g/kg ethanol produced enhanced HIC scores in WSP but not WSR mice at hour 8 post-injection as compared to baseline, indicating that acute dependence occurs in WSP

mice (Crabbe et al., 1991). This study also tested the effect of single i.p. injections of acetaldehyde (400 mg/kg), t-butanol (1.5 g/kg), pentobarbital (60 mg/kg), and diazepam (20 mg/kg, antagonized by flumazenil, 10 mg/kg) in separate groups of mice. All drugs first suppressed and then significantly enhanced HIC at later time points in WSP but not WSR mice, strongly implying that WSP mice are differentially sensitive to withdrawal convulsions produced by CNS depressant drugs (Crabbe et al., 1991).

The effects of acute withdrawal from ethanol, pentobarbital, and diazepam antagonized by flumazenil have been tested in another model of selectively bred lines, the DS and DR mice (Metten & Crabbe: unpublished data). These mice were selectively bred for sensitivity and resistance, respectively, to the ataxic effects of diazepam (20 mg/kg i.p.), as measured by duration of impairment on the rotarod (Gallaher et al., 1987). It was predicted that the DS line would be more sensitive to acute diazepam withdrawal as measured by HIC, and that this sensitivity would correlate with withdrawal severity from the other drugs as well. The doses of drugs followed Crabbe et al. (1991), except that 40 mg/kg instead of 20 mg/kg diazepam was used (the flumazenil dose remained the same), since it was felt that the DR line might not respond if too low a dose was used. Contrary to predictions, the DR line was significantly more affected by acute withdrawal than the DS line in response to all three drugs, indicating that there is a negative correlation between acute CNS depressant drug withdrawal severity and diazepam-induced ataxia. However, the results were consistent with the prediction that the withdrawal severities from all three drugs are genetically correlated.

Evidence has been collected that inbred strains are differentially sensitive to withdrawal convulsions produced by 4 g/kg ethanol (Metten &

Crabbe: unpublished data). Nine inbred strains of mice that had previously been tested for morphine effects on hypothermia, open-field activity, and quinine drinking were tested for acute ethanol withdrawal after a rest interval of at least one week. The tested strains were 129/J, A/HeJ, C57BL/6J, C57L/J, CBA/J, CE/J, DBA/2J, PL/J, and SJL/J. The measure used to index withdrawal was the peak withdrawal HIC minus the average baseline HIC. This peak-minus-baseline score was calculated for each animal. When these data were analyzed by one-way ANOVA, a significant effect of strain was found, supporting the hypothesis that inbred strains are differentially sensitive to acute withdrawal from ethanol. Consistent with other reports (e.g., Roberts et al., 1992), DBA/2J mice were found to have higher withdrawal index scores than C57BL/6J mice. In fact, DBA/2J mice scored the highest by this method. CE/J and C57L/J mice were intermediate, and C57BL/6J and the other strains scored lowest.

Rationale and Hypotheses

Study Goal

Taken together, the evidence indicates that there is substantial genetic influence on CNS depressant drug withdrawal severity. Furthermore, it suggests that genetic vulnerability to severe alcohol withdrawal (i.e., dependence) may predict vulnerability to withdrawal from other CNS depressants. One direct test of genetic correlation utilizes inbred strains (McClearn, 1991). The goal of these studies was to determine in a panel of 15 inbred strains whether acute withdrawal convulsion severities from ethanol, a barbiturate, and a benzodiazepine are genetically correlated. Significant genetic correlation would suggest

that the some of the same genes are mediating the responses to all three drugs.

Drugs and Doses

The drugs and doses chosen for testing were ethanol (4 g/kg), pentobarbital (60 mg/kg), and diazepam (20 mg/kg). The ethanol dose has been shown repeatedly to induce rapid loss of righting reflex and anesthesia, from which mice generally recover in 30 -180 minutes. Furthermore, this dose is habitually used in acute dependence studies in WSP/WSR mice, as indicated above. Metabolism of this dose has been shown to be complete by 4 hours after an i.p. injection (Kosobud & Crabbe, 1986).

Pentobarbital was chosen as the barbiturate because it has a comparable half-life to ethanol, and at moderate doses, is completely metabolized by roughly 2.5 to 3.5 hours following i.p. injection. The 60 mg/kg dose has been shown to induce rapid loss of righting reflex and anesthesia in mice. Recovery is somewhat more rapid than from ethanol; however, a higher dose cannot be used safely because 60 mg/kg is very close to the minimum lethal dose (MLD) in mice.

Diazepam was chosen as the benzodiazepine in part because it is the drug of choice for prevention of ethanol withdrawal seizures in humans, and because it has been shown to have dependence liability in humans and mice. Another reason for this choice is that few studies have demonstrated withdrawal convulsions in mice with other benzodiazepines, as mentioned earlier. Diazepam (or its active metabolites oxazepam and nordiazepam) have extremely long half-lives. Consequently, this drug was tested last, and withdrawal was precipitated using the antagonist flumazenil (10 mg/kg). The benzodiazepine doses were chosen based on

their demonstrated ability to produce precipitated withdrawal convulsions in WSP mice.

Hypotheses

The specific hypotheses tested were (1) that genetic variability among inbred strains in part determines differences in susceptibility to withdrawal convulsion severity after a single hypnotic dose of either ethanol, pentobarbital, or diazepam, (2) that among the inbred strains, genetically determined differences in ethanol withdrawal severity correlates with withdrawal convulsion severity induced by a single administration of pentobarbital, or diazepam (antagonized by flumazenil), and (3) that differences in severity of pentobarbital withdrawal correlate with those of diazepam.

Materials and Methods

Animals.

Subjects were adult male mice (50-74 days old at the beginning of the first experiment) from the following inbred strains: 129/J, A/HeJ, AKR/J, BALB/cJ, C3H/HeJ, C57BL/6J, C57BR/cdJ, C57L/J, CBA/J, CE/J, DBA/1J, DBA/2J, PL/J, SJL/J, and SWR/J. These particular inbred strains were chosen for their genetic diversity and availability from the supplier. The number of subjects tested per strain ranged between 8 and 16. The mice were ordered from Jackson Laboratories, Bar Harbor, Maine, and allowed at least one week to acclimate to their new housing. They were housed by strain, 4 animals per polycarbonate cage (28 x 17 x 11.5 cm); cages were lined with corn cob bedding and cleaned twice weekly. The colony was maintained on a 12 hour light:12 hour dark cycle (lights on at 06:00), and food and water were available *ad libitum*. The colony and testing room temperatures were maintained at 22±2 °C.

HIC Scoring.

Experiments were begun at 07:30. Mice were scored for HIC severity twice, 20-30 minutes apart, before i.p. drug administration, and at intervals after injection, as specified below for each drug. The scale employed has been published (Crabbe & Kosobud, 1990), and was modified from that of Goldstein (1972b). Each mouse was picked up by the tail and observed for convulsive signs. If no signs were present within 2 seconds, the mouse was spun gently by the tail through a 180 - 360 degree arc and again observed. A score was assigned based on the specific convulsive sign and whether the mouse was required to be spun, as specified in Table 1. All HIC scoring was performed by the same

Table 1

Handling-Induced Convulsion Rating Scale

Symptom	Score
Severe, tonic-clonic convulsion, with quick onset and long duration: spontaneous, or elicited by mild environmental stimulus such as lifting cage top	7
Severe, tonic-clonic convulsion when lifted by the tail, with quick onset and long duration, often continuing for several seconds after the mouse is released	6
Tonic, clonic convulsion when lifted by the tail, often with onset delayed by as much as 1 to 2 seconds	5
Tonic convulsion when lifted by the tail	4
No convulsion when lifted by the tail, but tonic-clonic convulsion after gentle 180 - 360 degree spin	3
Tonic convulsion elicited by spin	2
Only facial grimace after spin	1
No convulsion	0

experimenter (PM).

Drug Testing Order.

In order to conserve mice and resources, each mouse was tested serially with each of the proposed drugs, with a one to two week rest period between drugs. The order of drug testing was ethanol (Experiment 1), pentobarbital (Experiment 2), and diazepam/flumazenil (Experiment 3). Testing with all three drugs constituted one pass. Since the maximum number of animals that could be tested in one experimental pass was ~60, approximately 4 mice per strain were tested in each pass. Therefore, multiple passes were required to obtain sufficient numbers of animals per strain. The strain order of testing was randomized both across passes and across drugs within pass.

Drugs.

All drugs were freshly mixed the morning of each experiment. Ethanol (200 proof; Pharmco Products, Inc.) was prepared as 20% ethanol v/v in 0.9% physiological saline, and injected in a volume of 25.33 ml per kg body weight. Pentobarbital (Sigma) was prepared as pentobarbital sodium salt dissolved in 0.9% physiological saline. Diazepam and Ro15-1788 (flumazenil) were a gift of Dr. Edward J. Gallaher. Benzodiazepines were prepared in a vehicle containing 1.25 g 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals Incorporated) per 10 ml 0.9% physiological saline. Control animals for the diazepam study were injected with vehicle. Pentobarbital, diazepam, and flumazenil were injected in a 10 ml/kg volume.

Experimental Design

Experiments 1 (Ethanol) and 2 (Pentobarbital).

The designs of these two experiments were nearly identical and will be described together. The animals were weighed and assessed for HIC. After about 20 minutes, baseline HIC was scored for a second time. Immediately thereafter, all animals were injected i.p. with drug as rapidly as possible by three to four injectors. Injections were typically complete in 7.5 minutes/60 animals. Food and water were available *ad libitum*, and lights remained on, throughout each experiment.

For Expt. 1, HICs were assessed hourly at 2 through 12 hours after injection of ethanol (4 g/kg). Between HIC assessments, the animals were left undisturbed in the room. For the first groups of animals in Expt. 2 (pentobarbital), HICs were assessed hourly at 2 through 12 hours after pentobarbital injection (60 mg/kg); however, it became apparent after two passes that in some strains, HICs were incompletely suppressed 2 hours post-injection, so for the remaining passes, HICs were also assessed at 1 hour post-injection. At the end of each experiment, animals were returned to the colony room for one to two weeks before the next experiment.

Experiment 3 (Diazepam).

The animals were weighed and housed according to the above procedures. Animals within strain were divided pseudorandomly into two drug treatment groups. Half of the animals in each strain received diazepam (20 mg/kg) and half received vehicle. All animals were subsequently injected with the antagonist, providing a measure of precipitated withdrawal and of the effect of flumazenil alone for comparison. All injections were performed by one technician. Two

baseline HICs were obtained as above from two cages of mice at a time, followed immediately by the diazepam or vehicle injection. HICs were assessed at 30 and 55 minutes following injection of diazepam or vehicle to establish that HICs were depressed in the drug group. At 60 minutes after the first injection, all animals were injected within one minute with flumazenil (10 mg/kg) and scored for HICs at 1, 3, 5, 8, and 12 minutes later. This cycle of testing was repeated for the next two cages, and so on, with the injections staggered such that all mice in one pass were tested within 4 hours, thereby avoiding gross circadian effects on testing. Furthermore, strain order between passes was randomized so that some members of each strain were tested at all times across the four-hour period.

Acute Withdrawal Indices and Statistical Analyses.

Previously published procedures were employed to index and analyze acute withdrawal scores (Crabbe et al., 1991). For each experiment, the two pre-drug baseline scores for each animal were averaged. For the ethanol and pentobarbital experiments, acute withdrawal was indexed as the corrected area under the withdrawal curve. This measure corrects for differences in baseline HIC between strains by subtracting each animal's average baseline score from each post-drug score exceeding baseline (i.e., negative scores were discarded). The remaining (i.e., ≥ 0) corrected values were then summed to give the animal's withdrawal severity. Since two drug groups were required for the diazepam experiment, withdrawal severity was indexed for each animal in the diazepam treatment group as the area under its withdrawal curve (calculated as the sum of all HIC scores following flumazenil injection)

minus the strain mean of the area under the curve from the corresponding vehicle group (see results for further discussion).

Average baselines preceding ethanol and pentobarbital injections were analyzed statistically by one-way (Strain), and preceding diazepam injection by two-way (Strain X Drug) analyses of variance. Withdrawal severities for each drug were analyzed by one-way (Strain) analyses of variance. Results were taken to be significant at $\alpha = 0.01$ in order to minimize Type I error (non-rejection of a false null hypothesis). The proportion of total phenotypic variance in drug withdrawal accounted for by genetic factors was estimated as the Sum of Squares for the between groups factor (Strain) divided by the total Sum of Squares (Keppel, 1991).

Correlational analyses using Pearson's r were performed in order to determine whether strain mean acute withdrawal severities for the three drugs were correlated and whether acute and chronic ethanol withdrawal severities were correlated. Statistical significance for the genetic correlations was based on a two-tailed test, with $\alpha = 0.05$. A less stringent alpha level was chosen for correlations since reduction of the possibility of committing a Type II error (rejection of a "true" correlation) was desired. The percentage of the common phenotypic variance accounted for by genetic factors was estimated as the square of the correlation coefficient (Falconer, 1989).

Lastly, it is possible that repeatedly testing mice affected their ability to show the same degree of withdrawal on subsequent tests as they would have if naive. If there were no effect of repeated testing, then baseline HIC scores preceding each experiment should show no change across experiments. Finding a change would not indicate whether repeated handling, previous withdrawal episodes, or previous drug treatments were

responsible; however, other studies indicate that the effect may be due to repeated handling-induced convulsions (unpublished observations). The potential effects of repeated testing were examined using a two factor ANOVA with the between groups factor Strain and the repeated measures factor, Experiment. The dependent variable was the average of the two pre-drug baseline scores preceding each experiment. Simple main effects were analyzed to determine for which strains there were significant effects of repeated testing, and Tukey's HSD test was used to determine where they differed. All tests were based on $\alpha = 0.01$ to minimize Type I error.

Results

Experiment 1: Ethanol

Consistent with earlier reports, the inbred strains varied considerably in both pre-drug average baseline HICs and withdrawal convulsion severity following a single 4 g/kg injection of ethanol. Occasionally, mice were dropped from this and subsequent studies for apparent injection failure (i.e., failure to lose righting reflex within 5 minutes compared to other mice in the cage). DBA/1J mice were mistakenly not ordered for one pass, and extra 129/J, BALB/cJ, C57BR/cdJ, and SJL/J mice were tested in a separate pass. There were no withdrawal-induced deaths in any experiment. Final numbers of mice per strain ranged from 8 - 16, as depicted in Table 2.

The time-course of acute ethanol withdrawal for four strains is shown in Figure 1. These four strains provided typical examples of the four patterns of combined baseline and withdrawal responses: low baseline/low withdrawal (e.g., AKR/J), high baseline/low withdrawal (e.g.,

129/J), high baseline/high withdrawal (e.g., BALB/cJ), low baseline/high withdrawal (e.g., DBA/2J). Five strains (e.g., AKR/J and DBA/2J, dotted lines), had very low baseline scores, while others (e.g., BALB/cJ and 129/J, solid lines) had high baseline HICs (see Table 2, p. 66). The range of baseline HICs after averaging the two scores for each subject in this experiment was from 0 - 4, and differed among strains ($F_{(14,174)}$= 23.75). Ethanol depressed HIC scores in all mice showing baseline HICs greater than 0 at 2 hours after injection, followed by a rebound elevation in HIC in some strains. Some strains (e.g., BALB/cJ and DBA/2J) had substantial increases in HICs between 5 and 7 hours following ethanol, which returned to or near baseline by hour 12. Other strains (e.g., AKR/J and 129/J) had virtually no increase in HIC above baseline. The acute ethanol withdrawal time-courses for the other eleven strains tested are depicted in Figure 2. Strains differed significantly in withdrawal severity ($F_{(14,174)}$= 9.85), after accounting for differences in baseline HIC. The proportion of variance accounted for by genetic factors was 0.44 in this experiment.

Experiment 2: Pentobarbital

As in the ethanol experiment, the inbred strains varied in both pre-drug HICs and withdrawal convulsion severity after 60 mg/kg pentobarbital. The time-course of pentobarbital withdrawal for four strains is depicted graphically in Figure 3. These strains were chosen to allow comparison of the patterns for the two strains shown in both Figures 1 and 3, and to depict the low baseline/high withdrawal and high baseline/low withdrawal patterns. Overall, it can be seen that the peak withdrawal responses to pentobarbital occurred much earlier (between 2 and 4 hours)

Figure 1

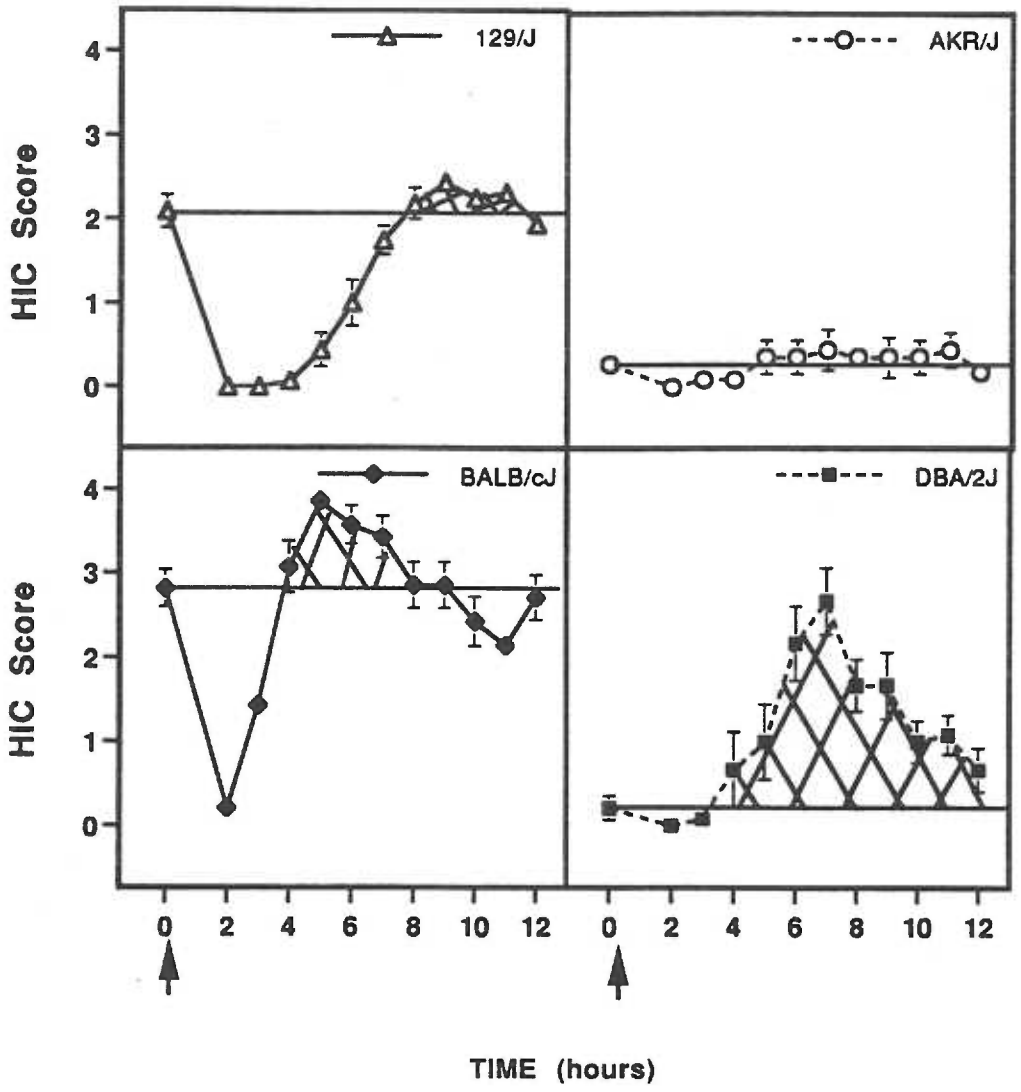

Figure 1. Time-course of ethanol withdrawal following a single i.p. injection of 4 g/kg ethanol. Symbols represent mean ± SE for each strain. Standard error bars not seen are smaller than the symbol. Y-axes: Handling-induced convulsion (HIC) score (see Table 1). X-axes: Time, in hours, following injection. Injection occurred at the arrows, immediately following pre-drug baseline HIC assessments, as discussed in the text. Withdrawal severity is depicted by the cross-hatched area. Significant withdrawal was shown by strains BALB/cJ and DBA/2J, but not 129/J or AKR/J.

Figure 2

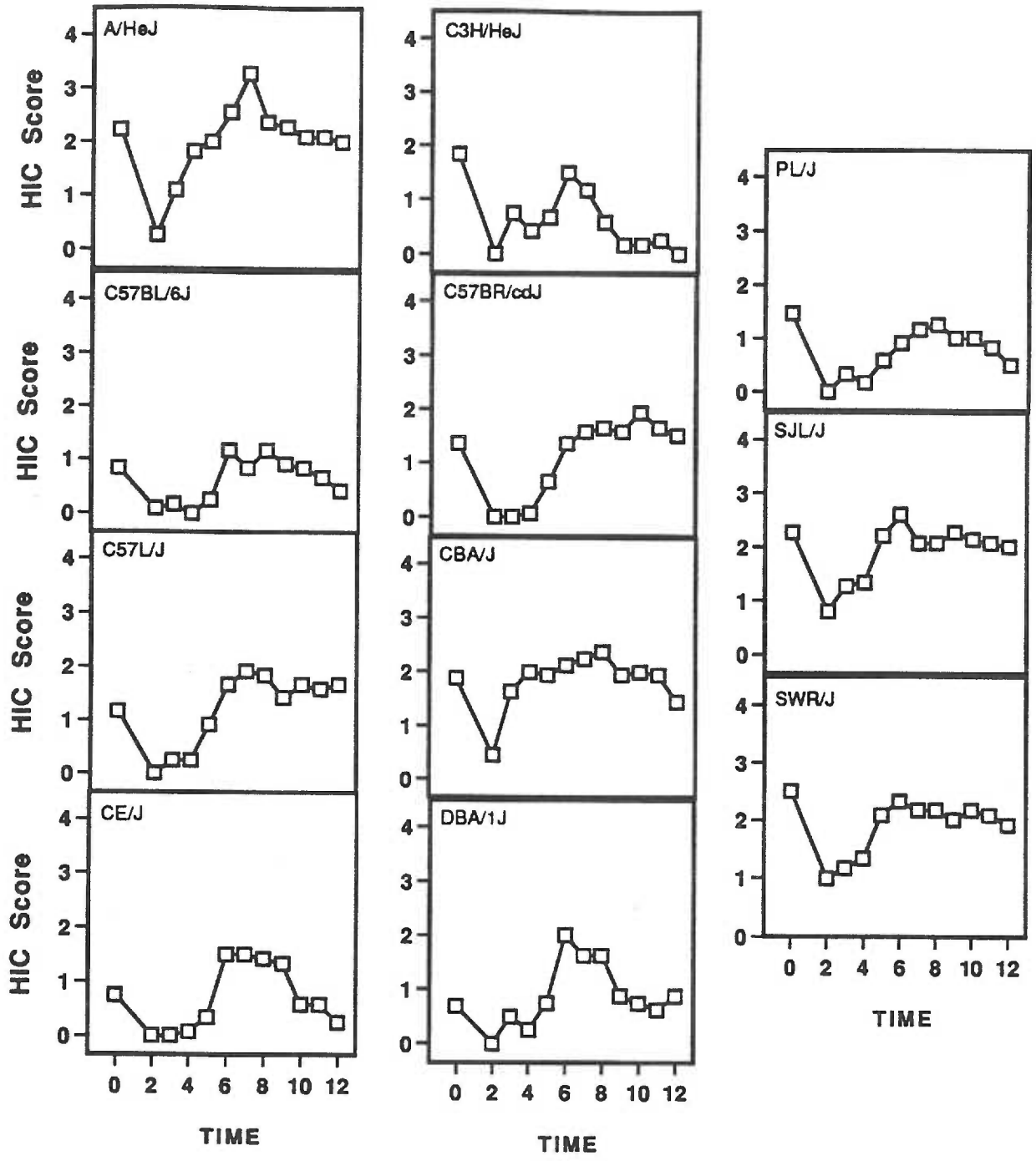

Figure 2. Time-course of ethanol withdrawal following a single i.p. injection of 4 g/kg ethanol. Square symbols represent mean for each strain. Standard errors were omitted for clarity, but ranged from 0 - 0.5. See Figure 1 for axis definitions.

Figure 3

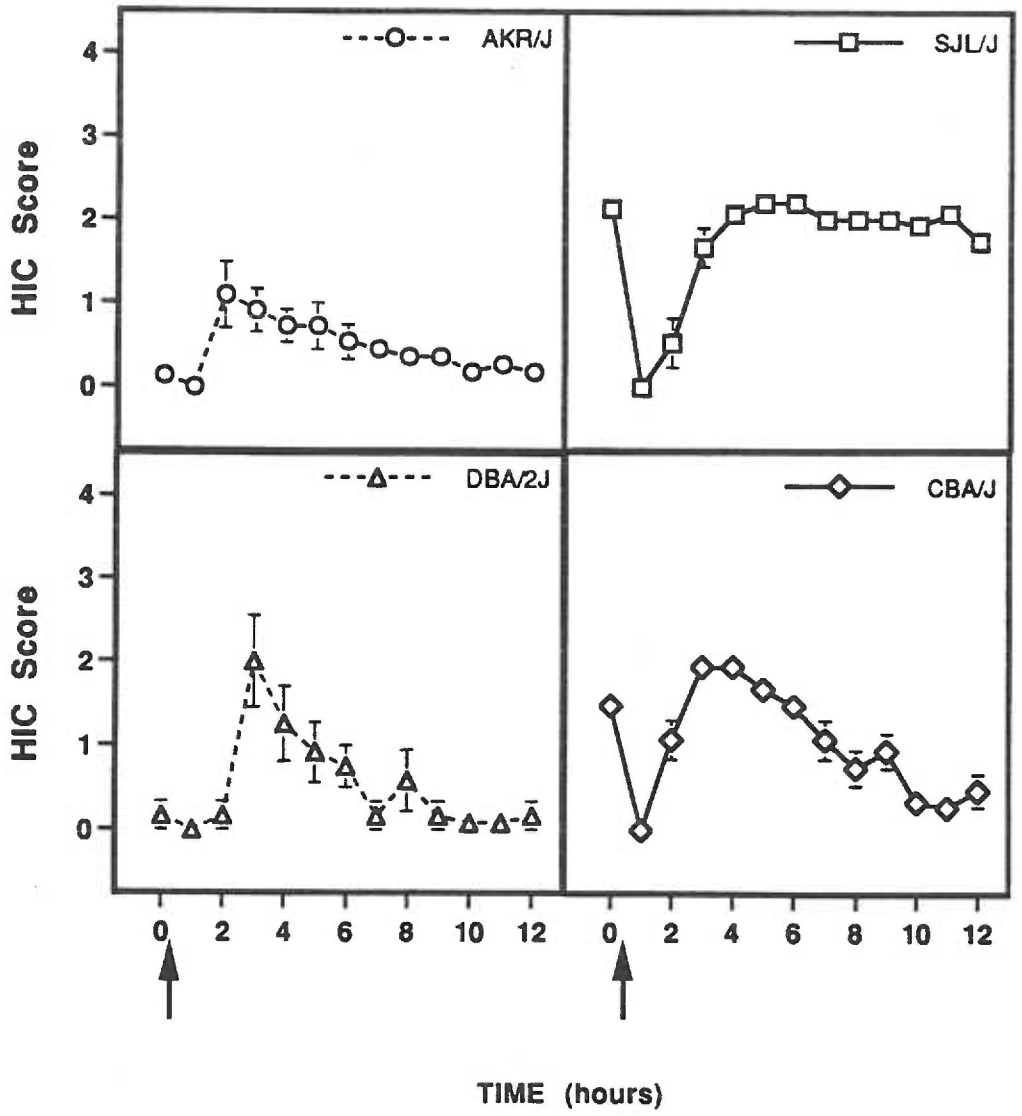

Figure 3. Time-course of pentobarbital withdrawal following a single i.p. injection of 60 mg/kg pentobarbital. Symbols represent mean ± SE for each strain. Standard error bars not seen are smaller than the symbol. Y-axes: Handling-induced convulsion (HIC) score (see Table 1). X-axes: Time, in hours, following injection. Injection occurred at the arrows, immediately following pre-drug baseline HIC assessments, as discussed in the text. Significant withdrawal was shown by strains AKR/J and DBA/2J, but not SJL/J or CBA/J.

than in the ethanol experiment. Six strains (e.g., AKR/J and DBA/2J, dotted lines), had very low baseline scores in this experiment, while other strains (e.g., SJL/J and CBA/J, solid lines) had high baseline HICs. Again, the range of baseline HICs after averaging was 0 - 4, and differed among strains ($F_{(14,169)}$= 45.02). Pentobarbital depressed HIC scores in all strains whose baseline HICs were greater than 0 at 1 hour after injection. Although some strains (e.g., SJL/J mice, square symbol) regained basal levels of HIC by 2 - 4 hours after injection, no increase above baseline was seen. CBA/J mice (diamond symbol) are an example of a strain which showed a moderate, but short-lived, rise above baseline. Other strains (e.g., DBA/2J and AKR/J, triangle and circle symbols, respectively) regained basal levels of HIC by 1 - 3 hours after injection and attained substantial peak withdrawal by 2 - 3 hours. Basal levels generally were regained by hours 7 - 8. Note that AKR/J mice had substantially higher pentobarbital withdrawal than they did in response to ethanol. Strains differed significantly ($F_{(14,169)}$= 3.73), after accounting for differences in baseline HIC, and the proportion of variance accounted for by genetic factors was 0.24 in this experiment.

Experiment 3: Diazepam

The inbred strains also differed significantly in both baseline and withdrawal convulsion severities following precipitation of withdrawal from 20 mg/kg diazepam. The time-course of precipitated diazepam withdrawal for four strains is depicted in Figure 4. These strains give examples of low and high withdrawal as shown by the cross-hatched area; one strain was shown in Figure 1, and one in Figure 3. The range of average baseline scores obtained was again 0 - 4. A significant main effect of strain on

Figure 4

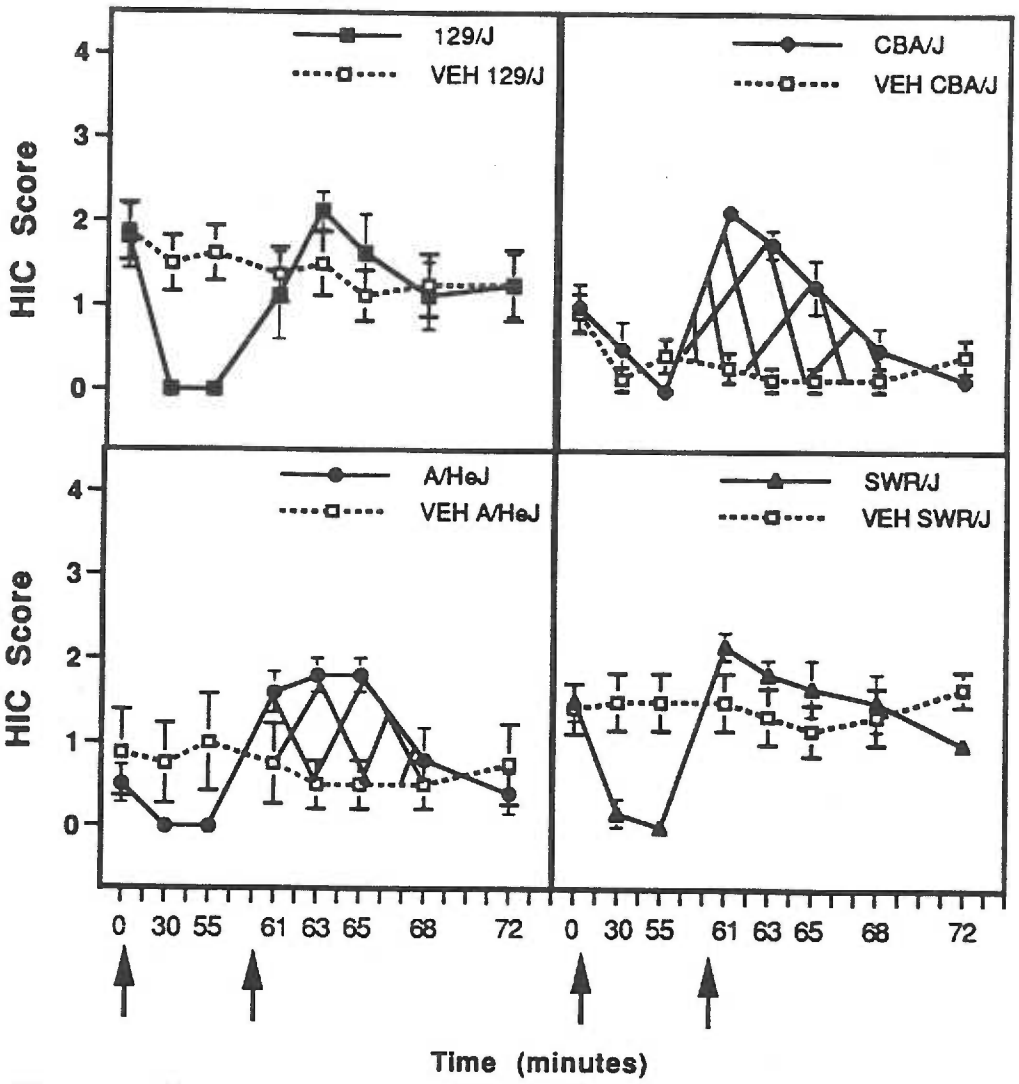

Figure 4. Time-course of diazepam withdrawal following a single i.p. injection of 20 mg/kg diazepam. Symbols represent mean ± SE for each strain. Standard error bars not seen are smaller than the symbol. Y-axes: Handling-induced convulsion (HIC) score (see Table 1). X-axes: Time, in minutes, following diazepam or vehicle injection. Axis breaks were omitted for clarity. Diazepam or vehicle injection occurred at the first arrows, immediately following pre-drug baseline HIC assessments, as discussed in the text. Flumazenil (10 mg/kg) was injected at the second arrow. Withdrawal severity is depicted by the cross-hatched areas on two of the plots, but omitted for clarity on the other two. Closed symbols represent the diazepam-treated animals. Significant withdrawal was shown by strains A/HeJ and CBA/2J, but not 129/J or SWR/J.

baseline HIC was detected ($F_{(14,152)}$= 23.14). As expected, neither a significant main effect of drug treatment group ($F_{(1,152)}$= 0.42, p = 0.51) nor Strain X Drug interaction ($F_{(14,152)}$= 0.32, p = 0.99) was found for the baseline measure (no drug had yet been given). In all strains having baseline HICs greater than 0, diazepam depressed HIC scores at 30 and 55 minutes following injection. Flumazenil injection restored HIC severity in the diazepam-treated animals to near baseline or higher levels in all strains.

Analysis of the vehicle group data revealed significant differences among strains for the sum of the post-flumazenil HIC scores ($F_{(14,72)}$= 5.80). These data indicated the need to control for basal differences in responding to flumazenil for the precipitated diazepam withdrawal groups. Therefore, within each particular strain, the mean of the sum of the vehicle group's post-flumazenil HIC scores was determined. To index diazepam withdrawal, the vehicle group strain mean was subtracted from the sum of the post-flumazenil HIC scores for each individual animal in the diazepam group. If the result was negative, a withdrawal score of zero was assigned (< 19% occurrence). Some strains, (e.g., CBA/J, solid diamonds and A/HeJ, solid circles) had severe withdrawal from diazepam, as shown by the cross-hatched areas in Figure 4. Other strains (e.g., 129/J, solid squares and SWR/J, solid triangles) had insignificant or slight withdrawal from diazepam. Strains differed significantly ($F_{(14,80)}$= 5.22) in diazepam withdrawal severity. The proportion of variance accounted for by genetic factors was 0.48 in this experiment. In addition, withdrawal severity was calculated as the difference from pre-drug HICs within animal, as in the ethanol and pentobarbital experiments. Inbred strains also differed significantly

($F_{(14,80)}$= 7.46) using this index. In fact, these two measures were significantly genetically correlated (r = 0.79).

Genetic Correlations

Figure 5 A-C shows the strain mean distributions for withdrawal severity following an acute injection of ethanol, pentobarbital, and diazepam, respectively. Note the different y-axis scales. With the exception of the ethanol withdrawal severity score for the DBA/2J strain, the approximate range of withdrawal severity scores for all three drugs was from 0 to 6. Clearly, the response of the DBA/2J strain to acute ethanol withdrawal exceeded that of the other strains, and exceeded this strain's response to the other two drugs. Using an arbitrary system of classification into low- (scores < 2.75) and high-withdrawal groups (scores ≥ 2.75), it can be seen that the following strains exhibited low withdrawal scores from all three drugs: 129/J, C3H/HeJ, C57BL/6J, PL/J, SJL/J, and SWR/J. High withdrawal scores from all three drugs was shown only by BALB/cJ and DBA/1J strains. The remaining strains showed a mixed response to the three drugs.

Scatterplots of the genotypic correlations using the strain mean withdrawal severities are shown in Figure 6 A-C. Ethanol withdrawal scores correlated significantly with corresponding pentobarbital withdrawal score means (r = 0.70, see Fig. 6A), indicating that there is substantial overlap in genes influencing acute withdrawal from these two drugs. Likewise, strain mean pentobarbital scores were significantly correlated with strain mean diazepam scores (r = 0.55), indicating that pentobarbital and precipitated diazepam withdrawal share a genetic basis (Fig. 6B).

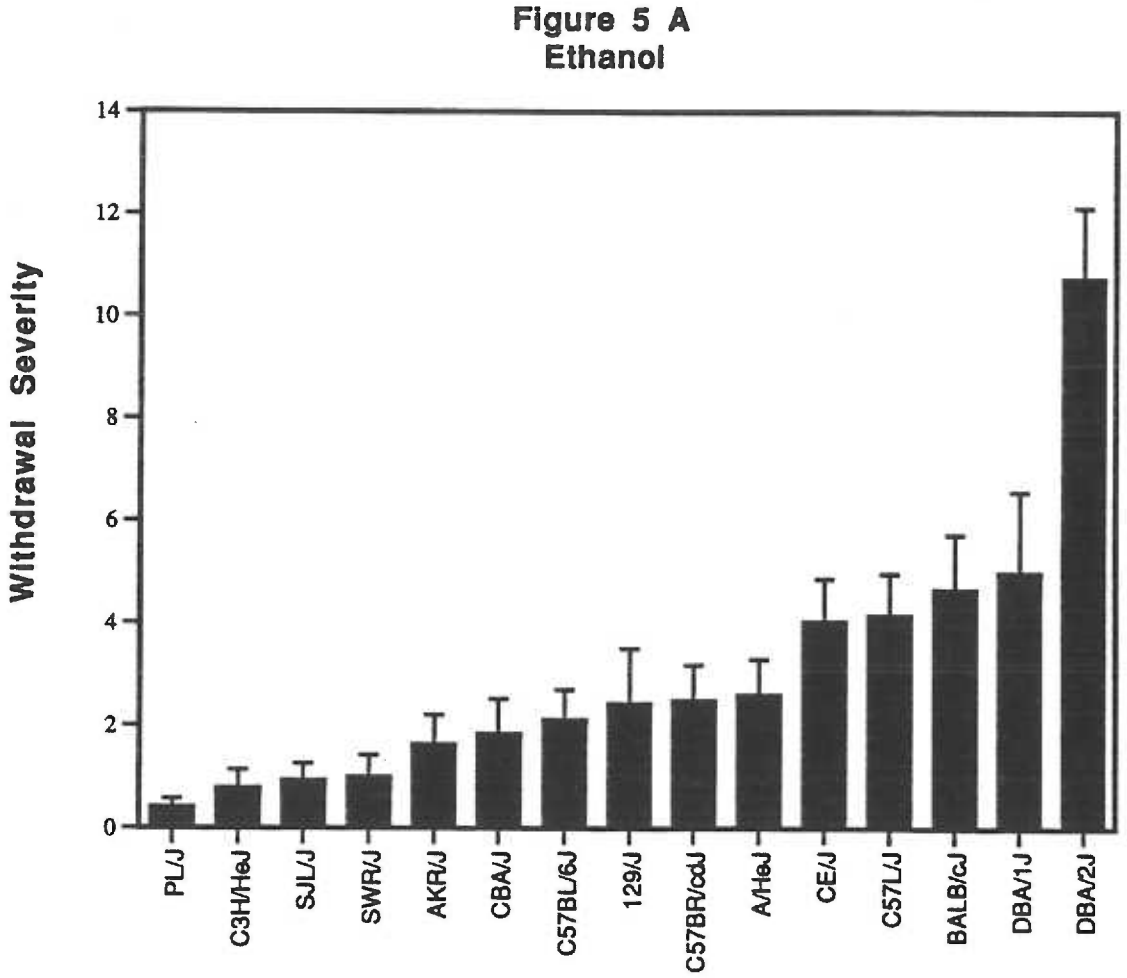

Figure 5 A. Strain mean (± SE) withdrawal severities following ethanol. Withdrawal severity scores were corrected areas under the withdrawal curve, computed as discussed in the text.

Figure 5B
Pentobarbital

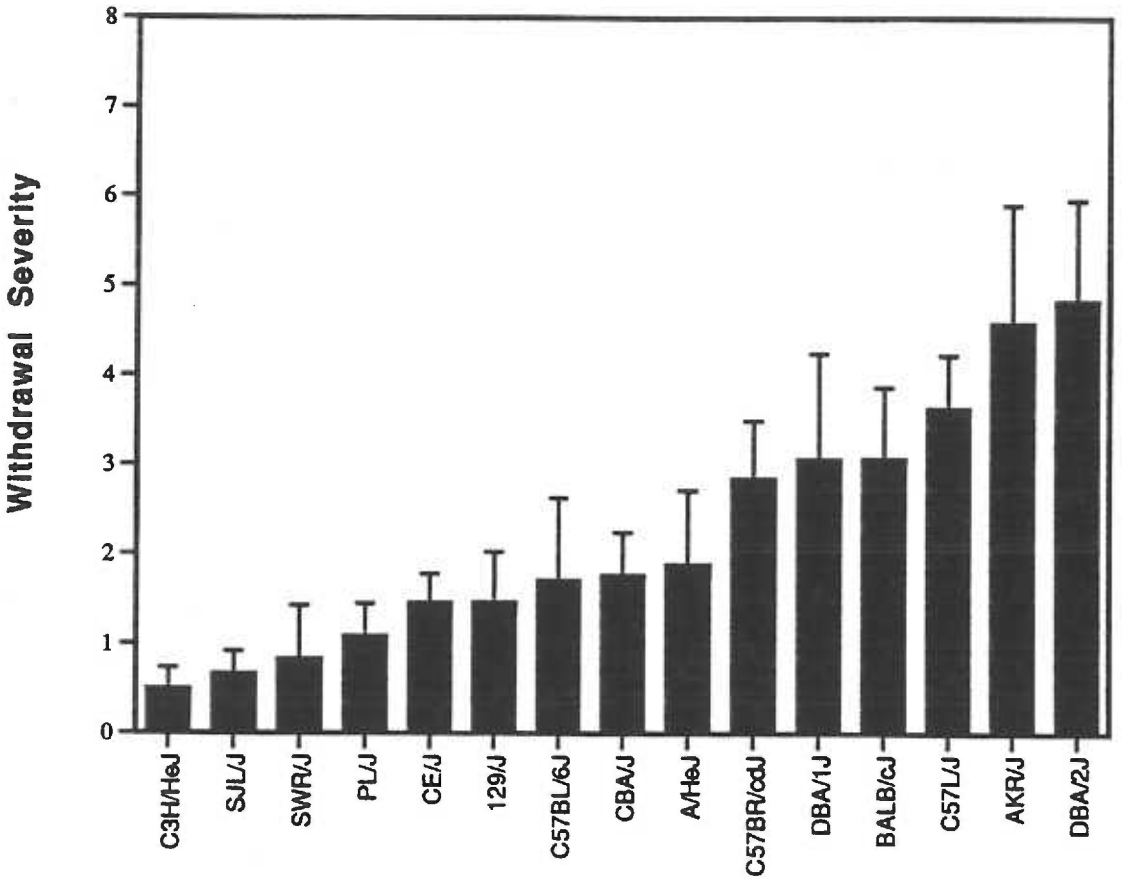

Figure 5 B. Strain mean (± SE) withdrawal severities following pentobarbital. Withdrawal severity scores were corrected areas under the withdrawal curve, computed as discussed in the text.

Figure 5 C
Diazepam

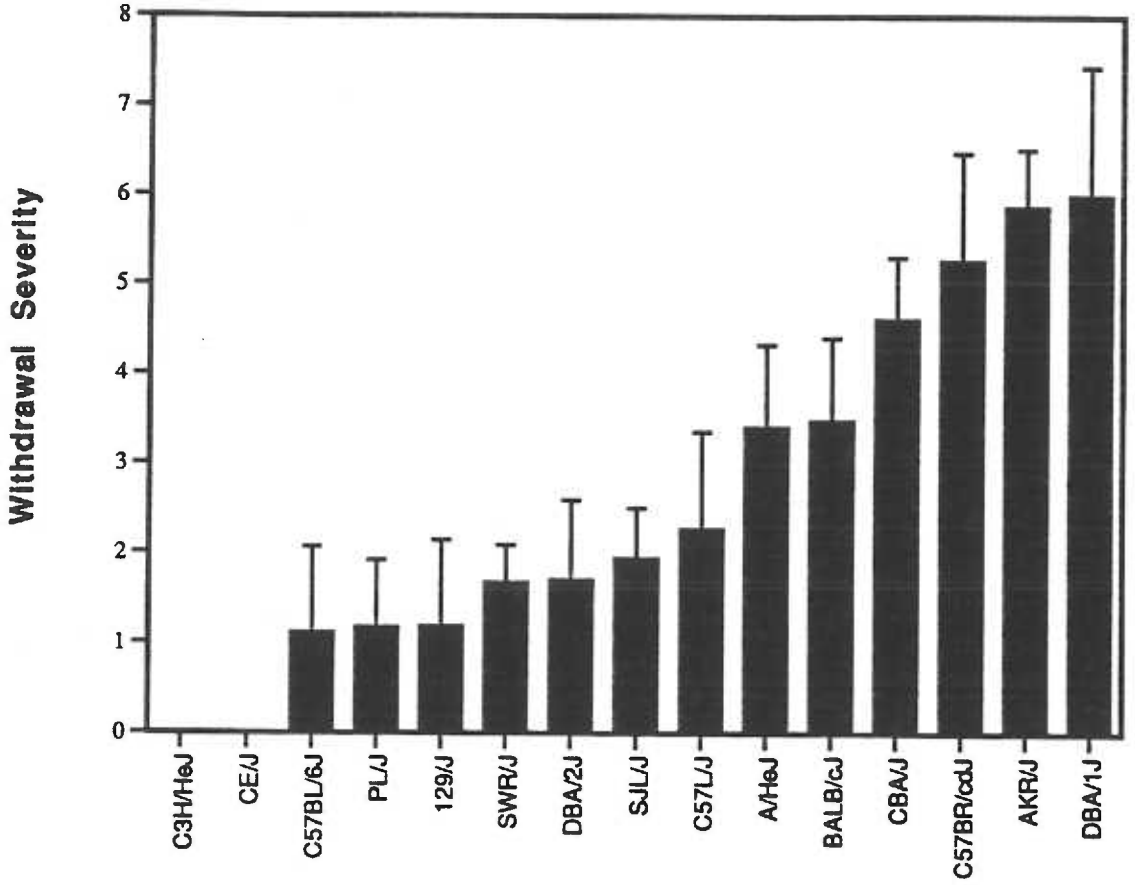

Figure 5 C. Strain mean (± SE) withdrawal severities following diazepam. Withdrawal severity scores for strains C3H/HeJ and CE/J were zero. Withdrawal severity scores were corrected areas under the withdrawal curve, computed as discussed in the text.

Figure 6A

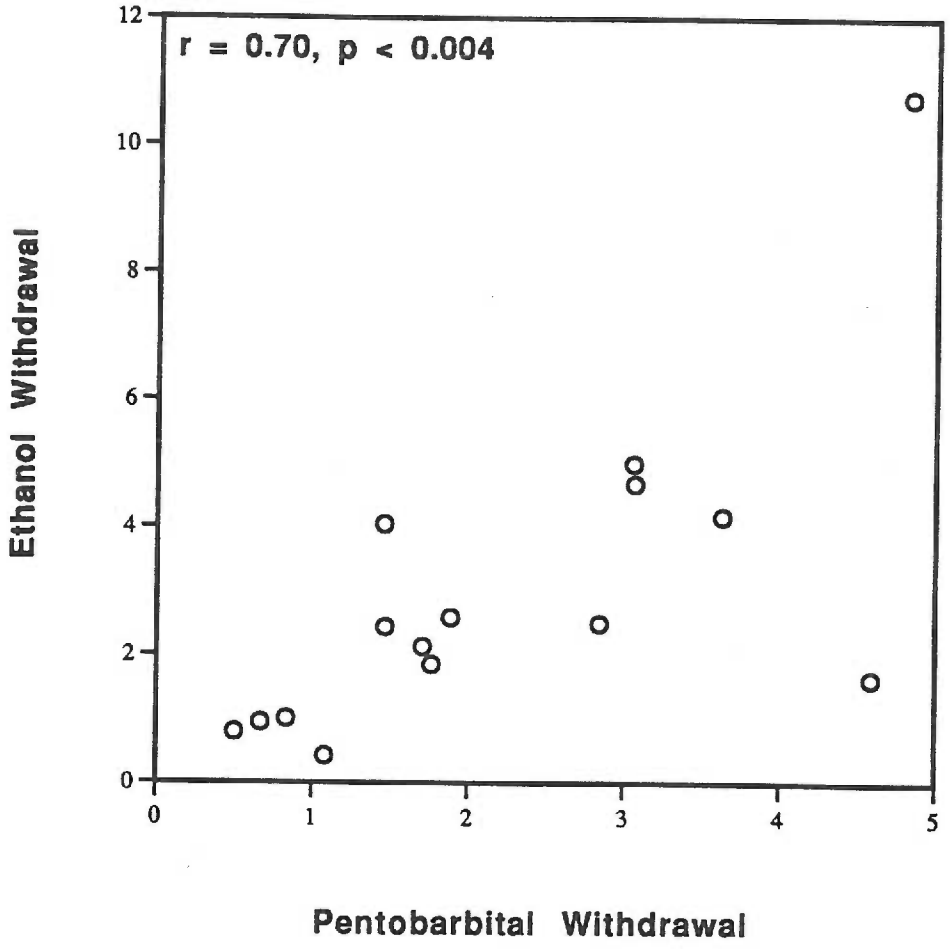

Figure 6 A. Scatterplot of strain means showing genotypic correlation of withdrawal severity scores of ethanol with pentobarbital. Each dot represents an inbred strain. Correlation coefficient and corresponding significance level are shown in the upper left hand corner of the plot.

Figure 6B

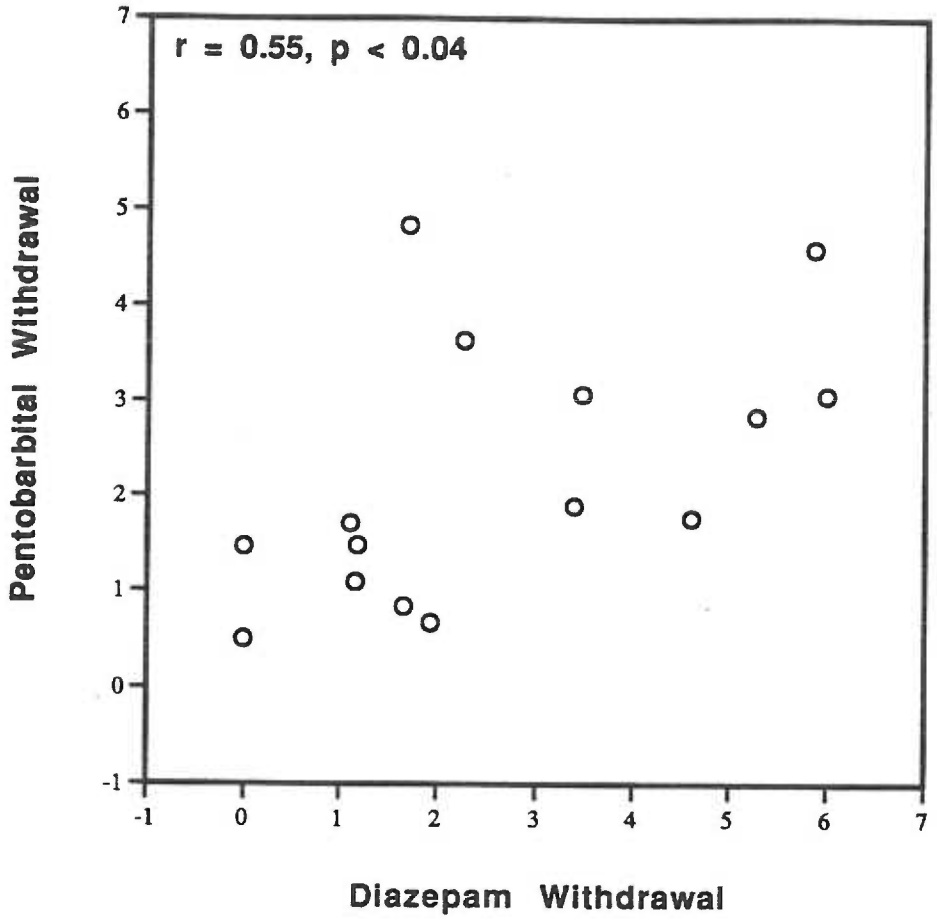

Figure 6 B. Scatterplot of strain means showing genotypic correlation of withdrawal severity scores of pentobarbital with diazepam. Each dot represents an inbred strain. Correlation coefficient and corresponding significance level are shown in the upper left hand corner of the plot.

Figure 6C

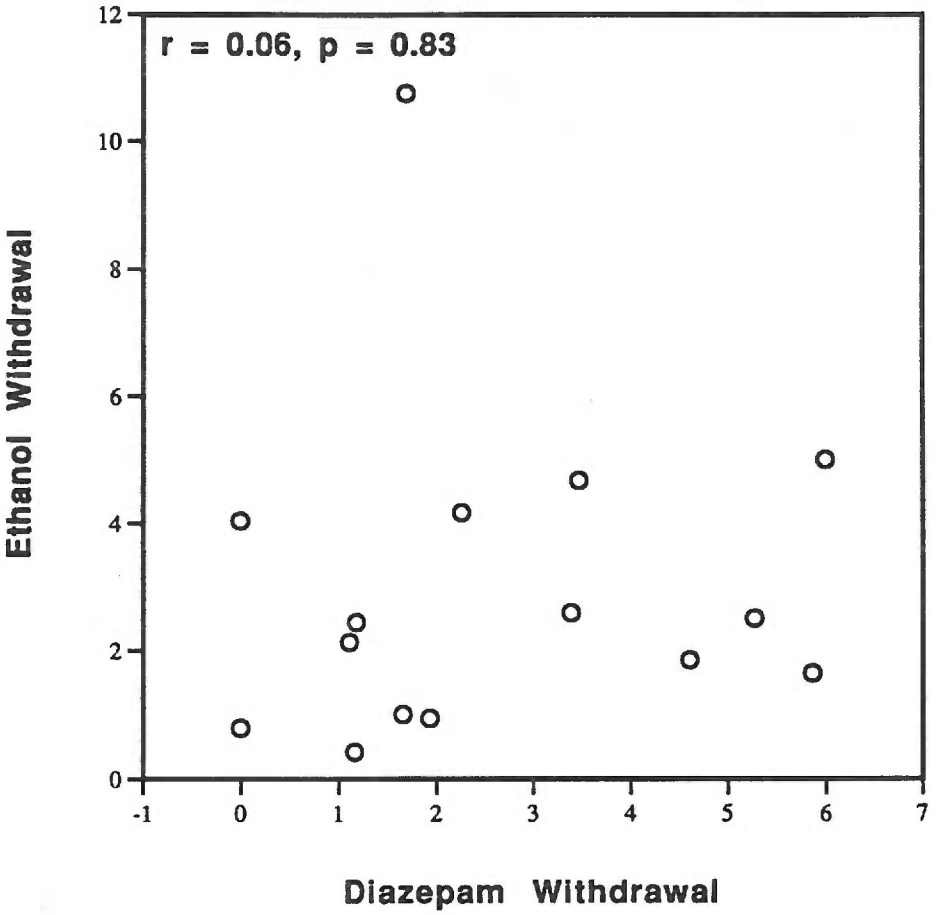

Figure 6 C. Scatterplot of strain means showing genotypic correlation of withdrawal severity scores of ethanol with diazepam. Each dot represents an inbred strain. Correlation coefficient and corresponding significance level are shown in the upper left hand corner of the plot.

Interestingly, strain mean ethanol and diazepam scores were not significantly correlated ($r = 0.06$, $p = 0.83$, Fig. 6C), indicating a lack of genetic commonality in mechanism. The proportions of variance accounted for by common genetic factors were 0.49, 0.30, and 0.004, respectively.

Since the DBA/2J strain had an extremely high score for ethanol withdrawal, correlations were also calculated after removal of this strain. The ethanol/pentobarbital and pentobarbital/diazepam withdrawal correlations remained significant ($r = 0.56$ and $r = 0.73$, respectively). Although still not significant, removing the DBA/2J strain mean improved the ethanol/diazepam correlation ($r = 0.31$, $p = 0.29$).

Correlations were also performed between drugs with the diazepam withdrawal severity strain means calculated in the same manner as those for ethanol and pentobarbital (see page 48). Results were essentially the same as shown: the pentobarbital/diazepam correlation was significant ($r = 0.71$) and the ethanol/diazepam correlation remained not significant ($r = 0.34$).

Effects of Repeated Testing

The effects of repeated testing on the baseline scores obtained before each experiment were examined. Table 2 reports baseline scores preceding each drug injection for each strain and experiment. Since baseline scores were not significantly different between diazepam drug treatment groups in Expt. 3, baseline scores were combined for these groups prior to analysis. Over all three drug tests, the between-groups factor, Strain, was significant. The effect of repeated testing was also

Table 2

Handling-Induced Convulsion Scores Before Drug Injections in Experiments 1 (Ethanol), 2 (Pentobarbital), and 3 (Diazepam)

(values are Mean ± SE)

	Ethanol		Pentobarbital		Diazepam	
Strain	Baseline	n	Baseline	n	Baseline	n
129/J	2.09±0.19	16	2.28±0.16	16	1.84±0.25	16
* A/HeJ	2.23±0.14	11	1.44±0.19	9	0.67±0.25	9
AKR/J	0.27±0.12	11	0.14±0.10	11	0.55±0.20	11
BALB/cJ	2.82±0.22	14	2.86±0.21	14	2.64±0.17	14
* C3H/HeJ	1.83±0.19	12	0.00±0.00	12	0.04±0.04	12
C57BL/6J	0.83±0.26	12	0.33±0.11	12	0.33±0.17	12
C57BR/cdJ	1.36±0.15	14	1.27±0.17	13	1.04±0.17	13
C57L/J	1.17±0.16	12	1.09±0.06	11	1.00±0.15	11
* CBA/J	1.88±0.07	16	1.47±0.12	15	0.97±0.17	15
* CE/J	0.75±0.14	12	0.04±0.04	12	0.00±0.00	12
DBA/1J	0.69±0.28	8	0.38±0.21	8	0.06±0.06	8
DBA/2J	0.21±0.14	12	0.17±0.17	12	0.18±0.18	11
* PL/J	1.46±0.11	12	1.00±0.16	12	1.13±0.20	12
SJL/J	2.27±0.11	15	2.13±0.08	15	2.43±0.10	14
* SWR/J	2.50±0.14	12	2.07±0.16	12	1.46±0.18	12

* Strains for which there was a significant effect of repeated testing as reported in Table 3.

significant, based on the within-subjects factor, Experiment ($F_{(2,334)}$= 61.13). The interaction term was also significant ($F_{(28,334)}$= 6.23), so the results were further examined by analysis of the simple main effects of Experiment (i.e., analysis of the effects of repeated testing on Baseline scores within each strain).

Table 3 shows the six strains for which there was a significant effect of repeated testing. Degrees of freedom for each simple main effects analysis, F values obtained, and the critical values of F at the $\alpha = 0.01$ significance level (Keppel, 1991) are in the first three columns of the table. The baseline scores of these strains were further examined using Tukey's HSD. Significant mean differences and the critical values ($\bar{d}_T$) with which they were compared make up the fourth and fifth columns. For example, when the A/HeJ strain was examined for the simple main effect of experiment, the F value obtained was 12.83. This value was compared to the critical value of 6.23 obtained from the statistical table for F distributions (Keppel, 1991, p. 502). Since 12.83 > 6.23, there was a significant effect of experiment (i.e., repeated testing). In order to determine which experiment(s) was (were) affected by earlier testing of this strain, Tukey's test was then performed. The value of baseline for the ethanol experiment exceeded that of the diazepam experiment by 1.39 in this strain. This number was then compared with the critical value, $\bar{d}_T$ = 0.93, calculated as the minimum pairwise difference between treatment means according to Keppel (1991, p. 174). Since 1.39 > 0.93, there was a significant decrease in baseline scores for the A/HeJ strain in the diazepam experiment compared to the ethanol experiment. Results for the other two possible pairwise mean comparisons (ethanol vs. pentobarbital and pentobarbital vs. diazepam) showed that these were not significant, and therefore are

Table 3

Strains Affected by Repeated Testing

Strain	df	F	Critical Value[1]	Significant mean difference[2]	$\bar{d}_T$[3]
A/HeJ	2, 16	12.83	6.23	1.39 (ET>DZ)	0.93
C3H/HeJ	2, 22	90.12	5.72	1.83 (ET>PB) 1.79 (ET>DZ)	0.51
CBA/J	2, 28	21.90	5.45	0.90 (ET>DZ) 0.50 (PB>DZ)	0.43
CE/J	2, 22	22.68	5.72	0.71 (ET>PB) 0.75 (ET>DZ)	0.41
PL/J	2, 22	5.97	5.72	0.46 (ET>PB)	0.45
SWR/J	2, 22	10.59	5.72	1.04 (ET>DZ)	0.74

[1] Value of F which is considered to be significant at $\alpha = 0.01$.

[2] Differences between means found to be significant using Tukey's HSD test at $\alpha = 0.01$. See footnote 3 and text. ET: Ethanol; PB: Pentobarbital; DZ: Diazepam.

[3] Critical value of Tukey's HSD test which is considered to be significant at $\alpha = 0.01$.

not given in Table 3.

The results of this analysis generally show that the baseline scores of six strains did change over time, and indicate the possibility that the severity of withdrawal from pentobarbital or diazepam shown by at least these strains may have been affected by earlier testing. Therefore, the inter-drug correlations were recalculated after removal of these six strains from the correlational analyses: only the ethanol/pentobarbital correlation remained significant ($r_{(7)} = 0.63$). The pentobarbital/diazepam correlation was no longer significant ($r_{(7)} = 0.42$), and the ethanol/diazepam correlation was also not significant ($r_{(7)} = -0.12$). The loss of significance of the pentobarbital/diazepam correlation is not surprising since the test for genetic correlation was weakened greatly by the reduction in sample size (from 15 to 9 strains).

A possible counter-argument to this analysis is that the HIC measure may be unreliable, and therefore, a significant difference between baseline scores among experiments may not be due to repeated testing, but to unreliability of the measure. To test this hypothesis, these data were subjected to split-half reliability analysis. Animals within strain were randomly assigned to either half of the data set, holding the sample sizes approximately equal, and strain means within each half were calculated for baseline and withdrawal severity scores for each drug. Split-half correlations were then performed within drug and within variable (e.g., the strain means for half A on the variable baseline preceding ethanol injection were correlated with the strain means for half B on the same variable). The results were that the baselines and withdrawal severities for each drug showed significant split-half reliability ($p < 0.01$), as depicted in Table 4A. The genetic correlations of withdrawal severities

were also subjected to split-half reliability analysis. The results are depicted in Table 4B. After correction by the Spearman-Brown correction (McNemar, 1966, p. 150), both randomly chosen halves of the data supported the genetic correlations between ethanol and pentobarbital withdrawal severities, and between pentobarbital and diazepam withdrawal, but not between ethanol and diazepam withdrawal.

Finally, retesting the animals for ethanol withdrawal at the end of the diazepam withdrawal experiment would have yielded test-retest reliability information for these data. Unfortunately, this idea did not occur until too late. Problems with the plan include: (1) there are six strains already affected by repeated testing (see Table 3); (2) the two drug-group design of the diazepam experiment meant that potential differences between differently-treated animals could have occurred, reducing the sample size for test-retest reliability; and (3) pharmacokinetic concerns regarding metabolism of diazepam and its active metabolites (see Discussion section) would have necessitated a considerable delay before final ethanol testing, potentially involving age or body weight effects.

Table 4

A: Split-Half Reliability Correlations Among Drug Variables

Variable[1]	Correlation Coefficient[2]	Split-Half Reliability[3]
Ethanol Baseline	0.898	0.95
Pentobarbital Baseline	0.956	0.98
Diazepam Baseline	0.912	0.95
Ethanol Withdrawal	0.820	0.90
Pentobarbital Withdrawal	0.643	0.78
Diazepam Withdrawal	0.854	0.92

B: Split-Half Reliability Correlations of Genetic Correlations

Half of Data	Genetic Correlation	Correlation Coefficient[4]	Split-Half Reliability[5]
A	Ethanol/Pentobarbital	0.528*	0.69*
B	Ethanol/Pentobarbital	0.763*	0.87*
A	Ethanol/Diazepam	-0.043	-0.09
B	Ethanol/Diazepam	0.153	0.27
A	Pentobarbital/Diazepam	0.425	0.60*
B	Pentobarbital/Diazepam	0.540*	0.70*

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[1] Baseline scores were the average of two scores for each animal, and Withdrawal scores were computed for ethanol and pentobarbital using within animal corrections and computed for diazepam using between group correction, as discussed in the text.

[2] Pearson's r ($d.f.$ = 13). All values are significant ($p < 0.01$).

[3] Correlation coefficients were modified for split half reliability according to the Spearman-Brown correction: $\frac{2r}{1+r}$. All values are significant ($p < 0.01$).

[4] Pearson's r ($d.f.$ = 13). Starred values are significant ($p < 0.05$).

[5] Correlation coefficients were modified for split half reliability according to the Spearman-Brown correction: $\frac{2r}{1+r}$. Starred values are significant ($p < 0.05$).

Discussion

Key Findings

The results of these experiments in inbred strains show clearly that severity of acute ethanol, pentobarbital, and diazepam withdrawal are genetically mediated phenomena, in independent confirmation of the results obtained in the WSP and WSR selected lines (Crabbe et al., 1991). Furthermore, these results confirm that severity of acute ethanol and pentobarbital withdrawal are genetically correlated, indicating some commonality of the genes involved. Genetic correlation of acute pentobarbital and precipitated diazepam withdrawal also was confirmed. However, the results show that acute ethanol and precipitated diazepam withdrawal were not genetically correlated in these inbred strains, as they were in the WSP and WSR mice (Crabbe et al., 1991).

It is important to emphasize several key findings. First, baseline and withdrawal measures were shown to be reliable based on split-half reliabilities (see Table 4). Furthermore, the inter-drug genetic correlations were shown to be reliable. Second, there was minimal loss of animals indicating that despite the substantial doses tested and successive withdrawal episodes, the animals were largely healthy. This is further upheld by the fact that body weights were not significantly affected by treatments (data not shown). Third, the ethanol/pentobarbital correlation was upheld whether or not strains were omitted due to the disproportionately intense ethanol withdrawal (DBA/2J) or to having been affected by repeated testing (Tables 2 & 3). Fourth, the ethanol/diazepam correlation was not significant whether diazepam withdrawal severity was calculated using between groups or within animal corrections (however, see below).

The differing magnitudes of withdrawal from the three drugs requires discussion. As can be seen from Figure 5, the magnitude of withdrawal ranged from 0 to about 6 (omitting the DBA/2J strain for ethanol only), and the strains which showed the most severe withdrawal from all three drugs tended to show the most severe withdrawal from ethanol. It could be argued that the doses of pentobarbital and diazepam were insufficient to induce the degree of dependence shown by 4 g/kg ethanol. While it is reasonable to increase the diazepam dose (e.g., from 20 mg/kg to 40 mg/kg; see section on acute withdrawal in genetic models) in order to test this hypothesis, increasing the pentobarbital dose is likely to result in significant loss of animals since 60 mg/kg is close to the MLD in mice. Furthermore, the reliability of the findings argues that the genetic correlations do not require the "same degree of dependence" inducement by the three drugs. This is particularly true for the pentobarbital/diazepam correlation, since the doses of these two drugs seem to have induced the same degree of dependence.

Contributions of these Data

These data make an important contribution to the inbred strain data base being compiled by several laboratories. They provide opportunities for further use in several key ways.

Inter-Study Genetic Correlations

First, the data may be used to test genetic correlations with other behaviors following administration of these drugs, such as hypothermia tolerance, locomotor activation or sedation, and conditioned place preference, as well as with withdrawal phenomena (e.g., Straub tail,

tremor) occurring from these and other drugs (Crabbe, Gallaher, Phillips, & Belknap, in press; Gallaher, Belknap, Jones, Cross, & Crabbe, manuscript in preparation). One hypothesis that is readily suggested is that acute withdrawal may predict chronic withdrawal. The present data are used in conjunction with those previously published by Crabbe et al. (1983a) in a test of this hypothesis in a special section below.

Quantitative Trait Loci Analysis

Second, these data may be subjected to quantitative trait loci (QTL) analysis as part of our efforts to map the genes responsible for withdrawal (Belknap, et al., 1993; Buck, Metten, Glenn, Belknap, & Crabbe, submitted; and, see Plomin & McClearn, 1993, for a brief discussion on the method plus issues of reliability, etc.). Previously, we reported the results of our testing of the BXD recombinant inbred (RI) strain series (derived from inbreeding F_2 progeny from the cross of C57BL/6J and DBA/2J mice) for acute ethanol withdrawal and the resultant QTL analysis (Belknap et al., 1993). This analysis suggested six loci on four chromosomes as potentially influencing acute ethanol withdrawal convulsions. In order to verify one candidate locus on chromosome 2, individual F_2 mice were then tested for acute ethanol withdrawal and genotyped using several markers that are known to be polymorphic between the two parental strains and whose map locations on chromosome 2 are fairly well-defined (Buck et al., submitted). The genotypes of the F_2 mice at one of these markers, *D2Mit9*, ~38-39 centiMorgans (cM) distal to the centromere, were significantly correlated with acute ethanol withdrawal convulsion severity. This indicates that this relatively small region, which is syntenic with (has the same marker loci in the same region as) human chromosome 2q24-q37,

encodes a gene which influences acute ethanol withdrawal severity. Confirmation in F_2 mice of more of the original QTL found in the BXD study is in progress.

Although it is highly unlikely that the inbred strain data will allow us to map any locus more precisely because the genotypic data base is not as dense and there are fewer degrees of freedom in the analysis, the inbred QTL will provide an independent test which could confirm some of the loci. Furthermore, these data are useful for selecting progenitor strains of any of the two dozen or so RI series available for testing. It would be useful to examine inbred strains and RI series which possess the same alleles as the C57BL/6J and DBA/2J strains for the same marker loci or closely linked markers to the putative QTL site.

Hypothesis Generation

Third, and perhaps most important, these data may be used to generate hypotheses which may be tested in future studies regarding the mechanisms underlying CNS depressant drug withdrawal phenomena and their genetic underpinnings. The studies in BXD RI strains provide an example: two loci within one cM of the marker locus *D2Mit9* have potential influences on withdrawal which are readily suggested. One locus (actually several tightly linked loci) encodes a cluster of α subunits of the neural sodium channel involved in action potentials. The panel of inbred strains tested in the present studies could be examined for the identity of the subtype of the α subunit of the sodium channel. If the neural sodium channel is involved in withdrawal convulsion severity, then it might be expected that strains having more severe withdrawal would have a different subtype in key brain regions than strains which display a mild

withdrawal response. Alternatively, expression levels of one or more subtypes could increase during ethanol withdrawal in more sensitive strains.

The other locus encodes glutamic acid decarboxylase (GAD), the rate-limiting enzyme in GABA synthesis. GABAergic agonists decrease, and antagonists increase, ethanol withdrawal severity (Buck & Harris, 1991; Crabbe, 1992; Crabbe, Merrill, & Belknap, in press), indicating that increased severity of withdrawal may be correlated with a decrease in GABA levels or availability. The inbred strains could be examined for differential GABA levels or GAD activity in a test of this hypothesis. Some data on this issue exists in the literature (e.g., Wong et al., 1974). In this study, no differences in GAD activity were found among six inbred strains.

The Ethanol/Diazepam Correlation

One advantage of testing a battery of inbred strains for phenotypes shown to be different between bi-directionally selected lines is that the number of genotypes is limited only by the number of inbred strains available. The number of genotypes of the selected lines is limited to the number of independently replicated lines (in this case, two replicates for a total of four genotypes). Lines which have been selected for many generations may differ in response for more phenotypes than those detected early in selection. The WSP and WSR selected lines were tested for acute CNS depressant drug withdrawal after 26 generations of selection pressure (Crabbe et al., 1991); therefore, it is possible that the reason that WSP mice showed enhanced acute withdrawal to all three of these drugs is due to the fixation of genes irrelevant to the selection trait in the homozygous state from random inbreeding. Another possibility is that

genes having a minor effect on the exacerbation of chronic ethanol withdrawal convulsions (the selection phenotype) but a major effect on diazepam withdrawal became fixed later in selection. Neither of these possibilities seems likely since the WSP/WSR lines differed in severity following precipitation of withdrawal from chronic diazepam (administered in food) in both generations 5 and 13 (Belknap et al., 1989). Furthermore, this line difference was present in both independently selected replicates. Nevertheless, the battery of inbred strains tested in the current studies did not show a genetic relationship between ethanol and precipitated diazepam withdrawal convulsion severity following a single administration of each of these drugs. Perhaps the pleiotropy shown by the selected lines occurred because the selection protocol (3 days of ethanol vapor inhalation) was intense enough to activate systems responding to withdrawal from CNS depressant drugs in general. Therefore, re-derivation of the selected lines using an acute ethanol withdrawal phenotype could potentially result in a lack of genetic correlation between acute ethanol and diazepam withdrawal. Finally, an additional possibility is that, among the alleles possessed by the inbred strains making up the HS/Ibg stock (from which the WSP/WSR lines were derived), are alleles affecting withdrawal which are not represented in the inbred strain panel tested in the present studies.

Several other possible explanations arise for the lack of genetic relationship of ethanol and diazepam withdrawal in the data reported here. One possibility is that an insufficient number of mice per strain (approximately half of the animals, see Table 2) were tested for diazepam withdrawal due to experimental design constraints (i.e., that we obtained an inaccurate estimate of the strain population means). However, the

estimate of total phenotypic variance explained by genetic factors in this experiment was 0.48 versus 0.44 in Expt. 1 and 0.24 in Expt. 2. These effect sizes are well within the range termed "large" by Cohen (1977). Furthermore, split-half reliabilities for the baseline and withdrawal variables were significant, arguing against the possibility that estimates of diazepam withdrawal severity were inaccurate (Table 4).

Another possibility is that the order of testing affected the diazepam withdrawal results. This appears to be the case for some strains, as evidenced by the significant mean differences in Baseline HICs shown in Table 3. One way of avoiding this would have been to employ a Latin Squares design; however, both the cost in number of animals required and the multiple drug groups required by the diazepam experiment made this design infeasible. Furthermore, the considerably longer half-life of diazepam necessitated that it be tested last (see pharmacokinetics section below). Another way to avoid potential problems with testing order would have been to test naive animals for acute withdrawal from only one drug, but again, the cost of doing so was prohibitive.

A third possibility is that the precipitation of diazepam withdrawal by flumazenil is inherently qualitatively different from the spontaneous withdrawal following metabolism of ethanol. At present, the only data of which we are aware that directly addresses this hypothesis (besides the present study) are those of Belknap et al. (1989; see p. 39) and Crabbe et al. (1991; see p. 39 - 40) in the WSP/WSR selected lines. These data would indicate there is not a difference between spontaneous and precipitated withdrawal. We have begun to collect data in WSP mice suggesting that precipitation of withdrawal convulsions may be unnecessary with some newer shorter-acting benzodiazepines, i.e., those

with half-lives in the 1 - 5 hour range, as opposed to diazepam's functional 30 - 60 hour half-life* (in humans; Harvey, 1985). Perhaps when the battery of inbred strains tested in these studies are tested in an unprecipitated model using one of these shorter-acting benzodiazepines, there may be a significant correlation in withdrawal severity with acute ethanol.

Acute and Chronic Withdrawal

One potential use of these data was to determine whether acute withdrawal from ethanol is genetically correlated with withdrawal from chronic ethanol vapor inhalation, using previously published inbred strain data. Table 5 lists the strains tested and scores reported in Crabbe et al. (1983a) that were examined for correlation with the acute ethanol withdrawal scores obtained in the present study (also listed). Chronic withdrawal severity was reported in two ways. First, strain mean withdrawal severities were calculated for ethanol-treated animals as the area under the withdrawal HIC curve minus the corresponding strain's mean area under the HIC curve for animals tested under pyrazole-only

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* Although the half-life of diazepam is reported to be in the longer range of action among the benzodiazepines, Gallaher et al. (1986) measured diazepam concentrations in the plasma of mice every few days during a period of chronic diazepam administration of doses ranging from about 350 - 1400 mg/kg/day, and found them to be negligible (0 to 229 ng/ml), indicating that diazepam itself may actually have a short half-life. Instead, the two metabolites of diazepam, nordiazepam ($t_{1/2}$ > 40 to 50 hours), and its metabolite, oxazepam ($t_{1/2}$ = 5 to 10 hours) had additive plasma levels totaling between 5 and 10 µg/ml (Gallaher et al., 1986). These metabolites have been reported to be active (i.e., if administered, they act like other benzodiazepines (Harvey, 1985). Therefore, these metabolites, and not diazepam directly, seem to be responsible for the long duration of action of this benzodiazepine.

conditions (Δ AUC 25). Second, chronic scores were further corrected for blood ethanol concentrations at the time of removal from the vapor chambers by linear regression (Δ AUC 25/BEC). The correlation between acute and chronic ethanol withdrawal severity scores corrected for pyrazole proved not to be significant ($r_{(11)} = 0.28$, $p = 0.36$; Figure 7). When chronic scores further corrected for BECs were correlated with the present data, they were also found not to be significant ($r_{(11)} = 0.26$, $p = 0.39$). Removal of the DBA/2 strain from these correlations did not improve them; in fact, the correlations became negative ($r_{(10)} = -0.23$, $p = 0.48$, and $r_{(10)} = -0.25$, $p = 0.44$, respectively). Since the data were available, correlations of acute pentobarbital and diazepam withdrawal with chronic ethanol withdrawal were also performed (data not shown). They also were found to be not significant.

Several possibilities exist for the lack of genetic correlation between acute and chronic ethanol withdrawal. First, the substrain designations differ for five strains compared to those tested in the present study (e.g., BALB/cAnN vs. BALB/cJ; Table 5). No differences between substrains was assumed for the purposes of the analysis; however, it remains to be demonstrated definitively that the substrains do not differ in either acute or chronic ethanol withdrawal. According to Bailey (1978), the separations of the DBA/2J from DBA/2N and C57BL/6J from C57BL/6N occurred ca. 1952 (approximately 70 and 90 generations before the initiation of the former and present studies, respectively). Differences between each pair of substrains could exist at an estimated 2% of all loci, based on mutation rates and degree of inbreeding prior to separation (Bailey, 1978).

Second, pyrazole alone has been shown to exacerbate handling-induced convulsions in some strains (e.g., C3H/HeN, 129/J, and PL/J) but

Table 5

Strains and Mean Withdrawal Scores following Chronic (left) and Acute (Right) Ethanol Withdrawal

Strain[1]	Δ AUC 25[2]	Δ AUC 25/BEC[3]	Strain[4]	Acute Withdrawal[5]
DBA/2N	36.75	33.37	DBA/2J	10.75
SWR/J	34.12	35.57	SWR/J	1.00
C3H/HeJ	32.21	29.90	C3H/HeJ	0.79
A/HeN	29.30	28.43	A/HeJ	2.59
DBA/1J	24.95	14.71	DBA/1J	5.00
SJL/J	23.80	15.30	SJL/J	0.93
CBA/J	11.40	10.43	CBA/J	1.84
C57BL/6N	11.20	21.63	C57BL/6J	2.13
CE/J	10.18	12.30	CE/J	4.04
PL/J	9.88	6.02	PL/J	0.42
BALB/cAnN	5.70	11.40	BALB/cJ	4.68
C57BR/cdJ	4.25	10.72	C57BR/cdJ	2.50
AKR/J	2.29	10.31	AKR/J	1.64

[1] Strains tested in Crabbe et al. (1983a)

[2] Strain mean Withdrawal Scores following chronic ethanol vapor. These scores were corrected for pyrazole (see text).

[3] Strain mean Withdrawal Scores following chronic ethanol vapor. These scores were corrected for pyrazole and blood ethanol concentrations following removal from the chamber (see text).

[4] Strains tested in the present study.

[5] Strain mean Withdrawal Scores following an acute injection of 4 g/kg ethanol (i.p.). These scores are also shown in Figure 5A.

Figure 7

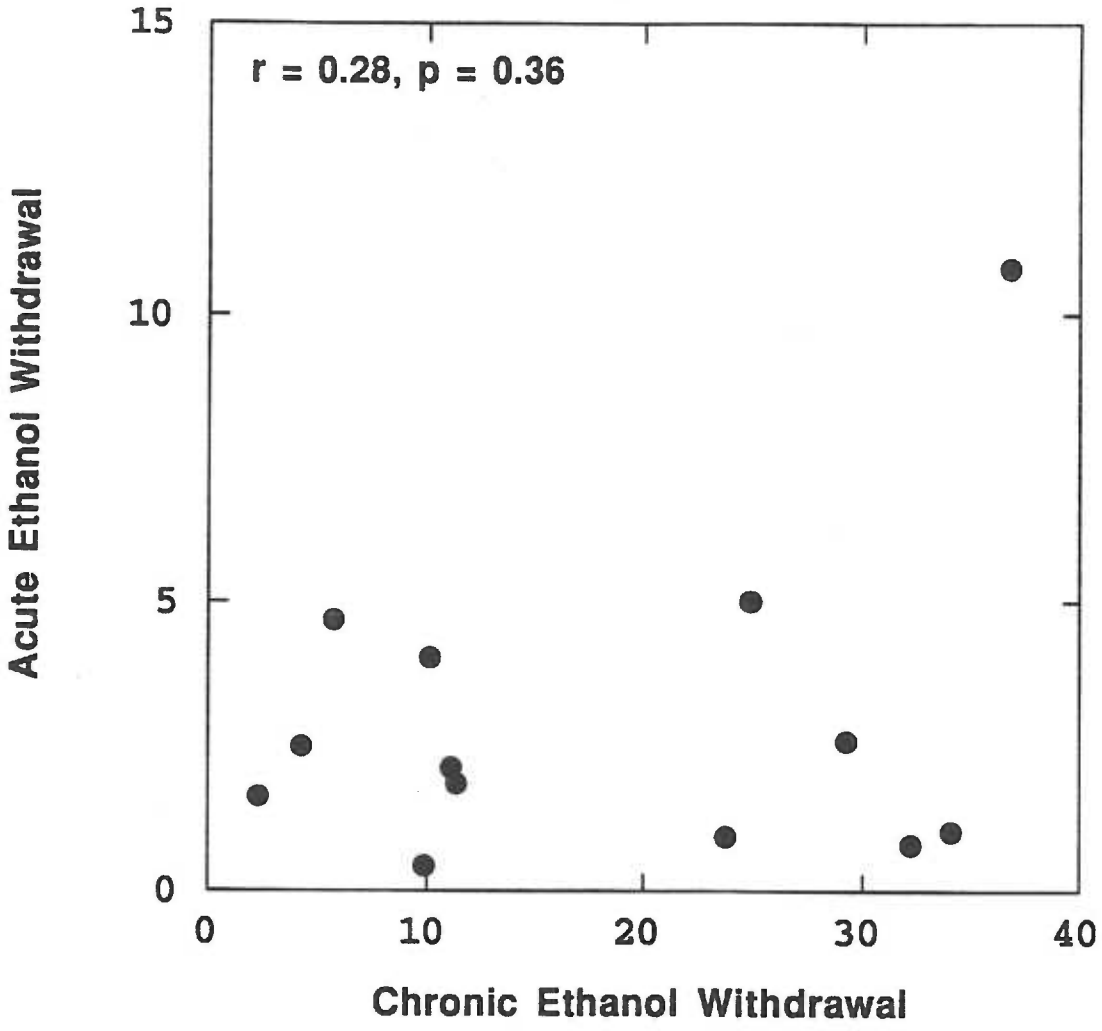

Figure 7. Scatterplot of strain means showing genotypic correlation of withdrawal severity scores of acute ethanol (present studies) with chronic ethanol (data from Crabbe et al., 1983a). Each dot represents an inbred strain. Correlation coefficient and corresponding significance level are shown in the upper left hand corner of the plot. Chronic scores were corrected for pyrazole only, as discussed in the text.

not others (e.g., C57BL/6N and DBA/2N; Crabbe et al., 1983a). Use of scores correcting for pyrazole should have addressed this concern adequately; however, an assessment of withdrawal following 24 hours of vapor inhalation without pyrazole should be performed in the identical substrains and correlated with both the acute and chronic data sets for an ideal test of the hypothesis that acute and chronic ethanol withdrawal are genetically correlated.

Additionally, the scale employed to assess HIC in the present study has been further articulated than in the previous study (7 points vs. 4), meaning that differences between strains for chronic ethanol withdrawal might have been greater than previously assessed. However, re-scaling the present acute data to the old scale did not increase the correlation between acute and chronic withdrawal ($r_{(11)} = 0.28$, $p = 0.36$). Finally, the sample sizes reported in the earlier study range from 3 - 5 per strain, which may be insufficient given the concerns listed above. Clearly, it is not possible presently to differentiate among these various possibilities. Therefore, the lack of demonstration of genetic correlation between acute and chronic ethanol withdrawal should not be taken as definitive.

Relationship of Results with Drug Pharmacokinetics

The hypothesis addressed by these studies assumes that there are no significant differences among inbred strains in brain drug levels attained or in rate of metabolism of each drug. Clinical literature suggests that drug levels but not rates of metabolism are correlated with withdrawal severity in humans. For example, Vinson and Menezes (1991) investigated in humans the relationship between breath alcohol levels at admission to drug treatment centers and severity of withdrawal using a

scoring system that rated, on a scale of 0 to 3, each of the following symptoms: tremor, sweating, nausea and vomiting, agitation, sleeplessness, confusion, hallucinations, tachycardia, hypertension, and fever. Every two to four hours following admission, each client at a nonmedical, social detoxification center was rated for these symptoms. Following each assessment, the resulting ten scores were summed to give a single withdrawal severity score. The area under the curve from a plot of these scores versus time was found to be related to breath alcohol levels ($R^2 = 0.26$). Furthermore, two groups have found blood alcohol levels at admission to be correlated with withdrawal severity, measured as the amount of chlordiazepoxide taken to abate symptoms in the initial 48 hours following admission (Clothier et al., 1985; Vinson & Menezes, 1991). However, when Clothier et al. (1985) investigated the possibility that rates of alcohol metabolism in humans were positively correlated with withdrawal severity, they found negative results. Rates of metabolism calculated from three to five blood alcohol levels in each of their 15 human subjects were not correlated with the amount of chlordiazepoxide taken in the initial 48 hour treatment period.

These data implicate alcohol level at admission but not rate of metabolism as a contributor to severity of withdrawal. Nevertheless, it is possible in the present studies that a strain that has a very rapid metabolism of a particular drug might have more severe handling-induced convulsions following withdrawal than a strain with slower metabolism. In agreement with clinical literature, Crabbe and colleagues (1983a) have reported that blood alcohol levels in inbred strains immediately following removal from three days of ethanol vapor inhalation are significantly correlated with severity of withdrawal. Furthermore, differences in severity

of withdrawal shown by the WSP and WSR selectively bred lines could not be attributed to differences in ethanol metabolism or absorption (Kosobud & Crabbe, 1986). In that study, no differences in blood ethanol concentrations were seen between the lines or replicates during ethanol vapor inhalation or in the first three hours after withdrawal from the vapor chambers, or following an acute injection of 3 g/kg ethanol (i.p.).

As part of another project, blood ethanol concentrations were analyzed at 30, 90, 150, and 210 minutes following a 4 g/kg i.p. dose administered to separate groups of animals of the same fifteen inbred strains as the present studies. Brain diazepam concentrations are presently being analyzed in a second set of animals at 30 minutes following an i.p. dose of either 8 or 16 mg/kg. Lastly, in yet another set of animals, brain pentobarbital samples are being collected at 30 minutes after 30 and 40 mg/kg i.p. These drug concentration data will be examined for genetic correlation with acute withdrawal. Preliminary analysis of the acute ethanol data suggest that severity of ethanol withdrawal is correlated with blood ethanol concentrations at 30 minutes ($r = 0.53$, $p < 0.04$), but not at other times. Brain diazepam concentrations at 30 minutes following either dose were not significantly correlated with severity of precipitated diazepam withdrawal ($r = 0.33$, $p = 0.23$ and $r = 0.07$, $p = 0.81$, respectively). Unpublished data collected earlier suggest that pentobarbital metabolism may influence severity of withdrawal, since DBA/2J mice may both metabolize pentobarbital faster and have more severe pentobarbital withdrawal than C57BL/6J mice. Brain pentobarbital levels measured at 15, 30, and 60 minutes following 40 mg/kg pentobarbital (i.p.) in C57BL/6J and DBA/2J mice yielded half-life estimates of 80 and 40 minutes, respectively. These results should be

interpreted cautiously since this is a comparison of only two strains. Clearly, additional analyses of the data will have to be done to determine whether drug metabolic differences can even partially explain strain differences in withdrawal severity.

Relationship of Results to Mechanistic Hypotheses

DBA/2J mice have been shown to have tonic and clonic seizures induced by picrotoxinin at lower doses than BALB/cByJ mice, as well as more effective picrotoxinin-inhibition of basal and pentobarbital-induced chloride flux (Schwartz et al., 1989). More intense ethanol, and slightly more intense pentobarbital, withdrawal was shown by DBA/2J than BALB/cJ mice in the present studies. This strain may possess more of the inhibitory $GABA_A$ receptor-coupled chloride channels than BALB/cByJ (or BALB/cJ) mice. This hypothesis seems plausible in light of the fact that BALB/cJ mice had dramatically more intense baseline convulsions than DBA/2J mice, indicating that they may have less natural inhibition of excitatory stimulation. Furthermore, if GABAergic mechanisms are involved in the rebound hyperexcitability seen in withdrawal, then these data would predict that DBA/2J would have more GABA receptors than BALB/cJ mice.

However, BALB/cJ mice have also been shown to have decreased augmentation by diazepam of GABA-mediated chloride flux (Mihic et al., 1992), and fewer diazepam binding sites (Robertson, 1979) compared to C57BL/6J mice. BALB/cJ mice had both more intense baseline and withdrawal severity from all three drugs than C57BL/6J mice in the present studies. More intense baseline convulsions would be expected; however, if GABA/BZ receptors are involved in withdrawal rebound, one would

predict from these data that C57BL/6J mice would have more intense withdrawal than BALB/cJ mice.

Although both of these studies imply that genetic mediation of GABA function may underlie the differences in several behaviors, including CNS depressant drug withdrawal, the results have limited generalizability because only two strains were tested in each study, and the behaviors measured were different (although related). The hypothesis that GABA receptor subunit composition and expression differences exist between inbred strains is being tested. The α_2, α_3, and δ subunits of the GABA receptor have been shown not to differ at the base pair level between C57BL/6J and DBA/2J mice, while the α_1 subunit does not differ at the amino acid level (Wang et al., 1992 a,b). No apparent strain differences in receptor subunit expression levels existed at the time of testing (3 weeks of age; Wang et al., 1992b).

It is intriguing that DBA/1J and DBA/2J do not show the same degree of withdrawal in response to the drugs tested (see Figure 4 A-C). DBA/2J mice clearly are affected more strongly by ethanol withdrawal than their closely-related substrain, and seem to be less affected by precipitated diazepam withdrawal. These substrains were separated earlier than the other pairs of substrains (ca. 1932; after ~50 generations of inbreeding), and so may differ by 100 or more genes (Bailey, 1978). It will be interesting to explore other areas where these two closely related strains have been shown to differ (and not). For example, DBA/1J mice have about a two degree hypothermic response to 2 g/kg ethanol at 30 minutes after injection, while DBA/2J mice are barely affected (Crabbe, Gallaher, et al., in press). These strains were tested as part of a larger project involving the other thirteen strains represented in the current studies, and as

mentioned earlier, correlation of all the data is planned. Previous work in inbred strains has indicated that strain means for chronic ethanol withdrawal severity are negatively correlated with strain means for initial sensitivity and magnitude of tolerance to ethanol-induced hypothermia (Crabbe et al., 1983a), which is in agreement with the prediction that DBA/2J mice would show less hypothermia than DBA/1J mice.

Summary

The present report provides clear, independent evidence that there is substantial genetic influence on withdrawal from these three CNS depressant drugs. Ethanol and pentobarbital withdrawal convulsions were strongly genetically correlated, indicating that they share genes underlying a common mechanism. Similarly, diazepam and pentobarbital withdrawal severities were significantly genetically correlated. Baseline handling-induced convulsion severity was also shown to be genetically mediated but was not predictive of withdrawal severity, as seen by the differing patterns of experimental time-courses (e.g., see Figures 1 and 2). Evidence was reviewed in the introduction that generally supports GABAergic or calcium-dependent mechanisms in withdrawal from these drugs. However, diazepam and ethanol withdrawal severities were found to be not significantly correlated in these studies. Reasons for this finding were discussed in view of the results obtained in the WSP/WSR selected lines. The correlations between acute withdrawal and chronic ethanol withdrawal using data from Crabbe, et al. (1983a) also were analyzed and discussed. Finally, potential problems with these data were discussed, including the potential influences of repeated testing, drug testing order, and pharmacokinetic differences between strains. However, the data were

found to be reliable using Spearman-Brown split-half reliability calculations, lending confidence in the results. Important future uses of these data were also presented.

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